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Synthesis of rotenoid derivatives with cytotoxic and topoisomerase II inhibitory activities

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ABSTRACT

6-Deoxyclitoriacetal (**1**) and a series of 11 further derivatives of it (**2**–**12**) were synthesized and evaluated for their cytotoxic and topoisomerase IIα inhibitory activities. Compounds bearing epoxide (**2**), morpholine (**6**) and benzylamine (**10**) moieties showed promising in vitro cytotoxic activities against four cancer cell lines, with IC_{50} values ranging from 0.38 to 0.73 μM. These three compounds also strongly inhibited topoisomerase II activity at 68.3–93.5% and showed a moderately high DNA intercalating property.

Cancer is one of the most common causes of premature death and morbidity. However, the rise in resistance to available chemotherapeutic agents, combined with their adverse side affects and high cost drives the search for new alternative anticancer compounds from natural products. These can then form the templates for further sequential chemical modifications to them or analogous compounds for improvement of their biological activities. This is an area of increasing interest, especially since some of the current effective commercial anticancer drugs currently available are derived from natural products,[1] such as psorospermin,[2] taxol[3] and vinblastine.[4]

Rotenoids consist of a four-fused tetrahydrochromeno[3,4*b*]chromene ring core (Fig. 1) from which specific modifications are built for each member. They are isolated from a fairly diverse array of medicinal plants in tropical countries often with a medical folklore tradition, and were originally applied as pesticides but now have also been investigated for conventional medical bioactivities, such as antibacterial, antimalarial, antifungal and anticancer properties.[5,6] The structural and biological interest in this group of compounds has encouraged attempts to isolate or modify the compounds for newly developed drugs.[7]

6-Deoxyclitoriacetal (**1**) (Fig. 1) is one of the rotenoid compounds extracted from roots of *Stemona collinsae* Craib. It exhibits a strong in vitro cytotoxicity against various human cancer cell lines, including the colon carcinoma (SW620), gastric carcinoma (KATO), lung carcinoma (CHAGO), breast carcinoma (BT474) and hepato carcinoma (HEP-G2).[8–10]

Chemical modifications of this compound in order to increase its cytotoxicity and to investigate the possible anticancer mechanism are our focus. A structure-based consideration of this compound found some of the interesting features of 6-deoxyclitoriacetal that appear to make it a good candidate anticancer agent that include (i) the molecule contains a planar moiety similar to doxorubicin (c) (Fig. 1), which is an important part for intercalating with DNA[11] and (ii) the molecule has a chromone ring system, like the flavonoids and coumarins, which has been shown to be an important feature for anticancer activity.[12] Therefore, one possible mechanism of its anticancer activity could be mediated via DNA intercalation or inhibition of the topoisomerase II process, like that reported for doxorubicin.[11]

Previous investigations have introduced different types of functional groups onto rotenoids, including doxyclitoriacetal, in an attempt to enhance the cytotoxic activity and develop more potent analogs. Firstly, coumarin-based compounds containing an epoxide group, for example, psorospermin (b), have been reported to display a good cytotoxic activity, exerting a good anticancer and

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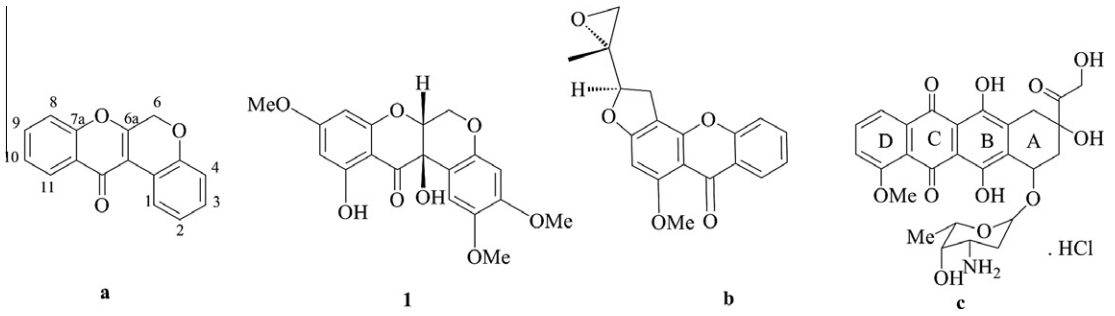


