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New benzoxanthone derivatives as topoisomerase inhibitors and DNA cross-linkers

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ABSTRACT

We synthesized 12 benzoxanthone derivatives classified as three different groups based on the tetracyclic ring shapes and evaluated their pharmacological activities to find potential anticancer agents. In the cytotoxicity test, most compounds showed effective cancer cell growth inhibition against the HT29 and DU145 cell lines. Among the compounds tested, compound **19** was the most effective in the cancer cell lines tested. Compound **9** showed dual inhibitory activities against DNA relaxation by topoisomerases I and II. The% inhibition of compound **9** on topoisomerase I was comparable to that of camptothecin. Compound **9** efficiently blocked topoisomerase II function by almost threefold than etoposide at 20 μM. Compound **19** had selective topoisomerase II inhibitory activity at 100 μM. The DNA cross-linking test revealed that only compounds **8** and **19**, which possess epoxy groups, cross-linked DNA duplex, while **14** did not. From the combined pharmacological results, we proposed that the target through which compound **19** inhibits cancer cell growth may be the DNA duplex itself and/or DNA–topoisomerase II complex.

1. Introduction

Xanthones[1] (**1**), which are mainly found as secondary metabolites from higher plants and microorganisms, have diverse pharmacological activities such as anti-hypertensive,[2] anti-thrombotic,[3] and anticancer activity,[4,5] based on their diverse structures. Simple and interesting structural scaffolds and diverse biological spectra of xanthones have led many scientists to isolate or synthesize xanthone analogues for the development of prospective drug candidates. Previously, we reported the synthesis and biological activities of some epoxy-tethered xanthone analogues.[5] The prepared compounds showed significant topoisomerase II inhibitory activities and the bis-oxiranylmethoxy-substituted compound (**2**) was the most efficient among the compounds tested.

A quinobenzoxazine, A-62176 (**3**), a fluoroquinolone analogue, is a catalytic inhibitor of topoisomerase II and a poison under certain conditions.[6,7] Hurley and co-workers reported that ring expansion of **3** increased the poison activity and reduced the catalytic function. FQA-CS (**4**) was the most effective topoisomerase II poison.[7]

Topoisomerases, generally classified as types I and II, are critical cellular enzymes necessary for cell proliferation that remove topological hurdles in the process of DNA replication.[8] Topoisomerase I mediates the breaking and rejoining of a single strand of the DNA duplex to relax supercoiled chromosomes. On the other hand, topoisomerase II relaxes DNA double helices by working on two strands. Due to the critical role of these enzymes in the cell proliferative process, topoisomerases have been one of the major targets in anticancer drug development.

Interstrand DNA cross-linking of two strands of DNA inhibits cascade of the cell proliferation, such as replication and transcription, and finally causes cell death.[9,10] Some antitumor compounds possessing two electrophilic functions, such as mitomycin C and nitrogen mustard, can act as DNA cross-linking agents, which is one of the important classes of antitumor drugs targeting DNA.[11,12]

In the continued effort to develop anticancer drug candidates that target topoisomerases and DNA, we designed a series of new benzoxanthone analogues possessing epoxy or thioepoxy groups. For this purpose we synthesized three different groups of benzoxanthones according to the position of the fourth phenyl ring to the xanthone core. We expected that the shapes of resulting benzoxanthones would affect their mode of interaction such as intercalation and/or binding to DNA and enzymes. Here, we report the synthesis of 12 benzoxanthone analogues and their pharmacological activities including cytotoxicity, topoisomerase inhibition, and DNA cross-linking.

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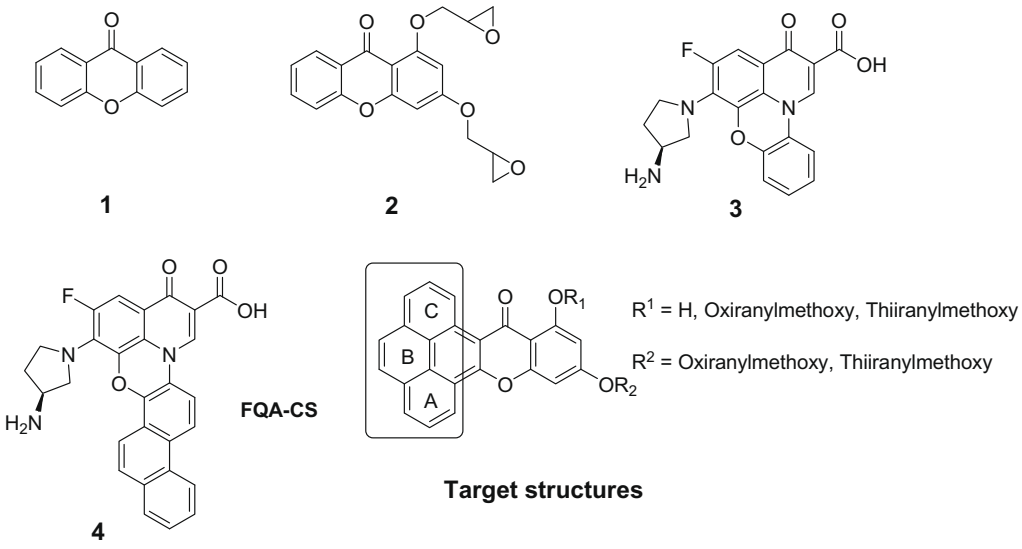


2. Results and discussion

2.1. Chemistry

Synthetic methods used for the preparation of benzoxanthone derivatives are shown in Scheme 1. Starting dihydroxybenzoxanthones **5**,[13] **10**,[14] and **15**[14] were synthesized by modifying or employing the reported methods. Coupling reactions of benzoxanthones with epichlorohydrin or epithiochlorohydrin were accomplished as reported previously by our research group.[5,15] First, coupling of the 8,10-dihydroxy-7*H*-benzo[*c*]xanthen-7-one (**5**) with chlorohydrins gave the monoepoxy- and bisepoxy-benzoxanthone compounds depending on the amount of K_2CO_3 and chlorohydrins used. One equivalent K_2CO_3 and three equivalents of epichlorohydrin or epithichlorohydrin provided monoepoxy-substituted **6** or monothioepoxy-substituted **7**, respectively. On the other hand, increment of K_2CO_3 and chlorohydrins by two and fivefold each furnished bisepoxy **8** (23.5% yield) or bisthioepoxy-benzoxanthone **9** (13.2% yield), respectively. Second, coupling reaction of 9,11-dihydroxy-12*H*-benzo[*a*]xanthen-12-one (**10**) with 5–6-fold of chlorohydrins in acetone in the presence of 3 equiv of Cs_2CO_3 afforded mono- and bisepoxy-benzoxanthone compounds **11**, **13**, **12**, and **14** as a mixture. Finally, 1,3-dihydroxy-12*H*-benzo[*b*]xanthen-12-one (**15**) with epithiochlorohydrin or epichlorohydrin in the presence of K_2CO_3 gave monothioepoxy or monoepoxy derivatives **16** and **17**. But replacement of K_2CO_3 with Cs_2CO_3 base afforded bisthioepoxy or bisepoxy derivatives **18** and **19**, respectively. In the ^{1}H NMR spectra, most mono-substituted benzoxanthones showed singlet peaks at δ ~13.00 corresponding to the hydroxyl protons adjacent to the carbonyl group in the benzoxanthone ring. These singlet peaks disappeared in the bisepoxy derivatives, which indicates introduction of two chlorohydrins groups at two hydroxyl groups. All other spectroscopic data were consistent with the proposed structures.

