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PAPER

Synthesis and evaluation of novel caged DNA alkylating agents bearing 3,4-epoxypiperidine structure†

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Previously, we reported that the 3,4-epoxypiperidine structure, whose design was based on the active site of DNA alkylating antitumor antibiotics, azinomycins A and B, possesses prominent DNA cleavage activity. In this report, novel caged DNA alkylating agents, which were designed to be activated by UV irradiation, were synthesized by the introduction of four photo-labile protecting groups to a 3,4-epoxypiperidine derivative. The DNA cleavage activity and cytotoxicity of the caged DNA alkylating agents were examined under UV irradiation. Four caged DNA alkylating agents showed various degrees of bioactivity depending on the photosensitivity of the protecting groups.

Introduction

The first documented use of a cancer therapeutic agent was a DNA alkylating agent, nitrogen mustard, in 1958.[1] This report showed that nitrogen mustard, which was created as a chemical weapon during World War II, exhibited drug potency for neoplastic disease. Starting with this first application of a cancer therapy, more efficient and harmless DNA alkylating agents have been created and used as means to treat cancer.[2,3] Even now, more efficient DNA alkylating agents with fewer side effects are desired.

Biologically active substances that are modified by a photo-labile protecting group to make them temporarily inactive and that can express their original bioactivity by photo irradiation are called "caged compounds".[4] Expression of the activity is confined to the time and position of irradiation. This behavior enables caged compounds to accomplish spatiotemporal control of bioactivity. Caging of DNA alkylating agents is anticipated to allow site-specific anticancer activity upon light irradiation and, accordingly, avoidance of systemic side effects such as myelosuppression. Many photo-inducible DNA damaging or cytotoxic compounds have been reported in the past.[5–11] However, to our knowledge, there are few reported cases of photo-triggered DNA alkylating agents.[12,13] Herein, we report novel caged DNA alkylating agents. We introduced four typical photo-labile protecting groups into a DNA alkylating agent 3,4-epoxypiperidine derivative, and examined DNA cleavage activity and cytotoxicity under UV irradiation.

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† Electronic supplementary information (ESI) available: NMR-spectra of new compounds, and UV-absorption spectra and result of agarose gel shift assay of **3** and **4a–4d** in the presence of radical scavenger. See DOI: 10.1039/c2ob25366f

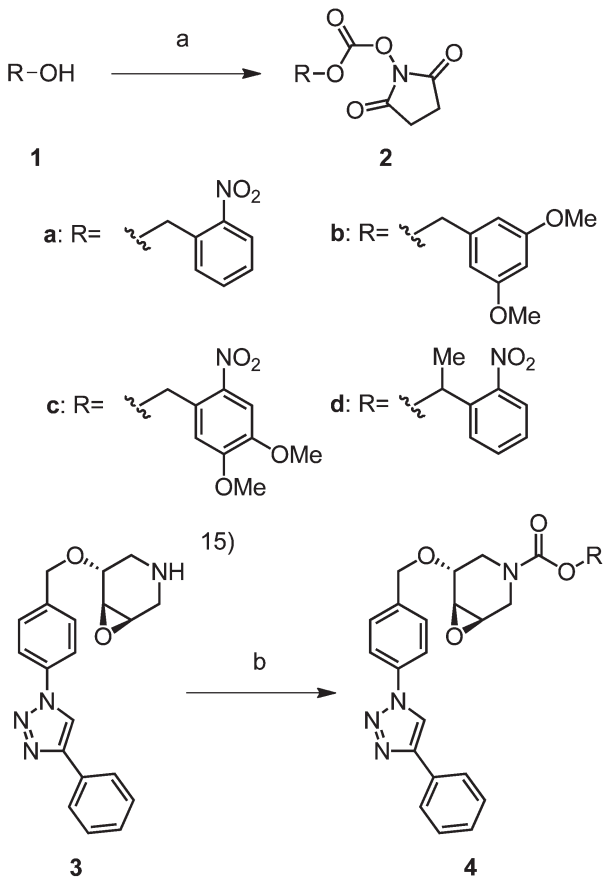
‡ These authors contributed equally to this study.

Results and discussion

Design and synthesis of caged DNA alkylating agents

Previously, we have hypothesized that the 4-hydroxy-1-azabicyclo-[3.1.0]hexane structure which is the active site of DNA alkylating natural products, azinomycins A and B, and the 3,4-epoxypiperidine structure could interconvert.[14] And indeed, we found that 3,4-epoxypiperidine derivatives possess potent DNA cleavage activity.[14] It is also found out that the secondary amine structure in the piperidine motif was important for the DNA cleavage activity, and therefore a modification at the piperidinic nitrogen atom of 3,4-epoxypiperidine derivatives caused less activity.[14] 3,4-Epoxypiperidine **3** bearing a phenyl triazolyl group, which was recently synthesized as a part of our 3,4-epoxypiperidine library,[15] has both the potent DNA cleavage activity and the chemical stability under isolated conditions, while most other 3,4-epoxypiperidine derivatives in the library are relatively unstable at concentrated solution conditions. These unique properties of 3,4-epoxypiperidine **3** encouraged us to prepare the corresponding caged DNA alkylating agents by a modification of the piperidinic secondary amine with photo-labile protecting group.