Figure 1. Chemical structures of the (a) rotenoid core (**1**) 6-deoxyclitoriacetal, (b) psorospermin and (c) doxorubicin hydrochloride.

topoisomerase II inhibitor activity. This is due to the intercalation of the psorospermin's xanthone group between the DNA helix base pair and that the epoxide ring undergoes alkylation reaction with the N7-guanine in the presence of topoisomerase II.[2] Secondly, several publications have revealed that the ring-opened epoxide with a good leaving group can enhance the cytotoxicity.[13] Introduction of either morpholine[14] or benzylamine[15] in part of the molecule also resulted in a higher potent cytotoxicity than the original compound. Finally, several studies suggested that the biological activities depended on the length of the side chain.[16] Therefore, we also studied the effect of the side chain length on the cytotoxic activity by varying the aliphatic chain spacer length in both the second and the third series.

In this Letter, we describe the synthesis, cytotoxicity and topoisomerase inhibitory activity of 11 substituted derivatives of 6-deoxyclitoriacetal with different functional groups at the C11-OH position (**2**–**12**) in an attempt to enhance the anticancer activity and potentially gain insight into the mechanism of action of 6-deoxyclitoriacetal and its derivatives. The reaction scheme for the modification of 6-deoxyclitoriacetal is shown schematically in Figure 2.

Initially the epoxy ring was introduced onto the C11-OH of the 6-deoxyclitoriacetal core by treating with epichlorohydrin in the presence of sodium hydride in dimethylformamide (DMF) at 80 °C, leading to compound **2**. Subsequently, the epoxide in the intermediate **2** was then ring-opened with small nucleophiles –Cl (**3**), –OH (**4**) and –OMe (**5**) to give the first series (**3**–**5**) to study the effect of the electron donor and acceptor on the cytotoxic activity. A similar reaction was performed using an amine in refluxing ethanol at 80 °C for 6–12 h leading to the second series (**6**–**9**) of compounds that consisted of cyclic and acyclic aliphatic amines. Lastly, compounds (**10**–**12**) were prepared by reacting **2** with a nucleophile containing an aromatic group. It should be noted that since the epichlorohydrin used was racemic, all products obtain were a mixture of two diastereomers and were evaluated as such.

The structures of the synthesized compounds were determined on the basis of their ^{1}H NMR, ^{13}C NMR and MS spectral data (shown in the Supplementary data), plus X-ray single crystal analysis for **3**.[17]

The in vivo activities of 6-deoxyclitoriacetal (**1**) against the colon carcinoma (SW620), gastric carcinoma (KATO), lung carcinoma (CHAGO), BT474 (breast carcinoma) and hepato carcinoma (HEP-G2) cell lines in tissue culture have already been reported.[9] In this work, all 6-deoxyclitoriaceal derivatives were tested for cytotoxic activity against the aforementioned five cell lines plus additionally the human oral carcinoma (KB), breast cancer (MCF-7) and human small lung (NCI-H187) for the evaluation of growth inhibition. Cell line growth was monitored using the MTT assay as reported,[16,18] and compared with doxorubicin as a positive control. IC_{50} values were classified by category as having a very high ($IC_{50} \leqslant 1$ μM), high ($1 \leqslant IC_{50} \leqslant 10$ μM), moderate ($10 \leqslant IC_{50} \leqslant 50$ μM), low

