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Original article

Ring fusion strategy for the synthesis of anthra[2,3-*d*]oxazole-2-thione-5,10-dione homologues as DNA topoisomerase inhibitors and as antitumor agents

Chun-Liang Chen [a, b], Fei-Lan Liu [b, c], Chia-Chung Lee [a, b], Tsung-Chih Chen [a, b], Wen-Wei Chang [d], Jih-Hwa Guh [e], Ahmed Atef Ahmed Ali [b, f], Deh-Ming Chang [b, c, **], Hsu-Shan Huang [a, b, d, *]

[a] *Graduate Institute of Cancer Biology and Drug Discovery, College of Medical Science and Technology, Taipei Medical University, Taipei 110, Taiwan*
[b] *Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 114, Taiwan*
[c] *Rheumatology/Immunology/Allergy, Taipei Veterans General Hospital, Taipei 112, Taiwan*
[d] *School of Pharmacy, National Defense Medical Center, Taipei 114, Taiwan*
[e] *School of Pharmacy, National Taiwan University, Taipei 100, Taiwan*
[f] *Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan*

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ABSTRACT

The efficient synthesis of mono-substituted anthraquinones and ring fusion into anthra[2,3-*d*]oxazole-2-thione-5,10-dione derivatives were developed, and all the compounds were tested for their cytotoxicity against PC-3 cancer cell lines. Compounds **8**, **14**, **17** and **23** were selected by the NCI and **12**, **17** and **19** were evaluated for topoisomerase I-mediated DNA relaxation. Among them, **17** appeared to be the most active compound of this series and not only showed higher inhibition when indicated from the low IC_{50} values against PC-3 cancer cell line but also attenuated the *in vitro* topoisomerase I-mediated DNA relaxation at low micromolar concentrations. All test compounds exhibited different cytostatic and cytotoxic activities for further developing potential anticancer drugs.

1. Introduction

DNA topoisomerase IB are eukaryotic ubiquitous enzymes that can relax negative and positive supercoiled DNA by breaking a single strand of the DNA duplex to prevent excessive supercoiling. The essential roles of these enzymes are regulating the topological rearrangement of DNA during transcription, replication and recombination [1,2]. Camptothecin (CPT) is a TOP I-targeting agent for anticancer treatment that can reversibly trap TOP1-DNA cleavage complexes [3]. However, hematological toxicity, diarrhea, chemical cystitis and clinical tolerability are major side effects limit the clinical use of CPT [4,5]. In spite of its established anticancer activity, CPT could inactivate within minutes at physiological pH by lactone E ring opening [6]. In previous works, our groups have found that various tricyclic and tetracyclic anthraquinone-derived analogs exhibited anticancer activities by acting as potent telomerase and/or TOP1 inhibitors [7–12]. Therefore, the development of new topoisomerase inhibitors remains an attractive goal in the cancer research, and its application could considered for further exploration.

The clinical use of daunorubicin and doxorubicin is limited due to the induced heart and bone marrow toxicity [13]. The precise mechanisms of action of these compounds have remained elusive, although their most important efficacy might attribute to intercalate with the DNA base pairs and topoisomerase complexes [14–16]. Recently, some anthrax[2,3-*b*]furan-5,10-diones, indolizinophthalazine-5,12-diones and naphthoindole-based analogues have been evaluated, and characterization of these compounds revealed significant cytotoxicity against several cancer cell lines *in vitro* by modulating the activity of topoisomerase I (Fig. 1) [8,9,17–20]. Since the planar anthraquinone-containing ring system could stack at the site of DNA, assemble the base-stacking interactions with base pairs, and inhibit the function of

* Corresponding author. Graduate Institute of Cancer Biology and Drug Discovery, College of Medical Science and Technology, Taipei Medical University, Taipei 110, Taiwan.
** Corresponding author. Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 114, Taiwan.
E-mail addresses: ming0503@ms3.hinet.net (D.-M. Chang), huanghs99@tmu.edu.tw, huanghs99@gmail.com (H.-S. Huang).

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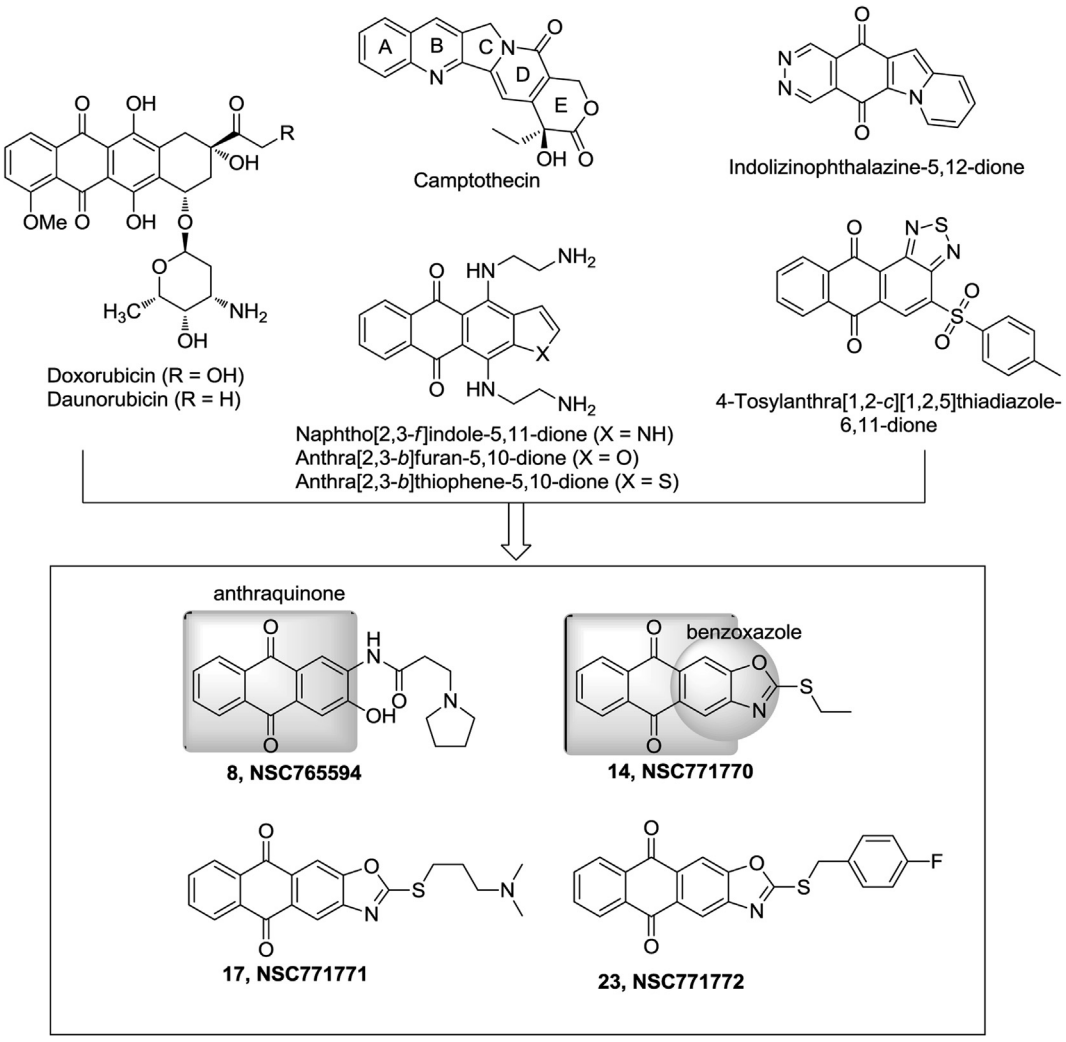


Fig. 1. Representative investigational structural sets used in the pharmacophore studies, some selective bioactive planar heterocyclic and anthraquinone-containing analogues.

topoisomerase I [17,20]. In addition, benzoxazoles have found to associate with DNA topoisomerase I inhibition and anti-cancer properties [21–23]. Thus, the strategy of this study is to infuse the oxazole moiety in the extended tricyclic planar ring system as potential topoisomerase inhibitors development.

