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Bioorganic & Medicinal Chemistry Letters 15 (2005) 2881–2885
Bioorganic & Medicinal Chemistry Letters

Evaluation of 3-substituted arginine analogs as selective inhibitors of human nitric oxide synthase isozymes

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Received 2 March 2005; revised 20 March 2005; accepted 22 March 2005
Available online 25 April 2005

Abstract—Nitric oxide (NO), a mediator of various physiological and pathophysiological processes, is synthesized by three isozymes of nitric oxide synthase (NOS). In developing candidate clinical drugs, it is very important not to inhibit endothelial NOS, because it plays an important role in maintaining normal blood pressure and flow. Here, we describe the design, synthesis and human NOS-inhibitory activities of *S*-methyl-L-isothiocitrulline-based 3-substituted arginine analogs. The 3*R**-methyl compound **4**, which has an *S*-methyl isothiourea moiety, inhibited nNOS and iNOS, but not eNOS (IC_{50} >1 mM). However, the 3*R**-methyl compound **7**, bearing a 5-iminoethyl moiety, did not inhibit any of the NOS isozymes, although L-*N*-iminoethylornithine (L-NIO) potently inhibited all three. A computational docking study was carried out to investigate the mechanism of the isozyme selectivity.

Nitric oxide synthases (NOS)[1] are a class of enzymes found in mammals and other species that utilize L-arginine to generate nitric oxide (NO). NO is an important signaling molecule involved in a wide range of physiological functions, as well as pathophysiological states.[2] Three NOS isozymes have been identified in various cells, that is, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). They all catalyze the same two-step oxidation of L-arginine, using the same cofactors, NADPH, FAD, FMN, hemin, and BH_4.[3–5] Those NOS isozymes are associated with different physiological functions: blood-vessel dilation (eNOS),[6] neuronal signal transmission (nNOS),[3] and immune response, such as cytotoxicity, against pathogens, and tumors (iNOS).[7] NO overproduction by NOS plays a role in a wide range of disorders, including septic shock, arthritis, diabetes, ischemia-reperfusion injury, pain, and various neurodegenerative diseases.[8,9] However, in developing NOS inhibitors to treat these disorders, it is very important to keep eNOS activity intact, because of its role in maintaining normal blood pressure and flow.[10] Therefore, the design of isozyme-selective NOS inhibitors to regulate NO synthesis in specific tissues is important. Structural analogs of L-arginine, the natural substrate of NOS, have been shown to inhibit the various forms of NOS, and nonamino acid inhibitors of NOS have also been reported.[11] However, the first generation of arginine analog inhibitors had low isozyme selectivity. For example, *S*-methyl-isothiocitrulline (L-MIT **1**)[12] and *N*-iminoethyl-L-ornithine (L-NIO **2**) are potent inhibitors of all three NOS isozymes. We attempted to design selective inhibitors based on arginine, and found that *S*-methyl-L-isothiocitrullinyl-L-phenylalanine (MILF **3**),[13,14] which has a phenylalanine moiety at the *C*-terminal of L-MIT, is an iNOS-selective inhibitor (Fig. 1). Our results suggested that the NOS isozymes have differences in the hydrophobic region near the binding site of the *C*-terminal of the substrate. To investigate the contribution of lipophilic substituents to the selectivity of NOS inhibition by arginine

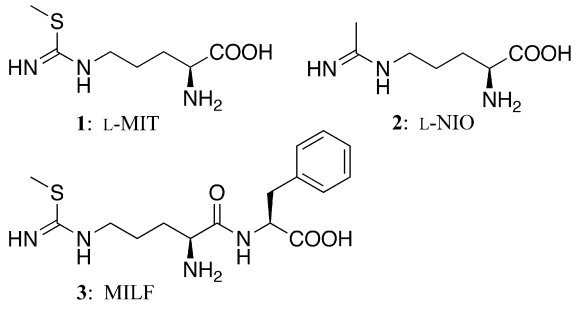

Figure 1. Structures of L-MIT, L-NIO, and MILF.