2.2. Pharmacological evaluation

2.2.1. Cytotoxicity test

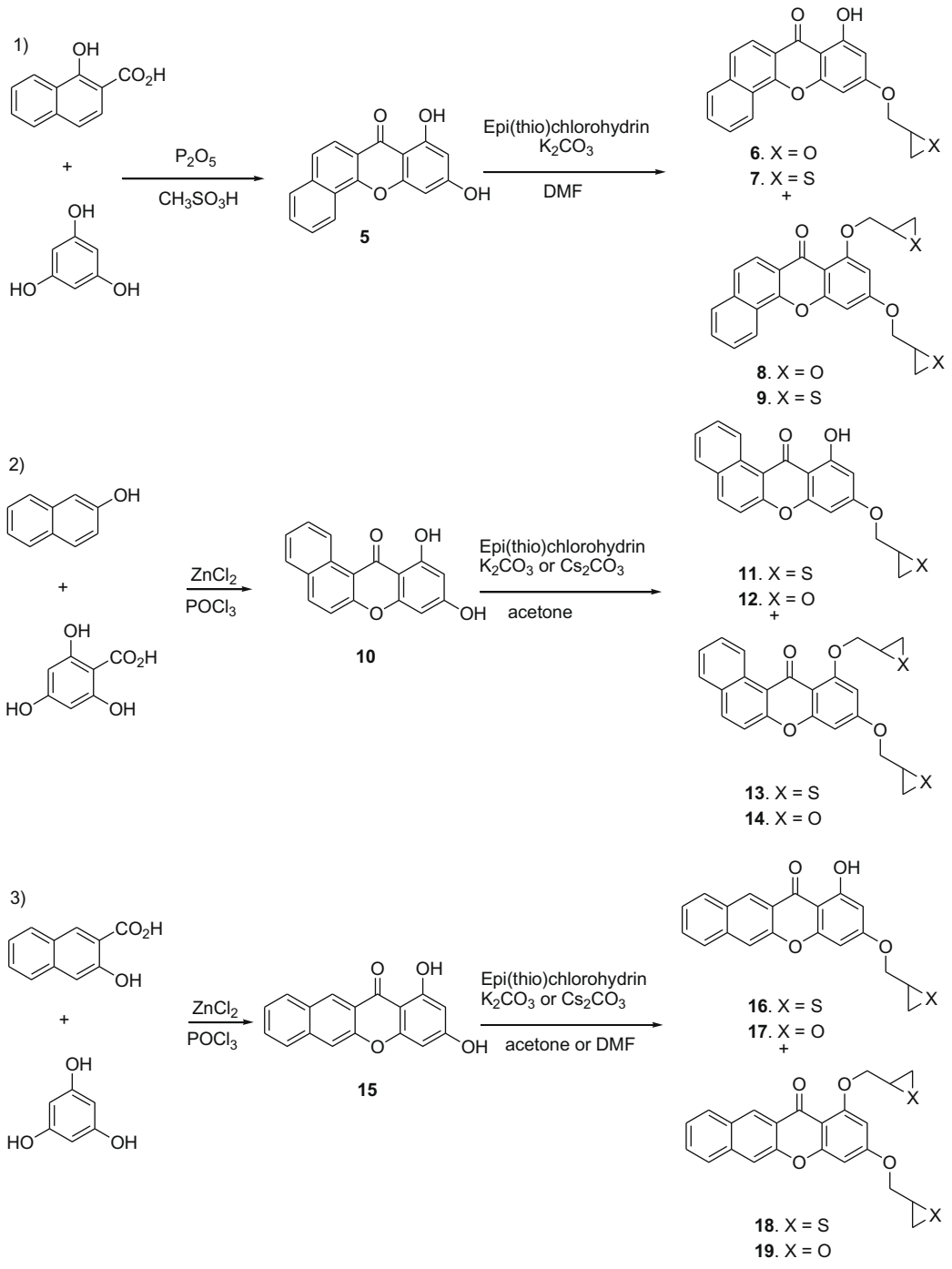
For the evaluation of cytotoxicity, five different cancer cell lines were tested: human breast adenocarcinoma cell line (MDA-MB231), human cervix tumor cell line (HeLa), human prostate tumor cell line (DU145), human colorectal adenocarcinoma cell line (HT29), and human myelogenous leukemia cell line (HL60). The typical MTT assay procedure was applied for this assessment.[16] The inhibitory activities of the compounds are shown in Table 1. Generally, most compounds effectively inhibited cancer cell proliferation. Among these, compounds **13** and **19** showed stronger suppression of the HT29 cancer cell line than did adriamycin, etoposide, and camptothecin, which were used as references. Compound **19**, especially, exhibited significant cytotoxicity in the tested cancer cell lines when compared to other compounds.

2.2.2. Topoisomerases I and II inhibition test

Topoisomerase relaxation inhibitory activities were evaluated using human topoisomerases I and II (Topogen) with camptothecin and etoposide as positive controls.[17] Data were analyzed and evaluated with LabWork 4.5 Software to calculate the inhibition ratio. Test results are indicated in Figure 1 and Table 2. Compound **9** showed comparable topoisomerase I inhibitory activity to camptothecin at the test concentrations. Interestingly, it was a better topoisomerase II inhibitor than etoposide, which was used as positive control, exhibiting about 2.5-fold higher efficiency at 20 μM. On the other hand, compound **19** exerted selective inhibitory activity on topo II function. It has almost the same capacity for topo II inhibition as the reference at 100 μM, but its inhibitory capacity was lower at 20 μM. All other compounds were inactive in the topoisomerase relaxation pathway. Although there were no clear correlations between topoisomerase inhibitory activities and structures of the benzoxanthone derivatives, the observed results suggest that the shape of the tetracyclic system and the incorporated functional groups is an important factor for interaction between the DNA–protein complex and benzoxanthone analogues during DNA unwinding process.