Four common protecting groups, *o*-nitrobenzyloxycarbonyl group (**a**),[16] 3,5-dimethoxybenzyloxycarbonyl group (**b**),[17] 3,4-dimethoxy-6-nitrobenzyloxycarbonyl group (**c**)[18,19] and α-methyl-*o*-nitrobenzyloxycarbonyl group (**d**),[20] were selected as photo-labile protecting groups. *o*-Nitrobenzyloxycarbonyl- (**a**), 3,4-dimethoxy-6-nitrobenzyloxycarbonyl- (**c**), and α-methyl-*o*-



Scheme 1 Reagents and conditions: (a) disuccinimidyl carbonate, triethylamine, MeCN, 66%–quant.; (b) **2**, triethylamine, CH_2Cl_2, 74–97%.

nitrobenzyloxycarbonyl group (**d**) would be sensitive to a relatively harmless 365 nm of wavelength, whereas 3,5-dimethoxybenzyloxycarbonyl group (**b**) which requires much shorter 254 nm of wavelength for the activation should be insensitive as long as 365 nm of wavelength is irradiated.[21]

First, active carbonates **2a–2d** were synthesized from the corresponding alcohols **1a–1d** by treatment with disuccinimidyl carbonate. Conversion of 3,4-epoxypiperidine **3** to caged DNA alkylating agents **4a–d** was accomplished by the treatment of **3** with the carbonates **2a–2d** (Scheme 1).

DNA relaxation assay

We examined by a relaxation assay of supercoiled plasmid DNA pBR 322 whether the caged DNA alkylating agents **4a–4d** could restore the native activity of **3** by UV irradiation. After the addition of each of the caged alkylating agents to a solution of plasmid DNA, UV irradiation (365 nm) was applied for the indicated period of time, and then incubated for 24 h at 37 °C. The alteration of plasmid DNA was analyzed by agarose gel electrophoresis (Fig. 1). As shown in Fig. 1a, activation of **4a–4d** by UV irradiation resulted in the transformation of the supercoiled plasmid DNA (form I) into an open circular DNA (form II). Fig. 1b–1f summarize the relationship between the UV irradiation period and DNA cleavage activity. The DNA cleavage activity is represented by the ratio of form I to form II of plasmid DNA. Without UV irradiation, all of the caged DNA alkylating agents **4a–4d** were inactive while the original 3,4-epoxypiperidine **3** converted form I to form II of plasmid. When UV irradiation was applied for 2 s, with 10 μM of caged alkylating agent, compound **4d** was activated, resulting in cleavage of approximately 50% of plasmid DNA, and **4a** and **4c** showed some activity. On the other hand, compound **4b** was not active toward plasmid DNA. After 5 s of irradiation, compound **4d** was activated completely, showing DNA cleavage activity equivalent to positive control **3**. In 5 s, compounds **4a** and **4c** expressed stronger DNA cleavage activity than under a 2 s irradiation period. Compound **4b** again showed no activity. When the irradiation period was prolonged to 15 or 60 s, compound **4b** was still inactive. The activity of **4a** grew depending on the irradiation period. In contrast, the activity of **4c** plateaued at about 50% even for an irradiation period of 60 s.

As observed above, the four caged DNA alkylating agents **4a–4d** showed different behavior toward UV irradiation. Most of this result can be explained as the differing photo-sensitivity of each four protecting groups. First of all, **4b** was inactive as we designed. A result that the activation of **4d** was much faster than that of **4a** and **4c** was consisted with a report by Hasan *et al.*[22] They described that mono-substitution at the α-position of the nitrobenzyl group makes abstraction of the benzylic hydrogen atom easier, consequently accelerating the photo-induced deprotection.[22]

The progress of the uncaging reaction is generally dependent on the irradiation period. The activation of **4a** and **4d** also irradiation-period-dependently proceeded and respectively completed within 1 min and 5 s. But in the case of **4c**, form-conversion of plasmid reached the plateau in mid course. Because the activity of **3** itself did not change whether nitrosobenzaldehyde co-existed or not (data not shown), it is considered that this phenomenon resulted from generation of 3,4-dimethoxy-6-nitrosobenzaldehyde from compound **4c** which potently absorbed UV rays, and conversion of **4c** into **3** was interrupted when the concentration of 3,4-dimethoxy-6-nitrosobenzaldehyde reached a certain degree.

Although we suppose that the 3,4-epoxypiperidine derivatives induced DNA cleavage *via* DNA alkylation, the mechanism is not confirmed yet. To exclude the other possibility of DNA cleavage pathway: radical-mediated DNA cleavage[10] induced by a photo irradiation, a plasmid DNA relaxation assay in the presence of hydroxyl radical scavenger (glycerol) or singlet oxygen scavenger (NaN_3) was carried out[10] (ESI, Fig. S1†). As a result, almost no change of DNA cleavage activity was observed despite the presence of scavenger. This result shows that the DNA cleavage activity of epoxypiperidine derivatives **3** and **4a–4d** is not derived from a radical pathway, and supports our assumption that epoxypiperidine derivatives work as DNA alkylating agents.

