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Synthesis and biological evaluation of molecular probes based on the 9-methylstreptimidone derivative DTCM-glutarimide

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ABSTRACT

Molecular probes based on 3-[(dodecylthiocarbonyl)methyl]glutarimide (DTCM-glutarimide) were synthesized and assessed for inhibitory activity against LPS-induced NO production. Among the probes examined, several derivatives exhibited potential for use in determining the target proteins of DTCM-glutarimide.

NF-κB is a transcription factor involved in the regulation of immune and inflammatory responses by induction of interleukins and iNOS,[1–3] and is constitutively active in cancer and inflammatory cells (i.e., macrophages). Activated NF-κB can increase viability and metastatic activity of cancer cells along with their malignant character. Therefore, NF-κB is considered an effective molecular target for antiinflammatory or anticancer agents.

9-Methylstreptimidone (**1**), a glutarimide antibiotic, was first isolated in 1974[4] and was reported to show antiinflammatory activity.[5] The natural product **1** inhibited NO production and iNOS expression in LPS-stimulated RAW264.7 cells, and induced apoptosis in Jurkat cells and adult T-cell leukemia cells, similar to other NF-κB inhibitors. Low fermentation yield of **1** interfered with detailed investigation of the inhibitory mechanism.

Here, we designed a new candidate inhibitor of NF-κB based on **1** (Fig. 1). Based on the design involving retention of glutarimide and replacement of the hydrophobic moiety with linear alkyl chains, simplified analogs of **1** were synthesized. Assessment of LPS-induced NO production, indicated that 3-[(dodecylthiocarbonyl)methyl]glutarimide (DTCM-glutarimide; **2**) was the most plausible candidate for the inhibitor.[6] In contrast to the remarkable inhibitory activity of **2** against LPS-induced expression of iNOS in RAW264.3 cells with low cytotoxicity similar to **1**, neither direct inhibition of LPS-induced nuclear translocation of NF-κB nor binding between NF-κB and DNA were observed.[7] Compound **2** also showed antiinflammatory activity in vivo.[7] Comparison with **1** suggested that **2** may have a different mode of action involving other cascades rather than inhibition of NF-κB signaling. Analysis of the differences in inhibitory mechanisms generated by small structural differences between **1** and **2** will yield interesting information from the viewpoint of drug discovery. Therefore, we examined the target protein of **2** with the biotin-avidin method or photolabeling method. Biotin-tagged molecules are widely utilized in bioanalytical applications to detect biomolecules due to the strong binding interaction with avidin. Many techniques for biotin-avidin assays have uncovered relationships between target molecules and reagents.[8,9] On the other hand, photoaffinity labeling reveals the structure and function of protein by utilizing aryl azide as a photoreactive agent,[10] photolysis of which provided nitrenes that form covalent bonds with target molecules, and capture even reversible binding interaction in the nonpolar moiety. Thus, biologically active molecular probes of **2**, possessing biotin and photoaffinity label, were examined in this study.

Molecular probes possessing a biotin or photoreactive group were designed and synthesized as shown in Figure 2.

Initially, two biotinylated probes, **3** and **4**, carrying different carbon chain lengths, were prepared to investigate the influence of the alkyl chain on inhibitory activity. In our previous study, derivatives possessing an amide instead of a thioester exhibited no inhibitory activity.[6] Therefore, we synthesized **5** as a negative control against **3** and **4**. Furthermore, photoaffinity probe **6** was synthesized to examine a different approach for determination of target protein.

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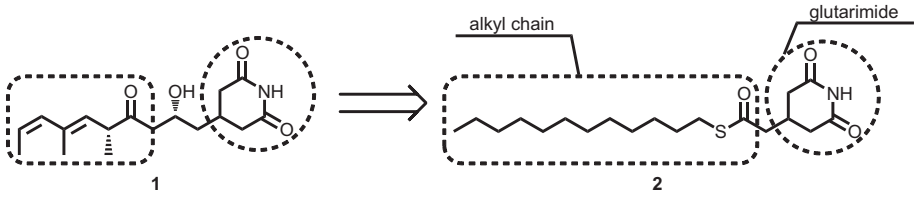


Figure 1. Structure of 9-methylstreptimidone (**1**) and DTCM-glutarimide (**2**).