2.2.3. DNA cross-linking test

Previously we reported that 1,3-bisepoxyxanthone (**2**) efficiently cross-linked DNA duplex due to the electrophilic nature of bisepoxy groups. DNA cross-linking property was measured using linearized pBR 322 DNA by alkaline agarose gel electrophoresis.[18] Only selected bis-substituted compounds were tested. The DNA cross-linking profiles for the benzoxanthone compounds are shown in Figure 2. Compounds **8** and **19** possessing epoxy groups showed strong DNA cross-linking activities, but others that had thiiranyl groups were inactive to nucleophiles. This observation was consistent with previous results.[5] Interestingly, compound **14** did not generate any DNA cross-linked adducts, although it had bisepoxy groups. These results implied that the intercalation pattern of the benzoxanthone was attributed to the DNA cross-linking process, which means that the location of the compound in the DNA–compound complex is important to provide



Scheme 1. Synthetic methods of benzoxanthone compounds.

proper distance necessary for nucleophilic attack of the DNA base to epoxy groups. Expansion of the phenyl ring toward the upper side of xanthone core might disfavor intercalation of benzoxanthone into DNA base pairs because of disrupted π–π stacking interaction between DNA base pairs and compound **14**.

3. Conclusions

We synthesized 12 benzoxanthone derivatives classified as three different groups based on the tetracyclic ring shapes and evaluated their pharmacological activities to find potential anticancer agents. In the cytotoxicity test, most compounds showed effective cancer cell growth inhibition against the HT29 and DU145 cell lines. Among the compounds, compound **19** was the most effective in the cancer cell lines tested. Generally, the prepared compounds were not active against topoisomerase functions. Compound **9**, however, showed dual inhibitory activities on DNA relaxation by topoisomerases I and II. The% inhibitory values of compound **9** on topoisomerase I were comparable to those of camptothecin at test concentrations. Compound **9** efficiently blocked topoisomerase II function by almost threefold than etoposide at 20 μM. Compound **19** has selective topoisomerase II inhibitory activity at 100 μM. The DNA cross-linking test revealed that only compounds **8** and **19** possessing epoxy groups cross-linked DNA duplex but **14** did not. These observations suggested that

Table 1
Cytotoxicities of compounds **6**–**9**, **11**–**14**, and **16**–**19** against various cancer cells

Compd/cells	IC_{50}[a] (μM)				
	HeLa	HT29	DU145	MDA-MB231	HL60
Adriamycin	0.71 ± 0.01	0.95 ± 0.00	1.19 ± 0.16	3.15 ± 0.23	0.94 ± 0.07
Etoposide	0.47 ± 0.07	1.42 ± 0.06	1.63 ± 0.08	2.87 ± 0.29	0.97 ± 0.07
Camptothecin	0.86 ± 0.03	0.85 ± 0.03	3.57 ± 1.03	0.92 ± 0.01	0.065 ± 0.0011
6	>50	18.93 ± 1.82	2.56 ± 0.37	0.73 ± 0.04	3.67 ± 0.71
7	>50	>50	10.84 ± 0.60	>50	22.58 ± 0.14
8	24.17 ± 0.46	8.20 ± 1.38	1.29 ± 0.06	7.43 ± 0.20	5.40 ± 0.12
9	25.60 ± 0.49	>50	5.66 ± 0.76	>50	37.04 ± 7.05
11	>100	3.58 ± 1.75	8.76 ± 2.21	29.13 ± 7.66	3.48 ± 0.80
12	>100	0.96 ± 0.05	1.40 ± 0.39	>50	2.31 ± 0.07
13	24.95 ± 1.74	0.75 ± 0.27	1.32 ± 0.16	18.86 ± 0.78	1.85 ± 0.00
14	10.15 ± 1.19	7.38 ± 2.98	3.44 ± 1.42	>50	6.47 ± 0.15
16	8.52 ± 0.44	5.40 ± 2.91	1.55 ± 0.03	14.84 ± 1.77	2.48 ± 0.15
17	>100	2.34 ± 0.07	36.20 ± 0.31	15.08 ± 0.21	9.40 ± 0.46
18	8.01 ± 0.76	1.66 ± 0.06	19.37 ± 4.26	5.16 ± 0.82	5.13 ± 0.72
19	2.19 ± 0.70	0.60 ± 0.31	3.01 ± 0.22	1.64 ± 0.06	5.13 ± 0.07

[a] Each data point represents mean ± SD from three different experiments performed in triplicate.

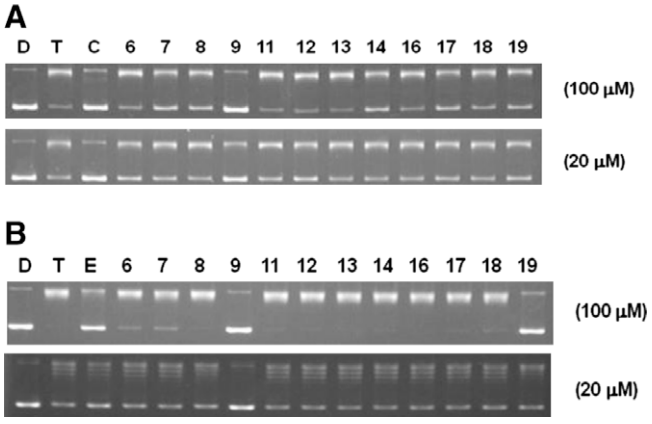


Figure 1. Topo I (A) and II (B) inhibitory activities of compounds. Compounds were examined at a final concentration of 20 and 100 μM as designated. (A) Lane D: pBR322 only, Lane T: pBR322 + Topo I, Lane C: pBR322 + Topo I + camptothecin, Lanes **6**–**19**: pBR322 + Topo I + compounds at designated concentrations. (B) Lane D: pBR322 only, Lane T: pBR322 + Topo II, Lane E: pBR322 + Topo II + etoposide, Lanes **6**–**19**: pBR322 + Topo II + compounds at designated concentrations.

Table 2
Topo I and II inhibitory activities of compounds **6**–**9**, **11**–**14**, and **16**–**19**

Compounds	Topo I (% inhibition)		Topo II (% inhibition)	
	Concentration			
	100 μM	20 μM	100 μM	20 μM
Camptothecin	78.3	75.5	—	—
Etoposide	—	—	54.4	27.9
6	1.7	10.6	9.0	5.5
7	15.6	2.5	13.9	0.0
8	11.9	0.0	5.7	0.1
9	79.0	56.5	71.9	72.7
11	0.0	2.4	5.3	0.0
12	0.0	9.7	3.7	0.0
13	0.0	0.0	4.4	8.4
14	18.0	0.0	1.0	0.0
16	0.5	0.0	0.0	7.2
17	13.7	2.6	0.0	0.0
18	4.3	0.0	1.9	13.5
19	12.3	8.8	60.1	8.9

DNA cross-linking occurs after intercalation of the tetracyclic ring into DNA base pairs and this process might be dependent on the shape of the tetracyclic ring system of benzoxanthone derivatives. From the combined pharmacological results, we proposed that the target of compound **19** action by which it inhibited cancer growth was the DNA duplex itself and/or DNA–topoisomerase II complex.