Cytotoxicity of caged DNA alkylating agents triggered by UV irradiation

The cytotoxicity of caged DNA alkylating agents against HepG2 cells was examined by MTT assay following UV irradiation for 5 s and incubation for 24 h at 37 °C (Fig. 2a and 3). All caged

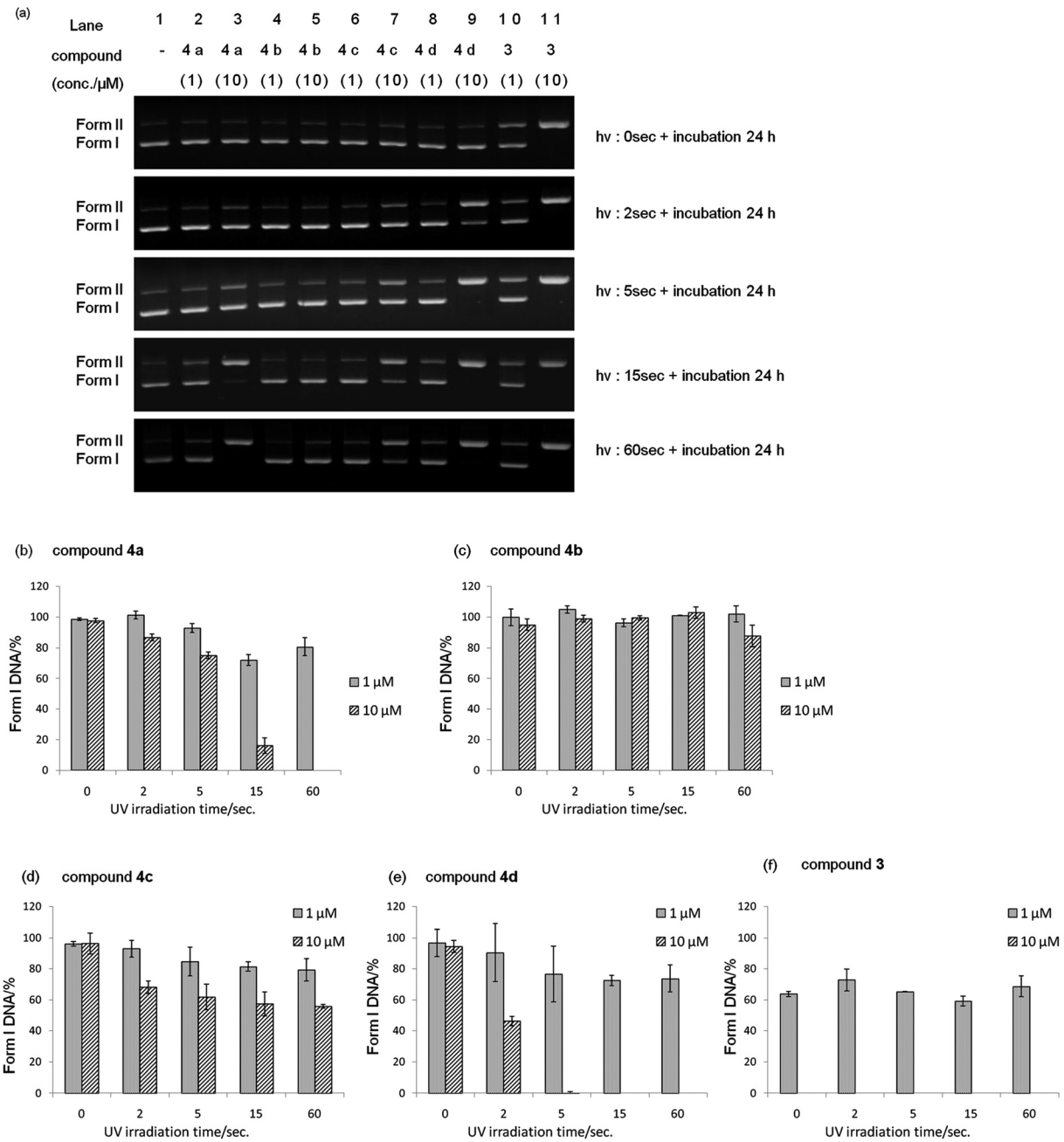


Fig. 1 Plasmid relaxation assay with caged DNA alkylating agents **4a**–**4d** and **3**. (a) Results of agarose gel shift assay. (b)–(f) Relationship between percentage of intact DNA and UV irradiation time. These are represented as the average of two times.

DNA alkylating agents **4a**–**4d** were atoxic without UV irradiation. Prominent enhancement of cytotoxicity of **4c** and **4d** at 100 μM were induced by UV irradiation, whereas that of **4a** and **4b** was not affected at all. Compounds **4c** and **4d** showed potent cytotoxicity equal to the parent compound **3** under irradiation conditions. Compound **3** itself did not show any change in activity by UV irradiation. This tendency was in complete agreement with that of the DNA relaxation assay, and indicated that **4c** and **4d** are excellent caged DNA alkylating agents which can express cytotoxicity triggered by UV irradiation.

In regard to this result, we were concerned about the possibility that nitrosobenzene derivatives produced from deprotection of photo-labile protective groups might affect cytotoxicity. Therefore, we synthesized compounds **5a** and **5b** as control compounds (Fig. 4a). These compounds release each nitrosobenzene derivative and noncytotoxic piperidine upon photo-irradiation. The control experiment was achieved in a similar manner to the cytotoxicity assay of **4a**–**4d**. Compounds **5a** and **5b** showed no cytotoxicity (Fig. 4), *i.e.*, nitrosobenzene derivatives had no potential toward cytotoxicity in our experimental system.

