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Synthesis of fluorescent and photoaffinity-labeled derivatives of bisphenol A and their inhibitory activity toward hypoxic expression of erythropoietin

Nobuhiro Maezawa,[a] Hiroshi Tsuchikawa,[a] Shigeo Katsumura,[a,*] Tomoko Kubo[b] and Susumu Imaoka[b,*]

[a]*Department of Chemistry and Open Research Center on Organic Tool Molecules, School of Science and Technology, Kwansei Gakuin University, Sanda, Hyogo 669-1337, Japan*
[b]*Department of Bioscience and Nanobiotechnology Research Center, School of Science and Technology, Kwansei Gakuin University, Sanda, Hyogo 669-1337, Japan*

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Abstract—Bisphenol A derivatives, possessing a fluorescent dye and a photo-reactive group, were synthesized from bisphenol A, and the inhibitory activity of the derivatives was evaluated against hypoxic response. The synthesized derivatives were found to inhibit the hypoxic expression of erythropoietin in Hep3B cells as well as bisphenol A.

Bisphenol A (BpA) is widely used as a major source of polycarbonate and epoxy resins in industry and is well known as one of the common environmental endocrine disruptors. This molecule is a xenoestrogen and affects the reproductive functions of animals.[1] In addition, as non-estrogenic effects, BpA was reported to induce developmental abnormalities in the neuronal systems of humans and animals.[2] The use of BpA, however, has not been restricted because of the lack of development of its alternatives. Thus, the elucidation of its mode of action and molecular mechanism is strongly desired. It is also important to pursue the behavior of BpA in the environment, cells, and organizations. Under these situations, the methods for detection and measurement of BpA have broadly been studied, and some methods have been reported; for example, the use of a specific monoclonal antibody against BpA[3] and the use of isotope-labeled BpA for detection.[4] However, the detection and the quantification of BpA are not necessarily facile, because these methods often require a special facility and instruments. In this paper, we describe the synthesis and evaluation of BpA derivatives possessing a fluorescent dye in order to detect BpA more simply and efficiently. In addition, we synthesized a BpA derivative possessing a photo-reacting group, namely a photoaffinity group. Furthermore, we report the synthesis of a valuable BpA derivative having both fluorescence and photoaffinity groups. These are expected to be useful tool molecules to elucidate the BpA behavior based on the evaluation of their inhibitory activity toward the hypoxic expression of *erythropoietin*.

In the previous study, we found that BpA inhibited the hypoxic expression of the *erythropoietin* (*EPO*) gene, which is known as a hematopoietic cytokine. In the structure–activity relationship study of BpA, we reported that the blocking of two phenol groups in BpA did not change the inhibitory effect on the induction of *EPO*, but the inhibition completely disappeared with the removal of the two central methyl groups.[5] Taking into consideration these previous results, we designed and synthesized three kinds of labeled compounds as shown in Figure 1, which have labels at the terminal of a C4 or C2 chain as a linker extended from the hydroxyl group of phenol. One of these compounds contains the 4-nitrobenzo-2-oxa-1,3-diazole (NBD) group as a fluorescence-labeled group[6] (**1**, **2**), and the second one contains the 3-(4-alkoxyphenyl)-3-trifluoromethyldiazirine group (**3**), which

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* Corresponding authors. Tel.: +81 79 565 8314; fax: +81 79 565 9077 (S.K.); tel.: +81 79 565 7673; fax: +81 79 565 9077 (S.I.); e-mail addresses: katsumura@ksc.kwansei.ac.jp; imaoka@ksc.kwansei.ac.jp