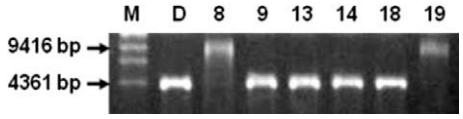


Figure 2. Cross-linking assay of selected compounds. Lane M: DNA HindIII marker, Lane D: linearized pBR322 plasmid DNA only, Lanes **8**, **9**, **13**, **14**, **18**, and **19**; linearized pBR322 + each compound at a final concentration of 100 μM.

In summary, oxiranylmethoxy- or thiiranylmethoxy-substituted benzoxanthone derivatives show potential for development as new anticancer agents following elaborate optimization of substituent functionalities.

4. Experimental

4.1. General

The solvents and reactants were of the best commercial grade available and were used without further purification unless noted. TLC plates were Kieselgel 60 F_{254} (art A715, Merck). Silica gel 60 (0.040–0.063 mm ASTM, Merck) was used for column chromatography. ^{1}H and ^{13}C NMR spectra were recorded on Varian NMR AS 400 MHz instrument. Chemical shifts (δ) are in parts per million (ppm) relative to tetramethylsilane as an internal standard, and coupling constants (J values) are in hertz. Mass spectral investigations were performed on a LCQ advantage-trap mass spectrometer equipped with an electrospray ionization (ESI) source or a GC-2010 (Shimadzu, USA) mass spectrometer equipped with an electron ionization (EI) source. Melting points were measured on a Barnstead International MEL-TEMP 1202D instrument without correction.

4.1.1. 8,10-Dihydroxy-7*H*-benzo[*c*]xanthen-7-one (5)

The mixture of phosphorous pentoxide (2.84 g, 0.02 mol) and methanesulfonic acid (40 mL) was stirred at 90 °C for 1 h until the mixture changed to a clear solution. To this solution were added 1-hydroxy-2-naphthoic acid (3.76 g, 0.02 mol) and phloroglucinol (3.02 g, 0.02 mmol). After stirring of the mixture at 90 °C for 30 min, the reaction mixture was poured into ice-water. The solid formed was left for 1 d and then filtered, washed with water

and dried under vacuum to give a red solid. Purification by silica gel column chromatography (eluant: ethyl acetate/*n*-hexane = 1:3) afforded compound **5** as a yellow solid (535 mg, 9.61%). R_f: 0.43 (ethyl acetate/*n*-hexane = 1:3); ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.16 (s, 1H), 6.35 (s, 1H), 7.52 (dd, J = 7.2, 6.8 Hz, 1H), 7.65 (dd, J = 7.2, 6.8 Hz, 1H), 7.99 (s, 2H), 8.17 (d, J = 8.0 Hz, 1H), 8.76 (s, 1H), 12.84 (s, 1H).

4.1.2. 8-Hydroxy-10-(oxiran-2-ylmethoxy)-7*H*-benzo[*c*]xanthen-7-one (6)

To a mixture of compound **5** (150 mg, 0.54 mmol) and K_2CO_3 (74.5 mg, 0.54 mmol) was added DMF (20 mL). After addition of epichlorohydrin (0.13 mL, 1.62 mmol), the reaction mixture was stirred for 15 h at 70 °C and then cooled to room temperature. The mixture was diluted with water (20 mL) and extracted with ethyl acetate. The combined organic layer was washed with brine and dried over Na_2SO_4. After evaporation of the solvent under reduced pressure, the residue was applied to silica gel column chromatography (eluant: ethyl acetate/*n*-hexane = 1:1) to afford compound **6** as a pale yellow solid (37 mg, 20.6%). Mp 266 °C; R_f 0.84 (eluant: Ethyl acetate/*n*-hexane = 1:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.80 (dd, J = 2.4, 4.8 Hz, 1H), 2.96 (dd, J = 4.6, 4.8 Hz), 3.40–3.42 (m, 1H), 4.05 (dd, J = 2.0, 10.9 Hz, 1H), 4.38 (dd, J = 2.6, 10.9 Hz, 1H), 6.42 (d, J = 2.0 Hz, 1H), 6.66 (d, J = 2.0 Hz, 1H), 7.69–7.75 (m, 2H), 7.74 (d, J = 8.6 Hz, 1H), 7.93 (d, J = 7.6 Hz, 1H), 8.16 (d, J = 8.6 Hz, 1H), 8.61 (d, J = 8.0 Hz, 1H), 12.93 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) 44.8, 50.0, 69.5, 93.7, 98.2, 105.0, 116.5, 120.6, 123.0, 123.9, 124.4, 127.3, 128.4, 130.0, 136.9, 154.0, 157.5, 163.5, 165.2, 180.9 ppm; LC-ESI: m/e 335.2 $[M+1]^+$.

4.1.3. 8-Hydroxy-10-(thiiran-2-ylmethoxy)-7*H*-benzo[*c*]xanthen-7-one (7)

To a mixture of compound **5** (100 mg, 0.36 mmol) and K_2CO_3 (49.7 mg, 0.36 mmol) was added DMF (20 mL). After addition of epithiochlorohydrin (117 mg, 1.08 mmol), the reaction mixture was stirred for 15 h at 70 °C and then cooled to room temperature. The mixture was diluted with water (20 mL) and extracted with ethyl acetate. The combined organic layer was washed with brine and dried over Na_2SO_4. After evaporation of the solvent under reduced pressure, the residue was applied to silica gel column chromatography (eluant: CH_2Cl_2) to afford compound **7** as a pale yellow solid (54 mg, 42.8%). Mp 214 °C; R_f 0.76 (eluant: CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.32 (dd, J = 2.4, 5.5 Hz, 1H), 2.60 (d, J = 5.5 Hz, 1H), 3.20–3.29 (m, 1H), 3.99 (dd, J = 6.8, 10.0 Hz, 1H), 4.25 (dd, J = 5.6, 10.0 Hz, 1H), 6.36 (d, J = 2.4 Hz, 1H), 6.59 (d, J = 2.4 Hz, 1H), 7.63–7.69 (m, 2H), 7.69 (d, J = 8.8 Hz, 1H), 7.88 (dd, J = 1.2, 8.8 Hz, 1H), 8.11 (d, J = 8.8 Hz, 1H), 8.55 (dd, J = 1.4, 8.2 Hz, 1H), 12.87 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) 31.0, 35.8, 73.2, 93.6, 98.2, 105.0, 116.5, 120.6, 123.0, 123.9, 124.4, 127.3, 128.4, 130.0, 136.9, 154.0, 157.6, 163.5, 165.1, 180.9 ppm; LC-ESI: m/e 351.2 $[M+1]^+$.