Additionally, caged DNA alkylating agent **4d** possessed an interesting property that its cytotoxicity varied depending on the interval periods between addition of **4d** and UV irradiation. This experiment was conducted as described in Fig. 2b. After 24 h of

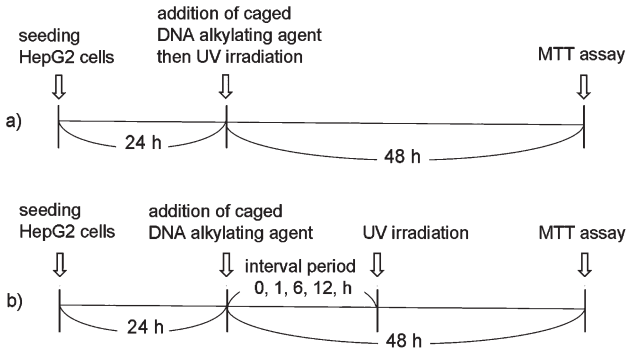


Fig. 2 Diagram of examination of cytotoxicity.

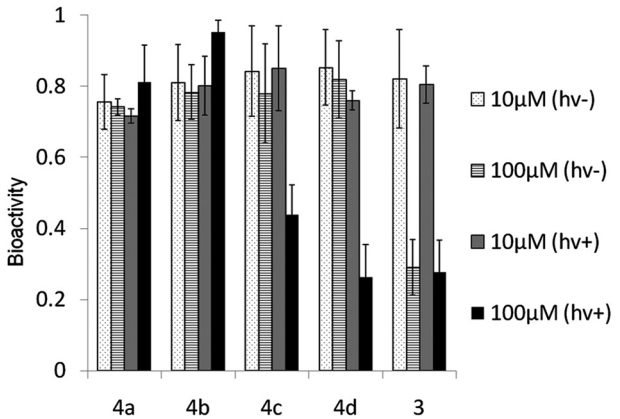


Fig. 3 MTT assay using caged DNA alkylating agents **4a**–**4d** and non-caged DNA alkylating agent **3** by treatment of UV irradiation for 5 s. The bioactivity is represented as a ratio to control.

HepG2 cells being seeded onto a 96-well plate, compound **4d** was added to each well, and the cells were further incubated for 48 h. UV irradiation was applied at 0, 1, 6 or 12 h (interval period) after addition of **4d**. The living cells were counted by MTT assay (Fig. 5). The cytotoxicity of **4d** was increased as the interval period was extended up to 12 h. In particular, the improvement of the activity was most remarkable at a concentration of 10 μM, and the increase in cytotoxicity was obviously relative to the interval period.

We believe that this behavior can be explained by membrane permeability because parent compound **3** had no cytotoxicity at 10 μM (Fig. 3); on the other hand, compound **4d**, whose amino group was covered by a relatively non-polar α-methyl-*o*-nitrobenzyloxycarbonyl group, showed potent cytotoxicity at the same concentration after an adequate interval and the following photo-irradiation (Fig. 5). It is known that several biologically active agents containing amino groups are less membrane-permeable due to their high polarity, and low membrane permeability provides a disadvantage in drug kinetics. To resolve this problem, conversion into prodrugs by modification on the polar amino group has been reported.[23–26] As in these cases, it is thought that improvement of bioactivity could be derived from altering the membrane permeability by introduction of a photo-labile protecting group.

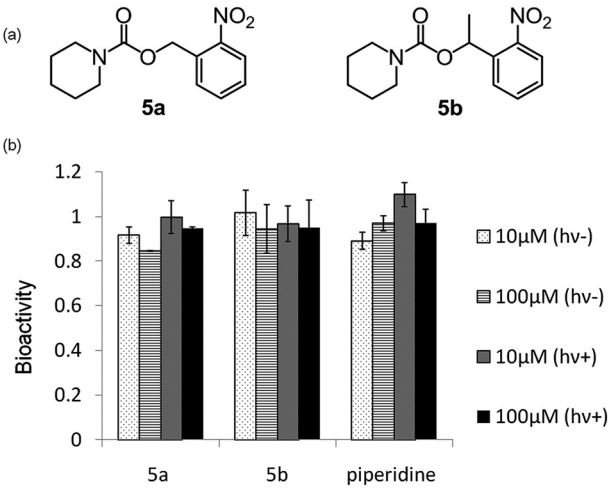


Fig. 4 (a) Structures of control compounds **5a** and **5b**. (b) MTT assay using control compounds **5a**, **5b** and piperidine by treatment of UV irradiation for 5 s. The cytotoxicity is represented as a ratio to control.

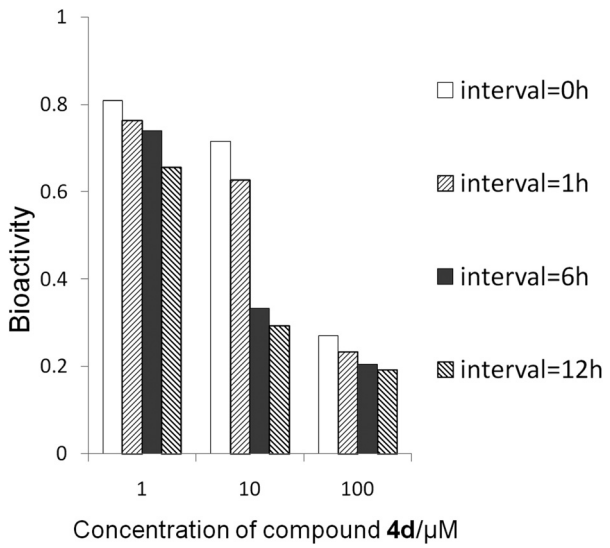


Fig. 5 The dependence between interval period from addition of caged DNA alkylating agent **4d** to UV irradiation and cytotoxicity. The cytotoxicity is represented as a ratio to control.

