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Novel xanthone-polyamine conjugates as catalytic inhibitors of human topoisomerase II α

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Abstract

It has been proposed that xanthone derivatives with anticancer potential act as topoisomerase II inhibitors because they interfere with the ability of the enzyme to bind its ATP cofactor. In order to further characterize xanthone mechanism and generate compounds with potential as anticancer drugs, we synthesized a series of derivatives in which position 3 was substituted with different polyamine chains. As determined by DNA relaxation and decatenation assays, the resulting compounds are potent topoisomerase II α inhibitors. Although xanthone derivatives inhibit topoisomerase II α -catalyzed ATP hydrolysis, mechanistic studies indicate that they do not act at the ATPase site. Rather, they appear to function by blocking the ability of DNA to stimulate ATP hydrolysis. On the basis of activity, competition, and modeling studies, we propose that xanthenes

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Supplementary data

Experimental details for the synthesis and characterization of the xanthone derivatives, as well as sources of materials and methods for biochemical assays and modeling studies, are available in the accompanying Supplementary Data.

interact with the DNA cleavage/ligation active site of topoisomerase II α and inhibit the catalytic activity of the enzyme by interfering with the DNA strand passage step.

Graphical Abstract

Keywords

Xanthone derivative; polyamines; DNA topoisomerase II α ; anticancer drug; catalytic inhibitor; DNA cleavage

Xanthenes are a class of heterotricyclic planar compounds that were originally identified as secondary metabolites in plants and microorganisms.^{1–3} These compounds display a wide range of biological activities and represent a prominent scaffold for agents with medicinal properties. Several xanthenes currently are under development as anticancer, anti-inflammatory or anti-thrombotic agents.^{2, 4–11} A number of these compounds display activity against type II topoisomerases.^{11–16} Furthermore, the primary cellular target of gambogic acid, a xanthone derivative in human phase II clinical trials as an anticancer drug, appears to be topoisomerase II α .^{12, 13}

Human type II topoisomerases play critical roles in a number of nucleic acid processes, including DNA replication, transcription, recombination, and chromosome segregation.^{17–22} These enzymes alter the topological state of the genetic material by transiently cleaving both strands of the double helix and passing a second double helix through the enzyme-generated DNA gate. This process is dependent on the presence of an ATP cofactor. ATP binding promotes the DNA strand passage event, while hydrolysis of the cofactor is required for enzyme turnover. Type II topoisomerases regulate DNA under- and overwinding, and more importantly, remove tangles and knots from the human genome.^{17–22} Humans encode two enzyme isoforms, topoisomerase II α and II β . Topoisomerase II α is essential for the survival of proliferating cells and is the isoform that is involved in DNA replication and the segregation of daughter chromosomes. In contrast, topoisomerase II β is not essential for cells survival, but is important for neurological development. Although the precise function

of topoisomerase II β is not well defined, it appears to play an important role in the expression of hormonally regulated genes.^{17–22}

Type II topoisomerases are the target for several commonly prescribed anticancer drugs, including etoposide, doxorubicin and mitoxantrone.^{17, 18, 22–27} These drugs act by increasing levels of covalent topoisomerase II-cleaved DNA complexes (*i.e.*, cleavage complexes), which are intermediates in the catalytic cycle of the enzyme. As result of drug action, the type II enzymes are converted to cellular toxins that fragment the genome. Drugs that act by this mechanism are called topoisomerase II poisons.^{17, 18, 22–27}

A second group of cytotoxic drugs that includes novobiocin, merbarone, aclarubicin, and ICRF-193 also targets type II topoisomerases. In contrast to the topoisomerase II poisons, these compounds are catalytic inhibitors that kill cells by robbing them of the essential activities of the type II enzyme. Catalytic inhibitors can block topoisomerase II activity by a variety of mechanisms, which can have important consequences for cells. Whereas drugs that block ATP binding (novobiocin), DNA cleavage, or strand passage (merbarone) inhibit the catalytic reactions of topoisomerase II, those that disrupt topoisomerase II-DNA binding (aclarubicin) impair both the structural and catalytic roles of the enzyme. Furthermore, drugs that allow ATP binding but block the hydrolysis of the high-energy cofactor (ICRF-193), trap the enzyme on the DNA.^{28–37}

Gambogic acid and several other xanthone derivatives are believed to inhibit human topoisomerase II α by binding to the ATPase active site of the enzyme and blocking interactions between the enzyme and the ATP cofactor.^{12, 15, 16, 38} This hypothesis is based on i) modeling studies that docked compounds to the ATPase site of topoisomerase II α , ii) *in vitro* experiments that demonstrated the inhibition of ATP hydrolysis by xanthone-based compounds, and iii) surface plasmon resonance studies that suggested that gambogic acid could bind the ATP domain of the human enzyme.

