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Syntheses of NAMDA derivatives inhibiting NO production in BV-2 cells stimulated with lipopolysaccharide

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Abstract—Sixteen derivatives of *N*-acetyl-3-*O*-methyldopamine (NAMDA), an inhibitor of BH_4 synthesis, were designed and synthesized. The ability of these derivatives to inhibit NO and BH_4 production by lipopolysaccharide-stimulated BV-2 microglial cells was determined. While NAMDA at 100 μM inhibited NO and BH_4 production by only about 20%, its catecholamide **8**, indole **23** derivative, **13**, and *N*-acetyl tetrahydroisoquinoline **25** inhibited the NO production by >50% at the same concentration. In particular, **13** and **25** inhibited both NO and BH_4 production to similar degrees, which suggested that these compounds might inhibit NO production by blocking BH_4-dependent dimerization of the newly synthesized iNOS monomer.

1. Introduction

Nitric oxide radical (NO) is a small, membrane-permeable, and yet reactive gaseous metabolite. It is produced from L-arginine by nitric oxide synthase (NOS), widely distributed in biosystems from plants to mammals. NO was originally recognized as the endothelium-derived relaxing factor in vasculature,[1] but was later found to serve as an important physiological mediator in cardiovascular, immune, and nervous systems.[2] While the small amount of NO produced intermittently by the endothelial NOS (eNOS, also referred to as type-3) and neuronal NOS (nNOS, type-1) serves as a key mediator in many physiological functions, the large amount of NO produced constantly by inducible NOS (iNOS, type-2) is associated with various diseases.[3]

Among the three isozymes, eNOS and nNOS are expressed constitutively and are activated by increased cytosolic Ca^{2+}. On the other hand, iNOS does not depend on Ca^{2+} concentration and constantly produces a large amount of NO.[4] The iNOS is not expressed under normal conditions but is induced in response to invading pathogens (e.g., bacteria, fungus, and virus) and various cytokines. The NO overproduced by iNOS is implicated in various diseases, such as stroke,[5] Alzheimer's disease,[6] Parkinson's disease,[7] artherosclerosis,[8] and septic shock.[9] Intensive efforts have been made to identify or synthesize selective inhibitors that control enzymatic activity of iNOS for effective attenuation of NO overproduction.[10]

The NO producing, functional NOS enzyme is a homodimer. Each monomer is composed of two domains: a reductase domain containing the binding sites for FMN, FAD, and NADPH and an oxygenase domain containing the binding sites for heme (iron protoporphyrin) and tetrahydrobiopterin (BH_4).[3,11] Binding of all these cofactors to their respective sites is essential for effective generation of NO. Among the cofactors, BH_4 plays an essential role in dimerization of the monomers and therefore in catalytic activity.[12] Thus, inhibition of iNOS dimerization even after an increased number of the monomers during iNOS induction would prevent the NO overproduction and reduce NO-related damages.

Keywords: NAMDA; Nitric oxide; Inhibition of NO; Inhibition of tetrahydrobiopterin; Nitric oxide synthase.
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In this connection, Cho et al. reported that *N*-acetyl-3-*O*-methyldopamine (NAMDA, **1**) attenuated NO generation via inhibition of BH_4 production in BV-2 microglial cells that had been stimulated with lipopolysaccharide (LPS).[13] However, large doses (1–3 mM) of NAMDA were required to observe this effect, obviating its application in clinical situations. Thus, toward effective inhibition of BH_4 and NO production, we designed and synthesized 16 derivatives using NAMDA as a lead compound. Based on the activities of these derivatives, we provide information on the structure–activity relationship (SAR) of NAMDA.

2. Chemistry

Chemical structure of NAMDA was modified in three directions: variation of acetamide to various alkyl-amino moieties (Method A), homologation by inserting one more carbon (Method B), and cyclization of the flexible acetamide group to construct a rigid NAMDA structure (Method C). This methodological scheme is shown in Figure 1.

First, to generate various alkyl-amino derivatives of NAMDA (Method A), a large amount of 3-*O*-methyldopamine hydrochloride (**4**) was required as a key intermediate of NAMDA derivatives. Compound **4** was obtained from 4-hydroxy-3-methoxybenzyl alcohol (**2**) by cyanation and hydrogenation using palladium as a catalyst, according to previously reported method.[14] As shown in Scheme 1, the reaction of **4** with naphthylmethyl thioacetamide gave the acetamidine **5** with 70% yield and trimethylorthoformate yielded formamide **6** with 71%, respectively. Alternatively, acetylation of 3-hydroxytryptamine produced the catecholamide **8** with 90% yield.