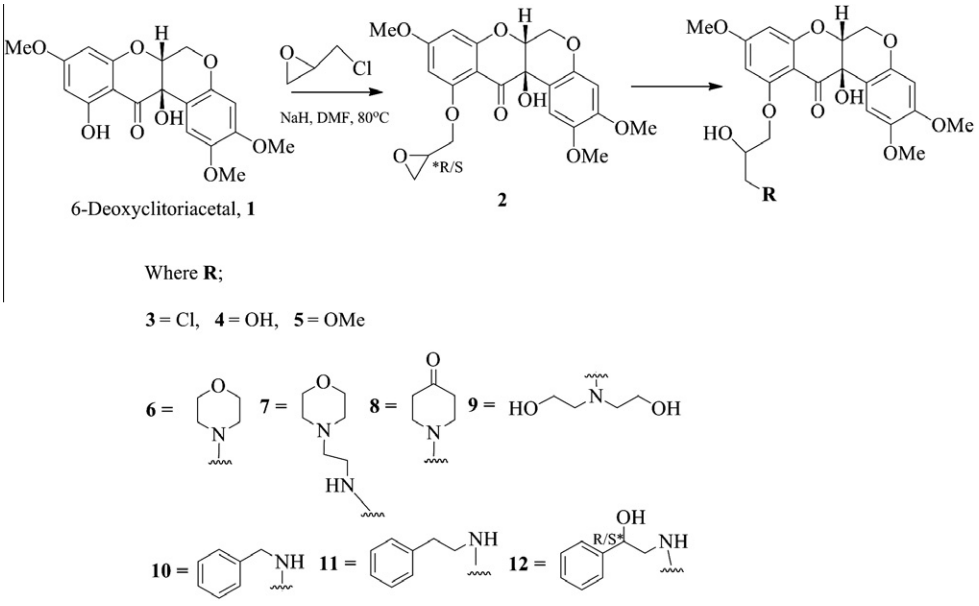
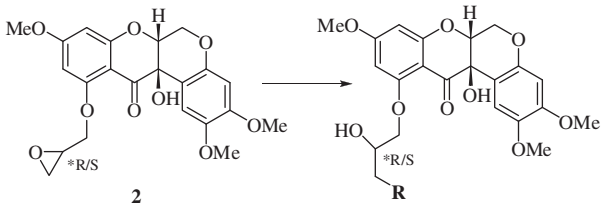
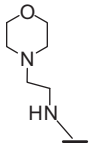
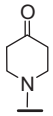
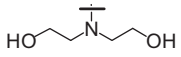
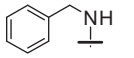
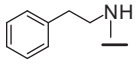
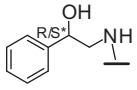


Figure 2. Schematic chart showing the synthesis route of the 11 derivatives from 6-deoxyclitoriacetal.[21]

Table 1
Cytotoxicity (as the inhibition of cell growth) and % topoisomerase II activity for compounds **1**–**12**



Compounds	R	% Topoisomerase II inhibition (100 μM)	IC_{50} (μM)[a]							
			KB	MCF7	NCI-H187	SW620	KATO	CHAGO	BT474	HEP-G2
Etop[b]	–	68.7	–	–	–	–	–	–	–	–
Dox[c]	–	72.1	2.01	42.52	0.03	0.23	0.33	0.83	0.07	0.26
1	–	72.1	13.28	23.65	3.12	>100	>100	>100	>100	>100
2	–	68.3	32.87	5.63	27.82	0.38	0.63	0.57	0.45	0.55
3	–Cl	58.3	14.77	24.91	6.61	1.09	1.09	>100	1.57	0.81
4	–OH	38.8	13.36	>100	13.94	8.04	>100	>100	>100	>100
5	–OMe	58.4	46.92	>100	11.69	>100	>100	>100	>100	>100
6		93.5	26.51	31.71	2.93	0.73	>100	0.61	0.34	0.61
7		77.1	26.31	36.82	4.70	0.54	1.94	40.23	0.45	0.56
8		54.2	>100	48.63	>100	>100	>100	>100	>100	>100
9		49.1	>100	>100	>100	>100	>100	>100	>100	>100
10		81.7	32.29	34.63	35.53	0.59	0.67	31.04	0.62	0.72
11		66.7	23.53	3.05	13.62	>100	>100	>100	>100	>100
12		60.4	46.44	>100	22.08	>100	>100	>100	>100	>100

[a] Data shown are derived from three independent trials.
[b] Etop = etoposide.
[c] Dox = doxorubicin.

($50 \leqslant IC_{50} \leqslant 100$ μM) or no (IC_{50} more than 100 μM) sensitivity to the test compound.

The IC_{50} values of the substituted compounds **2**–**12** are summarized in Table 1, along with that of the parental 6-deoxyclitoracetal (**1**) and doxorubicin for comparison. The parent 6-deoxyclitoracetal (**1**) exhibited a moderate cytotoxic activity against the KB and MCF-7 cell lines and a high activity against the NCI-H187 cell line but showed no significant activity against the other five tested cell lines. Interestingly, though, is the variation in response across the cell lines to the same compound, a trend which was observed with all of the 12 compounds screened. For the intermediate derivatives, the results showed that the compound **2** with the epoxide ring displayed the highest cytotoxicity, being active against all eight cell lines and at a higher level (lower IC_{50}) than the parent compound.