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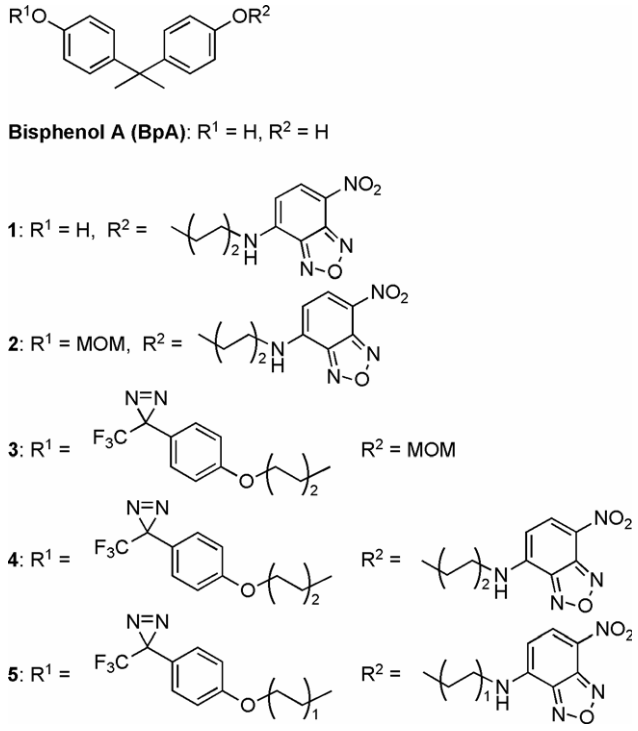
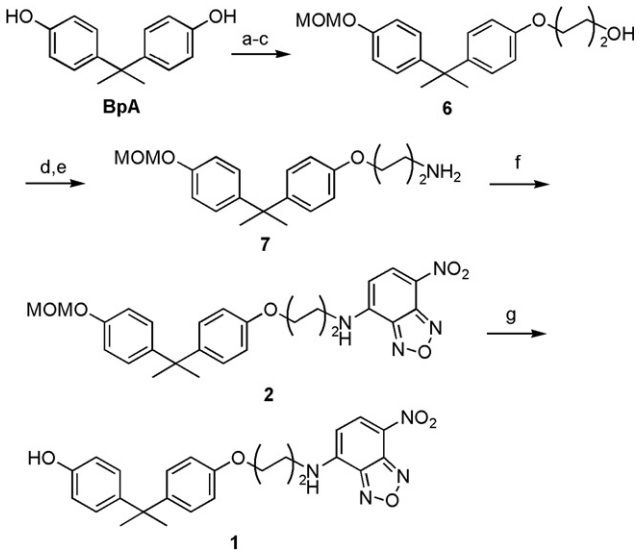


Figure 1. The structure of BpA and the derivatives.

can produce the corresponding carbene accompanied by liberating nitrogen by photoirradiation at 360 nm and can form a covalent bond with the neighboring function.[7] The third one contains both fluorescent and photoaffinity groups (**4**, **5**). The length of the linker was considered to avoid the interaction with the central methyl groups of BpA.

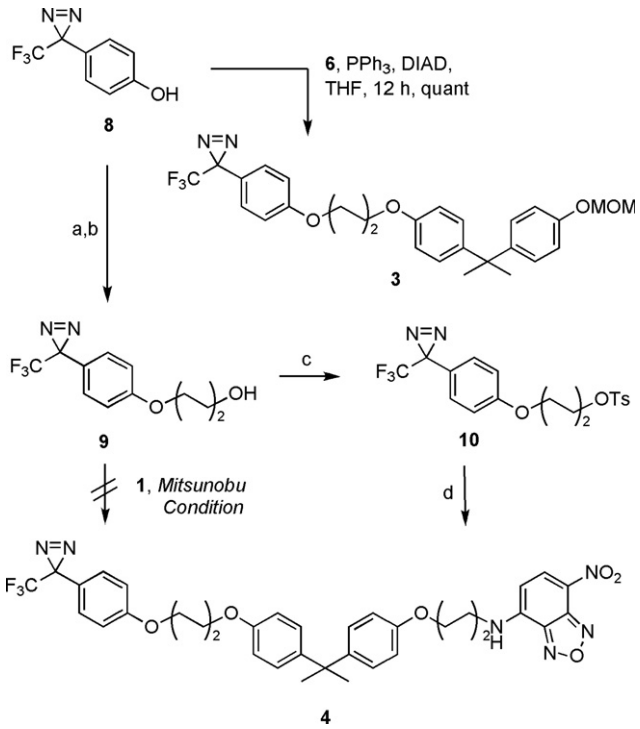
The synthesis was started from BpA as illustrated in Scheme 1. The C4 linker was introduced to the hydroxyl group of the mono-MOM ether of BpA by the Mitsunobu reaction using diisopropylazodicarboxylate (DIAD) with mono-protected butanediol,[8] followed by the removal of the TIPS group to produce alcohol **6** in 70% yield. The terminal hydroxyl group was converted into azide by reaction with diphenylphosphorylazide (DPPA), and primary amine **7** was obtained by treatment of the resulting azide with triphenylphosphine. Introduction of the NBD group was achieved by a nucleophilic substitution reaction of **7** with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBDCl) to provide compound **2** in 52% yield over three steps.[9] The subsequent deprotection of the MOM group under acidic condition gave fluorescence-labeled BpA **1** in 82% yield.[10]