According to previous and drug comparison studies, we found that some of the anthraquinone-linked heterocyclic derivatives and synthetic tetracycline scaffold might be interesting for cytotoxicity toward cancer cells or cancer polypharmacology [11,12,24–26]. Toward supporting the aforementioned hypothesis and exploration of structure activity relationships (SARs), the backbone of 2,3-heteroannelated anthraquinone derivatives is associated with DNA-intercalating and topoisomerase I inhibitory properties [8,9,18]. Interestingly, heteroannelated anthraquinones and substituted amidoanthraquinone homologues also show potent antiproliferative activities toward several cancer cells [11,12,26,27]. To study whether anthraquinone infuse with oxazole may enhance the topoisomerase I inhibitory and anticancer activity, we designed and synthesized 2-substituted amidoanthraquinones (**1**–**10**) and benzoxazole-containing derivatives (**12**–**28**), respectively. The cytotoxicity of these compounds against human hormone-refractory prostate cancer cell line (PC-3) evaluated by the SRB assay. Further to this research, the topoisomerase I mediated supercoiled pHOT DNA relaxation induced by these compounds was determined using the topoisomerase I assay kit [7,8]. Moreover, **8** (**NSC765594**), **14** (**NSC771770**), **17** (**NSC771771**) and **23** (**NSC771772**) were selected by the NCI for one dose screening program (Fig. 1). Considering their structure and side chain, the TOP1 inhibitory assay indicated that **17** exhibited TOP1 inhibitory activity. In this study, it is generally accepted that we provided insight into the interplay between the cytotoxicity and topoisomerase inhibition of these synthetic compounds on their anticancer activities.

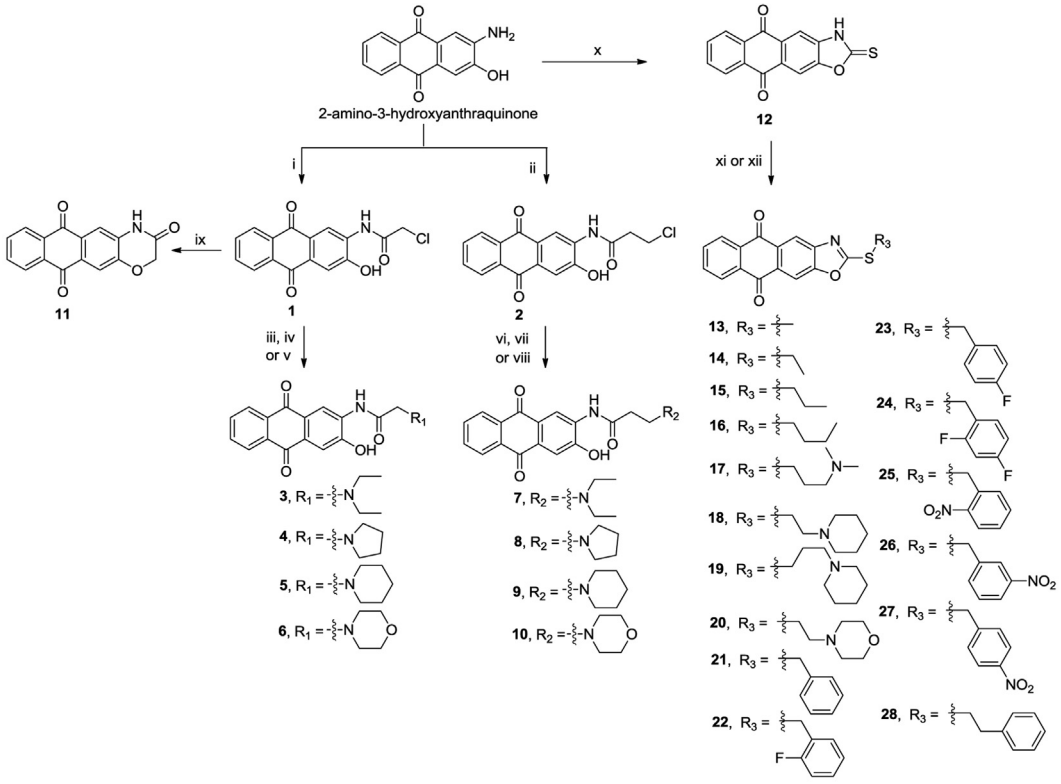
2. Chemistry

There has been continuing our interest in the synthesis of anthra[2,3-*d*]oxazole-2-thione-5,10-diones and their systematic dissection largely on account of their complementary activities. Compounds **1**–**2** were synthesized from 2-amino-3-hydroxyanthraquinone by reacting with chloroacetyl chloride and 3-chloropropanoyl chloride, respectively. Subsequently, **3**–**10** were prepared by amination with various amines obtained from **1** or **2** in THF to obtain products with different lengths of carbon side chains. Subsequent cyclization of **1** in the presence of methanolate to obtain **11** where the terminal chlorine atom on **1** is attached to

amino group. As shown in Scheme 1, **12** was synthesized by a one-step heterocyclic reaction of 2-amino-3-hydroxyanthraquinone with carbon disulfide in ethanol/water under base-catalyzed. Treating **12** with various amines in DMF gave the desired **13**–**28** through nucleophilic substitution of the chlorine atom by appropriate halides. In the series of designed analogues, we maintained the anthra[2,3-*d*]oxazole-2-thione-5,10-dione as a core structure, and the lead optimization focused on varying the electron-withdrawing or electron-releasing substituents at the thio-position of benzoxazole skeleton. The synthetic methods summarized in general procedure (I to XII) and ultimately furnished target compounds. All of the target compounds were determined by ^{1}H NMR, ^{13}C NMR and high-resolution mass (HRMS) spectra and the results are presented in the experimental part.