Next, to introduce the cyclic alkyl-amino derivatives to NAMDA, **4** was reacted with diphosgene to produce isocyanate and then addition of corresponding piperidine and morphorine produced **9** and **10** with 44% and 75% yield, respectively. Other compounds (**12**–**15**) were synthesized from 4-hydroxy-3-methoxybenzyl cyanide (**3**). Conversion of nitrile to carboxylic acid produced **11**. Further reactions for the amide formation were conducted by adding piperidine and pyrrolidine to obtain **12** and **13**, and following reduction of each carbonyl group yielded **14** and **15**, respectively, as shown in Scheme 2. Another homologation derivative, phenylpropylacetamide **19**, was prepared by condensation of **16** with acetonitrile, hydrogenation using palladium under acidic condition, and then acetylation (Scheme 3).

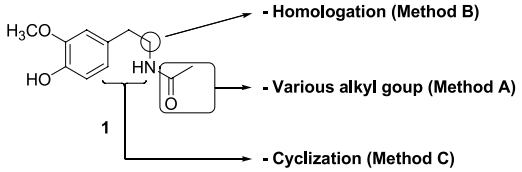
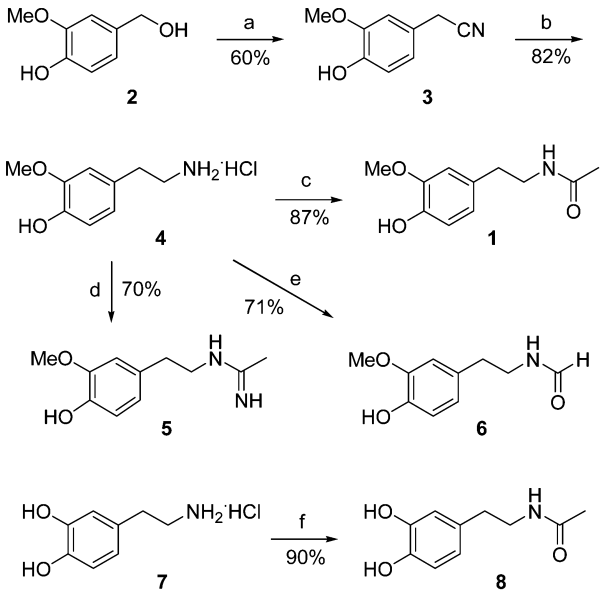
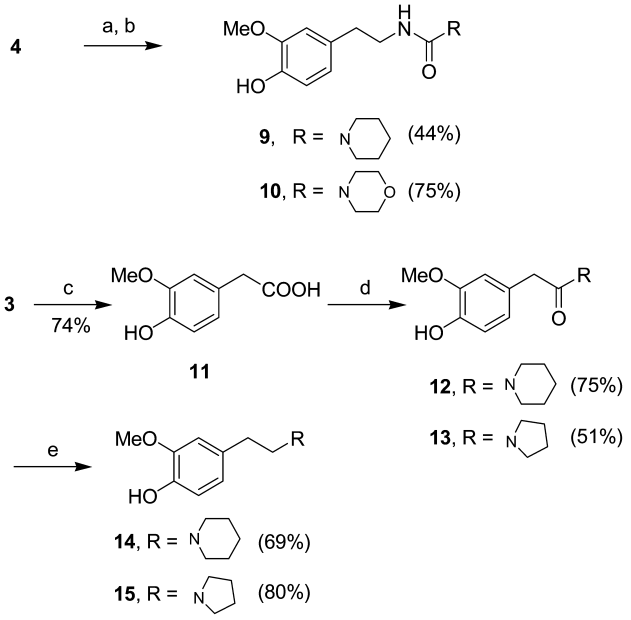


Figure 1. Structure and three methods for the modification of NAMDA.