Conclusion

In this paper, we described the synthesis and biological characteristics of novel caged DNA alkylating agents. The synthesis of compounds **4a**–**4d** was easily accomplished from 3,4-epoxypiperidine **3** by introduction of four types of photo-labile protecting groups. The results of the DNA relaxation assay indicated that **4c** and **4d** were successfully activated by UV irradiation within 5 s while **4b** scarcely showed DNA cleavage activity at the longest irradiation time (60 s). This tendency closely correlated with the result of the MTT assay. Additionally, cytotoxicity of **4d** varied depending on the timing of UV irradiation. It is considered that this result could be derived from the alteration of polarity by protection of the nitrogen atom, *i.e.*, the caging of epoxypiperidine **3** not only imparts a trigger function by UV

rays but also improves membrane permeability. These results suggested the utility of **4d** as a novel anticancer drug whose activity can be controlled spatiotemporally.

Experimental

General

Unless otherwise noted, all chemicals from commercial sources were used without further purification. CH_2Cl_2 and triethylamine were distilled over calcium hydride. All reactions were carried out under a nitrogen atmosphere. Melting points were measured on a Yanagimoto micro-melting point apparatus and are uncorrected. The ^1H and ^{13}C NMR spectra were recorded on JEOL AL-300 (^1H, 300 MHz; ^{13}C, 75.5 MHz) or JEOL ECS-400 (^1H, 400 MHz; ^{13}C, 100 MHz) instruments. Values of δ are in ppm relative to tetramethylsilane (0.00 ppm) or $CDCl_3$ (7.26 ppm) as internal standards. The IR spectra were recorded on a JASCO FT/IR-4200 spectrometer. FAB-mass or EI-mass were measured on a JEOL JMS-600 or JMS-700 mass spectrometer. Column chromatography was achieved using Fuji Silysia FL-100D or PSQ-100B silica gel. Photoirradiation at 365 nm was performed with a ZUV-C30H UV-LED lamp as a light source and ZUVL8H as a lens unit (Omron, Kyoto, Japan).

2-Nitrobenzyl-*N*-succinimidyl carbonate (2a)

To a solution of 2-nitrobenzyl alcohol (**1a**) (1.0 g, 6.5 mmol) and triethylamine (990 mg, 1.4 mL, 9.8 mmol) in acetonitrile (50 mL) was added disuccinimidyl carbonate (1.8 g, 7.2 mmol), and the reaction mixture was stirred at room temperature for 30 min. The reaction mixture was diluted with AcOEt. The organic layer was washed by water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by recrystallization (*n*-hexane–toluene = 4 : 1) to give **2a** (1.5 g, 73%) as colorless crystals: mp 120–123 °C; IR ν_{max}/cm^{-1} (KBr) 1812, 1788, 1741, 1526, 1360, 1258, 1235, 1201, 1076, 1058, 1047 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 2.84 (4H, s), 5.78 (2H, s), 7.52–7.57 (1H, m), 7.67–7.56 (2H, m), 8.19 (1H, d, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 25.44, 68.67, 125.35, 128.17, 129.38, 130.19, 134.43, 146.78, 151.33, 168.44; Mass (FAB) *m*/*z* 295 (M + H^+); HRMS (FAB) calcd for $C_{12}H_{11}N_2O_7$: 295.0566. Found: 295.0566.

3,5-Dimethoxybenzyl-*N*-succinimidyl carbonate (2b)

To a solution of 3,5-dimethoxybenzyl alcohol (**1b**) (500 mg, 3.0 mmol) and triethylamine (450 mg, 0.63 mL, 4.5 mmol) in acetonitrile (20 mL) was added disuccinimidyl carbonate (840 mg, 3.3 mmol), and the reaction mixture was stirred at room temperature for 20 min. The reaction mixture was diluted with CH_2Cl_2. The organic layer was washed by water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by recrystallization (*n*-hexane–toluene = 4 : 1) to give **2b** (0.92 g, quant.) as colorless crystals: mp 78–80 °C; IR ν_{max}/cm^{-1} (KBr) 1813, 1789, 1742, 1600, 1465, 1432, 1377, 1325, 1208, 1091, 1069; ^1H NMR (300 MHz; $CDCl_3$) δ 2.84 (4H, s), 3.80 (6H, s), 5.25 (2H, s), 6.46 (1H, d, J = 1.5 Hz), 6.52 (1H, d, J = 1.5 Hz); ^{13}C NMR (75 MHz; $CDCl_3$) δ 25.42, 55.41, 72.59, 101.24, 105.96, 135.35, 151.56, 161.02, 168.55; Mass (FAB) *m*/*z* 332 (M + Na^+); HRMS (FAB) calcd for $C_{14}H_{15}N_1O_7Na$: 332.0746. Found: 332.0742.