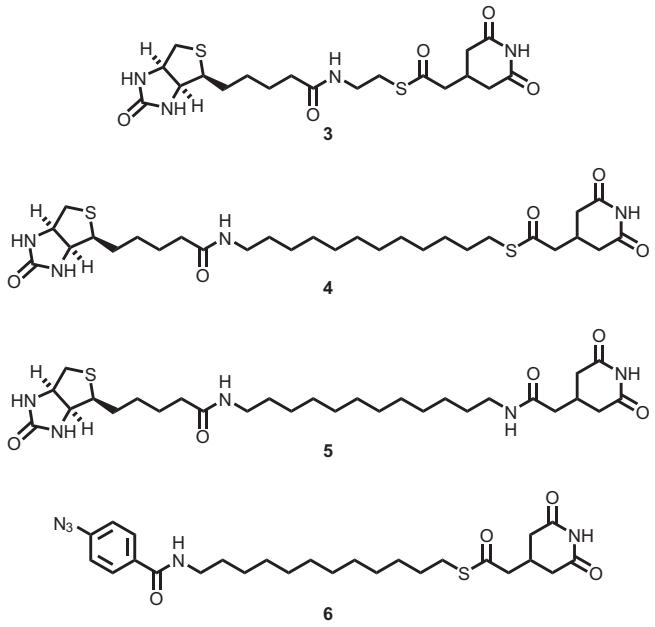


Figure 2. Synthetic probes possessing biotin (**3**, **4**, and **5**) or aryl azide (**6**).

Probes of **2** were synthesized as outlined in Scheme 1. Protected cystamine **7**[11] was treated with biotin succinimide ester[12] and triethylamine in DMF to afford the biotinylated derivative **8**. After cleavage of a trityl group, condensation of acid **13**, readily available from diethyl 1,3-acetonedicarboxylate,[13,14] afforded **3**.[20]

Table 1
Inhibitory activity (IC_{50}) for LPS-induced NO production and cytotoxicity (ED_{50}) of **3**–**6**

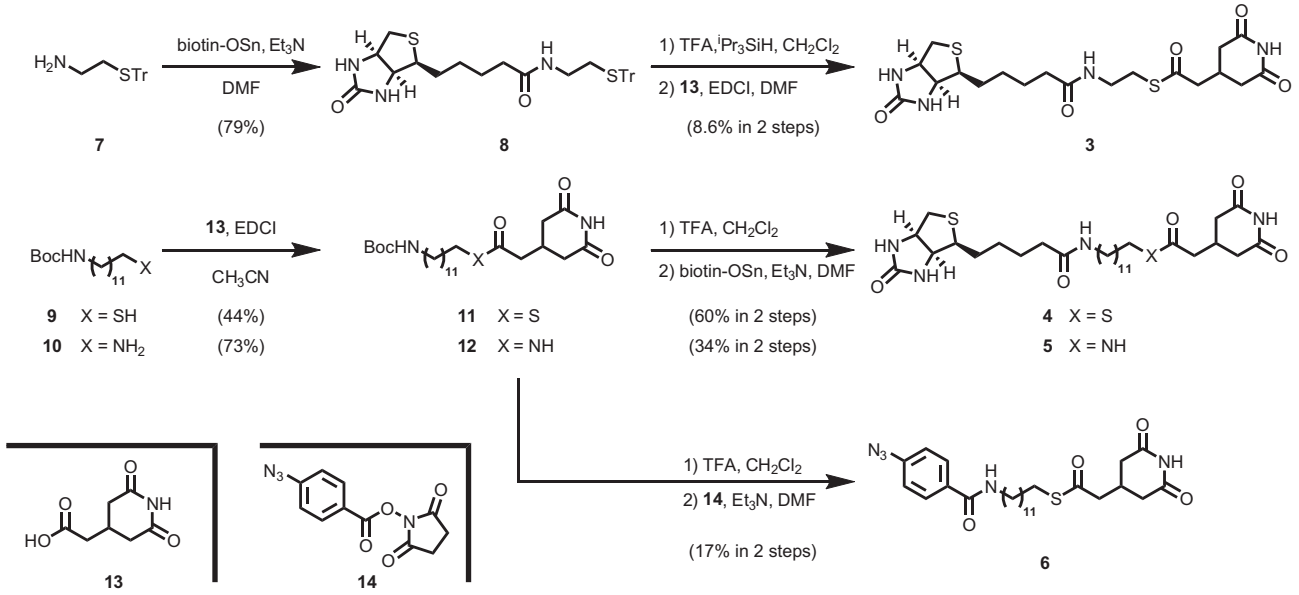
Compound	IC_{50} (μg/mL)	ED_{50} (μg/mL)
3	>30	>30
4	5	>30
5	>30	>30
6	4.5	>30