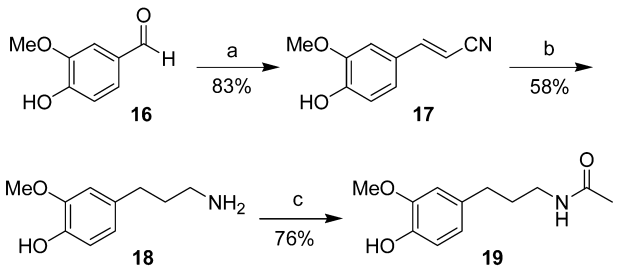


Scheme 1. Reagents and conditions: (a) NaCN, DMF, 130 °C, 20 h; (b) H_2, Pd/C, HCl, EtOH, rt, 12 h; (c) Ac_2O, Et_3N, CH_2Cl_2, rt, 30 min; (d) (*S*)-2-naphthylmethyl thioacetimidate hydrobromide, Et_3N, EtOH, rt, 3 h; (e) trimethylorthoformate, reflux, 2 h; (f) Ac_2O, Et_3N, CH_3CN, rt, 1 h.

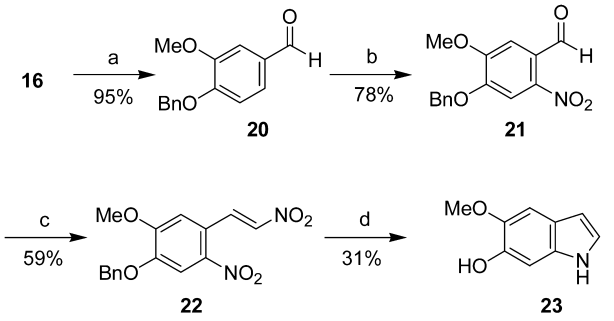


Scheme 2. Reagents and conditions: (a) diphosgene, $CHCl_3$, reflux, 8 h; (b) piperidine or morpholine, $CHCl_3$, rt, 2 h; (c) KOH, EtOH, reflux, overnight; (d) piperidine or pyrrolidine, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), *p*-dimethylaminopyridine (DMAP), DMF, rt, 3 h; (e) LAH, THF, reflux, 2 h.

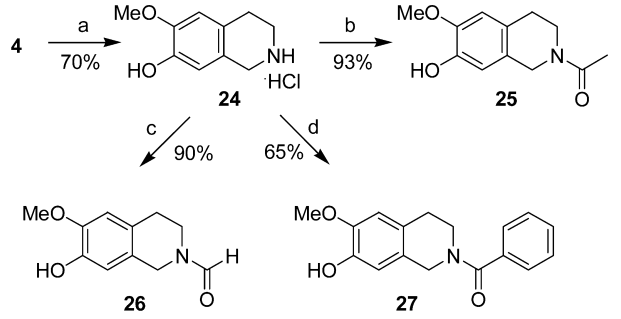
To obtain the cyclized derivatives of NAMDA (Method C), the modified Leimgruber–Batcho indole synthesis[15] (Scheme 4) and Pictet–Spengler tetrahydroisoquinoline synthesis[16] methods (Scheme 5) were used. Protection of hydroxyl group on **16** with benzyl bromide gave **20** and nitration produced regioselective 2-nitrobenzaldehyde **21**. Following Henry reaction, nitro-condensation, and palladium catalyzed reduction with ammonium for-



Scheme 3. Reagents and conditions: (a) KOH, CH_3CN, reflux, overnight; (b) H_2, Pd/C, HCl, EtOH, rt, 12 h; (c) Ac_2O, Et_3N, CH_2Cl_2, rt, 30 min.



Scheme 4. Reagents and conditions: (a) benzyl bromide, K_2CO_3, acetone, reflux, 4 h; (b) HNO_3, H_2SO_4, AcOH, rt, 1 h; (c) NH_4OAc, CH_3NO_2, reflux, 2 h; (c) Pd/C, $HCOONH_4$, formic acid, methanol, rt, 9 h.



Scheme 5. Reagetns and conditions: (a) HCHO, H_2O, HCl, 70 °C, 1 h; (b) Ac_2O, Et_3N, CH_2Cl_2, rt, 30 min; (c) trimethylorthoformate, concentrated HCl (cat), reflux, overnight; (d) Bz_2O, Et_3N, CH_2Cl_2, rt, 30 min.

mate in formic acid gave an indole **23**. Tetrahydroisoquinoline **24** was prepared by reaction of **4** with formaldehyde in acidic solution and **24** was derivatized further to yield **25** by reacting **24** with acetic anhydride, to yield **26** by reacting with trimethylorthoformate, and to yield **27** by reacting with benzoic anhydride.