4.1.4. 8,10-Bis(oxiran-2-ylmethoxy)-7*H*-benzo[*c*]xanthen-7-one (8)

To a mixture of compound **5** (100 mg, 0.36 mmol) and K_2CO_3 (99.4 mg, 0.72 mmol) was added DMF (15 mL). After addition of epichlorohydrin (0.14 mL, 1.8 mmol), the reaction mixture was stirred for 24 h at 70 °C and then cooled to room temperature. The mixture was diluted with water and extracted with ethyl acetate. The combined organic layer was washed with brine and dried over Na_2SO_4. After evaporation of the solvent under reduced pressure, the residue was applied to silica gel column chromatography (eluant: ethyl acetate/*n*-hexane = 2:1) to afford compound **8** as an orange solid (33 mg, 23.5%). Mp 154 °C; R_f 0.30 (eluant: ethyl acetate/*n*-hexane = 1:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.83 (dd, J = 2.4, 8.8 Hz, 1H), 2.97–3.01 (m, 2H), 3.19–3.22 (m, 1H), 3.41–3.45 (m, 1H), 3.50–3.53 (m, 1H), 4.05 (ddd, J = 1.2, 6.0, 11.2 Hz, 1H), 4.20 (dd, J = 4.0, 11.2 Hz, 1H), 4.38–4.43 (m, 2H), 6.48 (d, J = 2.2 Hz, 1H), 6.72 (d, J = 2.2 Hz, 1H), 7.65–7.70 (m, 2H), 7.70 (d, J = 8.8 Hz, 1H), 7.91 (dd, J = 2.2, 7.0 Hz, 1H), 8.24 (d, J = 8.8 Hz, 1H), 8.55 (dd, J = 2.0, 7.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) 44.8, 45.2, 50.0, 50.3, 69.3 (69.2), 69.5, 94.6, 97.6, 108.7, 118.9, 121.9, 122.7, 123.8, 124.1, 126.9, 128.2, 129.3, 136.4, 159.4, 160.8, 163.4, 152.3, 175.4 ppm; LC-ESI: m/e 391.2 $[M+1]^+$.

4.1.5. 8,10-Bis(thiiran-2-ylmethoxy)-7*H*-benzo[*c*]xanthen-7-one (9)

To a mixture of compound **5** (200 mg, 0.72 mmol) and K_2CO_3 (198.67 mg, 1.44 mmol) was added DMF (20 mL). After addition of epithiochlorohydrin (0.39 g, 3.59 mmol) the reaction mixture was kept stirring for 24 h at 70 °C and then cooled to room temperature. The mixture was diluted with water and extracted with ethyl acetate. The combined organic layer was washed with brine and dried over Na_2SO_4. After evaporation of the solvent under reduced pressure, the residue was applied to silica gel column chromatography (eluant: ethyl acetate/*n*-hexane = 2:1) to afford compound **9** as a white solid (40 mg, 13.2%). Mp 202 °C; R_f 0.59 (ethyl acetate/*n*-hexane = 1:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.39 (dd, J = 1.4, 5.0 Hz, 1H), 2.55 (d, J = 5.2 Hz, 1H), 2.68 (d, J = 6.4 Hz, 1H), 2.73 (d, J = 6.0 Hz, 1H), 3.30–3.35 (m, 1H), 3.47–3.53 (m, 1H), 3.97 (dd, J = 7.6, 10.1 Hz, 1H), 4.09 (dd, J = 6.6, 10.3 Hz, 1H), 4.31 (dd, J = 5.6, 10.3 Hz, 1H), 4.47 (dd, J = 4.6, 10.1 Hz, 1H), 6.42 (d, J = 2.4 Hz, 1H), 6.71 (d, J = 2.4 Hz, 1H), 7.66–7.69 (m, 2H), 7.72 (d, J = 8.6 Hz, 1H), 7.92 (d, J = 2.0, 8.8 Hz, 1H), 8.25 (d, J = 8.6 Hz, 1H), 8.57 (dd, J = 1.6, 8.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) 23.9, 24.7, 30.9, 31.1 73.2, 74.0, 94.5, 97.8, 108.7, 118.9, 121.9, 122.7, 123.8, 124.1, 126.9, 128.2, 129.3, 136.3, 152.3, 159.5, 160.8, 163.2, 175.4 ppm; LC-ESI: m/e 423.0 $[M+1]^+$ 445.1 $[M+Na]^+$.

4.1.6. 9,11-Dihydroxy-12*H*-benzo[*a*]xanthen-12-one (10)

The reaction mixture of 2,4,6-trihydroxybenzoic acid (1.88 g, 0.01 mol), 2-naphthol (1.44 g, 0.01 mol), $ZnCl_2$ (5.0 g, 0.036 mol), and $POCl_3$ (40 mL) was refluxed for 5 h at 80 °C, cooled to room temperature and then poured into iced water very slowly. The solid formed was kept for 1 d at room temperature, collected and washed with water to give a red solid. After drying under vacuum, the solid was applied to silica gel column chromatography (eluant: ethyl acetate/*n*-hexane = 1:3) to give a yellow solid (0.20 g, 7.2%). R_f: 0.28 (ethyl acetate/*n*-hexane = 1:3); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.26 (s, 1H), 6.38 (s, 1H), 7.42 (d, J = 8.8 Hz, 1H), 7.52 (dd, J = 7.6, 6.8 Hz, 1H), 7.69 (dd, J = 7.2, 7.2 Hz, 1H), 7.83 (d, J = 8.0 Hz, 1H), 8.04 (d, J = 8.8 Hz, 1H), 9.87 (d, J = 8.4 Hz, 1H).

4.1.7. 11-Hydroxy-9-(thiiran-2-ylmethoxy)-12*H*-benzo[*a*]xanthen-12-one (11) and 9,11-bis(thiiran-2-ylmethoxy)-12*H*-benzo[*a*]xanthen-12-one (13)

To a mixture of compound **10** (0.1 g, 0.36 mmol) and Cs_2CO_3 (0.36 g, 1.15 mmol), in anhydrous acetone (13 mL) was added epithiochlorohydrin (0.2 g, 1.84 mmol) in acetone (2 mL). The reaction mixture was stirred over night at 55–60 °C under nitrogen and then cooled to room temperature. The solid in the mixture was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (eluant: ethyl acetate/*n*-hexane = 1:3) to give compounds **11** (27.3 mg, 21.7%) and **13** (31.2 mg, 20.5%) as yellow solids. Compound **11**: Mp 167–168 °C; R_f: 0.66 (ethyl acetate/*n*-hexane = 1:3); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.37 (d, J = 5.2 Hz, 1H), 2.66 (d, J = 5.2 Hz, 1H), 3.28–3.44 (m, 1H), 4.03 (dd, J = 10.0, 6.8 Hz, 1H), 4.29 (dd, J = 10.0, 5.6 Hz, 1H), 6.40 (d, J = 2.0 Hz, 1H), 6.49 (d, J = 2.0 Hz, 1H), 7.51 (d, J = 9.2 Hz, 1H), 7.61 (dd, J = 8.0, 7.2 Hz, 1H), 7.78 (dd, J = 8.8, 7.2 Hz, 1H), 7.91 (d, J = 8.0 Hz, 1H), 8.14 (d, J = 8.0 Hz, 1H), 9.96 (d, J = 8.8 Hz, 1H), 13.48 (s, 1H); ^{13}C

