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Design and synthesis of carbamate and thiocarbamate derivatives and their inhibitory activities of NO production in LPS activated macrophages

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ABSTRACT

Series of carbamate and thiocarbamate derivatives were designed and synthesized and their inhibitory activities of NO production in lipopolysaccharide-activated macrophages were evaluated. Several thoicarbamate derivatives revealed promising inhibitory activity. The structure–activity relationship study of these compounds is also reported. Among these compounds, compound **12b** was the most potent with 6.5 μM of IC_{50}. They inhibited NO production through the suppression of iNOS protein and mRNA expression and nuclear translocation of p65.

Nitric oxide (NO) plays critical roles in various physiological processes,[1,2] including neurotransmission, smooth muscle contractility, platelet reactivity, and the cytotoxic activity of immune cells. NO is produced by neuronal nitric oxide synthase (nNOS) or mammalian endothelial NOS (eNOS) and serves as a second messenger molecule for homeostatic function in neuronal tissues and vascular endothelium, respectively.[3–6] Meanwhile, the overproduction of NO caused by inducible NOS (iNOS) has been linked to the pathogenesis of a number of disease states, including septic shock, neurodegenerative disorders, and various inflammatory processes.[7] It suggests the rationale for therapeutic agents that modulate overproduction of NO. Among several strategies for controlling the NO concentrations, main efforts have been directed to develop selective inhibitors of iNOS.

Early NOS inhibitors were mainly the arginine analogs such as *N*-monomethyl-L-arginine (L-NMMA), *N*-(iminoethyl)-L-ornithine (L-NIO), and *N*-nitro-L-arginine (L-NNA), which were potent but little selective against NOS isoforms.[8] Recently, many classes of non-arginine type iNOS inhibitors such as amino heterocycles,[9] amidines,[10] isoquinolinamines,[11] and isothiourea[12,13] have been reported but still limited their application in clinical due to their insufficient potency and/or subtype selectivity.

In our previous Letters, we reported urea, thiourea, and isothiourea derivatives as inhibitors for the NO production in LPS-activated macrophage.[14,15] It was suggested that introduction of appropriate lipophilic groups such as carbazole and phenoxazine to urea or thiourea moiety was necessary for the activity. Based on this finding, we investigated carbazole-linked carbamate and thiocarbamate derivatives depicted in Figure 1 as new inhibitors of NO production. Here we report the design and synthesis of a series of carbamates and thiocarbamates and their effects on the NO production and iNOS expression.

The preparation of the carbazole-linked carbamate and thiocarbamate derivatives is outlined in Schemes 1–3. N-alkylation of carbazole gave alcohol **2** which was mesylated to obtain compound **3**. Alkylation of 4-nitrophenol by treatment of compound **3** in the presence of NaH gave compound **4**. Following reduction of nitro compound **4** over 10% Pd/C under atmospheric pressure of hydrogen gas provided amine **5** which was reacted with thiophosgene to give isothiocyanate **6**. Addition of appropriate alcohol to isothiocyanate gave the target compounds **7**. Also, condensation of amine **5** with 4-nitrophenyl chloroformate offered carbamate **8**, which was converted to the desired thiocarbamates **9** by treating sodium thioalkoxide.

Other carbamates and thiocarbamates were obtained by substitution reaction of hydroquinone with mesylate **3** and following addition of the resulted alcohol **10** to the appropriate isocyanates or isothiocyanates offered the target compounds **11** and **12**.

The activities of the prepared compounds were evaluated for the inhibition of NO production in LPS-activated macrophages. Murine macrophage cell line, RAW 264.7 cells were stimulated with 1 μg/mL of LPS in the presence of samples for 20 h. The amounts of NO released into culture media were determined by the Griess method in the form of nitrite.[16]

The inhibitory activities of the tested compounds on the NO production are given in Table 1. Aminoguanidine, a well known