Scheme 1. Reagents and conditions: (a) MOMCl, NaH, DMF, rt, 1 h, 43%; (b) TIPSO$(CH_2)_4$OH, PPh_3, DIAD, THF, 3 h; (c) TBAF, THF, rt, 3 h, 70% (two steps); (d) $(PhO)_2P(O)N_3$, PPh_3, DIAD, THF, 3 h; (e) PPh_3, H_2O, 60 °C, 3 h; (f) NBDCl, Et_3N, rt, 30 min, 52% (three steps); (g) 2 M HCl, AcOH, rt, 3 days, 82%.

Next is the preparation of photoaffinity-labeled BpA derivative **3** (Scheme 2). The synthesis was commenced with 3-(4-hydroxyphenyl)-3-trifluoromethyl diazirine **8**, which was prepared according to the reported procedure.[11] The hydroxy group of phenol **8** was directly connected with BpA derivative **6** using our own method utilizing the Mitsunobu reaction to quantitatively produce **3**.[12] Thus, we realized the synthesis of both fluorescence-labeled and photoaffinity-labeled BpA derivatives.

The third derivative of labeled BpA was both fluorescent and photoaffinity-double-labeled BpA derivative **4**. The hydroxy group of phenol **8** was reacted with mono-protected butanediol by our method utilizing the Mitsunobu reaction, followed by treatment with TBAF to give the corresponding alcohol **9** in good yield. In this case, a C4 chain was also adopted as a linker part. Unfortunately, the direct coupling between photo-



Scheme 2. Reagents and conditions: (a) TIPSO $(CH_2)_4$OH, PPh_3, DIAD, THF, 1.5 h; (b) TBAF, THF, rt, 3 h; (c) TsCl, Et_3N, DMAP, CH_2Cl_2, rt, 2 h, 69% (three steps); (d) **1**, NaH, DMF, rt, 5 min, 64%.

affinity-labeled **9** and fluorescence-labeled **1** under the Mitsunobu conditions did not proceed,[12] and both compounds were recovered. The primary hydroxy group was then converted into the corresponding tosylate, which reacted with the sodium phenoxide of **1** to produce the desired **4** in 44% yield from **8**.[13] Thus, we succeeded in the preparation of double-labeled BpA derivative **4**. We also synthesized another double-labeled BpA derivative **5** which contained a C4 chain as the linker part by the same method.[14]

We then evaluated the biological activities of fluorescence-labeled derivative **1**, MOM ether **2**, photoaffinity-labeled derivative **3**, and fluorescence-photoaffinity-double-labeled derivatives **4** and **5**. After addition of BpA or these derivatives to Hep3B cells under hypoxia, respectively, we measured the expression of *EPO* by RT-PCR (Figs. 2 and 3).[15] *EPO* was strongly induced under hypoxia, and the addition of BpA completely suppressed *EPO* induction.[5] By addition of compound **1**, the expected inhibition effect on *EPO* expression was observed. Interestingly, the MOM ether **2** showed stronger inhibitory activity than **1**, and the magnitude of the suppression was comparable to that of BpA. Moreover, the double-labeled compound **4** also revealed the inhibitory activity almost equal to that of BpA, and compound **5** showed less inhibitory activity than compound **4** because compound **5** needed higher concentration (300 μM) than compound **4** (200 μM) to reveal the similar inhibitory effect. On the other hand, photoaffinity-labeled compound **3** did not show the inhibitory activity. These results strongly suggest that