Keywords: Nitric oxide; Nitric oxide synthase; Inhibitor; Arginine; Docking study.
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doi:10.1016/j.bmcl.2005.03.078

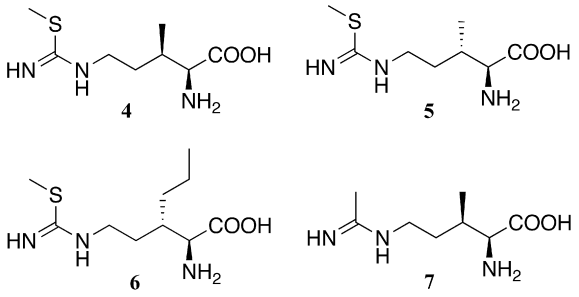


Figure 2. Design of 3-substituted arginine analogs.

analogs, we newly designed several 3-substituted arginine analogs **4**–**7** (Fig. 2).

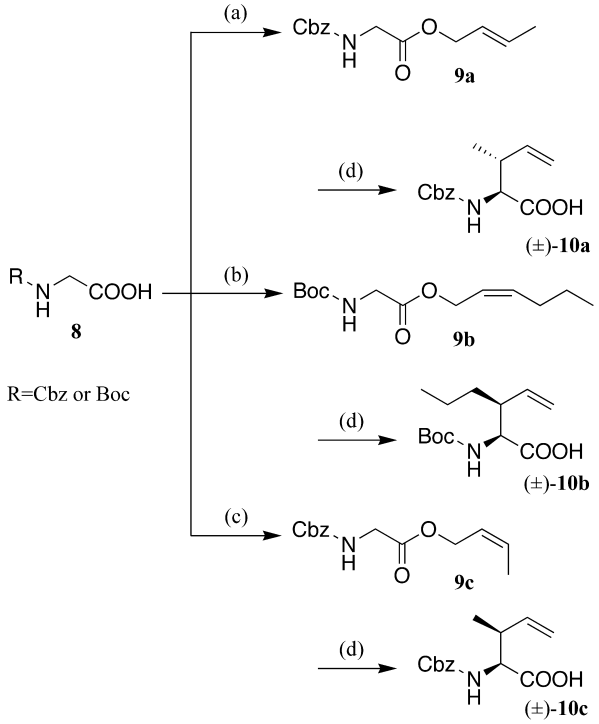
Here, we describe the synthesis of these 3-substituted arginine analogs and the evaluation of their inhibitory activity toward the three recombinant human NOS isozymes. A docking study was conducted to throw light on the effects of the substituents.

Our synthetic strategy was to use Claisen rearrangement to form the amino acid backbone bearing an alkyl group at the 3-position,[15,16] followed by conversion of the functional group. Protected glycine esters **9a**–**c** were obtained as precursors of Claisen rearrangement by condensation of the *N*-protected glycine **8** and the corresponding 2,3-unsaturated alcohol. Since *cis*-buten-1-ol was not commercially available, 2-butyn-1-ol was used and the terminal alkyne was reduced with Lindlar catalyst to the *cis* olefin for the preparation of **9c**. The 3-substituted amino acids were generated in the Claisen rearrangement with LHMDS and Al(O-*i*-Pr)$_3$, affording (±)-*N*-Cbz-3-substituted 4,5-unsaturated amino acids **10a**–**c**. In this rearrangement, the relative stereochemistry of the 3-substituent versus the 2-amino group was controlled by the geometry of the substrate (Scheme 1). *syn*-Form amino acid derivatives were obtained from trans substrates and *anti*-form from cis substrates.

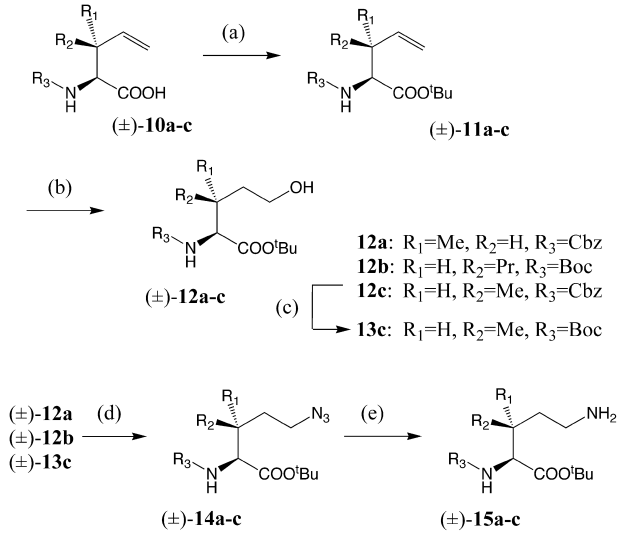
The carboxyl group was protected as the *tert*-butyl ester **11a**–**c**. The terminal olefin of the resulting **11a**–**c** was then converted to the primary alcohol **12a**–**c** by hydroboration followed by oxidation. Compound **12c** was deprotected by catalytic reduction and then protected with a Boc group to afford **13c**. Hydroxyl groups on **12a**–**b** and **13c** were converted to azide to afford **14a**–**c**. Azide compounds **14a**–**c** were easily converted to the ornithine derivatives **15a**–**c** by catalytic reduction (Scheme 2).