4,5-Dimethoxy-2-nitrobenzyl-*N*-succinimidyl carbonate (2c)

To a solution of 4,5-dimethoxy-2-nitrobenzyl alcohol (**1c**) (500 mg, 2.4 mmol) and triethylamine (360 mg, 0.49 mL, 3.5 mmol) in acetonitrile (20 mL) was added disuccinimidyl carbonate (660 mg, 2.6 mmol), and the reaction mixture was stirred at room temperature for 20 min. The reaction mixture was diluted with CH_2Cl_2. The organic layer was washed by water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The resulting mixture was purified by silica gel column chromatography (AcOEt–*n*-hexane = 1 : 2) to give **2c** (550 mg, 66%) as a pale yellow solid: mp 131–133 °C; IR ν_{max}/cm^{-1} (KBr) 1815, 1789, 1741, 1582, 1525, 1332, 1282, 1217, 1067; ^1H NMR (300 MHz; $CDCl_3$) δ 2.87 (4H, s), 3.98 (s, 3H), 4.07 (3H, s), 5.80 (2H, s), 7.06 (1H, s), 7.77 (1H, s); ^{13}C NMR (75 MHz; $CDCl_3$) δ 25.42, 56.43, 56.65, 69.14, 108.20, 108.60, 125.36, 139.07, 148.49, 151.36, 154.14, 168.44; Mass (FAB) *m*/*z* 377 (M + Na^+); HRMS (FAB) calcd for $C_{14}H_{14}N_2O_9Na$: 377.0597. Found: 377.0573.

1-(2-Nitrophenyl)ethyl-*N*-succinimidyl carbonate (2d)

To a solution of 2-nitroacetophenone (1.0 g, 6.1 mmol) in ethanol (30 mL) was added $NaBH_4$ (340 mg, 9.1 mmol) at 0 °C, and stirred at rt for 2 h. After addition of water, the resulting mixture was extracted twice with AcOEt. The combined organic layers were washed with water and brine. The organic layer was dried over Na_2SO_4 and concentrated to give 1-(2-nitrophenyl) ethanol (**1d**) as a crude compound. To the crude **1d** was added acetonitrile (30 mL), triethylamine (920 mg, 1.3 mL, 9.1 mmol), and disuccinimidyl carbonate (1.7 g, 6.7 mmol). The reaction mixture was stirred at room temperature for 1 h. The reaction mixture was diluted with CH_2Cl_2. The organic layer was washed by water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by recrystallization (*n*-hexane–toluene = 6 : 1) to give **2d** (1.6 g, 88%) as colorless crystals: mp 100–101 °C; IR ν_{max}/cm^{-1} (KBr) 1816, 1791, 1741, 1530, 1346, 1259, 1224, 1093; ^1H NMR (300 MHz; $CDCl_3$) δ 1.79 (3H, d, J = 6.5 Hz), 2.79 (4H, s), 6.39 (1H, q, J = 6.5 Hz), 7.51 (1H, ddd, J = 2.5, 7.0, 7.0 Hz), 7.71–7.75 (2H, m), 8.02 (1H, d, J = 8.0 Hz); ^{13}C NMR (75 MHz; $CDCl_3$) δ 22.07, 25.34, 75.92, 124.70, 126.90, 129.18, 134.27, 135.78, 147.14, 150.64, 168.43; Mass (FAB) *m*/*z* 309 (M + H^+); HRMS (FAB) calcd for $C_{13}H_{13}N_2O_7$: 309.0723. Found: 309.0714.

(3*RS*,4*RS*,5*RS*)-3,4-Epoxy-*N*-(2-nitrobenzyloxycarbonyl)-5-[4-(4-phenyl-1*H*-1,2,3-triazol-1-yl)benzyloxy]piperidine (4a)

To a solution of **3** (100 mg, 0.29 mmol) and triethylamine (44 mg, 60 μL, 0.44 mmol) in CH_2Cl_2 (3.0 mL) was added **2a**

(93 mg, 0.32 mmol), and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was diluted with AcOEt. The organic layer was washed by water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography (AcOEt–*n*-hexane = 1 : 1) to give **4a** (138 mg, 91%) as a white solid: mp 132–133 °C; IR ν_{max}/cm^{-1} (KBr) 1703, 1523, 1461, 1427, 1341, 1233, 1125, 1097; ^1H NMR (400 MHz; $CDCl_3$) δ 3.32–3.35 (2H, m), 3.47 (1H, ddd, J = 3.0, 14.5, 18.0 Hz), 3.65 (1H, ddd, J = 5.0, 14.0 Hz), 3.85–3.98 (3H, m), 4.62–4.82 (2H, m), 5.50–5.57 (2H, m), 7.36–7.63 (8H, m), 7.77 (2H, dd, J = 4.5, 8.0 Hz), 7.92 (1H, d, J = 8.0 Hz), 8.08 (1H, d, J = 8.0 Hz), 8.19 (1H, s); ^{13}C NMR (75 MHz; DMSO-d_6, 80 °C) δ 41.54, 41.65, 49.35, 51.15, 62.81, 69.66, 69.90, 119.04, 119.64, 124.07, 125.10, 127.72, 128.44, 128.65, 128.78, 130.04, 131.48, 133.31, 135.72, 138.44, 147.02, 147.27, 154.20; Mass (FAB) *m*/*z* 528 (M + H^+); HRMS (FAB) calcd for $C_{28}H_{26}N_5O_6$: 528.1883. Found: 528.1895; *Anal.* Calcd for $C_{28}H_{25}N_5O_6$: C, 63.75; H, 4.78; N, 13.28. Found: C, 63.81; H, 4.86; N, 13.22.

