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Synthesis and cytotoxic activity of a new series of topoisomerase I inhibitors

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Abstract—A series of structurally simple analogues of natural topopyrone C were synthesized and tested for cytotoxic and topoisomerase I inhibitory activities. The removal of the hydroxyl groups at the 5 and 9 positions resulted in an increased cytotoxic potency and ability to stabilize topoisomerase-mediated cleavage. In addition, the results suggest that some structural features, such as the pyrone ring and a polar group in position 11, are fundamental for topoisomerase I inhibitory effect. These structural requirements are also consistent with the cytotoxic activity.

Topoisomerase I (topo I) is a nuclear enzyme that catalyzes the relaxation of superhelical tension in DNA during replication and transcription. This process is initiated by a reversible covalent attachment of the enzyme to the DNA backbone, creating a transient single-strand break in the DNA duplex and preventing buildup of torsional energy. The formed DNA–topo I covalent binary complex, which normally undergoes religation, can be stabilized by drugs via formation of a ternary complex. Persistence of the stabilized DNA breaks leads to cell death.[1]

As cancer cells tend to overexpress this enzyme, which is required during various DNA functions, topo I inhibitors have emerged as important antineoplastic agents.[2] A diverse set of anticancer compounds, including camptothecins,[3] indolocarbazoles, and indenoisoquinolines,[4] are selective topo I inhibitors. These compounds bind to the transient topo I–DNA covalent complex and inhibit resealing of the single-strand break.[5] Other compounds, however, can inhibit topo I through an alternative mechanism,[4] such as inhibition of catalytic activity. Camptothecins (CPT) are the only topo I inhibitors in clinical use, but there are some disadvantages inherent to the use of CPT. Although CPT is cytotoxic in a wide range of animal tumors, its therapeutic efficacy is limited due to the rapid reversibility of the ternary DNA–enzyme–CPT complex and hydrolysis of the lactone to a hydroxyacid that binds to plasma proteins.[6] Moreover, some cancer cells develop resistance to CPT. In this context, there is a need for chemically stable compounds that act as topo I inhibitors and that are cytotoxic in cancer cell lines.

Recently, Kanai et al.[7] discovered four new specific inhibitors of topo I, topopyrones A–D (**1a**–**d**). All four compounds were isolated from the culture broth of a fungus, *Phoma* sp. BAUA2861, and two of them from *Penicillium* sp. BAUA4206. Structural elucidation showed that the compounds are of the anthraquinone type and contain a fused 1,4-pyrone moiety[8] (Chart 1).

Topopyrones A, B, C, and D selectively inhibit recombinant yeast growth, depending on the expression of human topo I. All these compounds showed significant cytotoxic effects (in the range 0.7–20 μM) against a panel of tumor cell lines when tested in vitro.[7] Structural

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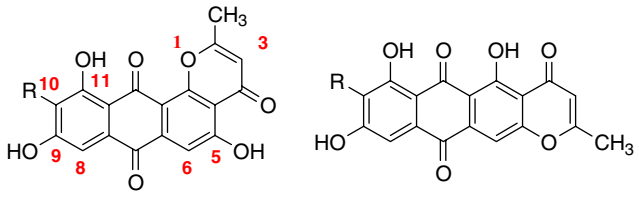


Chart 1.

and biological features of topopyrones make them attractive synthetic targets.

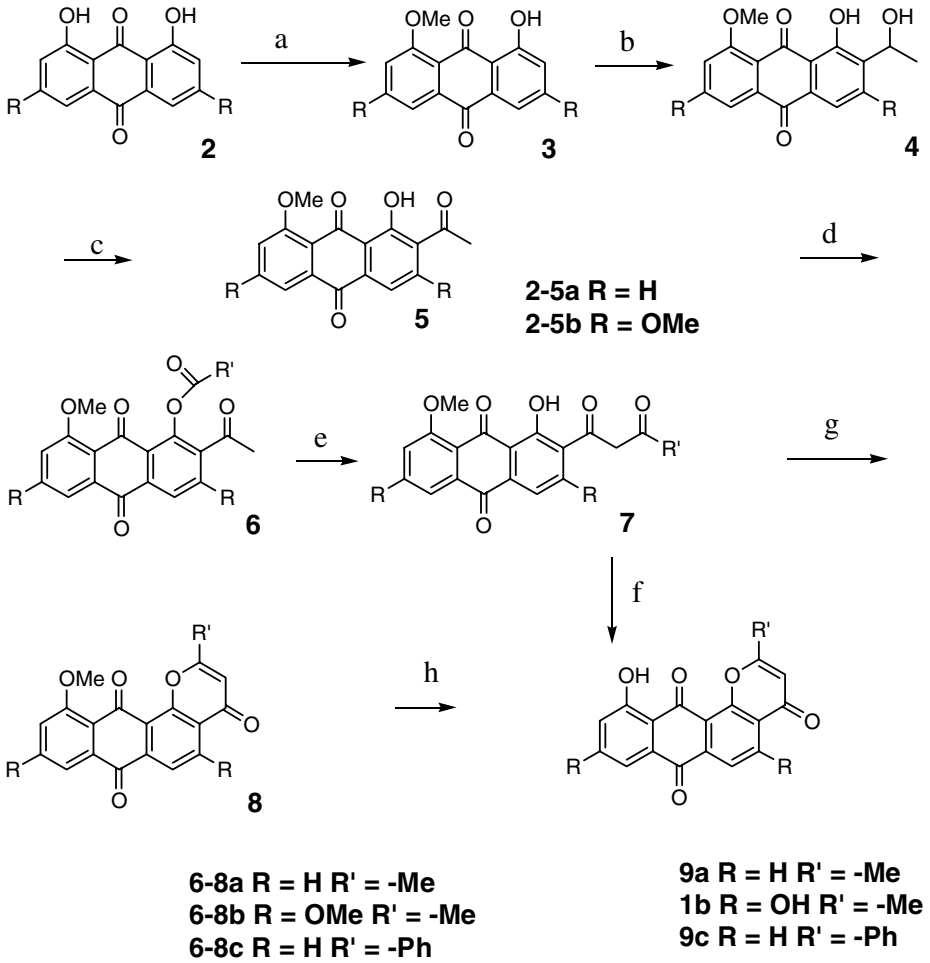
As a part of our ongoing project centered on the study of novel topo I inhibitors, we recently developed a method for total synthesis of topopyrone C.

