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Design and synthesis of biotinylated DHMEQ for direct identification of its target NF-κB components

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ABSTRACT

The design and synthesis of dehydroxymethylepoxyquinomicin (DHMEQ) derivatives were carried out to investigate the intracellular targets. The synthetic biotin probe exhibited membrane permeability and combined selectively with the target protein p65.

The NF-κB signaling pathway plays a central role in not only inflammation but also in the development of cancer cells. Therefore, inhibitors of NF-κB are novel potential candidate of chemotherapeutic agents for inflammatory and cancer diseases, as well as bioprobes for the characterization of intracellular biological responses and cell function.[1] Over the last decade, a number of structurally diverse small molecules that block the NF-κB signaling pathway have been identified. Especially, the epoxyquinol class NF-κB inhibitors, such as dehydroxymethylepoxyquinomicin (DHMEQ, **1**),[2] cycloepoxydon (**2**),[3] and panepoxydon (**3**),[4] exhibited remarkable inhibitory activity against NF-κB activation. Despite their structural similarity (Fig. 1), they showed completely different modes of action in the inhibition of NF-κB activation. Whereas **1** exhibits inhibitory activity by covalently binding to the NF-κB components, **2** and **3** show inhibition by interfering with the degradation of IκB-α and activation of IκB kinase (IKK). Furthermore, **1** showed potent anti-inflammatory and anticancer activities in various animal experiments,[5] so it is very important to identify its intracellular target.

Here, we describe the design and synthesis of a DHMEQ derivative carrying a biotin residue to examine its mode of action. Previously, we reported the synthesis of several NF-κB inhibitors (**4**–**5**) with the epoxyquinol structure and evaluation of the crucial factor for binding to NF-κB.[6] The salicylamide-conjugated *syn* epoxyquinol moiety of **1** bound to p65 components in NF-κB. Furthermore, alkylation of the phenol residue on DHMEQ had little influence on the activity.[7] Based on these results, we designed a biotinylated DHMEQ derivative (**6**).

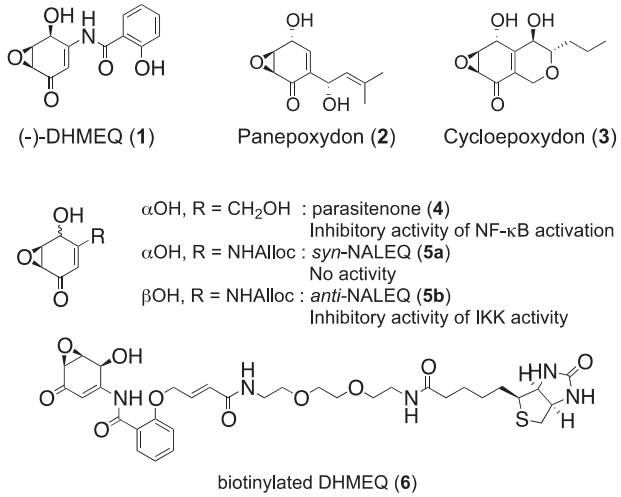

Figure 1. NF-κB inhibitors (**1**–**6**) carrying the epoxyquinol moiety and biotin conjugated DHMEQ derivative (**6**).

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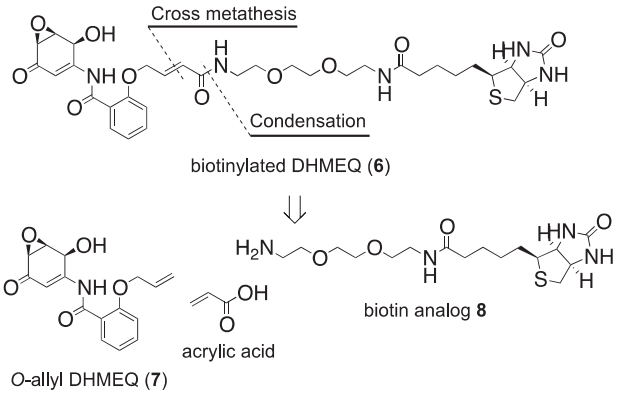


Scheme 1. Synthetic plan of biotinylated DHMEQ (**6**).