(3*RS*,4*RS*,5*RS*)-*N*-(3,5-Dimethoxybenzyloxycarbonyl-3,4-epoxy)-5-[4-(4-phenyl-1*H*-1,2,3-triazol-1-yl)benzyloxy]piperidine (4b)

To a solution of **3** (50 mg, 0.14 mmol) and triethylamine (22 mg, 30 μL, 0.22 mmol) in CH_2Cl_2 (1.5 mL) was added **2b** (49 mg, 0.16 mmol), and the reaction mixture was stirred at room temperature for 30 min. The reaction mixture was diluted with AcOEt. The organic layer was washed by water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The resulting mixture was purified by silica gel column chromatography (AcOEt–*n*-hexane = 1 : 1) to give **4b** (58 mg, 74%) as a white solid: mp 73–77 °C; IR ν_{max}/cm^{-1} (KBr) 1698, 1598, 1521, 1459, 1424, 1360, 1322, 1281, 1234, 1205, 1154, 1125, 1092, 1070, 1038; ^1H NMR (400 MHz; $CDCl_3$) δ 3.28–3.33 (2H, m), 3.46 (1H, dd, J = 3.0, 14.0 Hz), 3.63 (1H, dd, J = 5.0, 14.0 Hz), 3.75 (3H, s), 3.78 (3H, s), 3.85–3.98 (3H, m), 4.61–4.85 (2H, m), 5.05–5.01 (2H, m), 6.41 (1H, s), 6.50 (2H, s), 7.36–7.54 (5H, m), 7.72 (1H, d, J = 8.0 Hz), 7.77 (1H, d, J = 8.0 Hz), 7.92 (2H, d, J = 8.0 Hz), 8.20 (1H, s); ^{13}C NMR (75 MHz; DMSO-d_6, 80 °C) δ 41.54, 41.60, 49.38, 51.21, 54.83, 65.88, 69.65, 69.93, 99.40, 105.08, 119.04, 119.65, 125.10, 127.72, 128.45, 130.05, 135.72, 138.45, 138.81, 147.03, 154.65, 160.31; Mass (FAB) *m*/*z* 543 (M + H^+); HRMS (FAB) calcd for $C_{30}H_{31}N_4O_6$: 543.2244. Found: 543.2225.

(3*RS*,4*RS*,5*RS*)-*N*-(4,5-Dimethoxy-2-nitrobenzyloxycarbonyl)-3,4-epoxy-5-[4-(4-phenyl-1*H*-1,2,3-triazol-1-yl)benzyloxy]-piperidine (4c)

To a solution of **3** (50 mg, 0.14 mmol) and triethylamine (22 mg, 30 μL, 0.22 mmol) in CH_2Cl_2 (1.5 mL) was added **2c** (56 mg, 0.16 mmol), and the reaction mixture was stirred at room temperature for 30 min. The reaction mixture was diluted with AcOEt. The organic layer was washed by water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The resulting mixture was purified by silica gel column chromatography (AcOEt–*n*-hexane = 1 : 1) to give **4c** (62 mg, 74%) as a pale yellow foam: IR ν_{max}/cm^{-1} (KBr) 1704, 1580, 1522, 1459, 1433, 1362, 1328, 1278, 1247, 1220, 1123, 1093, 1067, 1037; ^1H NMR (400 MHz; $CDCl_3$) δ 3.30–3.33 (2H, m), 3.57 (1H, dd, J = 5.0, 13.0 Hz), 3.59 (1H, d, J = 5.0 Hz), 3.91 (6H, s), 3.92–4.07 (3H, m), 4.62–4.81 (2H, m), 5.42–5.64 (2H, m), 6.94 (1H, s), 7.37–7.40 (1H, m), 7.42–7.51 (4H, m), 7.71–7.76 (3H, m), 7.93 (2H, d, J = 8.0 Hz), 8.22 (1H, s); ^{13}C NMR (75 MHz; DMSO-d_6, 90 °C) δ 41.59, 41.74, 49.32, 51.23, 55.87, 55.91, 62.99, 69.66, 69.85, 99.65, 108.41, 111.02, 118.91, 119.55, 125.07, 126.50, 127.65, 128.37, 130.02, 135.70, 138.36, 139.54, 146.98, 147.81, 153.07, 154.27; Mass (FAB) *m*/*z* 588 (M + H^+); HRMS (FAB) calcd for $C_{30}H_{30}N_5O_8$: 588.2094. Found: 588.2097.

(3*RS*,4*RS*,5*RS*)-3,4-Epoxy-*N*-(1-(2-nitrophenyl)ethoxycarbonyl)-5-[4-(4-phenyl-1*H*-1,2,3-triazol-1-yl)benzyloxy]piperidine (4d)

To a solution of **3** (100 mg, 0.29 mmol) and triethylamine (44 mg, 60 μL, 0.44 mmol) in CH_2Cl_2 (3.0 mL) was added **2d** (88 mg, 0.29 mmol), and the reaction mixture was stirred at room temperature for 10 min. The reaction mixture was diluted with AcOEt. The organic layer was washed by water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The resulting mixture was purified by silica gel column chromatography (AcOEt–*n*-hexane = 1 : 1) to give a diastereomixture of **4d** (150 mg, 97%) as a white foam: IR ν_{max}/cm^{-1} (KBr) 1698, 1523, 1458, 1425, 1354, 1248, 1125, 1091, 1039; ^1H NMR (400 MHz; $CDCl_3$) δ 1.65–1.67 (3H, m), 3.32–3.87 (4H, m), 3.96–3.98 (2H, m), 4.52–4.89 (2H, m), 6.19–6.29 (1H, s), 7.33–7.92 (10H, m), 7.86–7.96 (3H, m), 8.17–8.22 (1H, m); Mass (FAB) *m*/*z* 542 (M + H^+); HRMS (FAB) calcd for $C_{29}H_{28}N_5O_6$: 542.2040. Found: 542.2047.