NMR (100 MHz, $CDCl_3$) 24.1, 31.0, 73.1, 92.7, 98.2, 105.7, 109.8, 117.8, 126.5, 127.0, 128.8, 129.8, 130.4, 131.0, 137.3, 156.7, 157.9, 163.8, 164.7, 183.2 ppm; GC–MS (EI): *m*/*e* 350 $[M]^+$. Compound **13**: Mp 225–226 °C; R_f: 0.35 (ethyl acetate/*n*-hexane = 1:3); ^1H NMR (400 MHz, $CDCl_3$) δ 2.38 (dd, *J* = 5.0, 1.6 Hz, 1H), 2.55 (d, *J* = 5.0 Hz, 1H), 2.66 (dd, *J* = 6.0 Hz, 1H), 2.74 (d, *J* = 6.0 Hz, 1H), 3.28–3.34 (m, 1H), 3.51–3.57 (m, 1H), 4.02 (dd, *J* = 10.4, 7.2 Hz, 1H), 4.05 (dd, *J* = 10.4, 7.2 Hz, 1H), 4.27 (dd, *J* = 10.4, 5.2 Hz, 1H), 4.50 (dd, *J* = 10.4, 4.8 Hz, 1H), 6.45 (d, *J* = 2.0 Hz, 1H), 6.57 (d, *J* = 2.0 Hz, 1H), 7.46 (d, *J* = 8.8 Hz, 1H), 7.58 (dd, *J* = 7.6, 7.2 Hz, 1H), 7.75 (dd, *J* = 8.8, 8.0 Hz, 1H), 7.88 (d, *J* = 8.0 Hz, 1H), 8.06 (d, *J* = 8.8 Hz, 1H), 10.06 (d, *J* = 8.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) 24.0, 24.8, 31.0, 31.3, 73.1, 74.4, 94.0, 98.4, 110.0, 115.9, 117.5, 126.1, 127.3, 128.5, 129.4, 130.6, 131.3, 135.9, 156.3, 158.4, 160.7, 162.8, 177.5 ppm; GC–MS (EI): *m*/*e* 422 $[M]^+$.

4.1.8. 11-Hydroxy-9-(oxiran-2-ylmethoxy)-12*H*-benzo[*a*]xanthen-12-one (12) and 9,11-bis(oxiran-2-ylmethoxy)-12*H*-benzo[*a*]xanthen-12-one (14)

To a mixture of compound **10** (0.1 g, 0.36 mmol) and Cs_2CO_3 (0.36 g, 1.15 mmol), in anhydrous acetone (20 mL) was added epichlorohydrin (0.2 g, 2.16 mmol) with a syringe. The reaction mixture was stirred for 2 d at 55–60 °C under nitrogen and then cooled to room temperature. The solid in the mixture was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (eluant: ethyl acetate/*n*-hexane = 1:3) to give compound **12** (24.3 mg, 20.3%) and compound **14** (32 mg, 22.1%) as yellow solids. Compound **12**: Mp 191–193 °C; R_f: 0.63 (ethyl acetate/*n*-hexane = 1:1); ^1H NMR (400 MHz, $CDCl_3$) δ 2.81 (dd, *J* = 4.8, 2.4 Hz, 1H), 2.97 (dd, *J* = 4.8, 4.4 Hz, 1H), 3.40–3.43 (m, 1H), 4.04 (dd, *J* = 11.2, 6.0 Hz, 1H), 4.35 (dd, *J* = 11.2, 3.2 Hz, 1H), 6.42 (d, *J* = 2.0 Hz, 1H), 6.52 (d, *J* = 2.0 Hz, 1H), 7.51 (d, *J* = 8.8 Hz, 1H), 7.61 (dd, *J* = 8.0, 3.2 Hz, 1H), 7.78 (ddd, *J* = 8.4, 8.4, 1.6 Hz, 1H), 7.91 (d, *J* = 8.0 Hz, 1H), 8.13 (d, *J* = 8.8 Hz, 1H), 9.96 (d, *J* = 8.4 Hz, 1H), 13.48 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) 44.9, 50.0, 69.4, 92.9, 98.2, 105.8, 113.5, 117.8, 126.5, 127.0, 128.8, 129.8, 130.4, 131.0, 137.3, 156.7, 157.9, 163.8, 164.8, 183.3 ppm; GC–MS (EI): *m*/*e* 334 $[M]^+$. Compound **14**: Mp 198–200 °C; R_f: 0.30 (ethyl acetate/*n*-hexane = 1:1); ^1H NMR (400 MHz, $CDCl_3$) δ 2.80 (dd, *J* = 4.8, 2.4 Hz, 1H), 2.96 (dd, *J* = 4.4, 4.4 Hz, 1H), 3.00 (dd, *J* = 4.8, 4.0 Hz, 1H), 3.15–3.17 (m, 1H), 3.38–3.42 (m, 1H), 3.52–3.55 (m, 1H), 4.02 (dd, *J* =10.8, 6.2 Hz, 1H), 4.20 (dd, *J* = 11.2, 4.4 Hz, 1H), 4.36 (dt, *J* = 11.2, 2.4 Hz, 1H), 4.47 (dt, *J* = 11.2, 4.2 Hz, 1H), 6.52 (d, *J* = 2.4 Hz, 1H), 6.57 (d, *J* = 2.4 Hz, 1H), 7.45 (d, *J* = 8.8 Hz, 1H), 7.56 (ddd, *J* = 7.2, 7.2, 1.2 Hz, 1H), 7.72 (ddd, *J* = 7.2, 7.2, 1.2 Hz, 1H), 7.87 (d, *J* = 7.2 Hz, 1H), 8.05 (d, *J* = 8.8 Hz, 1H), 10.05 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) 44.8, 45.3, 50.0, 50.5, 69.5, 69.7, 94.0, 97.9, 109.8, 115.9, 117.5, 126.1, 127.3, 128.5, 129.4, 130.6, 131.3, 135.9, 156.2, 158.3, 160.8, 162.9, 177.5 ppm; GC–MS (EI): *m*/*e* 390 $[M]^+$.