3. Biological studies

3.1. Cell culture

BV-2 cells, a murine microglial cell line, were grown and maintained in DMEM supplemented with 10% fetal calf serum and penicillin–streptomycin at 37 °C in a humidified incubator under 5% CO_2. For experiments, the cells were plated on polystyrene tissue culture dishes at a density of 2×10^5 cells/well in 24-well culture plates. After 24 h, the cells were changed into fresh medium and treated with bacterial LPS (0.2 μg/mL). The NAMDA derivatives (various concentrations dissolved in DMSO) were added at 15 min prior to the LPS treatment. After 24 h, the medium was taken to measure NO and BH_4 and lactate dehydrogenase (LDH) activity.

3.2. NO production

Accumulated nitrite, a stable oxidation metabolite of NO, was measured by the Griess reaction.[17] Briefly, 200 μL aliquots of the culture medium were mixed with 100 μL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% H_3PO_4) in a 96-well microtiter plate, and the absorbance was read at 540 nm. The effect of each compound on NO production was expressed as the percent of NO produced by the LPS-treated control cells.

3.3. BH_4 Production

BH_4 produced was determined according to the method reported previously.[18] To the 900 μL aliquot of culture medium, 100 μL of 1 M phosphoric acid and 200 μL of acidic iodine solution (0.5% I_2 and 1.0% KI in 0.2 M trichloroacetic acid) were added and incubated for 1 h in the dark. The oxidation reaction was terminated by addition of 0.1 mL of 0.1% ascorbic acid. The reaction mixture was then centrifuged for 15 min at 8000*g* and the supernatant was diluted with distilled water. BH_4 was separated isocratically with 5% methanol as mobile phase using HPLC and detected by a fluorescence detector (Waters, Boston, MA, USA). BH_4 standard curve was prepared every time. The BH_4 content was calculated using Waters 991 computerized integrator system as nanogram of BH_4 per milligram of cellular protein and expressed as percent of LPS-treated control.

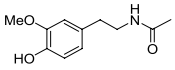
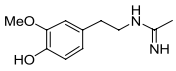
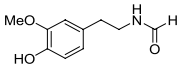
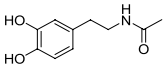
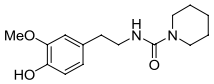
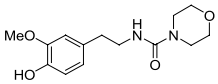
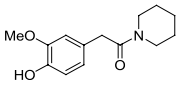
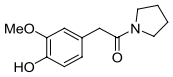
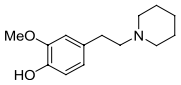
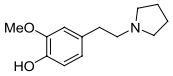
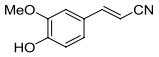
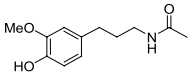
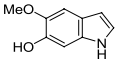
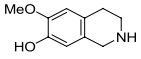
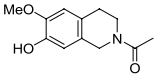
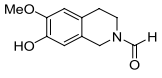
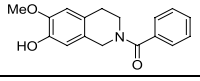
3.4. Determination of cytotoxicity by LDH activity

Cytotoxicity of the newly synthesized compounds was assessed by determining the activity of LDH released into the culture medium. LDH activity was determined using the CytoTox 96R Non-Radioactive Cytotoxicity Assay kit (source) and the absorbance was read at 490 nm using a spectrophotometer. Cytotoxicity values were expressed as percent of LDH released by the NAMDA derivatives compared with that released from LPS-treated control cells and thus, numbers approaching 100 indicate no toxicity.

4. Results and discussion

Sixteen new derivatives of NAMDA were synthesized. As compounds with cytotoxicity can give misleading

Table 1. Effects on the production of NO and BH_4 and cell survival[a]

No	Compound	Nitrite[b] (%)	LDH[c] (%)	BH_4[b] (%)
LPS		100	100	100
DAHP[d]				109
1		84.0	73.4	81.48
5		96.9	96.4	
6		74.8	90.6	
8		23.6	87.9	89.64
9		95.3	97.6	
10		99.1	88.5	
12		90.3	81.1	
13		56.1	68.8	38.46
14		85.9	78.2	
15		84.4	83.7	
17		54.0	100.4	
19		77.5	98.6	
23		54.9	115.9	
24		106.5	75.4	
25		45.6	106.8	46.23
26		128.8	108.1	
27		84.9	104.7	

[a] All values were obtained from triplicate experiments.
[b] Amount of nitrite and BH_4 in the presence of each drug in LPS-induced BV-2 cells, presented as percent of LPS-activated BV-2.
[c] Cytotoxicity of each drug assessed by lactate dehydrogenase (LDH) activity released into the medium, presented as percent of LPS-activated BV-2.
[d] DAHP (2,4-diamino-6-hydroxypyrimidine)—an inhibitor of GTP cyclohydrolase I.[20]

results on NO production and will not be a useful inhibitor, we examined their cytotoxicity by determining LDH activity released by the BV-2 cells that had been treated with each compound at 100 μM. As shown in Table 1, in general, none of the derivatives were significantly more toxic than NAMDA.

