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Inhibitory Effects of *N*-Amido-3,3-difluoropyrrolidin-2-ones on LPS-induced Nitric Oxide Production in RAW 264.7 Macrophages

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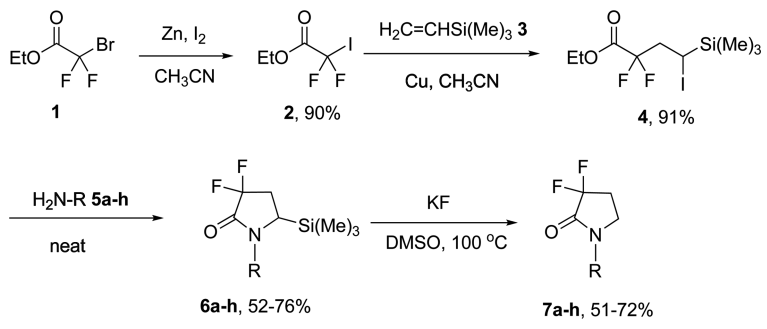
Introduction

Nitric oxide (NO) plays an important role in a variety of physiological processes such as platelet inhibition, blood pressure homeostasis, neurotransmission, immune responses, and especially, inflammation which is known as the central feature of many pathophysiological conditions in response to the tissue injuries and host defenses against the invading microbes.[1,2] Macrophages are the main pro-inflammatory cells responsible for invading pathogens by releasing many pro-inflammatory molecules, especially the short-lived free radical NO, a product of the oxidation of L-arginine to L-citrulline catalyzed by nitric oxide synthase (NOS). Three isoforms of NOS have been identified: two constitutive NOSs, such as endothelial NOS (eNOS) and neuronal NOS (nNOS) which are regulated by Ca^{2+}, and one inducible NOS (iNOS) which is independent of Ca^{2+} regulation.[3] Activated macrophages transcriptionally express iNOS, responsible for the profound production of NO.[4] The aberrant release of NO can lead to the amplification of inflammation as well as the tissue injury.[5] Therefore, the pharmacological interference in the expression of iNOS presents a promising chemotherapeutic strategy to control the potentially harmful pro-inflammatory activity of macrophages. Although many anti-inflammatory agents have been investigated,[6-14] there still remains a large demand for developing new and effective ones.

As previously reported, the difluoromethylene-contained compounds showed the significant physiological activities such as antibiotic,[15] anti-human immunodeficiency virus (HIV),[16] antihypertensive,[17] and anticancer effects.[18] However, there has been no report about the synthesis and biological evaluation of *N*-amido-2-pyrrolidinones derivatives so far. The aim of this study is to investigate the inhibitory activity of *N*-amido-3,3-difluoropyrrolidin-2-ones derivatives against the lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 macrophages with the hope that they would be recognized as the useful scaffolds for developing the new anti-inflammatory agents.

Results and Discussion

As shown in Scheme 1, the synthesis of *N*-amido-3,3-difluoropyrrolidin-2-ones **7** began with the conversion of ethyl bromodifluoroacetate **1** to ethyl iododifluoroacetate **2** in the presence of Zn and I_2. The resulting ethyl iododifluoroacetate **2** was reacted with vinyltrimethylsilane **3** under Cu (0) catalyst to afford ethyl 2,2-difluoro-4-iodo-4-(trimethylsilyl)butanoate **4**, which was then condensed with hydrazides **5** to give *N*-amido-3,3-difluoro-5-trimethylsilyl-2-pyrrolidinones **6**. Finally, the removal of trimethylsilyl group in the compounds **6** was accomplished by KF at 100 °C to give the desired *N*-amido-3,3-difluoropyrrolidin-2-ones **7** in good yields (Table 1). All of the products were identified by using ^{1}H NMR, ^{13}C NMR, ^{19}F NMR and MS spectra. While ^{19}F NMR spectra of compounds **6** showed typical AB splitting patterns ($^2J_{FF}$ = 262 Hz) because the two fluorides are not equivalent (diastereotopic groups), ^{19}F NMR spectra

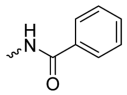
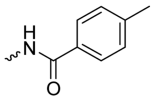
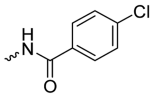
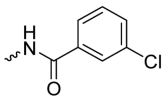
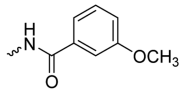
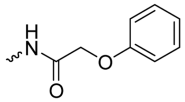
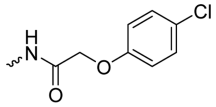
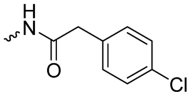


Scheme 1. Synthesis of *N*-amido-3,3-difluoropyrrolidin-2-ones **7**.

of compounds **7** appeared a nullified singlet (enantiotopic groups).