However, several lines of evidence suggest that the inhibition of topoisomerase II α by xanthone derivatives may be more complex. First, all of the ATPase studies reported for xanthone-based compounds were carried out in the presence of DNA.^{12, 15, 16} Because the ATPase activity of type II topoisomerases is stimulated by DNA binding and strand passage,^{39–41} interfering with DNA interactions could manifest itself as an indirect inhibition of ATP hydrolysis. Second, many xanthone-based compounds bind to DNA.^{13, 16} Thus, they may be able to interact with the DNA cleavage/ligation active site of type II topoisomerases. Third, some previously described xanthone derivatives display an IC₅₀ for inhibition of ATP hydrolysis that is >10-fold higher than observed for the inhibition of relaxation.^{15, 16} This makes it unlikely that the loss of overall catalytic activity could have resulted from interference with ATP interactions. Fourth, some xanthone-based compounds inhibit the DNA relaxation reaction of topoisomerase I. This is despite the fact that the type I enzyme has no binding site for ATP.⁴²

Therefore, to further examine the mechanism by which xanthenes inhibit topoisomerase II α , we synthesized a series of new xanthone polyamine conjugates, **2-5**, by inserting at the 3 position a side chain containing different polyamine moieties, including propandiamine

(compound **2**), butandiamine (compound **3**), spermidine (compound **4**), and spermine (compound **5**) (Fig. 1). Substitution at the 3 position is favored over the 1 position because of the proximity of the carbonyl.¹⁶ These polyamines were chosen because a previous study found that the presence of a secondary amine group in the side chain plays an important role in mediating topoisomerase II-drug interactions.^{43–45} Furthermore, the addition of a spermine side chain to the core of etoposide (generating F14512) greatly enhanced the ability of the drug to act as a topoisomerase II poison and to be taken up by cancer cells with active polyamine transport systems.^{43–47}

Compounds **1–5** were synthesized using the generalized scheme shown in Fig. 1. The key intermediate, 1-hydroxy-3-(oxiran-2-ylmethoxy)-9H-xanthen-9-one (**7**), was synthesized by an O- alkylation reaction of compound **6**. The synthesis of compound **7** was performed under microwave irradiation in order to shorten the reaction time. To join the nucleophilic chains to the xanthone core, intermediate **7** was coupled with butylamine and the N-Boc protected polyamines **12–15** to generate compounds **1** and **8–11**, respectively. In order to synthesize the final compounds **2** and **5** or **3** and **4**, *tert*-butyloxycarbonyl (Boc) groups were removed with 4 M HCl in dioxane or with trifluoroacetic acid (TFA) in CH₂Cl₂, respectively. All of the compounds were synthesized as racemic mixtures. The detailed syntheses and physical and chemical characterizations of the compounds are described in the accompanying Supplementary Data.

As a first step toward characterizing the activities of the xanthone derivatives shown in Fig. 1 against human topoisomerase II α , the effects of compounds **1–6** on enzyme-mediated DNA cleavage were determined (Fig. 2). Consistent with previous reports,^{12, 15, 16} none of the compounds displayed a significant ability to enhance DNA cleavage. Thus, these xanthone derivatives do not appear to act primarily as topoisomerase II poisons.