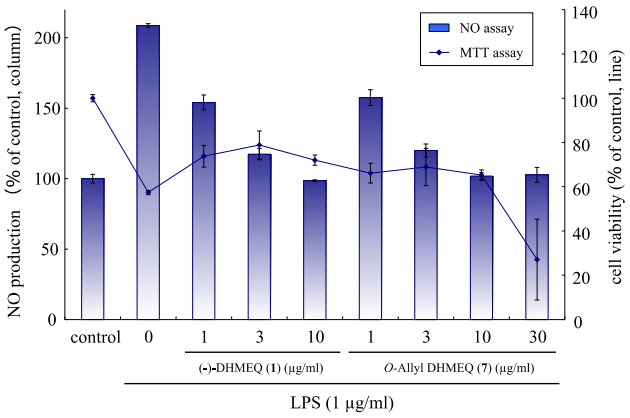


Figure 2. Effects on NO production in RAW264.7 cells and cell viability by (±)-**7**. Cells were treated with or without chemicals at indicated concentrations for 1 h, and stimulated with or without 1 μg/mL LPS for 24 h. NO secretion was assessed by Griess reaction. The cell viability was assessed by MTT exclusion.

First, we attempted to introduce the triethylene oxide linker directly to **1** under the Mitsunobu reaction conditions. However, the desired product was not obtained due to its low solubility in THF, toluene, and DMF. Therefore, an alternative synthetic route was designed, as shown in Scheme 1. Fortunately, **1** was stable under neutral conditions, and the linker was introduced via 'Cross-Metathesis reaction'. The synthesis commenced with acylation of 2,5-dimethoxyaniline (**9**) with *O*-allyl salicylic acid (**10**) solution to afford the amide **11** (Scheme 2). Oxidation by iodobenzene diacetate under neutral conditions afforded the bisacetal **12**, which was regioselectively hydrolyzed in AcOH-acetone, to yield the protected quinone **13** (80% in two steps). Epoxidation of **13** with H_2O_2 and NaOH in THF gave a 1:5 mixture of **13** and **14**, which on treatment with TsOH·H_2O in acetone yielded **14** as the sole product (60% in two steps). Compound **14** was deprotected, followed by selective reduction of the ketone at C5 with $NaBH(OAc)_3$ to afford the (±)-*O*-allyl DHMEQ (**7**).[8]

(−)-DHMEQ (**1**) suppressed the lipopolysaccharide (LPS)-induced expression of inflammatory mediators and cytokines, such as iNOS, COX-2, IL-6, and TNF-α, in the mouse macrophage cell line RAW264.7.[9] First, we evaluated the inhibitory activities of (±)-*O*-allyl DHMEQ (**7**), against cell viability and NO production. Compound **7** dose-dependently reduced NO production; at 10 μg/mL, **7** showed lower toxicity and almost the same activity as **1** (Fig. 2). Inhibition of NF-κB activation by (±)-**7** was confirmed by