Although synthesis of **4** may proceed in a similar way as in the case of **3**, the coupling reaction with **13** did not proceed smoothly. Thus, thiol **9** was synthesized from an alternative starting material. Thiol **9**, prepared by the known procedure from commercially available 12-aminododecanoic acid in six steps,[15,16] was successfully transformed into thioester **11**. Cleavage of a Boc group, followed by coupling with biotin under the same conditions as that of **3** afforded the probe **4**.[20]

Treatment of the intermediate **11** with *N*-succinimidyl 4-azidobenzoate **14**,[17] instead of biotin succinimide ester, effected production of **6**,[20] although 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide yielded no desired product.[18]

Subsequently, amide **5** was synthesized from the mono-Boc dodecanediamine (**10**).[19] Coupling of amine **10** with **13**, provided the corresponding amide **12**, which on deprotection followed by condensation with the biotin succinimide derivative yielded **5**[20] as a negative control.

To understand the validity of the synthetic probes, the synthesized derivatives **3**–**6** were examined by LPS-induced NO production in RAW264.7 cells, using DTCM-glutarimide as a positive control (Table 1).[21] Probes **3** and **5** have no inhibitory effect of



Scheme 1. Synthesis of compounds **3**, **4**, **5**, and **6**.

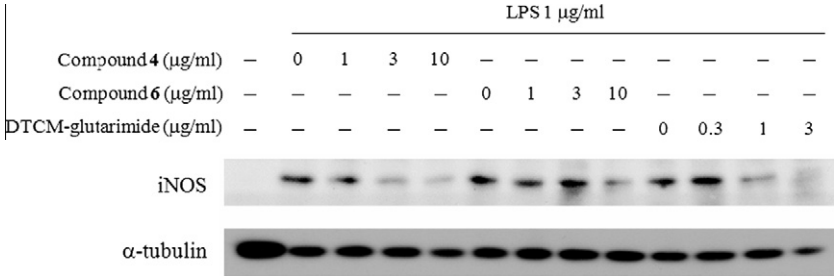


Figure 3. Inhibition of iNOS expression by synthetic probes **4** and **6**. Cells were incubated with or without indicated chemicals for 2 h, thereafter stimulated 1 μg/ml LPS for 24 h. Total cell lysates were subjected by Western blotting.

NO production. As expected, the length of the alkyl chain and the thioester moiety were crucial factors for the inhibitory effect. In contrast, the biotinylated probe **4** and the photoaffinity probe **6** inhibited NO production with low toxicity ($ED_{50} > 30$); their IC_{50} values were 5 and 4.5 μg/mL, respectively, comparable to that of **2**. On the basis of their activities against NO production, the probes **4** and **6** were evaluated by LPS-induced iNOS expression in RAW 264.7 cells. In Figure 3, the probes inhibited LPS-induced iNOS expression. These results indicated that alterations of NO production were caused by that of iNOS expression, and the effective probes **4** and **6** were not NO scavengers or influential factors to absorbance spectrum, but genuine inhibitors against signaling pathways induced by LPS stimulation. In addition, though quite a few biotinylated probes and photoaffinity probes extremely decrease their inhibitory activity within cells compared to original inhibitors, our synthetic probes **4** and **6** exhibited respectable potential against LPS-induced iNOS expression. Subsequently, **4** and **6** were submitted to electrophoretic mobility shift assay (EMSA) to confirm the same action as that of **2**. As expected (Fig. 4), **4** and **6** did not inhibit NF-κB induced by LPS as in the case of **2**.[7] From these results, synthetic probes will be utilized as powerful tools for activity-based protein profiling in future studies.

In conclusion, we have designed and synthesized biologically active probes to elucidate the inhibitory mechanism of **2**, which showed a different mode of action from that of **1**. Biotinylated probe **4** and photoaffinity probe **6** were potent inhibitors of LPS-induced NO production, and will be available to search for the target molecule of DTCM-glutarimide. Further investigations are currently underway in our laboratory.