These 3-substituted ornithines **15a**–**c** were treated with thiophosgene, then NH_3 in methanol, to give the thioureas **16a**–**c**, which were purified by HPLC. Treatment with iodomethane in CH_3CN converted **16a**–**c** to *S*-methyl isothiourea form, **17a**–**c**. Deprotection afforded the desired 3-substituted *S*-methyl-isothiourea-type arginine analogs **4**, **5**, **6**. To synthesize another type of arginine analog, **15a** was treated with ethyl acetimidate, and subsequent deprotection gave compound **7** (Scheme 3).

Human NOS isozymes were expressed in Sf-9 (a *Spodoptera frugiperda* insect cell line). Recombinant human

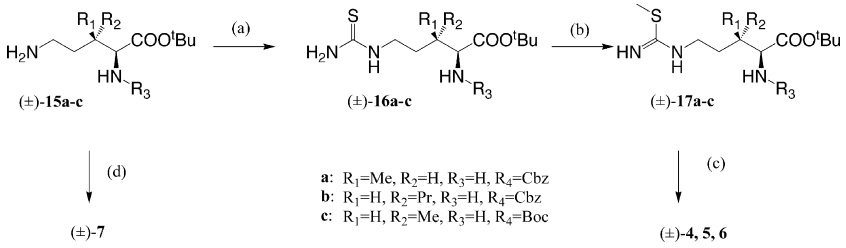


Scheme 1. Reagents and conditions: (a) crotyl alcohol, EDCI, DMAP, CH_2Cl_2, 0 °C, 95%; (b) *cis*-2-hexen-1-ol, EDCI, DMAP, CH_2Cl_2, 0 °C, 84%; (c) (i) 2-butyn-1-ol, EDCI, DMAP, CH_2Cl_2, 0 °C, (ii) Lindlar cat., MeOH, H_2, rt, 99%; (d) LHMDS, Al(O-*i*-Pr)$_3$, THF, −78 °C, **10a** 52%, **10b** 52%, **10c** 30%.



Scheme 2. Reagents and conditions: (a) isobutene, H_2SO_4, CH_2Cl_2, 0 °C, **11a** 72%, **11b** 31%, **11c** 98%; (b) (i) thexylborane, THF, 0 °C, (ii) H_2O_2, pH 7.0, **12a** 56%, **12b** 78%, **12c** 39%; (c) Pd/C, MeOH, $(Boc)_2O$, Et_3N, **13c** 53%; (d) (i) MsCl, pyridine, CH_2Cl_2, 0 °C, (ii) NaN_3, DMF, 50 °C, **14a** 67%, **14b** 42%, **14c** 9.1%; (e) for **14a**: Lindlar cat., MeOH, H_2, rt; for **14b**,**c**: Pd/C MeOH, H_2, rt, **15a**–**c** quant.

NOS activity was measured by monitoring the conversion of [^{14}C]-L-Arg to [^{14}C]-L-Cit.[17] The IC_{50} values of synthesized compounds were calculated from the concentration dependence of the inhibitory activity (Table 1). L-MIT **1** is a potent inhibitor of all the



Scheme 3. Reagents and conditions: (a) (i) thiophosgene, $CaCO_3$, CH_2Cl_2, H_2O, rt, (ii) NH_3, MeOH, 0 °C, **16a** 77%, **16b** 94%, **16c** 54%; (b) MeI, CH_3CN, rt, **17a** 80%, **17b** 84%, **17c** quant; (c) for **17a**: HCl, THF, reflux; for **17b**,**c**: (i) TFA, H_2O, phenol, thioanisole, 1,2-ethanedithiol, rt; for **17c**: (ii) (−)ion exchange column, **4**–**6** quant; (d) (i) for **15a**, ethyl acetimidate, K_2CO_3, MeOH, rt, 23%, (ii) HCl, THF, reflux, quant.