3. Biological results

All synthesized compounds were tested against PC-3 human prostate cancer cell line by using SRB assay. As illustrated in Table 1, where the IC_{50} value is the drug concentration required to achieve 50% growth inhibition. Our synthetic compounds showed IC_{50} ranging from 12.18 μM to more than 30 μM, which indicated that **1**–**10** and **11** have less cytotoxic effects toward the PC-3 cells. Conversely, the cytotoxicity observed, **17** and **19** showed the most potent cytotoxicity against the PC-3 cells among **12**–**28**. Thus, introducing the oxazole moiety having either *N*,*N*-dimethylpropanyl group or (piperidin-1-yl)propanyl group as side chain at the 2,3-position of the anthraquinone could enhance the anti-proliferative activity toward the PC-3 cells. In order to extend our anti-tumor characterization of the test compounds, we evaluated the abilities of compounds to regulate the topoisomerase I-mediated relaxation of supercoiled pHOT DNA by topoisomerase I assay. Having earlier demonstrated **17** and **19** as well as CPT as positive



[a]Reagents and conditions: (i) chloroacetyl chloride, THF, reflux, 2 hr. (ii) 3-chloropropionyl chloride, THF, reflux, 2 hr. (iii) diethylamine, DIPEA, THF, reflux, 8 hr. (iv) various amines, THF, reflux, 8 hr. (v) morpholine, THF, reflux, 3 hr. (vi) diethylamine, DIPEA, THF, reflux, 8 hr. (vii) various amines, THF, reflux, 8 hr. (viii) morpholine, THF, reflux, 3 hr. (ix) sodium methanolate, methanol, reflux, 5 hr. (x) carbon disulfide, potassium hydroxide, ethanol, H_2O, reflux, 3.5 hr. (xi) various halides, potassium hydroxide, DMF, reflux, 3 hr. (xii) various halides, potassium hydroxide, DMF, r.t., 3 hr.

Scheme 1. Synthesis of 2-substituted amidoanthraquinones, anthra[2,3-*b*][1,4]-oxazine-3,6,11-trione and anthra[2,3-*d*]oxazole-2-thione-5,10-dione derivatives.

Table 1
Cytotoxic effects of compounds **1**–**28** on PC-3 cell line determined by the SRB assay.

PC-3 cell line			
Compounds	$(IC_{50})^a$	Compounds	$(IC_{50})^a$
1	23.40	**15**	>30
2	26.67	**16**	>30
3	20.21	**17**	15.95
4	26.04	**18**	21.80
5	18.51	**19**	12.18
6	>30	**20**	>30
7	23.59	**21**	>30
8	>30	**22**	>30
9	>30	**23**	>30
10	>30	**24**	>30
11	>30	**25**	>30
12	>30	**26**	>30
13	>30	**27**	>30
14	>30	**28**	>30

[a] IC_{50} is the concentration of drug (μM) required to inhibit cell growth by 50% of the mean growth rate ($N = 3$).

control for their topoisomerase I inhibitory activities, attention next focused on determining possible reasons for their activities. In order to determine the effect of side chain substitution on the sulfur atom on the topoisomerase I inhibition, we evaluated **12** to act as a reference for the level of topoisomerase I inhibition activity. One conceivable hypothesis is that **12** showed no inhibition toward the topoisomerase I-mediated DNA relaxation (using concentrations of 25 and 50 μM). Through a series of experiments, **19** showed weak inhibitory activity at both 25 and 50 μM concentration. In comparison to **19** and CPT, **17** not only exhibited more potent inhibitory activity, but also completely blocked the topoisomerase I-mediated DNA relaxation at 25 and 50 μM. Attention turned to examining the activity of **17** attenuated the *in vitro* topoisomerase I-mediated DNA relaxation using concentrations of 12.5 and 6.25 μM dose-dependently (Figs. 2 and 3). These results indicated the side chain on the anthra[2,3-*d*]oxazole-2-thione-5,10-dione could modulate the topoisomerase inhibition activity, and that the cell cytotoxicity of **17** might correlate with its topoisomerase I inhibition activity.

As consequence of our series of antitumor comparison analysis, compounds **8**, **14**, **17** and **23** were selected by the NCI for their cytotoxic activities toward the NCI'60 human cancer cell line panel at a single dose of 10 μM. Attention next turned to **17** showed the highest mean value of growth inhibition and appeared to be the most active member of the tested compounds (Table S1). As a result of such complications, **17** was specifically effective against the leukemia cell lines MOLT-4 and SR achieving growth inhibition values of 86.23% and 71.97% respectively. The observed significant growth inhibition against the Leukemia cell line panel may be attributed to high topoisomerase I protein levels in leukemia cell lines [28], while the poor growth inhibition activities of **17** observed with the CNS cancer cell line panel may be due to the lower topoisomerase I protein levels in CNS cancer cell lines. Further efforts towards the identification of **17** might be correlated to its topoisomerase I inhibition activity. Instead, attention now turned to revealing **8**, **14** and **23** were less potent against the NCI's 60 human cancer cell lines showing mean values of growth inhibition less than 10%. Given that such a modification of the substituted benzoxazole derivatives are important compounds in both medicinal applications as well as synthetic organic chemistry [29,30]. Instead, a second recognized strategy to benzoxazole derivatives also possess DNA topoisomerase I inhibition and anticancer properties [21–23]. To test this approach, we succeeded to introduce the benzoxazole group with different side chains on the anthraquinone core structure and characterized the biological properties of these benzoxazole-fused anthraquinone derivatives. On the grounds that **17** have received attention for demonstrating not only regulated the topoisomerase I inhibition but also significantly affected anti-cancer activity. Similar to observations recorded for the oxazole-fused chromophore could be a novel lead structure for topoisomerase I inhibitors that such promising findings *in vitro* warrant future drug development to the greatest extent.

4. Discussion and conclusion

In summary, in an endeavor to further develop a series of heterocyclic anthra[2,3-*b*][1,4]oxazine-3,6,11-trione and anthra[2,3-*d*]oxazole-2-thione-5,10-dione derivatives were prepared and evaluated for biological activity. Among these compounds, **15**, **17** and **19** exhibited antiproliferative activity toward PC-3 cancer cell lines. Through a series of such promising experiments *in vitro*, employing various biological assays, **17** was evaluated with its topoisomerase I inhibition activity, combined with the fact that they could potentially possess difference antitumor profiles. The NCI's 60 human cancer cell line screening results revealed that **17** was the most active member of the tested compounds. One conceivable hypothesis is that the side chain moiety (*N*,*N*-dimethylpropanyl group) of **17** might regulate cytotoxic effects and topoisomerase I inhibition. As a result of such complications, further efforts towards the current study provides important information that can be the basis for further structural modifications of the anthra[2,3-*d*]oxazole-2-thione-5,10-dione derivatives to develop the rational design of the lead molecules and present more effective compounds for future drug discovery.

5. Materials and methods

5.1. Chemistry

Melting points were determined on a Büchi 545 melting point apparatus and are uncorrected. All reactions were monitored by TLC, which were performed on Silica Gel F_{254} plates (Merck). ^1H NMR and ^{13}C NMR spectra (Fig. S1–S56) were determined using Varian GEMINI-300 (300 MHz) or Agilent 400 MR DD2 (400 MHz) spectrometers. Mass spectra were recorded using Finnigan MAT-95XL (high resolution electron impact ionization, HREI) and Finnigan MAT 95S (high resolution electrospray ionization, HRESI) mass spectrometers. Reagents and solvents were purchased from Merck and Aldrich used without further purification.