Next, we examined the abilities of compounds **1–6** to inhibit the overall catalytic activity of topoisomerase II α using a DNA relaxation assay (Fig. 3). All of the compounds inhibited DNA relaxation and fell into three groups regarding potency: compounds **2** and **5** displayed an IC₅₀ $\approx$ 1 μ M, compounds **3** and **4** displayed an IC₅₀ $\approx$ 2.5–5 μ M, and compounds **1** and **6** displayed an IC₅₀ $\approx$ 100 μ M. From these results, it appears that the presence of the side chain containing a primary and secondary amine is critical. However, the number of secondary amines in the chain and their distance from the primary amine appear to be less important. These results notwithstanding, it should be noted that a previous study reported that compound **1** displayed an IC₅₀ $\approx$ 9 μ M for the inhibition of DNA relaxation catalyzed by topoisomerase II α .¹⁶ We could not recapitulate this finding in the present study. Given the low activity of compound **1** in our hands and the fact that the activity of compound **3** was similar to other derivatives we tested, we focused on compounds **2** and **4–6** for more detailed studies.

The ability of xanthone derivatives to inhibit the catalytic activity of topoisomerase II α was confirmed using a decatenation assay (Fig. 4). Although IC₅₀ values were ~2- to 3- fold higher than observed in the relaxation assay, the order of potency remained similar, with compounds **2, 4, 5** being much more potent than compound **6**.

Polyamines have the potential to bind to the double helix and alter topoisomerase II-DNA interactions in the absence of a specific interaction with the enzyme.^{48–50} Consequently, is possible that the xanthone derivatives inhibit the activity of topoisomerase II α in a bimodal fashion with the aromatic core of one molecule binding to the protein and the polyamine tail of another acting through a general effect on the DNA. Therefore, the importance of the linkage between the spermidine/spermine polyamine tails and the xanthone core (compound **6**) in a single molecule was examined.⁵¹ Independently, the IC₅₀ values for the inhibition of DNA relaxation by topoisomerase II α for spermidine and spermine were >1 mM (data not shown) and that of compound **6** was >100 μ M. As seen in Fig. 5, a 1:1 mixture of compound **6** and spermidine or spermine showed no ability to inhibit DNA relaxation at 10 μ M. This is compared to compounds **4** and **5** (which are essentially compound **6** coupled to spermidine and spermine, respectively), that displayed IC₅₀ values <5 μ M. Thus, the linkage between the xanthone core and the polyamine tails is critical for the potent inhibitory activity of these compounds.

As discussed above, the mechanism by which a compound inhibits the activity of topoisomerase II can have profound cellular consequences. Thus, it is important to understand which step of the topoisomerase II catalytic cycle is affected by xanthones. This catalytic cycle can be divided into six discrete steps: 1) DNA binding, 2) DNA bending, 3) DNA cleavage, 4) strand passage, 5) DNA religation, 6) release of the DNA substrate and enzyme turnover.^{17, 18, 52} ATP binding drives the strand passage step and ATP hydrolysis is required for enzyme turnover and the completion of the catalytic cycle.^{17, 18, 20, 21, 32, 53}

To determine whether the xanthone derivatives affect topoisomerase II activity at steps up to and including DNA scission, we reassessed the effects of the compounds **2**, **4–6** on enzyme-mediated DNA cleavage (Fig. 6). Normally, topoisomerase II α maintains very low levels of cleavage complexes in the presence of Mg²⁺, its physiological divalent cation.^{54, 55} This makes it difficult to determine the ability of the drug to inhibit this reaction step. Consequently, the experiments shown in Fig. 6 were carried out in the presence of Ca²⁺, which raises baseline levels of DNA cleavage ~10- to 15-fold.^{54, 56} No substantial inhibition was observed for xanthone concentrations up to 10 μ M, the range in which compounds inhibited overall catalytic activity. This finding strongly suggests that these compounds do not impair the overall catalytic activity (DNA relaxation or decatenation) of topoisomerase II α by inhibiting any of the reaction steps through DNA cleavage. However, this conclusion comes with the caveat that the DNA cleavage assay utilizes 150 nM enzyme as compared to the relaxation assay, which uses 3 nM. Thus, it could take higher concentrations of xanthone derivatives to inhibit the catalytic activity of topoisomerase II α under conditions of the cleavage assay. Therefore, as a control, the effects of compounds **2**, **4–6** on DNA relaxation catalyzed by 150 nM topoisomerase II α were determined (Fig. 7). Similar to the results of Fig. 3, compounds **2**, **4**, and **5** displayed complete (or near complete) inhibition of enzyme activity by 10 μ M. (Note that 10 μ M compound **6** displayed no ability to inhibit DNA relaxation under either assay condition).