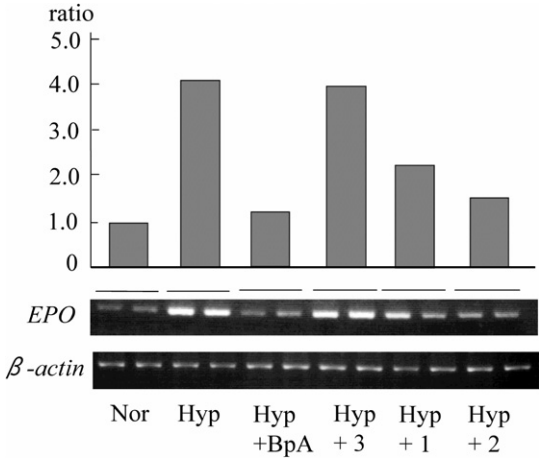


Figure 2. Inhibition of EPO induction under hypoxia by BpA and BpA derivatives (**1**–**3**). Hep3B cells were cultured for 6 h under hypoxia in the presence of BpA (200 μM), the derivative **1** (200 μM), derivative **2** (200 μM), and derivative **3** (200 μM). These chemicals were dissolved in DMSO. Expression of the *EPO* gene was detected by RT-PCR (reverse transcription-polymerase chain reaction). β-*Actin* was used as a control. Bands of amplified DNA fragments on agarose gel were quantified by NIH Image. Values in the graph are means of two different samples and are expressed as ratios of *EPO* mRNA and β-*actin* mRNA. Control value is set at 1.0. Nor, normoxia; Hyp, hypoxia; Hyp + BpA, addition of bisphenol A under hypoxia; Hyp + **3**, addition of derivative **3** under hypoxia; Hyp + **1**, addition of derivative **1** under hypoxia; and Hyp + **2**, addition of derivative **2** under hypoxia.

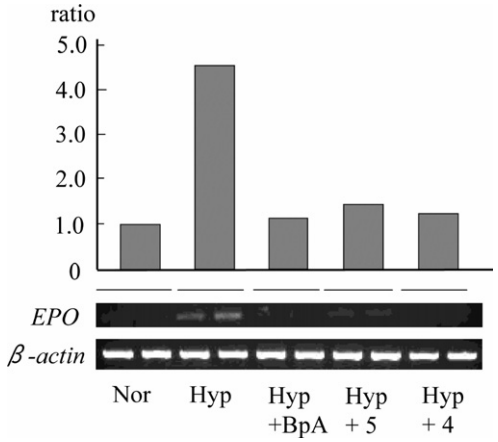


Figure 3. Inhibition of EPO induction under hypoxia by BpA and BpA derivatives (**4** and **5**). The conditions are same in the legend of Figure 2 except the concentration of the derivative **5** (300 μM). Nor, normoxia; Hyp, hypoxia; Hyp + BpA, addition of bisphenol A under hypoxia; Hyp + **5**, addition of derivative **5** under hypoxia; and Hyp + **4**, addition of derivative **4** under hypoxia.

the synthesized BpA derivatives **2** and **4** would be recognized in cells as well as BpA. Thus, they are considered to be simpler and more practical tool molecules for the detection of BpA. Furthermore, they are expected to identify the target molecule of BpA by photoaffinity labeling and to disclose the behavior and distribution of BpA in cells.

In conclusion, we synthesized the BpA derivatives which possess a fluorescent dye and a photo-reactive group. To the best of our knowledge, these molecules are the first examples which possess fluorescence and affinity labels in the BpA molecule. Furthermore, we observed that these derivatives (**1**, **2**, **4**, and **5**) inhibited the hypoxic expression of *EPO* in Hep3B cells as well as BpA, and hence they are expected to be powerful tool molecules to elucidate BpA behavior.