5.2. General synthetic methods

5.2.1. General procedure (I): preparation of compound *1*

Chloroacetyl chloride (0.44 mL, 4 mmol) was added dropwise with stirring to a solution of 2-amino-3-hydroxyanthraquinone (0.71 g, 3 mmol) in THF (15 mL). The reaction mixture was refluxed for 2 h then cooled to room temperature for 1 h. The precipitate was collected, washed with dichloromethane and recrystallized from ethanol.

5.2.2. General procedure (II): preparation of compound *2*

3-Chloropropanoyl chloride (0.5 mL, 4 mmol) was added dropwise with stirring to a solution of 2-amino-3-hydroxyanthraquinone (0.71 g, 3 mmol) in THF (15 mL). The reaction mixture was refluxed for 2 h then cooled to room temperature for 1 h. The precipitate was collected, washed with dichloromethane and recrystallized from ethanol.

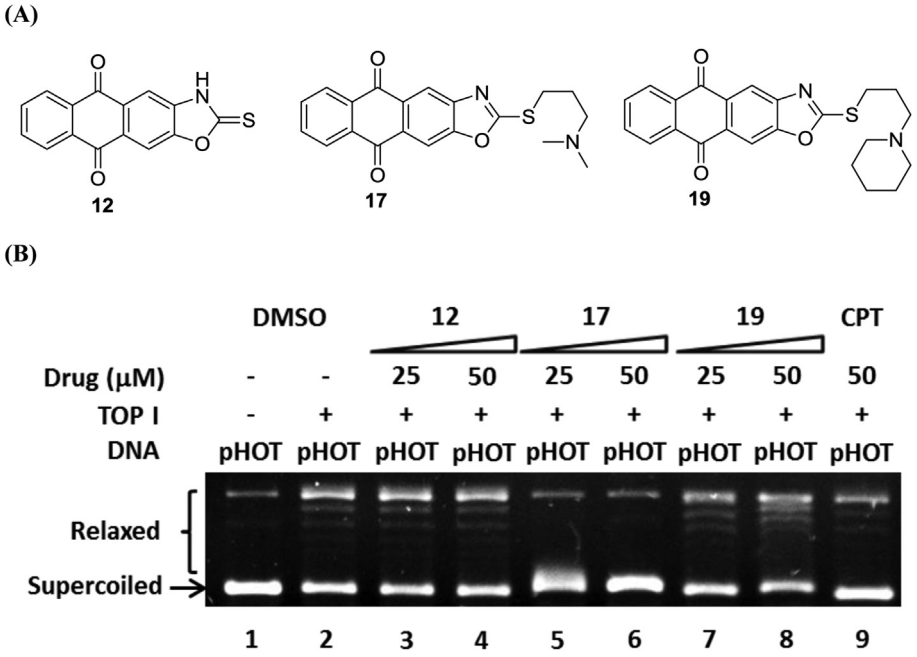


Fig. 2. Chemical structures and topoisomerase I inhibition activities of compounds **12**, **17**, and **19**. (A) Chemical structures of compounds **12**, **17**, and **19**. (B) Effects of compounds **12**, **17**, and **19** on the topoisomerase I mediated supercoiled pHOT DNA relaxation. Lane 1: untreated supercoiled pHOT DNA. Lane 2: pHOT DNA treated with topoisomerase I in the absence of drugs. Lanes 3–4 (compound **12**), 5–6 (compound **17**) and 7–8 (compound **19**) are the pHOT DNA treated with topoisomerase I in the presence of drugs using the concentrations of 25 or 50 μM respectively. Lane 9 is the pHOT DNA treated with topoisomerase I in the present of CPT using the concentration of 50 μM.

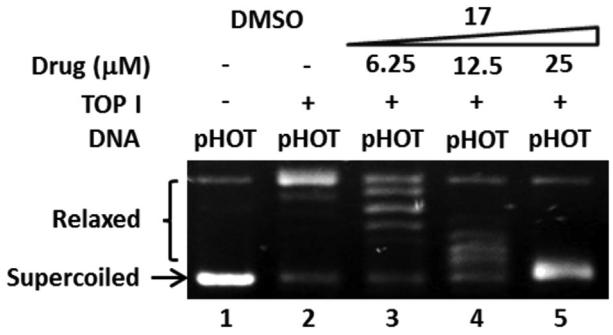


Fig. 3. Effect of compound **17** on topoisomerase I mediated supercoiled pHOT DNA relaxation. Lanes 1: untreated supercoiled pHOT DNA. Lanes 2: pHOT DNA treated with topoisomerase I in the absence of drug. Lanes 3–5 are the pHOT DNA treated with topoisomerase I in the presence of compound **17** using the concentrations of 6.25, 12.5 or 25 μM.

*5.2.3. General procedure (III): preparation of compound **3***

DIPEA (0.5 mL, 3.9 mmol) and diethylamine (0.73 mL, 10 mmol) were added dropwise with stirring to a solution of compound **1** (0.94 g, 3 mmol) in THF (15 mL). The reaction mixture was refluxed for 8 h. The mixture was concentrated and extracted with ethyl acetate, dried over $MgSO_4$, and then the solvent was removed in vacuo. The precipitate was filtered and recrystallized from ethanol.

*5.2.4. General procedure (IV): preparation of compounds **4**–**5***

Pyrrolidine/piperidine (10 mmol) was added dropwise with stirring to a solution of compound **1** (0.94 g, 3 mmol) in THF (15 mL). The reaction mixture was refluxed for 8 h and cooled to room temperature for 1 h. The precipitate was collected on a filter, washed with dichloromethane and recrystallized from ethanol.

*5.2.5. General procedure (V): preparation of compound **6***

Morpholine (0.87 mL, 10 mmol) was added dropwise with stirring to a solution of compound **1** (0.94 g, 3 mmol) in THF (15 mL). The reaction mixture was refluxed for 3 h and cooled to room temperature for 1 h. The mixture was concentrated and extracted with ethyl acetate, dried over $MgSO_4$, and then the solvent was removed in vacuo. The precipitate was filtered off, washed with dichloromethane and recrystallized from ethanol.

*5.2.6. General procedure (VI): preparation of compound **7***

DIPEA (0.5 mL, 3.9 mmol) and diethylamine (0.73 mL, 10 mmol) was added dropwise with stirring to a solution of compound **2** (0.99 g, 3 mmol) in THF (15 mL). The reaction mixture was refluxed for 8 h. The mixture was concentrated and extracted with ethyl acetate, dried over $MgSO_4$, and then the solvent was removed in vacuo. The precipitate was filtered and recrystallized from ethanol.

*5.2.7. General procedure (VII): preparation of compounds **8**–**9***

Pyrrolidine/piperidine (10 mmol) was added dropwise with stirring to a solution of compound **2** (0.99 g, 3 mmol) in THF (15 mL). The reaction mixture was refluxed for 8 h and cooled to room temperature for 1 h. The precipitate was collected on a filter, washed with dichloromethane and recrystallized from ethanol.