However, in our hands, the compound showed moderate cytotoxic activity (IC_{50} = 29.50 μM) compared to the reference CPT topotecan (IC_{50} = 1.18 μM) when tested on the human non-small cell lung cancer NCI-H460 cell line, a cell model selected for its sensitivity to topo I inhibitors. This finding prompted us to investigate the structural requirements for antitumor activity of this class of compounds.

A series of simple analogues were synthesized following our previously developed synthetic strategy. Although the number of compounds tested was limited, some structural features important to the topo I inhibitory effect can be inferred. We report here the synthesis, the assessment of the cytotoxic activity, and the stabilizing activity of the DNA–topo I complex by the derivatives of this series.

Topopyrone C (**1b**) was synthesized as previously described.[9] The synthetic approach was based on the use of the Marschalk alkylation reaction of 1-hydroxy-3,6,8-trimethoxyanthraquinone[10] followed by a Baker–Venkataraman chain elongation[11] and an acid-catalyzed cyclization for the construction of the pyrone framework.

In an effort to define the structural elements in topopyrone C essential for binding to the topo I–DNA covalent binary complex, we have been altering topopyrone C at



Scheme 1. Reagents and conditions: (a) $TsOCH_3$, Na_2CO_3, tetraglyme, 120 °C, 2 h, 81%, or $TsOCH_3$, Na_2CO_3, MW, 210 W, 15 min, 81–88%; (b) 1—NaOH, $Na_2S_2O_4$, CH_3OH, N_2, 0 °C, 2—CH_3CHO, N_2, 20 °C, 3 h, 3—H_2O_2, 0 °C, 30–41%; (c) PCC, H_5IO_6, CH_3CN, 0 °C, 30 min, then rt, 3 h, 60–100%; (d) Ac_2O, Py, reflux, 10 h, 88–92% or PhCOCl, K_2CO_3, $CH_3COC_2H_5$, reflux, 4 h, 93%; (e) LiH, THF, N_2, reflux, 20 h, 55–82%; (f) HBr, AcOH, 8 h, reflux, 53–76%; (g) TFA, 76–94%; (h) BBr_3, CH_2Cl_2, −60 °C, 90 min, 25–69% or HBr, AcOH, 6 h, reflux, 83%.

single sites deemed to be critical for inhibitory activity. The first goal of the present investigation was to define the role of the hydroxyl groups in positions 5, 9, and 11 and to determine whether they could be deleted with retention of activity. Thus, the synthetic strategy previously described was followed to obtain the simplified derivatives **9a** and **9c**, starting from commercially available 1,8-dihydroxyanthraquinone (Scheme 1). The compounds were tested for growth inhibition ability against the human non-small cell lung cancer NCI-H460 cell line.[12] Cytotoxicity was assessed by growth inhibition assay after a 1-h drug exposure.[13] Interestingly, the cytotoxic activity of **9a** and **9c** was higher than that of topopyrone C, revealing that the presence of the two hydroxyl groups in positions 5 and 9 was detrimental for activity (Table 1). In contrast, the hydroxyl group in position 11 seemed to play an important role: in fact, methylation of the OH in position 11 led to a drop in activity (**8a** vs **9a**, **8c** vs **9c**, see also **8b**).[14]

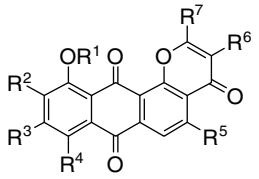
Topo I-mediated DNA cleavage assay was used to investigate the ability of the compounds to stimulate DNA damage.[15] Purified human topo I was used with SN38 as the reference drug (Fig. 1). Compound **9a** induced the same sequence selectivity of DNA cleavage by topo I shown by CPT SN38. This result indicates that, although less potent than a CPT, compound **9a** stabilizes in a similar way the binary DNA–topo I cleavable complex. The formation of a stable ternary complex is deemed to be the reason for the cytotoxic activity of CPT.