Acknowledgments

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9. Data for **2**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.48 (d, J = 8.4 Hz, 1H), 7.16 (AB-q, J = 7.3 Hz, 2H), 7.13 (AB-q, J = 7.1 Hz, 2H), 6.93 (AB-q, J = 8.8 Hz, 2H), 6.85 (AB-q, J = 8.8 Hz, 2H), 6.44 (br s, 1H), 6.19 (d, J = 8.4 Hz, 1H), 5.15 (s, 2H), 4.64 (t, J = 5.6 Hz, 2H), 3.60 (t, J = 6.3 Hz, 2H), 3.47 (s, 3H), 1.95–2.05 (m, 2H), 1.64 (s, 6H); ESI HRMS *m/z* calcd for $C_{27}H_{30}N_4O_6$ $[M-H]^-$ 505.2087, found 505.2092.
10. Data for **1**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.48 (d, J = 8.4 Hz, 1H), 7.15 (AB-q, J = 7.4 Hz, 2H), 7.09 (AB-q, J = 8.8 Hz, 2H), 6.82 (AB-q, J = 8.8 Hz, 2H), 6.73 (AB-q, J = 8.5 Hz, 2H), 6.47 (br s, 1H), 6.18 (d, J = 8.4 Hz, 1H), 4.04 (t, J = 5.6 Hz, 2H), 3.51–3.60 (m, 2H), 3.47 (s, 3H), 1.94–2.07 (m, 2H), 1.63 (s, 6H); ESI HRMS *m/z* calcd for $C_{25}H_{26}N_4O_5$ $[M-H]^-$ 461.1825, found 461.1811.
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13. Data for **4**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.48 (d, J = 8.5 Hz, 1H), 7.16 (AB-q, J = 9.0 Hz, 2H), 7.12 (AB-q, J = 9.0 Hz, 4H), 6.88 (AB-q, J = 8.5 Hz, 2H), 6.82 (AB-q, J = 8.5 Hz, 2H), 6.78 (AB-q, J = 8.5 Hz, 2H), 6.19 (br s, 1H), 6.18 (d, J = 8.5 Hz, 1H), 3.98–4.06 (m, 6H), 3.57–3.62 (m, 2H), 1.93–2.04 (m, 8H), 1.64 (s, 6H); ESI HRMS *m/z* calcd for $C_{37}H_{37}F_3N_6O_6$ $[M+Na]^+$ 741.2624, found 741.2641.
14. Data for **5**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.51 (d, J = 8.8 Hz, 1H), 7.16 (AB-q, J = 8.5 Hz, 2H), 7.15 (AB-q, J = 9.0 Hz, 2H), 7.13 (AB-q, J = 8.5 Hz, 2H), 6.83 (AB-q, J = 8.8 Hz, 2H), 6.83 (AB-q, J = 8.5 Hz, 2H), 6.82 (AB-q, J = 8.5 Hz, 2H), 6.55 (br s, 1H), 6.27 (d, J = 8.8 Hz, 1H), 4.28–4.31 (m, 6H), 3.89 (dt, J = 4.9, 5.0 Hz, 2H), 1.63 (s, 6H); ESI HRMS *m/z* calcd for $C_{33}H_{29}F_3N_6O_6$ $[M+Na]^+$ 685.1998, found 685.1977.
15. The detailed experimental conditions are as follows: Hep3B cells were cultured in DMEM containing 10% FCS, and the FCS concentration was reduced to 0.1% at 24 h before the treatment with chemicals. For hypoxic treatment, the cells were incubated in 5% O_2, 5% CO_2, and 90% N_2 balanced with a modulator incubator chamber (Napco 7101, Winchester, VA) or were incubated in a sealed 2.5-L box with an Anero Pack for cells. Hep3B cells were incubated for 6 h under hypoxia in the presence of BpA and derivatives (200–300 μM). Total RNA was extracted from Hep3B cells and a reaction mixture containing 1 μg of RNA and 200 U of reverse transcriptase was reacted according to the condition as follows: incubation for 10 min at 25 °C and 60 min at 42 °C, followed by heating for 10 min at 70 °C to stop the reaction. Polymerase chain reaction (PCR) was performed using a reaction mixture containing 10 pmol of each primer, 1.5 U of Ampli Taq, and 100 ng of cDNA according to the following protocol: 10 min at 96 °C and then 35 cycles of 30 s at 96 °C, 30 s at 56 °C, and 1 min at 72 °C. Primers for β-*actin* were 5′-CAAGAGATGGCC ACGGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCC TGTCGGCA-3′ (antisense). Primers for *EPO* were 5′-G CCAGAGGAACTGTCCAGAG-3′ (sense) and 5′-TTC TCCAGGTCATCCTGTCC-3′ (antisense). The cycle number is within the linear range of amplification. Bands of amplified DNA fragments on agarose gel were quantified by NIH Image.