Concerning the epoxide ring-opened derivatives with small substituent (**3**–**5**), the presence of –Cl group (**3**) as both a strong electron withdrawing and a good leaving group resulted in the highest cytotoxicity against seven of the eight cell lines (very high for one, high for four and medium for two cell lines), with IC_{50} values ranging from 0.85 to 24.91 μM, but with no significant activity against the CHAGO cell line. The –OH bearing compound **4** as only an electron withdrawing group displays a lower cytotoxicity than that seen with compound **3**, with no significant activity against six cell lines and only moderate activity against the KB and NCI-H187 cell lines. However, this is slightly higher than that seen for the compounds having electron donor substituted groups of $–NH_2$ (**4**) and –OMe (**5**), which, although only showing moderate activity against the same two cell lines as compound **3**, they do so at a higher IC_{50} value. These results tentatively indicate that the ability of Cl as a leaving group is an important influence on the cytotoxicity, although the difference in electron withdrawing ability of the substituent group may also be important. However, all of these analogs exhibit lower activities than that of the epoxide containing compound **2**. There are examples in the literature that the replacement of the unstable epoxide ring with a leaving group resulted in moderate activities because of the electron withdrawing property of the substituents.[13]

Among the compounds with aliphatic amine substituents (**6**–**9**), some fairly consistent variation was seen. The compound with a morpholine substituent (**6**) showed the highest cytotoxic activity (four very high, one high, two moderate and one cell line with no significant activity), with IC_{50} values in the range of 0.33–31.71 μM for the seven responsive cell lines. This finding is in agreement with the literature, where the introduction of a morpholine core in part of the molecule was found to enhance its cytotoxic activity.[14] When the oxygen group is replaced with a carbonyl group, the piperidone (**8**) displays very poor cytotoxicity with only moderate activity against one (MCF-7) cell line and no significant activities against other seven cell lines, suggesting that the oxygen ether group is preferred in this position.

As the length of the aliphatic chain spacer is increased, as seen in comparing the morpholinoethyl derivative (**7**) to **6**, the cytotoxic activities in general are slightly decreased. Exceptions include the significantly large decrease and increase in the cytotoxicity level seen in the CHAGO and KATO cell lines, respectively. However, the presence of the bis(hydroxyethyl)amino group (**9**) lead to a dramatic decrease in the cytotoxicity, with no significant activity seen against all eight cell lines. These results tentatively indicate that the steric effect is not favored at this position. This also corresponds with previous work where longer and branched side chains were reported to reduce the intercalation ability of the compounds into DNA base pairs and hence decreased the observed level of cytotoxicity due to the steric effects.[19]

With respect to compounds with an aromatic substituent with different spacer lengths, (**10**–**12**), the compound with a benzylamine core (**10**) showed the highest cytotoxicity, with a very high cytotoxicity against four cell lines and moderate cytotoxicity against the other four cell lines. However, in contrast, compounds **11** and **12** with longer spacers displayed lower cytotoxicities than compound **10**, showing no significant toxicity against five (**11**) or six (**12**) cell lines and only moderate cytotoxicity against two cell lines (**12**), although compound **11** did show high cytotoxicity against one cell line. The result is in agreement with the previously mentioned unfavorable steric effects.[19] Moreover, when a polar hydroxyl-substituent was introduced, a lower cytotoxic activity resulted.

Doxorubicin is clinically used but has some resistance and so new compounds are required. Although most of the 11 derivatives of **1** evaluated here were found to mostly be significantly less effective than doxorubicin, some were seen to be more effective. Thus, considering the% inhibition level of topoisomerase II, compound **1** was as effective as doxorubicin, whilst compounds **6**, **7**, and **10** were more effective. Considering the IC_{50} value for the inhibition of the MTT assay, compounds **1**–**3**, **6**, **7**, **10**, and **11** showed lower IC_{50} values than doxorubicin for the MCF7 cell line, as did compounds **2** and **6** for the CHAGO cell line. However, importantly, the rather ineffective compound **1** was significantly improved in some of the derivatives paving the way for further future modifications to enhance the activity or specificity of new compounds or overcome resistance mechanisms.