Taken together, the results shown in Figs. 6 and 7 indicate that the xanthone derivatives act by inhibiting topoisomerase II α in a reaction step that follows DNA cleavage. Consequently, we examined the effects of compounds **4**, and **5** on DNA strand passage mediated by the

enzyme (Fig. 8). A DNA catenation assay was employed that utilizes a high concentration (150 nM) of topoisomerase II α and replaces the ATP cofactor with the non-hydrolyzable analogue, APP(NH)P. Because APP(NH)P cannot be hydrolyzed by topoisomerase II α , this assay monitors enzyme activity through the strand passage step.^{52, 57} By using a DNA catenation assay, a single catalytic event moves the DNA substrate away from the bands of relaxed DNA. Relaxed, rather than negatively supercoiled, DNA was used for this assay because it is a preferred substrate for catenation. Similar to the results in Fig. 7, compounds **4** and **5** all displayed IC₅₀ values <10 μ M. Because these compounds showed little ability to inhibit DNA cleavage (Fig. 6), the step that immediately precedes the strand passage step, we conclude that the xanthone derivatives decrease the overall catalytic activity of topoisomerase II α by inhibiting the ability of the enzyme to carry out DNA strand passage.

Xanthenes can inhibit the strand passage step in three different ways: they can bind at the ATP active site and interfere with topoisomerase II α -ATP interactions, they can bind at the DNA cleavage/ligation active site and interfere directly with DNA movement, or they may bind outside of either active site and cause deleterious conformational changes in the enzyme. On the basis of modeling studies and the ability of compounds to inhibit enzyme-catalyzed ATP hydrolysis, previous studies suggested that xanthone derivatives acted by inhibiting topoisomerase II α -ATP interactions.^{12, 15, 16, 38} Therefore, we assessed the effects of compound **4** on ATP hydrolysis catalyzed by topoisomerase II α . As seen in Fig. 9, compound **4** inhibited ATP hydrolysis with an IC₅₀ $\approx$ 10 μ M. However, as with earlier studies, ATPase assay mixtures contained DNA. As discussed above (and shown in Fig. 9), rates of ATP hydrolysis are stimulated $\sim$ 5-fold by the presence of DNA. Thus, decreased rates of ATP hydrolysis observed in the presence of compound **4** could be due to interactions of the xanthone derivative at either the DNA or ATP sites. In order to distinguish between these two possibilities, the effects of compound **4** on ATP hydrolysis were examined in the absence of DNA (Fig. 9). Under this condition, no inhibition was observed up to 50 μ M xanthone. Thus, we conclude that the xanthone-induced decrease in the rate of ATP hydrolysis in the presence of DNA is not due to a direct inhibition of ATP binding. Rather, it is observed because xanthone derivatives inhibit the ability of DNA to stimulate the rate of ATP hydrolysis. Consistent with this conclusion, ATPase rates generated in the presence of supercoiled plasmid asymptotically approached those seen in the absence of DNA as the concentration of compound **4** increased (Fig. 9).

To further define the site of interaction of xanthone derivatives on topoisomerase II α , we used a competition assay to determine whether these compounds could be inhibiting DNA strand passage (and ATP hydrolysis) by acting at the DNA cleavage/ligation active site. The competition assay determined the ability of compounds **2**, **4** and **5** (which do not inhibit the DNA cleavage step) to block DNA cleavage enhancement by etoposide. This drug has been shown to bind at the DNA cleavage/ligation active site of human type II topoisomerases.⁵⁸ As seen in Fig. 10, all three compounds competed with etoposide, diminishing the ability of the anticancer drug to induce topoisomerase II α -mediated DNA scission. These data strongly suggest that the xanthone derivatives interact in the vicinity of the DNA cleavage/ligation active site of the human type II enzyme, despite the fact that they have little effect on the cleavage reaction. A similar conclusion has been drawn for the binding of some

quinolone-derivatives that do not enhance DNA cleavage to eukaryotic or prokaryotic type II topoisomerases.^{59, 60}