4.1.9. 1,3-Dihydroxy-12*H*-benzo[*b*]xanthen-12-one (15)

The reaction mixture of 3-hydroxy-2-naphthoic acid (2.0 g, 10.63 mmol), phloroglucinol (1.34 g, 10.63 mmol), $ZnCl_2$ (3.30 g, 24.24 mmol), and $POCl_3$ (40 mL) was refluxed for 5 h at 80 °C, cooled to room temperature and then poured into iced water very slowly. The solid formed was kept for 1 d at room temperature, collected and washed with water to give a brown solid. After drying under vacuum, the solid was applied to silica gel column chromatography (eluant: ethyl acetate/*n*-hexane = 1:3) to give a yellow solid (0.29 g, 9.8%). R_f: 0.47 (ethyl acetate/*n*-hexane = 1:3); ^1H NMR (400 MHz, DMSO-d_6) δ 6.16 (s, 1H), 6.35 (s, 1H), 7.52 (dd, *J* = 7.2, 6.8 Hz, 1H), 7.65 (dd, *J* = 7.2, 6.8 Hz, 1H), 7.99 (s, 2H), 8.17 (d, *J* = 8.0 Hz, 1H), 8.76 (s, 1H), 12.84 (s, 1H).

4.1.10. 1-Hydroxy-3-(thiiran-2-ylmethoxy)-12*H*-benzo[*b*]xanthen-12-one (16)

To a mixture of compound **15** (0.05 g, 0.18 mmol) and K_2CO_3 (0.07 g, 0.54 mmol) in anhydrous acetone (18 mL) was added epithiochlorohydrin (0.07 g, 0.65 mmol) in acetone (2 mL). The reaction mixture was stirred overnight at 55–60 °C under nitrogen and then cooled to room temperature. The solid in the mixture was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (eluant: ethyl acetate/*n*-hexane = 1:3) to give compound **16** (13.1 mg, 20.8%) as a yellow solid. Mp 189–190 °C; R_f: 0.61 (ethyl acetate/*n*-hexane = 1:3); ^1H NMR (400 MHz, $CDCl_3$) δ 2.38 (d, *J* = 5.2 Hz, 1H), 2.67 (d, *J* = 6.0 Hz, 1H), 3.31–3.34 (m, 1H), 4.05 (dd, *J* = 10.4, 7.2 Hz, 1H), 4.30 (dd, *J* = 10.4, 6.0 Hz, 1H), 6.35 (d, *J* = 1.6 Hz, 1H), 6.47 (d, *J* = 1.6 Hz, 1H), 7.53 (dd, *J* = 7.6, 7.2 Hz, 1H), 7.64 (dd, *J* = 8.0, 7.2 Hz, 1H), 7.84 (s, 1H), 7.92 (d, *J* = 8.4 Hz, 1H), 8.06 (d, *J* = 8.0 Hz, 1H), 8.86 (s, 1H), 12.94 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) 24.0, 30.9, 73.2, 93.8, 96.3, 97.3, 113.5, 120.4, 126.0, 127.3, 127.9, 129.5, 129.8, 130.0, 137.0, 152.6, 158.4, 164.3, 166.0, 181.6 ppm; GC–MS (EI): *m*/*e* 350 $[M]^+$.

4.1.11. 1,3-Bis(thiiran-2-ylmethoxy)-12*H*-benzo[*b*]xanthen-12-one (18)

To a mixture of compound **15** (0.05 g, 0.18 mmol) and Cs_2CO_3 (0.23 g, 0.74 mmol) in anhydrous acetone (18 mL) was added epithiochlorohydrin (0.12 g, 1.11 mmol) in acetone (2 mL). The reaction mixture was stirred overnight at 55–60 °C under nitrogen and then cooled to room temperature. The solid in the mixture was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (eluant: ethyl acetate/*n*-hexane = 1:3) to give compound **18** (13.1 mg, 17.2%) as an off-white solid. Mp 196–197 °C; R_f: 0.39 (ethyl acetate/*n*-hexane = 1:3); ^1H NMR (400 MHz, $CDCl_3$) δ 2.37 (d, *J* = 5.2 Hz, 1H), 2.53 (d, *J* = 4.8 Hz, 1H), 2.65 (d, *J* = 6.0 Hz, 1H), 2.71 (d, *J* = 6.0 Hz, 1H), 3.28–3.31 (m, 1H), 3.46–3.50 (m, 1H), 3.98 (dd, *J* = 10.0, 7.2 Hz, 1H), 4.06 (dd, *J* = 10.0, 7.2 Hz, 1H), 4.25 (dd, *J* = 10.0, 5.6 Hz, 1H), 4.44 (dd, *J* = 10.0, 4.4 Hz, 1H), 6.34 (d, *J* = 2.4 Hz, 1H), 6.51 (d, *J* = 2.4 Hz, 1H), 7.46 (dd, *J* = 8.4, 7.2 Hz, 1H), 7.58 (dd, *J* = 8.0, 7.2 Hz, 1H), 7.75 (s, 1H), 7.87 (d, *J* = 8.0 Hz, 1H), 8.02 (d, *J* = 8.4 Hz, 1H), 8.84 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) 23.9, 24.6, 31.0, 31.1, 73.1, 73.8, 94.4, 96.8, 107.3, 112.8, 123.6, 125.6, 127.1, 128.4, 128.9, 130.0 (×2), 136.4, 151.7, 160.4, 161.3, 164.1, 176.2 ppm; GC–MS (EI): *m*/*e* 422 $[M]^+$.

4.1.12. 1-Hydroxy-3-(oxiran-2-ylmethoxy)-12*H*-benzo[*b*]xanthen-12-one (17)

To a mixture of compound **15** (0.1 g, 0.36 mmol) and K_2CO_3 (0.2 g, 1.44 mmol) in anhydrous DMF (5 mL) was added epichlorohydrin (0.19 g, 2.05 mmol) with a syringe. The reaction mixture was stirred overnight at 75 °C under nitrogen and then cooled to room temperature. The mixture was diluted with water and extracted with ethyl acetate (two times). The combined organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (eluant: ethyl acetate/*n*-hexane = 1:3) to give compound **17** (13.0 mg, 10.8%) as a yellow solid. Mp 201–202 °C; R_f: 0.58 (ethyl acetate/*n*-hexane = 1:1); ^1H NMR (400 MHz, $CDCl_3$) δ 2.82 (dd, *J* = 4.4, 2.8 Hz, 1H), 2.98 (dd, *J* = 4.4, 4.4 Hz, 1H), 3.41–3.44 (m, 1H), 4.04 (dd, *J* = 10.8, 6.0 Hz, 1H), 4.39 (dd, *J* = 11.2, 3.2 Hz, 1H), 6.36 (d, *J* = 2.0 Hz, 1H), 6.50 (d, *J* = 2.0 Hz, 1H), 7.52 (dd, *J* = 7.2, 6.8 Hz, 1H), 7.64 (dd, *J* = 7.2, 6.8 Hz, 1H), 7.84 (s, 1H), 7.92 (d, *J* = 8.4 Hz, 1H), 8.07 (d, *J* = 8.4 Hz, 1H), 8.85 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$+CD_3OD) 44.8, 50.0, 69.4, 94.0, 97.2, 113.4, 113.5, 120.3,

126.0, 127.3, 127.8, 129.6, 129.8, 129.9, 137.0, 152.1, 158.4, 163.9, 166.1, 181.7 ppm; GC–MS (EI): *m*/*e* 334 $[M]^+$.