*5.2.8. General procedure (VIII): preparation of compound **10***

Morpholine was added dropwise with stirring (0.87 mL, 10 mmol) to a solution of compound **2** (0.99 g, 3 mmol) in THF (15 mL). The reaction mixture was refluxed for 3 h and cooled to room temperature for 1 h. The mixture was concentrated, extracted with ethyl acetate, dried over $MgSO_4$, and then the solvent was removed in vacuo. The precipitate was filtered off, washed with dichloromethane and recrystallized from ethanol.

*5.2.9. General procedure (IX): preparation of compound **11***

A solution of sodium methanolate (0.54 g, 10 mmol) in methanol (5 mL) was added to a solution of compound **1** (0.94 g, 3 mmol) dissolved in methanol (15 mL). The reaction mixture was refluxed for 5 h and cooled to room temperature for 1 h. The precipitate was collected on a filter, washed with water and recrystallized from hot ethanol.

*5.2.10. General procedure (X): preparation of compound **12***

A mixture of 2-amino-3-hydroxyanthraquinone (0.47 g, 2 mmol), potassium hydroxide (0.22 g, 4 mmol) and carbon disulfide (0.6 mL, 10 mmol) in ethanol/water (4:1, 25 mL) was refluxed for 3.5 h. The reaction mixture was cooled to room temperature, and the formed precipitate was filtered and washed with dichloromethane.

*5.2.11. General procedure (XI): preparation of compounds **13**–**24**, **28***

Methyl iodide/ethyl iodide/propyl chloride/butanyl chloride/3-chlorodimethylamine/1-(2-chloroethyl)piperidine/1-(3-chloropropyl)piperidine/1-(2-chloroethyl)morphiline/benzyl bromide/2-fluorobenzyl bromide/4-fluorobenzyl bromide/3,5-difluorobenzyl bromide or (2-bromoethyl)benzene (5 mmol) was added to a solution of potassium hydroxide (0.22 g, 4 mmol) and compound **12** (0.56 g, 2 mmol) in DMF (15 mL), and the mixture was refluxed for 3 h. The reaction mixture was poured into ice water (20 mL) for 1 h. The precipitate was collected on a filter, washed with hexane, and recrystallized from ethanol.

*5.2.12. General procedure (XII): preparation of compounds **25**–**27***

2-Nitrobenzyl bromide/3-nitrobenzyl bromide or 4-nitrobenzyl bromide (5 mmol) was added to a solution of potassium hydroxide (0.22 g, 4 mmol) and compound **12** (0.56 g, 2 mmol) in DMF (15 mL). The mixture was stirred at r.t. for 3 h, and then poured into ice water (20 mL) for 1 h. The precipitate was collected on a filter, washed with hexane, and recrystallized from ethanol.

*5.2.13. 2-(Chloroacetamido)-3-hydroxy-anthraquinone (**1**)*

The pure compound was obtained as a yellow powder (yield 72%). Mp 194–195 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 4.50 (s, 2H), 7.61 (s, 1H), 7.84–7.87 (m, 2H), 8.10–8.16 (m, 2H), 8.92 (s, 1H), 9.86 (s, 1H), 11.78 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 43.71, 112.19, 119.01, 126.35, 126.84, 126.96, 130.67, 132.01, 133.54, 133.73, 134.34, 134.62, 152.85, 165.97, 181.85, 182.27. HRMS (ESI) m/z calcd for $[M+H]^+$: 316.0377, found: 316.0370.

*5.2.14. 2-(3-Chloropropionamido)-3-hydroxy-anthraquinone (**2**)*

The pure compound was obtained as a yellow powder (yield 78%). Mp 198–199 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 3.04 (t, J = 6.3 Hz, 2H), 3.89 (t, J = 6.3 Hz, 2H), 7.62 (s, 1H), 7.84–7.89 (m, 2H), 8.13–8.18 (m, 2H), 8.97 (s, 1H), 9.73 (s, 1H), 11.64 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 34.55, 39.17, 112.40, 119.42, 126.28, 126.84, 126.95, 130.32, 132.58, 133.61, 133.79, 134.31, 134.57, 152.91, 169.65, 181.95, 182.33. HRMS (EI) m/z calcd for $[M]^+$: 329.0455, found: 329.0454.

*5.2.15. 2-[2-(Diethylamino)acetamido]-3-hydroxy-anthraquinone (**3**)*

The pure compound was obtained as a yellow powder (yield 46%). Mp 244–245 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 1.04 (t, J = 7.2 Hz, 6H), 2.62 (q, J = 7.2 Hz, 4H), 3.24 (s, 2H), 7.58 (s, 1H), 7.83–7.87 (m, 2H), 8.10–8.15 (m, 2H), 9.03 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 12.56, 48.59, 58.62, 111.88, 116.79, 126.32, 126.90, 127.01, 130.16, 132.54, 133.67, 133.88, 134.38, 134.65, 152.77, 170.93, 182.01, 182.49. HRMS (EI) m/z calcd for $[M]^+$: 352.1423, found: 352.1425.

*5.2.16. 2-[2-(Pyrrolidinyl)acetamido]-3-hydroxy-anthraquinone (**4**)*

The pure compound was obtained as a red powder (yield 55%). Mp 198–199 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 1.76–1.80 (m, 4H), 2.68 (br 4H), 3.38 (s, 2H), 7.49 (s, 1H), 7.81–7.86 (m, 2H), 8.08–8.14 (m, 2H), 8.98 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ ppm 23.58, 53.86, 59.33, 111.57, 116.12, 123.93, 126.31, 126.41, 129.95, 132.47, 133.13, 133.52, 133.65, 134.09, 154.87, 168.98, 181.06, 182.30. HRMS (EI) m/z calcd for $[M]^+$: 350.1267, found: 350.1272.

*5.2.17. 2-[2-(N-piperidin)acetamido]-3-hydroxy-anthraquinone (**5**)*

The pure compound was obtained as a red powder (yield 63%). Mp 237–238 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 1.42–1.43 (m, 2H), 1.57–1.58 (m, 4H), 2.51 (t, J = 4.8 Hz, 4H), 3.19 (s, 2H), 7.58 (s, 1H), 7.84–7.87 (m, 2H), 8.11–8.15 (m, 2H), 9.01 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 23.58, 26.15, 54.54, 62.52, 111.91, 116.97, 126.60, 126.98, 127.09, 130.20, 132.52, 133.70, 133.87, 134.51, 134.76, 152.40, 169.75, 182.13, 182.51. HRMS (ESI) m/z calcd for $[M+H]^+$: 365.1501, found: 365.1487.

*5.2.18. 2-[2-(N-morpholin)acetamido]-3-hydroxy-anthraquinone (**6**)*

The pure compound was obtained as a yellow powder (yield 58%). Mp 264–265 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 2.57 (t, J = 4.2 Hz, 4H), 3.23 (s, 2H), 3.66 (t, J = 4.2 Hz, 4H), 7.57 (s, 1H), 7.84–7.86 (m, 2H), 8.09–8.14 (m, 2H), 8.98 (s, 1H), 9.99 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 53.50, 62.04, 66.84, 111.80, 117.02, 126.67, 126.88, 126.98, 130.11, 132.29, 133.59, 133.75, 134.39, 134.63, 152.06, 169.15, 182.01, 182.32. HRMS (EI) m/z calcd for $[M]^+$: 366.1216, found: 366.1222.