Finally, to determine the feasibility of xanthone binding at the cleavage/ligation active site of topoisomerase II α , compound **4** was docked into the topoisomerase II cleavage complex by molecular modeling. Both the R and S enantiomers were used for modeling studies. Fig. 11 shows the results with the R enantiomer. Our findings suggest that the heterocyclic moiety of the xanthone derivative preferentially locates in a region similar to that of the 4'-demethylepipodophyllotoxin core of etoposide.⁵⁸ The polyamine chain extends toward the DNA major groove, as was observed in previous computations of F14512 and other polyamine-conjugates.⁴⁵ Compound **4** has the potential to form several favorable interactions with both the enzyme and the DNA. The hydroxyl group along the polyamine chain points toward Gln778, while the central nitrogen atom is in close enough proximity to form hydrogen bonds with the backbone of either Lys814 or Ala816. The terminal amine is stabilized by the DNA, as it lays in between the phosphates of DNA bases C₊₃ and A₊₄.

In conclusion, xanthone derivatives represent a potent class of topoisomerase II inhibitors with anticancer potential. Previously, these compounds were believed to inhibit enzyme activity by interfering with the binding of the high-energy ATP cofactor. However, results of the present study strongly suggest that xanthone-polyamine conjugates act at the DNA cleavage/ligation active site of human topoisomerase II α and impair catalytic activity by blocking the DNA strand passage step of the topoisomerase II catalytic cycle. Having a greater understanding of xanthone mechanism may allow for future development of more active derivatives that utilize this important medicinal scaffold.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Structures and synthetic pathway of the compounds utilized in this study. Reagents and Conditions: **(a)** $ZnCl_2$, $POCl_3$, 70 °C, 3 hours, 69% yield; **(b)** epichlorohydrin, K_2CO_3 , DMF, 80 °C, 5 hours, mw, 32% yield; **(c)** DMF, 50 °C, 26 hours, 32-80% yield; **(d)** CF_3COOH , CH_2Cl_2 , 0 °C, 2 hours or HCl in dioxane, 0 °C, 2-5 hours, 33-60% yield. Boc = $(CH_3)_3COCO$. * = hydrochloride salt; ** = trifluoroacetate salt.

Fig. 2.

Effects of xanthone derivatives on DNA cleavage mediated by topoisomerase II α . Results for compounds **1-6** (2.5 μM , blue; 10 μM , red; 50 μM , yellow; 100 μM , green) on the generation of enzyme-mediated double-stranded DNA breaks are shown. Due to solubility issues, compound **5** was only used up to 10 μM . DNA cleavage in the presence of 100 μM etoposide (purple) is shown for comparison. DNA cleavage levels were calculated relative to control reactions that contained no drug (TII, orange) and were set to 1. Error bars represent standard deviations for 2-3 independent experiments. The inset shows an ethidium bromide-stained gel of a typical DNA cleavage experiment carried out in the presence of 10 μM compounds **1-6** and 100 μM etoposide. The positions of negatively supercoiled (form I, FI), nicked (form II, FII) and linear (form III, FIII) DNA are indicated.

Fig. 3.

Effects of xanthone derivatives on DNA relaxation catalyzed by topoisomerase II α . Results for compounds **1-6** are shown. Assays containing intact negatively supercoiled DNA in the absence of topoisomerase II α (DNA) or negatively supercoiled DNA treated with topoisomerase II α in the absence of xanthone derivatives (TII) are shown as controls. The positions of negatively supercoiled (form I, FI) and nicked (form II, FII) DNA are indicated. Gels are representative of 2-4 independent experiments.

Fig. 4.

Effects of xanthone derivatives on DNA decatenation catalyzed by topoisomerase II α .

Results for compounds **2**, **4-6** are shown. Assays containing intact kDNA in the absence of topoisomerase II α (DNA) or kDNA treated with topoisomerase II α in the absence of xanthone derivatives (TII) are shown as controls. The positions of intact kDNA at the origin (kDNA), decatenated nicked kDNA minicircles, and decatenated supercoiled (SC) kDNA minicircles are indicated. Gels are representative of 3 independent experiments.

Quantification of results is shown in the bar graphs at the bottom. Levels of decatenation in the absence of xanthone derivatives was set to 100%. Error bars represent standard deviations for 3 independent experiments.

Fig. 5.