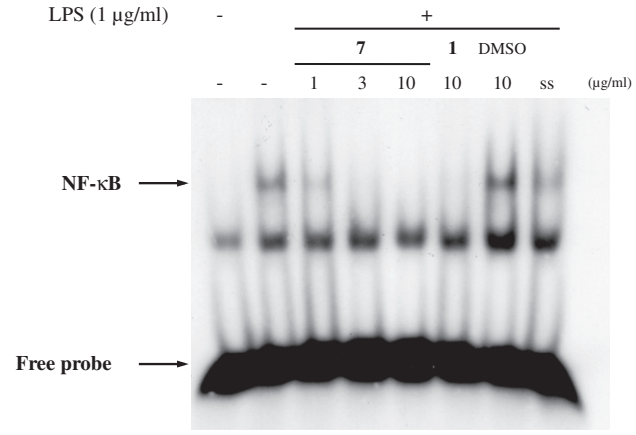
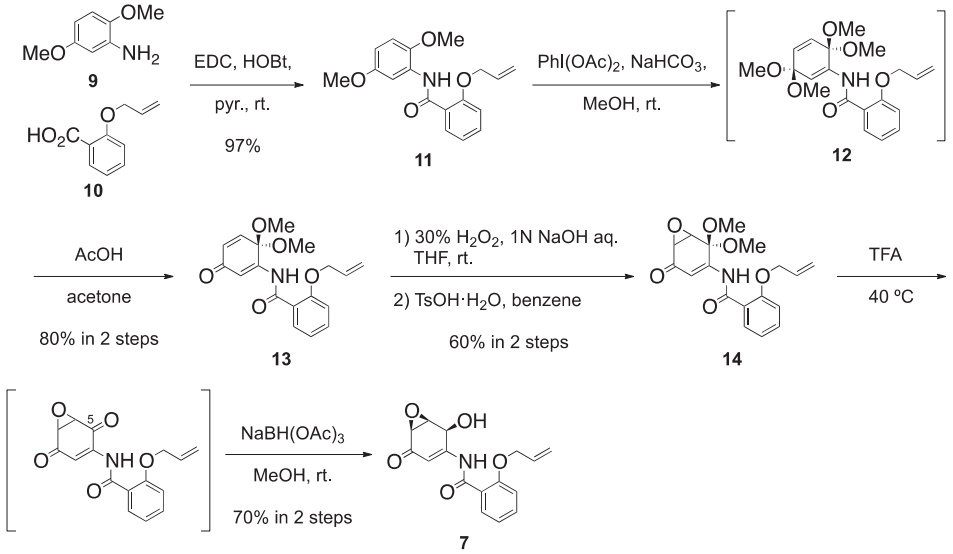
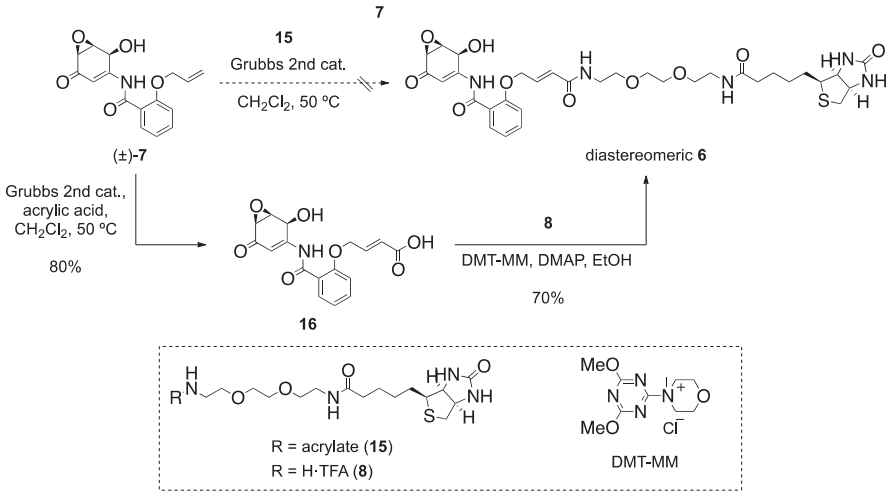


Figure 3. Effects of (±)-**7** on NF-κB activation in RAW264.7 cells. RAW264.7 cells were treated with or without **7**, then stimulated or not 1 μg/mL LPS for 30 min, and thereafter, total nuclear extracts containing equal amounts of protein (5 μg) were then analyzed by electrophoretic mobility shift assay (EMSA) for DNA binding activity of NF-κB using a ^{32}P-labeled oligonucleotide with the high affinity site 5′-AGTTGAGGGGACTTTCCCAGGC-3′. The inducible NF-κB complex is indicated. A section of the fluorogram from a native gel is shown. 'ss' means super shift.



Scheme 2. Synthesis of *O*-allyl DHMEQ (**7**).



Scheme 3. Synthesis of biotinylated DHMEQ (**6**).

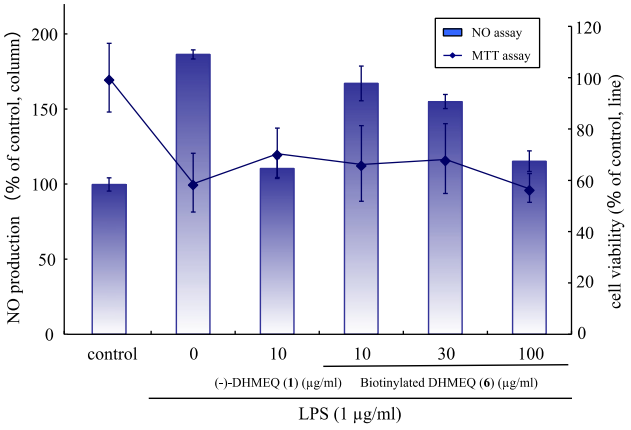


Figure 4. Effects on NO production in RAW264.7 cells and cell viability by diastereomeric mixture of biotinylated DHMEQ **6**. Cells were treated with or without chemical at indicated concentrations for 1 h, and stimulated with or without 1 μg/mL LPS for 24 h. NO secretion was assessed by Griess reaction. The cell viability was assessed by MTT exclusion.