We also tested the newly synthesized compounds for their inhibitory effects on LPS-induced NO overproduction in BV-2 microglial cells. As shown in Table 1, the inhibitory effect of NAMDA (100 μM) was 84.0% of that produced by the LPS-treated control cells. This was similar to that reported previously.[13] Compounds numbered **8**, **13**, **17**, **19**, **23**, and **25** had a stronger inhibitory effect than NAMDA.

Among the compounds (**5**, **6**, **8**, **9**, **10**, **12**, **13**, **14**, and **15**) synthesized by Method A, **5** and **6** have structural similarities with NAMDA and produced similar inhibitory effects on NO production as NAMDA. Compound **8**, a demethylated catechol amide, had a strong inhibitory effect and decreased the NO production by up to 23.6% of untreated LPS-activated control. The azacycloalkyl substituted NAMDA derivatives (**9**, **10**, **12**, **14**, and **15**) synthesized by Method B had similar inhibitory effects on NO production as NAMDA.

However, for an unknown reason, compound **13** inhibited the NO production to a greater degree (39.5% of that produced by LPS-treated control cells). Both the cinnamonitrile **17** and one carbon inserted NAMDA homologue **19** showed some improvement of the inhibitory effect. The cyclized compounds synthesized by Method C had interesting effects on NO production: while the compounds with less polar group (**23**, **25**, and **27**) exerted greater inhibition, those with polar group (**24**, **26**) had no inhibitory effect. Both the lipophilic indole **23** and *N*-acetyltetrahydroisoquinoline **25** derivatives are expected to be permeable and to strongly inhibit the NO production. However, the indole derivative **23** was not stable under the atmospheric conditions and needs to be further modified for stabilization.

As we sought for inhibitors of NO production that were significantly more effective than NAMDA, and the BH_4 assay method was rather time- and labor-consuming, we selected only those with >50% inhibitory effects on NO production (e.g., **8**, **13**, and **25**) and determined their effects on BH_4 production. Among these three compounds, **8** had a <20% inhibitory effect, similar to NAMDA. Compounds **13** and **25** on the other hand showed >50% inhibitory effects. The finding that the inhibitory effect of **8** on NO production (>75%) was much greater than that on the BH_4 production suggested that **8** might inhibit NO production at other steps. This may be explained by the fact that catecholamines bind at both the L-arginine and BH_4 binding sites and therefore both compete with the substrates and antagonize NO production.[19] Compounds **13** and **25** exhibited similar degrees of inhibition on both NO and BH_4 production. Thus, these compounds may suppress NO production by inhibiting BH_4 production, and thus, iNOS dimerization. As NAMDA is known to be travel across

blood–brain barrier (BBB) and compounds **13** and **25** have similar lipophilicity to NAMDA, it is noteworthy that compounds **13** and **25** are expected to travel across BBB.

In conclusion, we designed and synthesized 16 NAMDA derivatives by three different methods and identified lead compounds that more effectively inhibited NO production than NAMDA in LPS-stimulated BV-2 cells. Compounds **8**, **13**, **17**, **23**, and **25** inhibited NO production by 25% to >50%. Among these compounds, *N*-acetyltetrahydroisoquinoline **25** appeared to be the most promising candidate for mechanistic studies on BH_4-related inhibition of NO production, perhaps via inhibition of NOS dimerization. Because various alkyl groups can be modified at the first position on tetrahydroisoquinoline, it provides opportunities for synthesizing other derivatives with potentially stronger inhibitory effects on BH_4 and NO production. Syntheses of tetrahydroisoquinoline derivatives are currently being pursued in our laboratory. Further mechanistic studies on the effect of these compounds on GTP cyclohydrolase I activities, the first and the rate-limiting enzyme involved in BH_4 synthesis, are also under investigation. Novel inhibitors of NO overproduction will be useful to control inflammatory damages caused by activated microglia and macrophages.

Acknowledgments

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