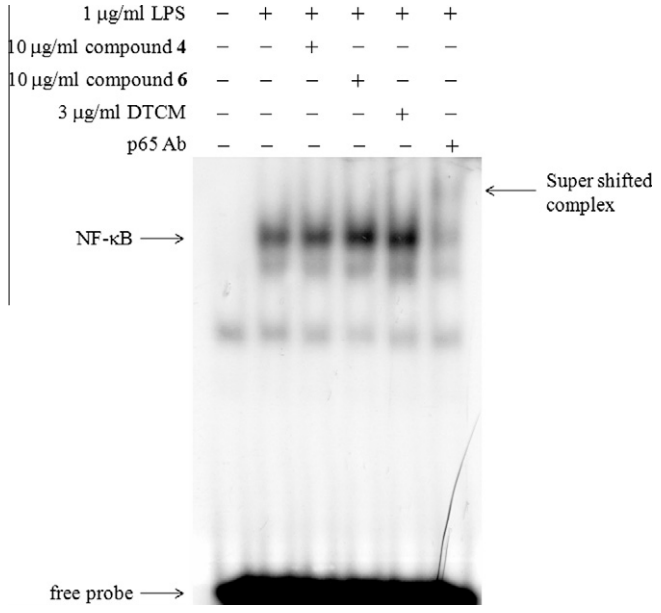


Figure 4. Effects of probes **4** and **6** on NF-κB activation in RAW264.7 cells. Cells were treated with or without indicated chemicals for 2 h, and thereafter stimulated 1 μg/ml LPS for 30 min. The nuclear proteins were then extracted and used for EMSA.

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20. *Spectroscopic data for* **3**: IR (KBr) ν 3282, 3080, 2926, 2858, 1697, 1645, 1265 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 1.44 (2H, m), 1.62 (4H, m), 2.19 (2H, t, J = 7.3 Hz), 2.38 (2H, m), 2.70 (5H, m), 2.71 (1H, d, J = 12.7 Hz), 2.92 (1H, dd, J = 4.9,12.7 Hz), 3.06 (2H, br t, J = 6.3 Hz), 3.21 (1H, ddd, J = 4.4, 5.6, 8.8 Hz), 3.36 (1H, t, J = 6.3 Hz), 3.37 (1H, t, J = 6.8 Hz), 4.31 (1H, dd, J = 4.4, 7.8 Hz), 4.48 (1H, ddd, J = 1.0, 4.9, 7.8 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 26.8, 29.1, 29.51, 29.55, 29.8, 36.8, 37.8, 39.8, 41.1, 57.0, 61.6, 63.4, 166.1, 174.7, 176.2, 198.3. HRMS (FAB) calcd for $C_{19}H_{29}N_4O_5S_2$ $[M+H]^+$: 457.1579. Found: m/z 457.1571. Spectroscopic data for **4**: IR (KBr) ν 3308, 2922, 2851, 1686, 1632, 1265 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.22–1.78 (26H, m), 2.19 (1H, t, J = 6.9 Hz), 2.20 (1H, t, J = 7.4 Hz), 2.36 (2H,m), 2.63 (2H, br d, J = 6.5 Hz), 2.74 (1H, d, J = 12.5 Hz), 2.75 (2H, m, overlapped with 1H signal), 2.90 (2H, br t, J = 7.4 Hz), 2.93 (1H, dd, J = 5.2, 12.5 Hz), 3.17 (1H, ddd, J = 4.7, 7.0, 7.8 Hz), 3.23 (1H, t, J = 6.5 Hz), 3.24 (1H, t, J = 7.0 Hz), 4.33 (1H, m), 4.52 (1H, m), 4.98 (1H, s), 5.59 (1H, s), 5.63 (1H, s), 8.64 (1H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 25.4,