electrophoretic mobility shift assay (EMSA), as shown in Figure 3. Compound **7** inhibited the NF-κB activation at 3 μg/mL. These observations suggested that alkylation of the phenol residue in **1** may not influence its activity. Therefore, the linker was introduced into the allyl phenol ether by metathesis ligation.

Although coupling reaction of **7** with the acrylated biotin analog **15** provided no desired **6**, **7** was preliminary converted into the corresponding acryl structure with acrylic acid in the presence of Grubbs 2nd catalyst to yield **16** (80%, Scheme 3). Finally, biotinylated DHMEQ (**6**) was synthesized by condensation with biotin analog **8** (70%) as a diastereomeric mixture.[8]

Biotinylated DHMEQ (**6**), which is a mixture of the corresponding diastereomers, was also evaluated for its inhibitory activities on cell viability and NO production in RAW264.7 cells. Compound **6** showed weaker inhibitory activity against NO production at 100 μg/mL than that of (−)-DHMEQ (Fig. 4). These observations suggested that **6** might be able to pass through the cell membrane. (−)-DHMEQ (**1**) specifically binds to a cysteine residue in both canonical and noncanonical NF-κB components.[10] DHMEQ does not bind to other proteins such as bovine serum albumin; and it binds only to cysteine residue in p65, cRel, RelB, and p50, which residue is essential for DNA binding. In the case of p65, **1** covalently binds to the 38th cysteine residue at 1 equiv addition.[10]

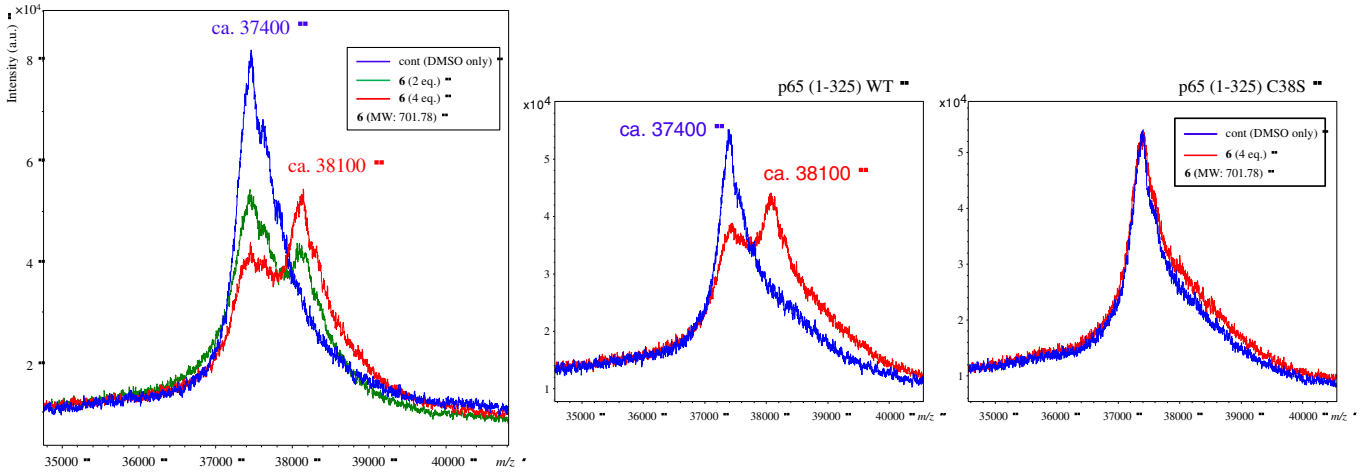


Figure 5. MALDI-TOF MS analysis of p65 (1–325) with diastereomeric **6**. Recombinant p65 (1–325) and p65 (1–325) C38S were treated with or without 2 or 4 equiv of **6**. The proteins (20 μM) were treated with indicated equivalent of **6** for 1 h at 4 °C. After incubation, the proteins were used for MALDI TOF-MS analysis.[11]

Diastereomeric **6** also binds to p65; the mass-to-charge ratio began to shift about the molecular weight of **6** (**6**: MW. 701.78) at 2 equiv, and at 4 equiv, over half of p65 was substituted by **6** (Fig. 5). Mutated p65, in which the 38th cysteine residue was changed to a serine residue, was not substituted by **6** at 4 equiv. These results suggested that **6** binds covalently to the 38th cysteine residue.