*5.2.19. 2-[3-(Diethylamino)propionamido]-3-hydroxy-anthraquinone (**7**)*

The pure compound was obtained as a red powder (yield 50%). Mp 278–279 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 1.05 (t, J = 7.2 Hz, 6H), 2.60–2.70 (m, 6H), 2.83 (t, J = 6.2 Hz, 2H), 7.49 (s, 1H), 7.80–7.86 (m, 2H), 8.09–8.14 (m, 2H), 9.02 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 10.65, 33.20, 45.57, 48.00, 111.44, 117.28, 124.32, 126.29, 126.41, 129.56, 133.14, 133.30, 133.49, 133.66, 134.07, 154.55, 171.03, 181.20, 182.17. HRMS (EI) m/z calcd for $[M]^+$: 366.1580, found: 366.1587.

*5.2.20. 2-[3-(N-pyrrolidinyl)propionamido]-3-hydroxy-anthraquinone (**8**)*

The pure compound was obtained as a red powder (yield 57%). Mp 239–240 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 3.18–3.19 (m, 4H), 3.30–3.37 (m, 6H), 3.56 (t, J = 6 Hz, 2H), 8.17 (s, 1H), 8.53–8.55 (m, 2H), 8.80–8.84 (m, 2H), 9.71 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ ppm 23.10, 34.75, 50.59, 52.91, 111.41, 117.14, 124.33, 126.29, 126.41, 129.55, 133.14, 133.40, 133.52, 133.67, 134.09, 154.41, 170.94, 181.15, 182.21. HRMS (EI) m/z calcd for $[M]^+$: 364.1423, found: 364.1427.

*5.2.21. 2-[3-(N-piperidin)propionamido]-3-hydroxy-anthraquinone (**9**)*

The pure compound was obtained as a pale red powder (yield 64%). Mp 234–235 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 1.44 (s, 2H), 1.64 (br, 4H), 2.54 (s, 4H), 2.63 (d, J = 5.2 Hz, 2H), 2.67 (d, J = 5.2 Hz, 2H), 7.56 (s, 1H), 7.85–7.86 (m, 2H), 8.11–8.15 (m, 2H), 9.05 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ ppm 23.74, 24.81, 32.82, 53.30, 53.62, 111.34, 117.50, 125.25, 126.36, 126.49, 129.37,

133.09, 133.12, 133.37, 133.84, 134.15, 153.01, 171.14, 181.39, 181.97. HRMS (EI) *m/z* calcd for $[M]^+$: 378.1580, found: 378.1574.

*5.2.22. 2-[3-(N-morpholin)propionamido]-3-hydroxy-anthraquinone (**10**)*

The pure compound was obtained as a red powder (yield 54%). Mp 251–252 °C. ^{1}H NMR (300 MHz, acetone-d_6): δ ppm 2.63–2.68 (m, 4H), 2.75–2.92 (m, 4H), 3.78 (t, J = 4.5 Hz, 4H), 7.73 (s, 1H), 7.86–7.89 (m, 2H), 8.20–8.25 (m, 2H), 9.22 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 33.23, 53.12, 54.00, 66.31, 111.86, 118.14, 126.48, 127.05, 129.85, 133.53, 133.69, 133.89, 134.44, 134.69, 152.64, 171.83, 182.15, 182.46. HRMS (EI) *m/z* calcd for $[M]^+$: 380.1372, found: 380.1376.

*5.2.23. Anthra[2,3-b][1,4]oxazine-3,6,11-trione (**11**)*

The pure compound was obtained as a yellow powder (yield 75%). Mp 203–204 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 4.30 (s, 2H), 7.262 (s, 1H), 7.264 (s, 1H), 7.79–7.82 (m, 2H), 8.07–8.11 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 67.03, 79.55, 113.16, 115.99, 126.93, 126.97, 127.90, 129.24, 133.66, 133.93, 134.42, 134.58, 148.89, 166.56, 181.56, 182.32. HRMS (ESI) *m/z* calcd for $[M-H]^+$: 278.0453, found: 278.0458.

*5.2.24. Anthra[2,3-d]oxazole-2-thione-5,10-dione (**12**)*

The pure compound was obtained as a red powder (yield 85%). Mp 194–195 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 7.73 (s, 1H), 7.77 (s, 1H), 7.84–7.87 (m, 2H), 8.14–8.17 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 104.09, 111.33, 126.79, 126.89, 126.97, 130.29, 133.65, 134.05, 134.17, 134.40, 152.72, 155.79, 182.40, 183.15, 188.41. HRMS (ESI) *m/z* calcd for $[M-H]^-$: 280.0068, found: 280.0071.

*5.2.25. 2-(Methylthio)anthra[2,3-d]oxazole-5,10-dione (**13**) [31]*

The pure compound was obtained as a pale yellow powder (yield 57%). Mp 234–235 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 2.84 (s, 3H), 7.80–7.83 (m, 2H), 8.32–8.35 (m, 3H), 8.52 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 14.58, 108.78, 117.81, 127.40, 127.49, 130.93, 131.72, 133.88, 133.95, 134.10, 134.16, 146.96, 155.57, 171.41, 182.52, 182.58. HRMS (ESI) *m/z* calcd for $[M+H]^+$: 296.0381, found: 296.0388.

*5.2.26. 2-(Ethylthio)anthra[2,3-d]oxazole-5,10-dione (**14**)*

The pure compound was obtained as a pale yellow powder (yield 40%). Mp 176–177 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 1.55 (t, J = 7.4 Hz, 3H), 3.39 (q, J = 7.5 Hz, 2H), 7.78–7.80 (m, 2H), 8.29–8.32 (m, 3H), 8.46 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 14.60, 26.99, 108.66, 117.60, 127.26, 127.34, 130.57, 131.38, 133.55, 133.64, 134.03, 134.09, 146.75, 155.08, 170.73, 182.35, 182.42. HRMS (ESI) *m/z* calcd for $[M+H]^+$: 310.0538, found: 310.0530.

*5.2.27. 2-(Propylthio)anthra[2,3-d]oxazole-5,10-dione (**15**)*

The pure compound was obtained as a gray powder (yield 35%). Mp 149–150 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1.13 (t, J = 7.2 Hz, 3H), 1.86–1.98 (m, 2H), 3.37 (t, J = 7.2 Hz, 2H), 7.78–7.84 (m, 2H), 8.31–8.37 (m, 3H), 8.50 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 13.30, 22.86, 34.78, 108.97, 118.03, 127.66, 127.74, 131.15, 131.97, 134.16, 134.24, 134.33, 144.40, 147.30, 155.64, 171.38, 182.83, 182.90. HRMS (ESI) *m/z* calcd for $[M+H]^+$: 324.0694, found: 324.0699.