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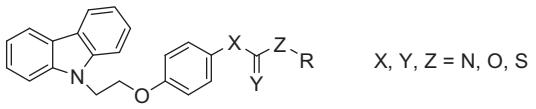
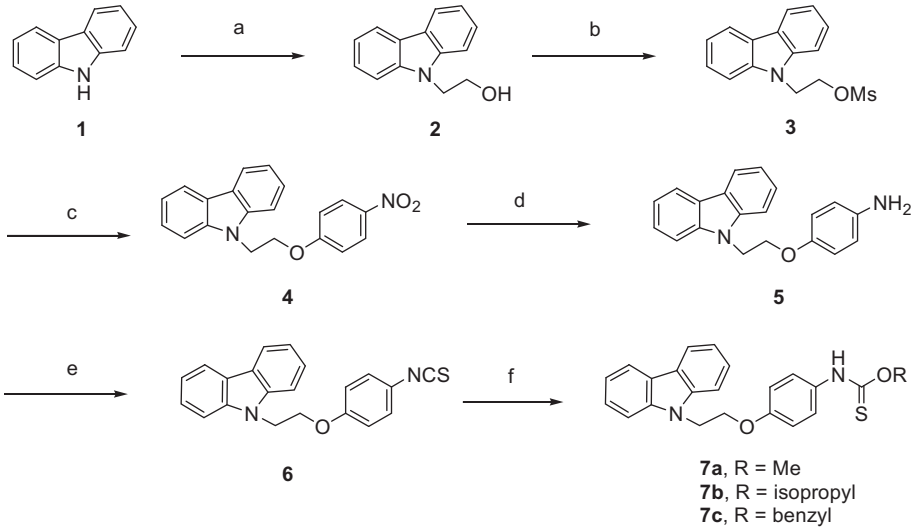


Figure 1. Representative structure of carbamate and thiocarbamate.

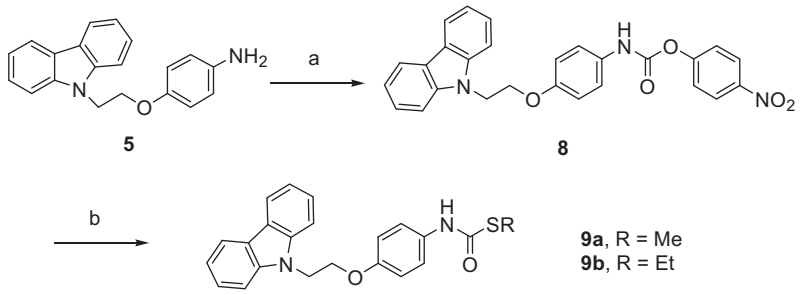
specific inhibitor of iNOS was used as positive control that showed 74% inhibition of NO production at 100 μM.

Effect of *O*-alkyl substituent of thiocarbamates **7a–c** on the inhibitory activity was evaluated. When methyl substituent (**7a**) was replaced with isopropyl (**7b**) or benzyl (**7c**) group, the inhibitory activity was decreased. This result suggested that bulky substituents were inappropriate for the activity. The activity of thiocarbamate **9a**, which has the reverse position of S and O comparing with thiocarbamate **7a**, was lower than those of the corresponding compound **7a**. Methyl substituted **9a** showed a little bit higher activity than ethyl substituted compound **9b**. Regarding N-substituents of compounds **11** and **12**, the activities of alkoxycarbonylmethyl substituted derivatives (**11b**, **11c**, and **12b**) were slightly higher than ethyl substituted derivatives **11a** and **12a**. Among these compounds, thiocarbamate **12b** was the most potent showing 6.5 μM of IC_{50} value.

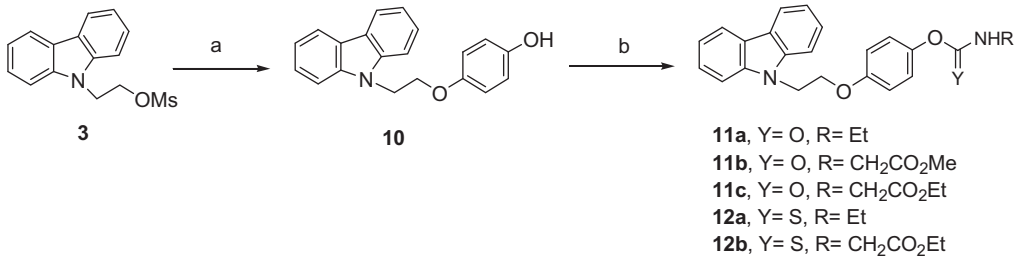
For the further biological study of our compounds, we examined the effects of **7a** and **12b** on the expression of iNOS protein and mRNA in LPS-activated RAW 264.7 cells. The amounts of iNOS protein was analyzed in Western blot analysis after 20 h incubation with compounds during LPS (1 μg/mL) activation of macrophages.[17] Compounds **7a** and **12b** significantly reduced the amounts of iNOS at 20 μM (Fig. 2). At RT-PCR analysis,[18] the expression level of iNOS mRNA was increased by LPS-activation for 6 h. Both compounds **7a** and **12b** suppressed the induction of



Scheme 1. Preparation of carbazole-linked thiocarbamate derivatives. Reagents: (a) 2-iodoethanol, NaH, DMF; (b) MsCl, TEA, DMF; (c) 4-nitrophenol, NaH, DMF; (d) H_2, 10% Pd/C, THF/MeOH; (e) thiophosgene, K_2CO_3, $CHCl_3$; (f) ROH, NaH, DMF.