With respect to the cell lines, some dramatic variations in their different susceptibilities to each compound were evident. This serves as a warning to the use of just a few cell lines for the screening of cytotoxicity, and potentially alludes to the cancer type as well as acquired drug resistance in culture. For example, the SW 620, KATO, CHATO, BT474 and HEP-G2 cell lines were typically highly sensitive to compounds with epoxide rings, but gained resistance upon the addition of the electron donor groups, or an aromatic substituent with longer spacers. However, the same effect was not observed with the KB and NCI-H187 cell lines that, in general, showed a lower sensitivity to compounds with epoxide rings but retained some sensitivity to those with electron donor groups, or to compounds with aromatic substituents with longer spacers. Further characterization may prove useful in allowing composition of a better cell line panel for screening new compounds against particular cancer types or for understanding resistance mechanisms.

The DNA replication process appears to be the key target involved in the mechanism of action of anticancer drugs that act as intercalators and/or topoisomerase II inhibitors, including doxorubicin and psorospermin. In order to study the possibly anticancer mechanism of 6-deoxyclitoriacetal (**1**) and its derivatives (**2**–**11**), we performed a single topoisomerase IIα inhibition assay.[20] The topoisomerase IIα relaxation assay was carried out using human topoisomerase IIα (GE health care) and using etoposide as the reference (100 μM) standard and positive control. As shown in Figure 3, the% inhibition was classified into strongly and weakly active, depending on whether its% inhibition was lower or higher than the activity of the reference control, etoposide (68% inhibition).

The ranked topoisomerase IIα inhibitory activity for all derivatives was broadly consistent with the observed cell line cytotoxic activity in Table 1. That is the derivatives with a strong cytotoxic activity (**2**, **6**, **7**, and **10**) also displayed a strong topoisomerase IIα inhibitory activity with more than 80% inhibition at 100 μM. Compounds **4**, **5**, **8**, and **9**, which were inactive against almost all of the eight cell lines tested, were also inactive against topoisomerase IIα showing no inhibitory activity at 100 μM. Indeed, compound **6** showed the strongest topoisomerase IIα inhibition (over 90%) compared to etoposide as the reference (68.7%), the parent compound (**1**) (72.1%) at 100 μM. However, note that the concentration used in the topoisomerase IIα inhibition assay (100 μM) may well be above the actual IC_{50} values for this inhibition, and certainly is higher than that reported for the inhibition of cell proliferation for many of these compound-cell line combinations. Reducing the concentration of the strongest topoisomerase IIα inhibitor (**6**) 5-fold from 100 to 20 μM caused an almost 2-fold decrease in the% topoisomerase IIα inhibition (Fig. 4). Although, the correlation of the cytotoxic and topoisomerase IIα inhibitory activity is not perfectly clear, in terms of the IC_{50} especially, compound **6** exhibited a strong topoisomerase IIα inhibitory activity and showed nearly as good a cytotoxic activity compared to doxorubicin, as the reference standard and positive control. These results could suggest that these series of 6-deoxyclitoriacetal derivatives may not act via the same mechanism as doxorubicin, and may be differentially modified by cell metabolism. However, these results may provide advanced opportunities to design new chemotherapeutic agents.

DNA intercalation is an important mode of control of gene expression and DNA replication, as well as mutagenesis.[2,11] Generally, the local T_m will increase when intercalative binding occurs because of the stabilization of the base stacking, and this can be used to indicate the intercalating affinity of small molecules into the double helix, and so potential cytotoxicity. The 6-deoxclitoriacetal derivatives of **2**, **6**, **7**, and **10** were selected to evaluate their effect upon the ct-DNA T_m as they showed a high cytotoxic activity and topoisomerase IIα inhibition compared to the parental **1** (Table 1). The melting temperature curves of ct-DNA in the absence and presence of 6-deoxclitoriacetal derivatives **1**, **2**, **6**, **7**, and **10** are summarized in Table 2 and Figure 5. The T_m of ct-DNA in the presence of the tested compounds was about 57.3–68.0 °C compared with the absence (56.4 °C). A slight increase in the T_m (0.9 °C increase to 57.3 °C) is observed in the presence of the parent compound **1**, indicating a low binding affinity in DNA intercalation, [22] while the T_m of the ct-DNA increased further upon the addition of the compounds **2**, **6**, **7**, and **10** (Table 2). The highest change in T_m (ΔT_m = +10.7 °C) was observed in the presence of the benzylamine bearing compound **10**, and this may be due to the non-covalent π–π interaction of the DNA bases by the benzene ring, in addition to the intercalation. For the other derivatives (epoxide **2** and heterocyclic **6** and **7**), the electrostatic attraction between the epoxide (**2**) and protonable