Table 1. IC_{50} values (μM) for inhibition of human NOS isozymes[a]

Entry	Compound	Substituent		nNOS[b]	iNOS[b]	eNOS[b]
		3-Position	5-Position			
1	**1**	H	*S*-Me-isothiourea	0.06	0.3	0.4
2	**3**[c,d]	H	*S*-Me-isothiourea	36	3.9	>1000[f]
3	(±)-**4**[e]	(*R**)-Me	*S*-Me-isothiourea	38	110	>1000[f]
4	(±)-**5**[e]	(*S**)-Me	*S*-Me-isothiourea	3.5	4.5	11
5	(±)-**6**[e]	(*S**)-Pr	*S*-Me-isothiourea	500	370	>1000[f]
6	**2**	H	Iminoethyl	9	4	6
7	(±)-**7**[e]	(*R**)-Me	Iminoethyl	>1000[f]	>1000[f]	>1000[f]

[a] Human NOS activity was measured by monitoring the conversion of [^{14}C]-Arg to [^{14}C]-L-Cit.
[b] Human NOS isozymes were expressed in Sf-9 cells.
[c] Data from Ref. 13.
[d] Dipeptide compound.
[e] The relative configuration at the 2-position of all compounds is *S**.
[f] Not inhibited by 50% at concentrations up to 1 mM inhibitor.

NOS isozymes, with slight selectivity for nNOS, while MILF **3** is an iNOS-selective inhibitor (entries 1 and 2). This difference between **1** and **3** could be explained by the existence of a hydrophobic region in the iNOS active site at a position corresponding to the *C*-terminal of the ligand.

The 3*R**-methyl derivative **4** had similar nNOS inhibitory activity (IC_{50} = 38 μM) to MILF **3** (36 μM). However, the iNOS and eNOS inhibitory activities were very low (iNOS 110 μM, eNOS >1000 μM) (entry 3). In contrast, the 3*S**-methyl derivative **5** inhibited all the NOS isozymes (nNOS 3.5 μM, iNOS 4.5 μM, eNOS 11 μM) (entry 4). The 3*S**-propyl derivative **6** had very weak inhibitory activity for all of the NOS isozymes (nNOS 500 μM, iNOS 370 μM, eNOS >1000 μM) (entry 5). Surprisingly, **7** had no inhibitory activity for any of the NOS isozymes (entry 7). In contrast, L-NIO **2** potently inhibited all of them (entry 6). Therefore, the 3*R* substituent appears to have a critical negative effect on substrate recognition. The 3*R**-methyl derivative **4** was slightly selective for nNOS over iNOS, and did not inhibit eNOS, whereas the 3*S**-methyl derivative **5** had inhibitory activity for all of the NOS isozymes. This suggests that *R*-configuration at the 3-position results in lower potency than *S*-configuration (entries 3 and 4). The difference between the 3*R**-methyl compound and 3*S**-methyl compound is remarkable. Comparing the methyl **5** and propyl **6** substituents, it seems that the propyl group is too large to bind to the NOS L-arginine-binding site (entries 4 and 5). In order to investigate substitution effects at the 3-position of L-MIT in detail, we conducted docking calculations.

To study the binding mode of compounds **4** and **5** to the active site, we calculated the minimum energy conformations of **4** and **5** when they were docked into models based on the crystal structures of rat nNOS (PDB code 1LZX) and human eNOS (PDB code 3NOS) using the software package MACROMODEL 8.0, and we compared the results with those obtained for L-MIT **1** (Fig. 3). As shown in Figure 3a and b, there is a high degree of similarity among the binding modes to nNOS of **1** (green), **4** (blue), and **5** (red). Next, we investigated the binding modes to eNOS of **1**, **4**, and **5**. In the case of **4**, the carboxyl group cannot be overlapped with that of **1** (Fig. 3c and d), owing to rotation of the alkyl chain resulting from the presence of the 3*R*-methyl group at the 3-position. On the other hand, the binding mode of **5** is similar to that of **1**. Thus, in the case of the nNOS active site, the carboxyl groups and amino groups of **4** and **5** were superimposed in the binding pocket, while such superimposition did not occur in the case of the eNOS active site. This result is consistent with the difference of eNOS inhibitory activity between **4** and **5**. It appears that a 3*R*-methyl group interferes with docking to the eNOS active site and thus reduces eNOS-inhibitory activity.