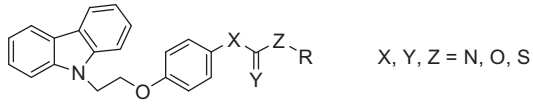


Scheme 2. Preparation of carbazole-linked thiocarbamate derivatives. Reagents: (a) 4-nitrophenyl chloroformate, MC; (b) NaSR, MC.



Scheme 3. Preparation of carbazole-linked carbamate and thiocarbamate derivatives. Reagents and conditions: (a) hydroquinone, NaH, DMF; (b) (i) RNCO, TEA, toluene or RNCO, K_2CO_3, THF, (ii) RNCS, TEA, toluene or RNCS, 18-crown-6, KH, THF.

Table 1
Inhibitory activities of carbazole-linked carbamates and thiocarbamates on the NO production in LPS-activated macrophages



Compds	X	Y	Z	R	Inh[a] (%)	IC_{50}[b] (μM)
7a	NH	S	O	Me	60	12.0 ± 0.6
7b	NH	S	O	*i*-Pro	20	
7c	NH	S	O	Bn	18	
8	NH	O	O	4-NO_2–C_6H_4	22	
9a	NH	O	S	Me	44	
9b	NH	O	S	Et	26	
11a	O	O	NH	Et	10	
11b	O	O	NH	CH_2CO_2Me	33	
11c	O	O	NH	CH_2CO_2Et	35	
12a	O	S	NH	Et	10	
12b	O	S	NH	CH_2CO_2Et	73	6.5 ± 0.5
Aminoguanidine					74	

[a] Values mean the inhibition (%) of NO production at 20 μM of compounds relative to the LPS control ($n = 3$).
[b] Values are means ± SD of three experiments.

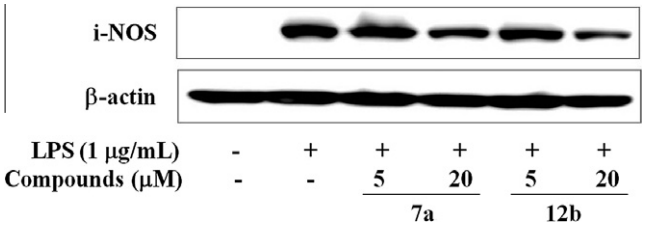


Figure 2. Effects of the tested compounds on the expression of iNOS protein in LPS-activated macrophages. RAW 264.7 cells were treated for 20 h with compounds **7a** and **12b** (5, 20 μM) during LPS (1 μg/mL) activation. Cell lysates were prepared and the iNOS and β-actin protein levels were determined by Western blotting. β-Actin was used as an internal control. The results shown are the representative of three independent experiments.

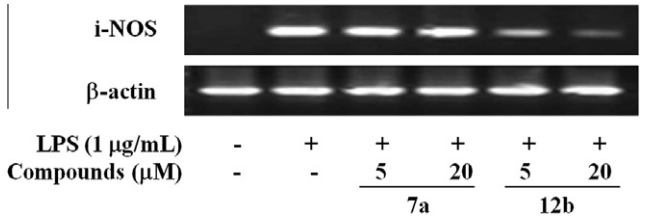


Figure 3. Effects of the tested compounds on the expression of iNOS mRNA in LPS-activated macrophages. RAW 264.7 cells were treated for 6 h with compounds **7a** and **12b** (5, 20 μM) during LPS (1 μg/mL) activation. The mRNA levels of iNOS and β-actin were determined by RT-PCR from total RNA extracts. β-Actin was used as an internal control. The results shown are the representative of three independent experiments.

iNOS mRNA at 20 μM. Compound **12b** revealed remarkable inhibition even at 5 μM (Fig. 3). These results indicated that the inhibition of NO production by thiocarbamate derivatives was resulted from the suppression of iNOS protein and mRNA.