In conclusion, we prepared biotinylated DHMEQ derivative **6** which reacted with specific cysteine residue in p65. Additional biological studies regarding the mechanism of action of **1** such as identification of intracellular targets are currently underway in our laboratory.

Acknowledgments

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8. *Selected data*. Compound **8**: HRMS (ESI-MS) calcd for $C_{16}H_{14}NO_5$ $(M-H)^-$ 300.0872, obsd *m*/*z* 300.0867. ^{1}H NMR ($CDCl_3$): δ 3.12 (br s, 1H), 3.47 (dd, 1H, *J* = 2.0, 4.0 Hz), 3.83 (dd, 1H, *J* = 2.8, 4.0 Hz), 4.65 (br s, 1H), 4.68 (d, 2H, *J* = 3.7 Hz), 5.36 (m, 2H), 6.12 (m, 1H), 6.85 (s, 1H), 6.93 (d, 1H, *J* = 8.0 Hz), 7.03 (t, 1H, *J* = 8.0 Hz), 7.42 (t, 1H, *J* = 8.0 Hz), 8.09 (d, 1H, *J* = 8.0 Hz), 10.41 (br s, 1H). ^{13}C NMR ($CDCl_3$): δ 53.5, 53.7, 65.5, 70.53, 107.5, 112.8, 119.9, 120.6, 121.8, 131.8, 132.7, 134.3, 149.6, 156.5, 164.7, 193.2. Compound **6**: HRMS (ESI-MS) calcd for $C_{33}H_{42}N_5O_{10}S$ $(M-H)^-$ 700.2652, obsd *m*/*z* 700.2672. ^{1}H NMR (CD_3OD): δ 1.19-1.75 (m, 6H), 2.09 (t, 2H, *J* = 7.2 Hz), 2.58 (d, 1H, *J* = 12.4 Hz), 2.81 (dd, 1H, *J* = 4.4, 12.4 Hz), 3.08 (m, 1H), 3.22 (t, 2H, *J* = 5.6 Hz), 3.34 (t, 2H, *J* = 5.6 Hz), 3.34 (dd, 1H, *J* = 2.4, 4.4 Hz), 3.42 (t, 2H, *J* = 5.6 Hz), 3.47 (t, 2H, *J* = 5.6 Hz), 3.50 (s, 4H), 3.78 (dd, 1H, *J* = 2.8, 4.4 Hz), 4.18 (dd, 1H, *J* = 4.4, 7.6 Hz), 4.38 (dd, 1H, *J* = 4.4, 7.6 Hz), 4.69 (dd, 1H, *J* = 1.2, 2.4 Hz), 4.96 (d, 2H, *J* = 4.4 Hz), 6.16 (d, 1H, *J* = 15.6 Hz), 6.90 (dt, 1H, *J* = 4.4, 15.6 Hz), 6.95 (dd, 1H, *J* = 1.2, 2.4 Hz), 7.02–7.07 (m, 2H), 7.47 (dt, 1H, *J* = 2.0, 8.8 Hz), 7.98 (dd, 1H, *J* = 2.0, 8.0 Hz). ^{13}C NMR (CD_3OD): δ 26.8, 29.4, 29.7, 36.7, 40.2, 40.4, 41.0, 55.9, 56.1, 56.9, 58.3, 63.3, 66.4, 69.2, 70.4, 70.5, 71.2, 71.3, 108.5, 114.5, 122.4, 122.9, 126.3, 133.2, 135.7, 138.3, 155.5, 157.7, 166.4, 167.6, 170.6, 176.6, 193.3.
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11. Although the stability of **6** is still unclear, **6** in DMSO solution showed no decomposition stored at −40 °C for 2 weeks. Biotinylation of **1** caused reduction of the binding ability to p65. The newly produced derivatives in optically active forms should be required for comparison of correct biological activity with that of (−)-DHMEQ.