N-2-Nitrobenzyloxycarbonylpiperidine (5a)

To a solution of piperidine (100 mg, 1.17 mmol) in CH_2Cl_2 (5 mL) was added triethylamine (177 mg, 0.24 mL, 1.76 mmol) and **2a** (300 mg, 1.17 mmol) at rt. The reaction mixture was stirred for 15 min at room temperature, diluted with Et_2O, and washed with water and brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography (AcOEt–*n*-hexane = 1 : 2) to give **5a** (250 mg, 72%) as a yellow oil; IR ν_{max}/cm^{-1} (KBr): 2983, 2857, 1702, 1527, 1469, 1433, 1343, 1283, 1147, 1092, 1076, 1026; ^1H NMR (400 MHz; $CDCl_3$) δ 1.54–164 (6H, m), 3.46 (4H, brs), 5.53 (2H, s), 7.47 (1H, dt, J = 1.5, 8.0 Hz), 7.56 (1H, dd, J = 1.0, 8.0 Hz), 7.64 (1H, dt, J = 1.5, 8.0 Hz), 8.07 (1H, dd, J = 1.0, 8.0 Hz); ^{13}C NMR (75 MHz; DMSO-d_6, 80 °C) δ 23.3, 24.8, 44.1, 62.5, 124.0, 128.5, 128.8, 131.8, 133.2, 147.4, 153.6; Mass (FAB) *m*/*z* 265 (M + H^+); HRMS (FAB) calcd for $C_{13}H_{17}N_2O_4$: 265.1188. Found: 265.1194.

N-1-(2-Nitrophenyl)ethoxycarbonyl piperidine (5b)

To a solution of piperidine (100 mg, 1.17 mmol) in CH_2Cl_2 (5 mL) was added triethylamine (177 mg, 0.24 mL, 1.76 mmol) and **2d** (360 mg, 1.17 mmol) at rt. The reaction mixture was

stirred for 15 min at room temperature, diluted with Et_2O, and washed with water and brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography (AcOEt–*n*-hexane = 1 : 2) to give **5a** (310 mg, 95%) as a yellow oil; IR ν_{max}/cm^{-1} (KBr): 2938, 2857, 1698, 1526, 1468, 1430, 1350, 1283, 1262, 1234, 1147, 1086, 1069, 1023; ^1H NMR (400 MHz; $CDCl_3$) δ: 1.53–1.59 (6H, m), 1.64 (3H, d, J = 6.5 Hz), 3.38–3.47 (4H, m), 6.25 (1H, q, J = 6.5 Hz), 7.38–7.42 (1H, m), 7.58–7.63 (2H, m), 7.91 (1H, dd, J = 1.5, 8.0 Hz); ^{13}C NMR (75 MHz; DMSO-d_6, 80 °C) δ: 21.6, 23.6, 25.2, 44.3, 68.0, 123.8, 127.2 128.5, 133.4, 137.4, 147.7, 153.5; Mass (FAB) m/z 279 ($M + H^+$); HRMS (FAB) calcd for $C_{14}H_{19}N_2O_4$: 279.1345. Found: 279.1327.

Relaxation assay of supercoiled plasmid DNA

To a solution of supercoiled pBR 322 DNA (0.15 μg) in pH 7.0 TE buffer (9 μL) was added a DMSO solution of the compounds (1 μL, 10 μM or 100 μM). The reaction mixture was photo-irradiated for 2, 5, 15 or 60 s, and the mixture was incubated for 24 h at 37 °C. The resulting DNA analysis was conducted using electrophoresis (tris-acetate-EDTA buffer, ethidium bromide 1.3 μM solution) on 0.7% native agarose gel at 7.4 v cm^{-1} for 30 min.

Relaxation assay of supercoiled plasmid DNA in the presence of hydroxyl radical or singlet oxygen scavengers

To a solution of supercoiled pBR 322 DNA (0.15 μg) in pH 7.0 TE buffer (9 μL) was added a DMSO solution of the compounds (1 μL, 10 μM or 100 μM) and glycerol (10 mM) or NaN_3 (10 mM). The reaction mixture was photoirradiated for 15 s, and the mixture was incubated for 24 h at 37 °C. The resulting DNA analysis was conducted using electrophoresis (tris-acetate-EDTA buffer, ethidium bromide 1.3 μM solution) on 0.7% native agarose gel at 7.4 V cm^{-1} for 30 min.

Assessment of cytotoxicity of caged DNA alkylating agents toward HepG2 cells under UV irradiation

The human hepatoblastoma cell lines HepG2 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g L^{-1} glucose, supplemented with 10% (v/v) fetal bovine serum (FBS), 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Compounds **3** and **4a–4d** were dissolved in DMSO (100 μM, 1 mM or 10 mM). Each 100 μL of cell suspension was seeded into a 96-well flat-bottomed microplate at a density of 10 000 cells per well and incubated at 37 °C in a 5% CO_2 humidified atmosphere for 24 h. Compound solution (1 μL) was added (for evaluation of membrane permeability, interval times of 0–12 h were used), then UV rays were irradiated for each respective time. After 48 h (for evaluation of membrane permeability, after difference time calculated by interval time to 48 h), 10 μL of MTT solution was added to each well, followed by further incubation for 4 h. 100 μL of 2-propanol was added. The optical densities were observed using a Microplate Reader (BIO-RAD Model 550) at 595 nm.

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