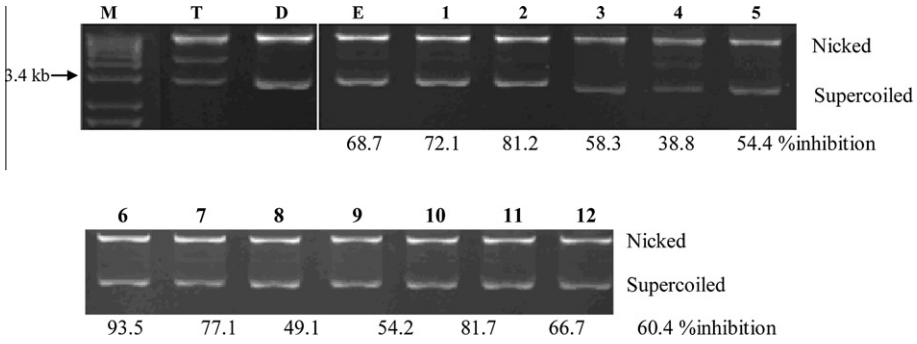


Figure 3. Inhibitory effect of the tested compounds on human DNA topoisomerase II activity. Supercoiled pBR322 plasmid DNA was mixed with 0.3 U topoisomerase II (TopoII) and the test compound (100 μM) dissolved in DMSO (lanes 4–16). Lane 1: marker; lane 2, TopoII + plasmid DNA; lane 3, DNA only; lane 4, TopoII + plasmid DNA + etoposide and lanes 5–16, TopoII + plasmid DNA + test compounds **1**–**12**, respectively. Gels shown are representative of two independent assays.

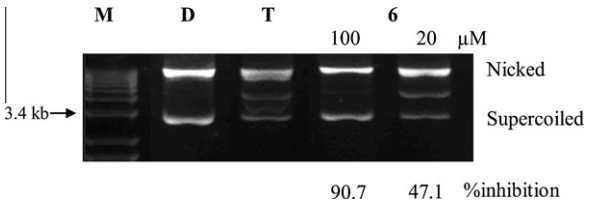


Figure 4. Inhibitory effects of the morpholine derivative (**6**) at two different concentrations on human DNA topoisomerase II activity. Supercoiled PBR322 plasmid DNA was mixed with 0.3 U topoisomerase II (TopoII) and the test compounds. Lane 1, marker; lane 2, plasmid DNA only; lane 3, TopoII + plasmid DNA; lanes 4 and 5, TopoII + plasmid DNA + compound **6** at (lane 4) 100 and (lane 5) 20 μM, respectively. Gels shown are representative of two independent assays.

Table 2
The melting temperature data for compounds **1**, **2**, **6**, **7**, and **10**

Compounds	T_m (°C)	ΔT_m (°C)[a]
CT only	56.4	—
1	57.3	—
2	59.3	2.0
6	65.1	7.8
7	66.1	8.8
10	68.0	10.7

[a] Difference between T_m values of ct-DNA in the presence of the tested compound and **1**.