To elucidate the action mechanism of compounds **7a** and **12b** for the inhibitory consequence of iNOS expression in activated macrophages, we analyzed whether compounds **7a** and **12b** affect the LPS-induced translocation of NF-κB. NF-κB is located in the cytoplasm as inactive dimer composed of p65 and p50 subunits that is physically associated with inhibitor κB (I-κB). Macrophage activation evokes I-κB kinase complex-induced phosphorylation of I-κBα, which leads to its degradation and subsequent translocation

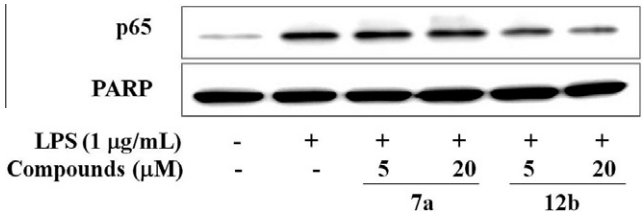


Figure 4. Effects of the tested compounds on the nuclear translocation of p65 in LPS-activated macrophages. Cells were pretreated with compounds **7a** and **12b** (5, 20 μM) for 30 min and followed by further incubation with LPS (1 μg/mL) for 15 min. The protein levels of nuclear p65 were determined by Western blotting. PARP was used as an internal control. Images are the representative of three independent experiments that shows similar results.

of NF-κB to the nucleus. The nuclear NF-κB activates the transcription of iNOS, leading to the induction of its mRNA expression.

To examine whether compounds **7a** and **12b** prevented the nuclear translocation of the p65 subunit of NF-κB, the nuclear levels of p65 were determined. Treatment with compounds **7a** and **12b** decreased the nuclear translocation of LPS-induced p65 subunit. Compound **12b** treatment showed markedly decreased the nuclear level of p65 at 5 and 20 μM as shown in Figure 4. PARP was used as the internal control of nuclear extract. These observations suggest that compounds **7a** and **12b** inhibit the LPS-induced activation of NF-κB by suppressing the translocation to the nucleus in macrophage cells.

In conclusion, we prepared a series of carbamate and thiocarbamate derivatives and evaluated their inhibitory activities of NO production in LPS-activated macrophages. Some of the prepared compounds revealed promising inhibitory activity. They suppressed the release of NO into culture media through the suppression of iNOS protein and mRNA expression and also inhibited the nuclear translocation of p65 subunit of NF-κB. Further study of the other biological activities related with the overproduction of NO, and the detailed mechanism for the activities of the representative compounds are in progress. Our carbamate and thiocarbamate derivatives might serve as useful lead compounds for the development of therapeutic agents for the management of NO-related diseases.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.03.010.

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16. Cell culture and nitrite assay in LPS-activated RAW 264.7 cells—Cells in 10% fetal bovine serum (FBS)-DMEM medium, were plated in 48-well plates (1×10^5 cells/mL), and then incubated for 24 h. The cells were replaced with fresh media with 1% FBS, and then incubated for 20 h in the presence or absence of test compounds with LPS (1 μg/mL). NO production in each well was assessed by measuring the accumulated nitrite in culture supernatant. Samples (100 μL) of media were incubated with Griess reagent (150 μL) for 10 min at room temperature in 96 well microplate. Absorbance at 570 nm was read using an ELISA plate reader. A standard calibration curve was prepared using sodium nitrite as a standard. A dose–response curve was prepared, and the results were typically expressed as IC_{50} values.
17. Western blot analysis of iNOS protein expression—RAW 264.7 cells (8×10^5 cells/60 mm dish) were stimulated with LPS (1 μg/mL) in the presence or absence of test compounds. After incubation for 20 h, the cells were washed and lyzed with lysis buffer. Twenty μg protein of cell lysates was applied on 8% SDS–polyacrylamide gels and transferred to PVDF membrane by a standard method. The membrane was probed with antibody for anti-iNOS (BD Biosciences, Franklin Lakes, NJ) and anti-β-actin (Sigma Chemical Co., St. Louis, MO). The bands were visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's instruction.
18. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of iNOS mRNA expression—RAW 264.7 cells (8×10^5 cells/60 mm dish) were stimulated for 6 h with LPS (1 μg/mL) in the presence or absence of test compounds. After washing twice with phosphate buffered saline, total RNA was isolated from cell pellet, using an RNA isolation reagent (Trizol, Invitrogen, Carlsbad, CA). Two microgram of RNA was reverse transcribed into cDNA using reverse transcriptase and random hexamer. The PCR samples, contained in the reaction mixture, were comprised of mixture buffer, dNTP, Taq DNA polymerase (Promega, Madison, WI) and primers (sense and antisense). The sense and antisense primers for iNOS were 5′-ATGTCCGAAGCAAACATCAC-3′ and 5′-TAATGTCCAGGAAGTAGGTG-3′, respectively. The sense and antisense primers for β-actin were 5′-TGTGATGGTGGGAATGGGTCAG-3′ and 5′-TTTGATGTCACGCACGATTTCC-3′, respectively. The PCR amplification was performed under following conditions; 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, using thermal cycler (Gene Amp PCR system 2400, Applied Biosystems, Foster City, CA). The amplified PCR products were separated on a 2% agarose gel.