Covalent linkage of the C3 polyamine moiety to the 1,3-dihydroxy-9h-xanthen-9-one core is critical for the inhibition of DNA relaxation catalyzed by topoisomerase II α . The effects of a 1:1 mixture of 1,3-dihydroxy-9h-xanthen-9-one (compound **6**) + spermidine (left panel) or + spermine (right panel) on the DNA relaxation activity of human topoisomerase II α is shown. Assays containing intact negatively supercoiled DNA in the absence of topoisomerase II α (DNA) or negatively supercoiled DNA treated with topoisomerase II α in the absence of xanthone derivatives (TII) are shown as controls. The positions of DNA markers are as shown in Figure 3. Gels are representative of 2 independent experiments. Quantification of results is shown in the graphs at the bottom. Data for compound **6** + spermidine (Spmd, left) and compound **6** + spermine (Spm, right) are shown as open circles. Control reactions in the presence of compound **4** (left) and **5** (right) are shown as closed circles. Error bars represent standard error of the mean for 2 independent experiments.

Fig. 6.

Inhibition of topoisomerase II α -mediated DNA cleavage by xanthone derivatives. Results for compounds **2**, **4-6** are shown. Reactions were carried out in the presence of CaCl₂ to enhance baseline levels of DNA cleavage by the enzyme. Assays containing negatively supercoiled DNA in the absence of topoisomerase II α (DNA) or negatively supercoiled DNA treated with topoisomerase II α in the absence of xanthone derivatives (TII) are shown as controls. The positions of DNA markers are as shown in Figure 2 inset. Gels are representative of 3 independent experiments. Quantification of results is shown in the graphs at the bottom. Levels of DNA cleavage in the absence of xanthone derivatives was set to 100%. Error bars represent standard deviations for 2-3 independent experiments.

Fig. 7.

Effects of xanthone derivatives on DNA relaxation catalyzed by 150 nM topoisomerase II α . Results for compounds **2**, **4-6** are shown. Assays containing intact negatively supercoiled DNA in the absence of topoisomerase II α (DNA) or negatively supercoiled DNA treated with topoisomerase II α in the absence of xanthone derivatives (TII) are shown as controls. The positions of DNA markers are as in Figure 3. Gels are representative of 2-4 independent experiments.

Fig. 8.

Effects of xanthone derivatives on DNA strand passage mediated by topoisomerase II α .

Results for compounds **4** and **5** are shown. Assays monitored the catenation of relaxed DNA in the presence of the non- hydrolyzable ATP analogue APP(NH)P so that the enzyme could only carry out one round of DNA strand passage. Assays containing relaxed DNA in the absence of topoisomerase II α (DNA) or relaxed DNA treated with topoisomerase II α in the absence of xanthone derivatives (TII) are shown as controls. The positions of relaxed DNA (Rel) and catenated DNA at the origin (Cat) are indicated. Gels are representative of at least 3 independent experiments. Quantification of results is shown in the graphs at the bottom. Levels of catenation in the absence of xanthone derivatives was set to 100%. Error bars represent standard deviations for at least 3 independent experiments.

Fig. 9.

Effects of compound **4** on ATP hydrolysis catalyzed by topoisomerase II α . Assays carried out in the presence or absence of negatively supercoiled DNA are shown as closed or open circles, respectively. ATPase activity rate in the presence of DNA and in the absence of compound **4** was set to 100%. Error bars represent standard error of the mean for 2 independent experiments.

Fig. 10.

Ability of xanthone derivatives to inhibit the enhancement of topoisomerase II α -mediated DNA cleavage by 100 μ M etoposide. Results for compounds **2**, **4**, and **5** (5 μ M, blue; 10 μ M, red; 25 μ M, green; 50 μ M, yellow; 100 μ M, orange) on the generation of enzyme-mediated double-stranded DNA breaks are shown. Due to solubility issues, compound **5** was only used up to 10 μ M. DNA cleavage in the presence of 100 μ M etoposide (purple) in the absence of xanthone derivatives set to 100%. Error bars represent standard deviations for 2-3 independent experiments.

Fig. 11. Molecular modeling of compound **4**. Docking of compound **4** into the DNA cleavage/ligation active site of topoisomerase II α . (Top). Superposition between etoposide (pdb: 3QX3) and the docking pose of compound **4** into the topoisomerase II DNA cleavage complex (Bottom).