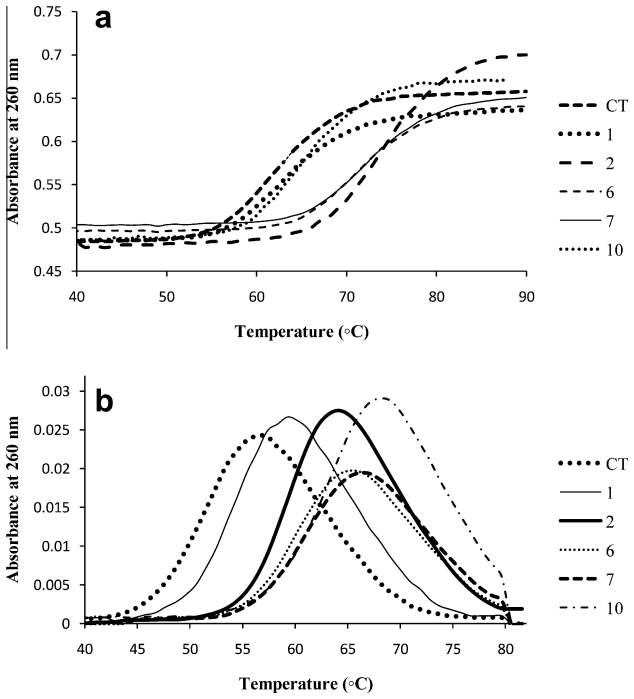


Figure 5. Effects of five different 6-deoxyclitoriacetal derivatives (**1**, **2**, **6**, **7**, and **10**) on the thermal denaturation of calf thymus DNA, showing the (a) T_m curves and (b) derivative curves of T_m at 260 nm. Data shown are representative of two independent assays.

nitrogen (**6** and **7**) with the anionic DNA may also be important. The intercalative ability of the tested compounds is in the order of aromatic > heterocyclic > epoxy substituents by comparing the ΔT_m. It's possible that the functional groups attached at C-11 of 6-deoxyclitoriacetal may enhance the DNA binding affinity. These results are also consist with the trends of cytotoxic and topoisomerase II inhibitory activities. To conclude, the 11 investigated 6-deoxyclitoriacetal derivatives may show DNA-intercalation and be important targets for cancer chemotherapy.

These results show that 6-deoxyclitoriacetal (**1**) and its derivatives bearing epoxide (**2**), morpholine (**6**) and benzylamine (**10**) moieties showed promising cytotoxic activities against seven cancer cell lines with IC_{50} values ranging from 0.45 to 35.53 μM, strongly inhibited topoisomerase II activity and displayed a reasonably strong intercalating property, as determined by the increased T_m of the ct-DNA. The anticancer activities of these compounds may occur via inhibition of topoisomerase IIα and DNA intercalation. However, this preliminary correlation certainly requires further work for conformation, including to extensively further investigate the structure–activity relationships of the 6-deoxyclitoriacetal derivatives. It also becomes of interest as to the different sensitivities and especially resistant levels of the different cell lines. Likewise, there are still a diverse number of other compounds in this range still to be screened, and others yet to be made, to further test the preliminary findings reported here.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.06.052.

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18. *Cytotoxicity assay:* Cytotoxic activity, as inhibition of cell line growth, was evaluated by the MTT assay. Cell lines were cultured according to the supplier's instructions at 2–4 $\times$ 10^4 cells per well in media containing 10% (v/v) fetal bovine serum and incubated under 5% (v/v) CO_2 at 37 °C, overnight. The compounds were added in each well and three days later the MTT solution was added to a final concentration of 0.5 mg/mL, incubated for 4 h and then the medium in each well was discarded and replaced with 150 μL of DMSO and 25 μL of glycine pH 10.5. The absorbance of each well was determined by an Automatic Elisa Reader System at 540 nm.
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20. *In vitro topoisomerase II assay*: The reaction mixture contained 10 mM Tris–HCl (pH 7.9), 175 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2.5% (v/v) glycerol, 1 mM ATP, 0.5 mM dithiothreitol, 30 μg/mL bovine serum albumin, 0.2 μg pBR322, 0.6 U DNA topoisomerase IIα and the test compounds in a final volume of 20 μL. The reactions were incubated for 30 min at 37 °C and terminated by the addition of 3 μL of solution containing 0.77% (w/v) sodium dodecyl sulfate and 77 mM EDTA. Samples were mixed with 2 μL of solution containing 30% (w/v) sucrose, 0.5% (w/v) bromophenol blue and 0.5% (w/v) xylene cyanol, and subjected to electrophoresis on a 1% (w/v) agarose gel at 1.5 V/cm for 10 h with a running buffer of Tris–borate–EDTA. 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 quantified using an image analyzer and Syngene software.
21. Experimental procedures and spectral data for compounds **2**–**12** can be found in the supplementary data.
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