*5.2.28. 2-(Butylthio)anthra[2,3-d]oxazole-5,10-dione (**16**)*

The pure compound was obtained as a gray powder (yield 30%). Mp 142–143 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 0.99 (t, J = 7.4 Hz, 3H), 1.54 (sext, J = 7.4 Hz, 2H), 1.86 (pent, J = 7.4 Hz, 2H), 3.38 (t, J = 7.2 Hz, 2H), 7.78–7.80 (m, 2H), 8.30–8.32 (m, 3H), 8.47 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 13.54 21.81, 31.09, 32.29, 108.63, 117.58, 127.26, 127.34, 130.55, 131.38, 133.56, 133.64, 134.03, 134.09, 146.77, 155.09, 170.98, 182.38, 182.44. HRMS (ESI) *m/z* calcd for $[M+H]^+$: 338.0851, found: 338.0828.

*5.2.29. 2-((3-(Dimethylamino)propyl)thio)anthra[2,3-d]oxazole-5,10-dione (**17**)*

The pure compound was obtained as a pale yellow powder (yield 32%). Mp 115–116 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 2.03–2.12 (m, 2H), 2.29 (s, 6H), 2.50 (t, J = 6.9 Hz, 2H), 3.44 (t, J = 6.9 Hz, 2H), 7.77–7.83 (m, 2H), 8.29–8.35 (m, 3H), 8.48 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 27.09, 30.53, 45.23, 57.91, 108.69, 117.75, 127.37, 127.45, 130.87, 131.67, 133.87, 133.96, 134.05, 134.10, 146.99, 155.37, 171.05, 182.48, 182.55. HRMS (ESI) *m/z* calcd for $[M+H]^+$: 367.1116, found: 367.1098.

*5.2.30. 2-((2-(Piperidin-1-yl)ethyl)thio)anthra[2,3-d]oxazole-5,10-dione (**18**)*

The pure compound was obtained as a pale yellow powder (yield 50%). Mp 246–247 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1,69 (br, 2H), 2.07 (br, 4H), 3.19 (br, 4H), 3.45 (t, J = 7.2 Hz, 2H), 3.94 (t, J = 7.8 Hz, 2H), 7.70–7.83 (m, 2H), 8.35 (s, 1H), 8.27–8.34 (m, 2H), 8.43 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 21.15, 22.74, 26.15, 53.74, 56.06, 109.08, 117.85, 127.46, 131.20, 131.72, 133.64, 133.78, 134.29, 146.18, 155.44, 169.42, 182.25, 182.45. HRMS (ESI) *m/z* calcd for $[M+H]^+$: 393.1273, found: 393.1283.

*5.2.31. 2-((3-(Piperidin-1-yl)propyl)thio)anthra[2,3-d]oxazole-5,10-dione (**19**)*

The pure compound was obtained as a yellow powder (yield 20%). Mp 256–257 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1.43–1.47 (m, 2H), 1.55–1.62 (m, 4H), 2.04–2.11 (m, 2H), 2.40 (br, 4H), 2.48 (t, J = 7.1 Hz, 2H), 3.41 (t, J = 7.1 Hz, 2H), 7.77–7.80 (m, 2H), 8.28–8.32 (m, 3H), 8.44 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 24.35, 25.89, 26.44, 30.81, 54.56, 57.44, 108.65, 117.68, 127.34, 127.43, 130.75, 131.59, 133.80, 133.88, 134.05, 134.11, 146.98, 155.31, 171.27, 182.49, 182.57. HRMS (ESI) *m/z* calcd for $[M+H]^+$: 407.1429, found: 407.1404.

*5.2.32. 2-((2-Morpholinoethyl)thio)anthra[2,3-d]oxazole-5,10-dione (**20**)*

The pure compound was obtained as a yellow powder (yield 36%). Mp 154–155 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 2.56 (t, J = 4.5 Hz, 4H), 2.84 (t, J = 6.9 Hz, 2H), 3.54 (t, J = 6.9 Hz, 2H), 3.71 (t, J = 4.5 Hz, 4H), 7.75–7.78 (m, 2H), 8.25–8.29 (m, 3H), 8.40 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 30.26, 53.33, 56.90, 66.80, 108.66, 117.68, 127.32, 127.40, 130.84, 131.62, 133.77, 133.86, 134.03, 134.09, 146.84, 155.32, 171.09, 182.37, 182.45. HRMS (ESI) *m/z* calcd for $[M+H]^+$: 395.1066, found: 395.1073.

*5.2.33. 2-(Benzylthio)anthra[2,3-d]oxazole-5,10-dione (**21**)*

The pure compound was obtained as a pale yellow powder (yield 19%). Mp 214–215 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 4.63 (s, 2H), 7.33–7.37 (m, 3H), 7.48–7.51 (m, 2H), 7.79–7.82 (m, 2H), 8.32–8.36 (m, 3H), 8.53 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 36.84, 108.83, 117.93, 127.41, 127.49, 128.27, 128.98, 129.23, 129.40, 130.97, 131.71, 133.52, 133.84, 133.92, 134.12, 134.18, 135.46, 146.87, 155.42, 170.33, 182.52, 182.60. HRMS (ESI) *m/z* calcd for $[M+H]^+$: 372.0694, found: 372.0681.

*5.2.34. 2-((2-Fluorobenzyl)thio)anthra[2,3-d]oxazole-5,10-dione (**22**)*

The pure compound was obtained as a gray powder (yield 26%). Mp 200–201 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 4.65 (s, 2H), 7.07–7.16 (m, 2H), 7.30–7.35 (m, 1H), 7.57–7.62 (m, 1H), 7.77–7.83 (m, 2H), 8.29–8.36 (m, 3H), 8.51 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 36.07, 108.81, 115.74, 116.03, 117.93, 127.38, 127.46, 130.91,

131.01, 131.38, 131.42, 131.71, 133.82, 133.90, 134.08, 134.15, 146.74, 155.39, 161.12, 164.41, 170.01, 182.39, 182.48. HRMS (ESI) *m*/*z* calcd for $[M+H]^+$: 390.0600, found: 390.0580.

5.2.35. 2-((4-Fluorobenzyl)thio)anthra[2,3-d]oxazole-5,10-dione (**23**)

The pure compound was obtained as a pale yellow powder (yield 32%). Mp 196–197 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 4.58 (s, 2H), 7.01–7.07 (m, 2H), 7.45–7.49 (m, 1H), 7.76–7.80 (m, 2H), 8.27–8.33 (m, 3H), 8.48 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 36.07, 108.83, 115.75, 116.04, 117.93, 127.40, 127.48, 130.92, 131.03, 131.41, 131.71, 133.81, 133.90, 134.11, 134.18, 146.74, 155.40, 161.11, 164.40, 170.03, 182.43, 182.51. HRMS (ESI) *m*/*z* calcd for $[M+H]^+$: 368.0600, found: 390.0583.

5.2.36. 2-(2,4-Difluorobenzyl)anthra[2,3-d]oxazole-5,10-dione (**24**)

The pure compound was obtained as a gray powder (yield 20%). Mp: 201–202 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 4.57 (s, 2H), 6.73–6.79 (m, 1H), 7.03–7.08 (m, 2H), 7.80–7.83 (m, 2H), 8.32–8.36 (m, 3H), 8.53 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 35.78, 103.50, 103.75, 104.00, 108.92, 111.99, 112.06, 112.18, 112.25, 117.97, 127.35, 127.43, 130.89, 131.54, 133.56, 133.64, 134.14, 134.21, 139.36, 146.38, 155.29, 161.70, 161.82, 164.18, 164.31, 169.29, 182.34, 182.41. HRMS (ESI) *m*/*z* calcd for $[M+H]^+$: 408.0506, found: 408.0481.