26.4, 27.3, 28.1, 28.2, 28.5, 28.8, 28.9, 29.00, 29.02, 29.1, 29.2, 35.2, 36.6, 38.4, 47.6, 55.5, 59.2, 61.1, 162.7, 171.8, 172.5, 197.1. HRMS (ESI) calcd for $C_{29}H_{49}N_4O_5S_2$ $[M+H]^+$: 597.3144. Found: *m*/*z* 597.3146. Spectroscopic data for **5**: IR (KBr) *ν* 3302, 3076, 2923, 2850, 1698, 1640, 1543, 1267 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 1.24–1.61 (26H, m), 2,04 (2H, t, *J* = 7.4 Hz), 2.10 (2H, d, *J* = 6.7 Hz), 2.27 (2H, m), 2.44 (2H, m, overlapped with 1H signal), 2.58 (1H, d, *J* = 12.5 Hz), 2.81 (1H, dd, *J* = 5.0, 12.5 Hz), 3.01 (4H, br t, *J* = 6.7 Hz), 3.09 (1H, ddd, *J* = 4.5, 6.1, 8.5 Hz), 4.12 (1H, m), 4.30 (1H, m), 6.35 (1H, s), 6.42 (1H, s), 7.72 (1H, t, *J* = 5.4 Hz), 7.87 (1H, t, *J* = 5.4 Hz), 10.70 (1H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 25.4, 26.42, 26.44, 27.2, 28.77, 28.80, 29.05, 29.10, 29.2, 35.3, 37.0, 38.4, 39.1, 55.5, 59.2, 61.1, 162.7, 169.7, 171.8, 172.9. HRMS (ESI) calcd for $C_{29}H_{50}N_5O_5S$ $[M+H]^+$: 580.3533. Found: *m*/*z* 580.3524. Spectroscopic data for **6**: IR (KBr) *ν* 3343, 3090, 2919, 2851, 2124, 1677, 1629, 1605, 1536, 1500, 1280 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.31 (18H, m), 1.60 (2H, m), 2.36 (2H, m), 2.63 (2H, d, *J* = 6.1 Hz), 2.76 (2H, m, overlapped with 1H signal), 2.90 (2H, br t, *J* = 7.4 Hz), 3.43 (1H, t, *J* = 7.2 Hz), 3.45 (1H, t, *J* = 7.0 Hz), 6.06 (1H, s), 7.06 (2H, d, *J* = 8.3 Hz), 7.76 (2H, d, *J* = 8.3 Hz), 7.88 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 27.1, 27.8, 28.9, 29.2, 29.37, 29.44, 29.5, 29.6, 29.8, 29.9, 37.3, 40.3, 48.0, 119,1, 128.8, 131.5, 143.3, 166.6, 171.1, 196.7. HRMS (ESI) calcd for $C_{26}H_{38}N_5O_4S$ $[M+H]^+$: 516.2645. Found: *m*/*z* 516.2642.

21. The assays to determine inhibition of LPS-induced NO production, iNOS expression, NF-κB activation were performed as described below.

Cell culture

Mouse macrophage RAW264.7 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 200 μg/ml kanamycin (Sigma, St. Louis, MO, USA), 100 units/ml penicillin G (Sigma), 600 μg/ml L-glutamine (Sigma), and 2.25 g/L $NaHCO_3$ at 37 °C under 5% CO_2 plus air.

NO production assay

Cells in complete medium (1×10^5 cells/ml) were seeded in a 96-well plate (Corning Inc., Corning, NY, USA), with each well receiving 100 μl of the cell suspension. On the next day, the cells were treated with each chemical for 2 h and then stimulated with 1 μg/ml LPS for 20 h. Then 100 μl Griess reagent solution was added to each well. The concentration of NO was obtained by measuring the absorbance at 570 nm with a microplate reader.

MTT assay

Cells in complete medium (1×10^5 cells/ml) were seeded in a 96-well plate, with each well receiving 100 μl of the cell suspension. On the next day, the cells were treated with each chemical for 2 h and then stimulated with 1 μg/ml LPS for 20 h. Then, 10 μl of MTT (3-[4,5-dimethylthiazol-2–yl]-2,5-diphenyltetrazoliumbromide) solution was added to each well, and the cells were incubated for 4 h at 37 °C under 5% CO_2 plus air. Subsequently, the culture supernatant was replaced with 100 μl DMSO to dissolve the formazan crystals made from MTT by the enzymatic action of succinic dehydrogenase in the mitochondria of live cells. The absorbance at 570 nm was measured with a microplate reader.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the method of Andrews and Faller. The binding reaction mixture contained nuclear extract (5 μg of protein), 1 μg poly(dI-dC), and 10,000 cpm ^{32}P-labeled probe (oligonucleotide containing NF-κB) in binding buffer (15 mM Tris–HCl[pH 7.0], 75 mM NaCl, 1.5 mM EDTA, 1 mM DTT, 7.5% glycerol, and 1.5% NP-40). Samples were incubated for 20 min at room temperature (RT) in this mixture. DNA/protein complexes were separated from free DNA on 4% native polyacrylamide gel in TBE buffer (22.5 mM Tris–HCl[pH8.3] and 0.5 mM EDTA). The DNA probes used for NF-κB binding were purchased from Promega (Madison, WI, USA).