The activities of the synthetic compounds **7a-h** were evaluated by the inhibitor of NO production in LPS-activated macrophages. RAW 264.7 cells, a murine macrophages cell line, were incubated with or without LPS (1 μg/mL) for 18h in the presence or absence of each synthetic compound **7**. The amounts of NO released into culture media were determined by the Griess method in the form of nitrite. As shown in Table 1, all of compounds revealed the similar inhibitory activities with the IC_{50} values from 8.7 to 18.2 μM and also exhibited no cytotoxicity under MTT assay (Figure 1). Compared to the other derivatives, compound **7h** containing *p*-chlorophenylacetamido substrate showed the most potent with the IC_{50} value of 8.7 μM, a level approximately three times greater than the positive control L-N^G-Monomethyl Arginine Citrate (L-NMMA)[19] (Table 1, entries 8 and 9), in the inhibition of NO production.

Table 1. Biological activity of *N*-amido-3,3-difluoropyrrolidin-2-ones **7**

Entry	R	Compound **7** (Yield %)	IC_{50} (μM)[a,b]
1		**7a** (56)	12.1 ± 0.01
2		**7b** (51)	18.2 ± 0.04
3		**7c** (63)	11.5 ± 0.04
4		**7d** (63)	12.4 ± 0.08
5		**7e** (72)	16.2 ± 0.19
6		**7f** (58)	11.7 ± 0.04
7		**7g** (61)	13.7 ± 0.02
8		**7h** (67)	8.7 ± 0.05
9	–[c]	L-NMMA	25.5

[a]Inhibitory activity of NO production of compound **7**. [b]Values mean ± S. D. of the three independent experiments. [c]Reference compound.

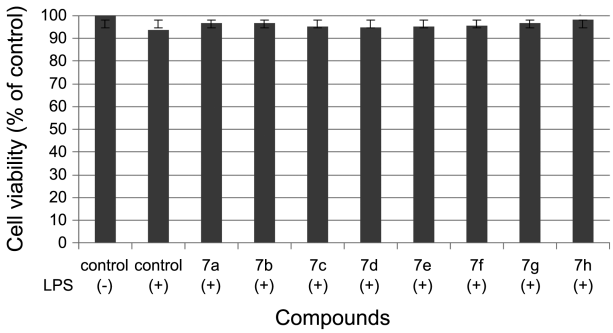


Figure 1. Effects of compounds on the cell viability of in RAW 264.7 cell (2.0×10^5 cell/mL).

Conclusions

In summary, the good inhibitory activities of the NO production from the LPS-treated RAW 264.7 macrophages by *N*-amido-3,3-difluoropyrrolidin-2-ones derivatives were investigated. Therefore, *N*-amido-3,3-difluoropyrrolidin-2-ones would be recognized as the useful scaffolds for developing the new anti-inflammatory agents. The study of the biological mechanism of the inhibitor of NO production is under our consideration.

Experimental

Synthesis. Compounds **7a-h** were synthesized by following the previous reported method.[20]

Cell Culture. RAW 264.7 cells, a murine macrophages cell line, were obtained from American Type Culture Collection (Manassas, VA). The mammalian cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 25 mM HEPES (pH 7.5), 100 U/mL penicillin and 100 μg/mL streptomycin. The RAW 264.7 cells were plated at a density of 1×10^6, pre-incubated for 24 h at 37 °C, and maintained in a humidified atmosphere containing 5% CO_2. For all experiments, the cells were grown to 80-90% confluence, and subjected to no more than 20 cell passages.

Nitrite Analysis. Accumulated nitrite (NO_2^-) in the media obtained from the cell culture was determined by using the colorimetric assay and based on the Griess reaction. The samples (100 μL) were reacted with 100 μL Griess reagent including 0.1% naphthylethylenediamine dihydrochloride, and 1% sulphanilamide in 5% phosphoric acid (6 mg/mL) at room temperature for 10 min., and then the NO_2^- concentration was determined by measuring the absorbance at 570 nm. The standard curve was constructed by using the known concentrations of sodium nitrite.

Statistical Analysis. Data was analyzed by using Student's t-test. Differences were significantly considered when $P < 0.05$.

MTT Assay. MTT is the pale yellow substrate reduced by the living cells to yield the dark blue formazan product. This process requires the active mitochondria, and even the fresh dead cells do not reduce significant amounts of MTT. The

cells were cultured in 96-well flat bottom plate at the concentration of 2.0×10^5 cell/mL. After 12 h of preconditioning, the cells were treated with various concentrations of the compounds for 24 h. The culture media were aspirated. Then 100 μL of MTT dye (1 mg/mL in phosphate-buffered saline) was added to the cultures and further incubated for 4 h at 37 ^oC. The formazan crystals formed by the dye reduction of viable cells were dissolved by using acidified isopropanol (0.1 N HCl). The index of cell viability was calculated by measuring the optical density (OD) of the color produced by the MTT dye reduction at 570 nm.

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