In summary, we have designed and synthesized L-MIT-based compounds as 3-substituted arginine analogs in order to find selective NOS inhibitors. The 3*R**-methyl compound **4** had no inhibitory effect on eNOS, though it inhibited both nNOS and iNOS. Compound **7** did not inhibit any of the NOS isozymes. The 3*S**-methyl compound **5** potently inhibited all of the isozymes. A large substituent, such as the *n*-propyl group in **6**,

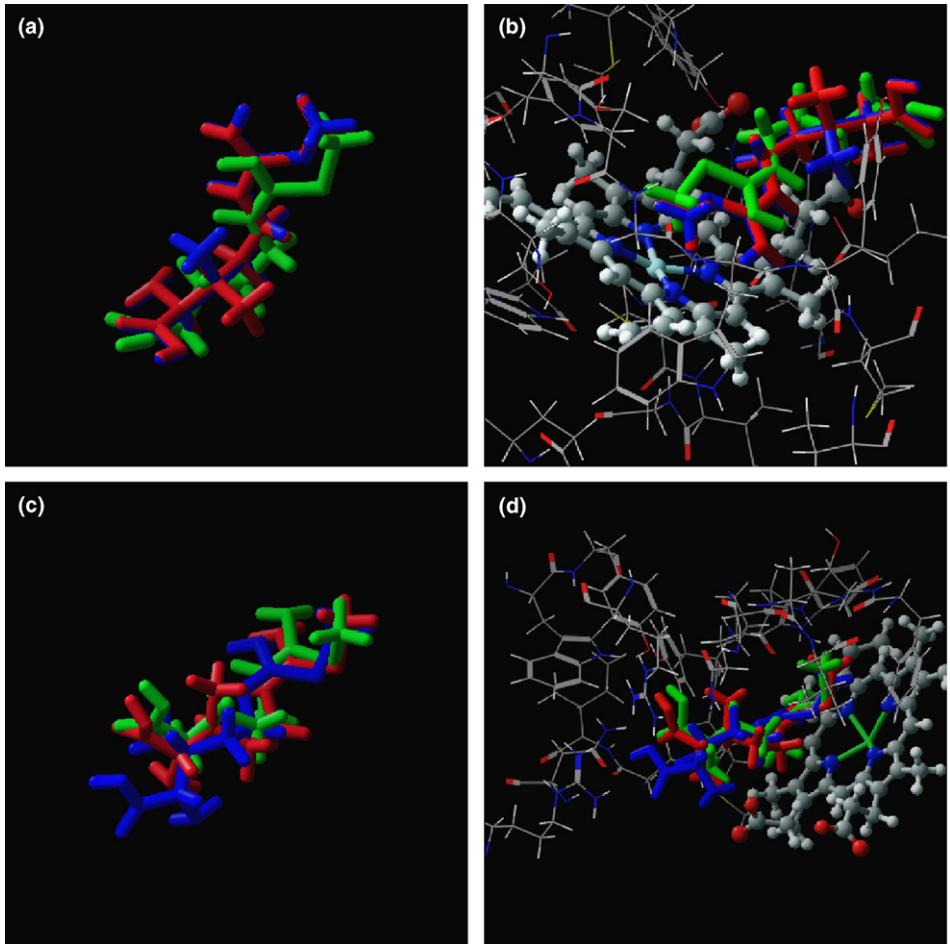


Figure 3. Superposition of the minimum energy conformations of **1** (green) and **4** (blue) and **5** (red) in the nNOS (a, b) and eNOS (c, d) active sites. (a) nNOS protein is not shown for the sake of clarity. (b) View of the conformation of the inhibitors docked into the nNOS catalytic center. Residues within 5 Å from the ligand are displayed as wire graphics and heme is displayed as ball and stick. (c) eNOS protein is not shown for the sake of clarity. (d) View of the conformation of the inhibitors docked into the eNOS catalytic center. Residues within 5 Å from the ligand are displayed as wire graphics and heme is displayed as ball and stick.

decreased the inhibitory effect on all of the NOS isozymes. Since the prepared compounds were racemic, there might be a difference of activity between the optical isomers. Further investigation of the structure–activity relationship is in progress.

Acknowledgements

We thank Dr. Jung-Min Park and Professor Tetsuo Nagano of the University of Tokyo for providing human NOS baculovirus and for advice about the assay. We also thank Dr. Takayoshi Suzuki and Professor Naoki Miyata of this university for the docking study. We are grateful to Uehara Memorial Foundation for financial support.

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