5.2.37. 2-((2-Nitrobenzyl)thio)anthra[2,3-d]oxazole-5,10-dione (**25**)

The pure compound was obtained as a yellow powder (yield 46%). Mp: 216–217 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 4.94 (s, 2H), 7.47–7.53 (m, 1H), 7.61–7.67 (m, 1H), 7.77–7.82 (m, 2H), 7.93 (dd, J = 7.5 Hz, J = 1.2 Hz, 1H), 8.15 (dd, J = 8.4 Hz, J = 1.2 Hz, 1H), 8.29 (s, 1H), 8.30–8.35 (m, 2H), 8.50 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 34.34, 108.88, 117.82, 125.62, 127.35, 127.40, 129.43, 130.81, 131.51, 132.49, 132.99, 133.59, 133.70, 133.97, 134.13, 134.16, 146.42, 147.85, 155.41, 170.37, 182.32, 182.50. HRMS (ESI) *m*/*z* calcd for $[M+H]^+$: 417.0545, found: 417.0551.

5.2.38. 2-((3-Nitrobenzyl)thio)anthra[2,3-d]oxazole-5,10-dione (**26**)

The pure compound was obtained as a pale yellow powder (yield 36%). Mp: 213–214 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 4.68 (s, 2H), 7.55 (t, J = 7.8 Hz, 1H), 7.79–7.82 (m, 2H), 7.89 (d, J = 7.8 Hz, 1H), 8.15–8.19 (m, 1H), 8.3–8.35 (m, 3H), 8.41 (t, J = 1.8 Hz, 1H), 8.52 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 35.69, 108.95, 118.02, 123.15, 124.14, 127.36, 127.45, 129.79, 130.98, 131.60, 133.59, 133.67, 134.16, 134.23, 135.23, 137.96, 146.34, 148.45, 155.33, 169.06, 182.32, 182.39. HRMS (ESI) *m*/*z* calcd for $[M+H]^+$: 417.0545, found: 417.0531.

5.2.39. 2-((4-Nitrobenzyl)thio)anthra[2,3-d]oxazole-5,10-dione (**27**)

The pure compound was obtained as a gray powder (yield 43%). Mp: 232–233 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 4.67 (s, 2H), 7.71 (d, J = 8.8 Hz, 2H), 7.80–7.83 (m, 2H), 8.21–8.24 (m, 2H), 8.31–8.36 (m, 3H), 8.52 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 35.66, 108.96, 117.99, 124.02, 127.37, 127.45, 130.08, 130.97, 131.58, 133.55, 133.63, 134.18, 134.25, 143.22, 146.29, 147.63, 155.32, 169.01, 182.32, 182.39. HRMS (ESI) *m*/*z* calcd for $[M+H]^+$: 417.0545, found: 417.0533.

5.2.40. 2-(Phenethylthio)anthra[2,3-d]oxazole-5,10-dione (**28**)

The pure compound was obtained as a pale yellow powder (yield 25%). Mp:145–146 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 3.19 (t, J = 7.5 Hz, 2H), 3.62 (t, J = 7.8 Hz, 2H), 7.28–7.38 (m, 6H), 7.79–7.82 (m, 2H), 8.32–8.36 (m, 3H), 8.52 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 33.80, 35.52, 108.71, 117.81, 127.01, 127.37, 127.45, 128.75, 128.80, 130.89, 131.67, 133.85, 133.93, 134.05, 134.11, 139.12, 146.93, 155.35, 170.66, 182.45, 182.51. HRMS (ESI) *m*/*z* calcd for $[M+H]^+$: 386.0851, found: 386.0827.

5.3. Cell cultures and sulforhodamine B (SRB) assay

PC-3 cell line (human hormone-refractory prostate cancer cell line) was obtained from the American Type Culture Collection (Rockville, MD) [32,33]. The cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (v/v) and streptomycin (100 μg/mL)/penicillin (100 units/mL). Cells were cultured in a humidified incubator at 37 °C in 5% CO_2. Twenty-four hours before adding the compounds to be tested, the cells were plated in 96-well plates with 5% fetal bovine serum at a density of 5×10^3 cells per well. To assess the *in vitro* cytotoxicity of the synthesized compounds, each compound was dissolved in DMSO and, each compound was dissolved in DMSO and diluted into the complete medium before addition to cell cultures. Then every test compound was added to the cell cultures to incubate the cells with the designated concentrations of test compounds. After the incubation with the vehicle (DMSO) or the test compounds for 48 h, cells were fixed with 10% TCA, and SRB at 0.4% (w/v) in 1% acetic acid was added to stain cells. Unbound SRB was away by 1% acetic acid and SRB bound cells were solubilized with 10 mM Trizma base. The absorbance was measured at a wavelength of 515 nm. Using the following absorbance measurements, such as time zero (T_0), control growth (C), and cell growth in the presence of compound (T_x), the percentage growth was calculated at each of the compound concentrations levels. Percentage growth inhibition was calculated as: $100 - [(T_x - T_0)/(C - T_0)] \times 100$ for concentrations for which $T_x \geq T_0$. IC_{50} was determined at the drug concentration, which resulted in 50% reduction of total protein increased in control cells during the compound incubation.

5.4. DNA topoisomerase I assay

The DNA topoisomerase I activity was determined by evaluating the relaxation of supercoiled DNA pHOT using the topoisomerase I assay kit (TopoGEN, Inc., USA) which was used according to the previously described protocol with modifications [7,8]. The test compounds and CPT were dissolved in DMSO to form 10 mM stock solutions. Prepared mixtures each containing 0.25 μg of the plasmid pHOT DNA and 2 units of the recombinant human DNA topoisomerase I (TopoGEN INC., USA) were incubated with the prepared 0.5% DMSO (negative control), CPT (positive control) or compounds in the buffer (10 mM Tris–HCl, pH 7.9; 1 mM EDTA; 0.15 M NaCl; 0.1% BSA; 0.1 mM Spermidine; 5% glycerol) at 37 °C for 45 min. The reactions were quenched by the addition of sodium dodecyl sulfate (final 1% concentration) and proteinase K (final 50 μg/mL concentration) at 37 °C for 15 min. To the reaction mixtures, the loading buffer containing 0.25% bromophenol blue and 50% glycerol was added 1/10 volume in reactions mixtures. These samples were then electrophoresed on 1% agarose gel at using 60 V for 1.5 h with TAE (Tris–acetate–EDTA) as the buffer. The gels were stained with ethidium bromide for 10 min and destained with water for 20 min after electrophoresis.

5.5. In vitro NCI-60 human cell line cytotoxicity testing

As a primary screen, four compounds were selected by the NCI to evaluate their cytotoxicity activity against the NCI-60 cell line panel. The detailed methods used for the cytotoxicity testing have been described elsewhere [34–36]. Briefly, certain cellular protein

levels were determined after 48 h of drug exposure by SRB (Sulforhodamine B) colorimetry to monitor the change in cell growth.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.09.016.

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