4.1.13. 1,3-Bis(oxiran-2-ylmethoxy)-12*H*-benzo[*b*]xanthen-12-one (19)

To a mixture of compound **15** (0.15 g, 0.54 mmol) and Cs_2CO_3 (0.53 g, 1.63 mmol) in anhydrous acetone (30 mL) was added epichlorohydrin (0.25 g, 2.7 mmol) with syringe. The reaction mixture was stirred overnight at 55–60 °C under nitrogen and then cooled to room temperature. The solid in the mixture was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (eluant: ethyl acetate/*n*-hexane = 1:3) to give compound **19** (15.3 mg, 7.3%) as a yellow solid. Mp 181–182 °C; R_f: 0.26 (ethyl acetate/*n*-hexane = 1:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.80 (dd, *J* = 4.8, 2.4 Hz, 1H), 2.96 (dd, *J* = 4.0, 2.4 Hz, 1H), 2.99 (dd, *J* = 5.2, 4.4 Hz, 1H), 3.21–3.25 (m, 1H), 3.35–3.41 (m, 1H), 3.47–3.52 (m, 1H), 4.00 (ddd, *J* = 10.4, 5.2, 2.0 Hz, 1H), 4.18 (dd, *J* = 11.2, 4.4 Hz, 1H), 4.36 (dt, *J* = 9.6, 2.4 Hz, 1H), 4.40 (dt, *J* = 11.2, 2.4 Hz, 1H), 6.37 (d, *J* = 2.4 Hz, 1H), 6.49 (d, *J* = 2.4 Hz, 1H), 7.45 (ddd, *J* = 8.0, 6.8, 1.2 Hz, 1H), 7.56 (ddd, *J* = 8.0, 6.8, 1.2 Hz, 1H), 7.72 (s, 1H), 7.85 (d, *J* = 8.0 Hz, 1H), 8.01 (d, *J* = 8.4 Hz, 1H), 8.82 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) 44.8, 45.2, 50.0, 50.3, 69.0, 69.5, 94.5, 96.6, 107.3, 112.8, 122.7, 125.5, 127.1, 128.3, 128.9, 130.0 (×2), 136.3, 151.7, 160.3, 161.2, 164.1, 175.9 ppm; GC–MS (EI): *m*/*e* 390 $[M]^+$.

4.2. Human topoisomerase I relaxation assay

All test compounds were dissolved in DMSO at a concentration of 20 mM as stock solutions and kept at −20 °C until used. DNA topo I inhibition assay was performed as described previously with minor modifications. The activity of DNA topo I was determined by assessing the relaxation of supercoiled pBR322 DNA. The mixture of 100 ng of plasmid pBR322 DNA and 0.4 units of recombinant human DNA topoisomerase I (TopoGEN Inc., USA) was incubated without and with the prepared compounds at 37 °C for 30 min in relaxation buffer [10 mM Tris–HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin, 1 mM spermidine, 5% glycerol]. The reaction (final volume = 10 μL) was terminated by adding 2.5 μL of stop solution containing 5% sarcosyl, 0.0025% bromophenol blue, and 25% glycerol. DNA samples were then electrophoresed on 1% agarose gels at 15 V for 7 h with TAE running buffer. Gels were stained for 30 min in an aqueous solution of ethidium bromide (0.5 μg/mL). DNA bands were visualized by transillumination with UV light and were quantified using AlphaImager™ (Alpha Innotech Corp.).

4.3. Human topoisomerase IIα relaxation assay

DNA topo II inhibitory activity of compounds was measured as follows. A mixture of 200 ng of supercoiled pBR322 plasmid DNA and 1 unit of human DNA topoisomerase IIα (Usb Corp., USA) was incubated without and with the prepared compounds in assay buffer [10 mM Tris–HCl (pH 7.9) containing 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, and 15 μg/mL bovine serum albumin] for 30 min at 30 °C. The reaction (final volume = 20 μL) was terminated by the addition of 3 μL of 7 mM EDTA. Reaction products were electrophoresed on 1% agarose gels at 25 V for 4 h with TAE running buffer. Gels were stained for 30 min in an aqueous solution of ethidium bromide (0.5 μg/mL). DNA bands were visualized by transillumination with UV light and supercoiled DNA was quantified using AlphaImager™ (Alpha Innotech Corp.).

4.4. Cytotoxicity test

For the evaluation of cytotoxicity, five different cancer cell lines were used: human breast adenocarcinoma cell line (MDA-MB231), human cervix tumor cell line (HeLa), human prostate tumor cell line (DU145), human colorectal carcinoma cell line (HCT116), and human myelogenous leukemia cell line (HL60). Cancer cells were cultured according to supplier's instructions. Cells were seeded in 96-well plates at a density of 2–4 × 10^4 cells per well and incubated overnight in 0.1 mL of media supplied with 10% fetal bovine serum (Hyclone, USA) in 5% CO_2 incubator at 37 °C. On day 2, culture medium in each well was exchanged with 0.1 mL aliquots of medium containing graded concentrations of compounds. On day 4, 5 μL of the cell counting kit-8 solution (Dojindo, Japan) were added to each well and then incubated for 4 h under the same conditions. The absorbance of each well was determined by an Automatic Elisa Reader System (Bio-Rad 3550) at a wavelength of 450 nm. For determination of IC_{50} values, the absorbance readings at 450 nm were fitted to the four-parameter logistic equation. Adriamycin, etoposide, and camptothecin were purchased from Sigma and used as positive controls.

4.5. DNA cross-linking assay

DNA cross-linking property of each compound was tested using linearized pBR322 plasmid DNA and denaturing 1.2% alkaline agarose gel electrophoresis. The linear plasmid DNA was obtained by treating circular pBR322 plasmid DNA with EcoRI (Invitrogen, USA) and purified by ethanol precipitation. Alkaline agarose gel (1.2%) was prepared with the solution (pH 8.0) containing 50 mM NaCl and 1 mM EDTA. 0.5 μg of the linearized pBR322 was incubated with designated concentrations of compound for 2 h at room temperature in buffered 10 mM Tris–HCl and 1 mM EDTA adjusted to pH 8.0. After the mixture of DNA and compound was loaded with agarose loading dye, the gel was soaked in an alkaline running buffer containing 50 mM NaOH and 1 mM EDTA. The gel was run in fresh alkaline running buffer then neutralized for 1 h in neutralizing buffer solution containing 100 mM Tris and 150 mM NaCl adjusted to pH 7.6 with refreshing every 20 min. The gel was subsequently stained with ethidium bromide solution (2.5 μL of 10 mg/mL ethidium bromide in 50 mL of neutralizing buffer solution). The gel was visualized by UV transillumination and photographed using ChemiImager™ Ready (Alpha Innotech Corp.).

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