In accord with these findings, compound **9a** was further modified by introducing either lipophilic or hydrophilic moieties on the skeleton of the molecule (Scheme 2). Treatment of **9a** with $Cu(NO_3)_2$ in trifluoroacetic acid gave compound **10** in a 20% yield. The monobromo derivative **11** was obtained by treatment of **9a** with NBS and *p*-toluenesulfonic acid, whereas the dibromo-derivative **13** was obtained in an 83% yield using Br_2 and acetamide in acetic acid. Compound **12** was synthesized to improve the water solubility of this very lipophilic class of compounds. Conversion of **9a**–**12** was achieved using dimethylaminoethyl chloride hydrochloride in tetraglyme.

The introduction of a dimethylaminoethyl chain linked to the oxygen at position 11 led to a more water-soluble derivative (**12**), which retained good cytotoxic activity. The incorporation of a nitro or a bromo group ortho or para to the 11-OH resulted in the maintenance of activity. The most active derivative was compound **13**, with an additional bromo group on the pyrone ring. In order to investigate the ability of the compounds to stimulate DNA damage, topopyrone C analogues were submitted to a test of topo I-mediated DNA cleavage. All the compounds analyzed appeared less potent than SN38 in producing DNA damage. Compared to SN38, no change in sequence selectivity was observed. Compound **13** was the most effective of the series, whereas **9a**, **11**, and **12** revealed a lower intensity of DNA cleavage. The activity was markedly reduced in **8a**. This trend is consistent with the changes in the cytotoxic activity. The only exception seems to be compound **10**, which is not active on topo I, although showing some cytotoxic activity. This effect could reflect a steric hindrance which influences the drug interaction in the ternary complex. The cytotoxic effect of this compound is likely related to a topo I-independent mechanism.

Another goal of the present study was to assess the role of the pyrone ring. Compounds **3a**, **14**, and **15** where a simple methoxy group replaces the pyrone ring (Scheme 3) were tested for their cytotoxic activity and compared with the corresponding topopyrones (**9a**, **12**, and **10**). The results showed that removal of the pyrone ring led to complete loss of antiproliferative activity in all three cases (Table 2). Moreover, analysis of the topo I-mediated DNA cleavage assay showed that these compounds were substantially not able to induce DNA damage.

Table 1. In vitro cytotoxic activity of topopyrone C and analogues on H460 cell lines



Compound	R^1	R^2	R^3	R^4	R^5	R^6	R^7	IC_{50}[a] (μM)
1b	H	H	OH	H	OH	H	Me	29.50
8a	Me	H	H	H	H	H	Me	>260
8b	Me	H	OMe	H	OMe	H	Me	>100
8c	Me	H	H	H	H	H	Ph	>100
9a	H	H	H	H	H	H	Me	5.50
9c	H	H	H	H	H	H	Ph	7.06
10	H	H	H	NO_2	H	H	Me	9.10
11	H	H	H	H	H	Br	Me	6.50
12	$(CH_2)_2N(Me)_2$	H	H	H	H	H	Me	7.15
13	H	Br	H	H	H	Br	Me	4.95
Topotecan								1.18

[a] The IC_{50} values are means of three inhibition assays.

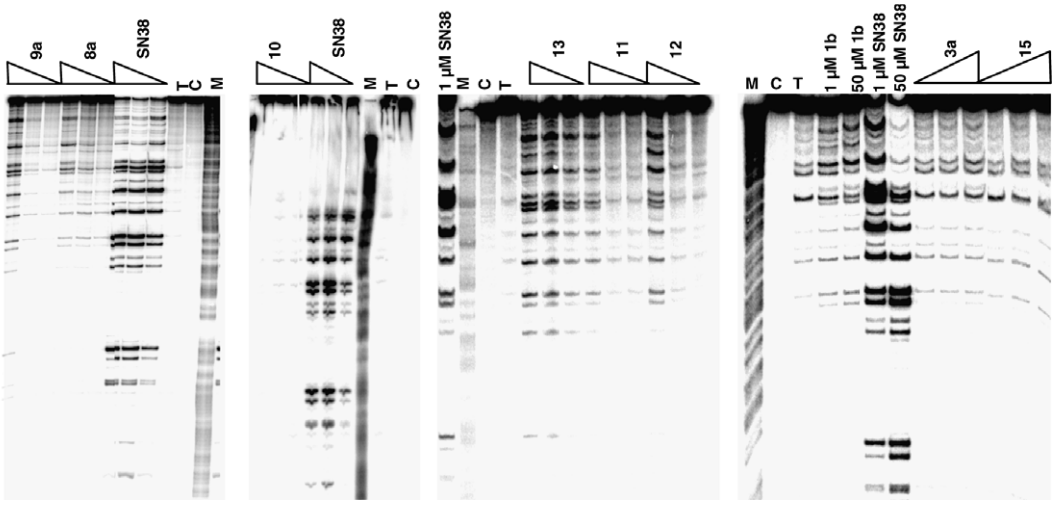
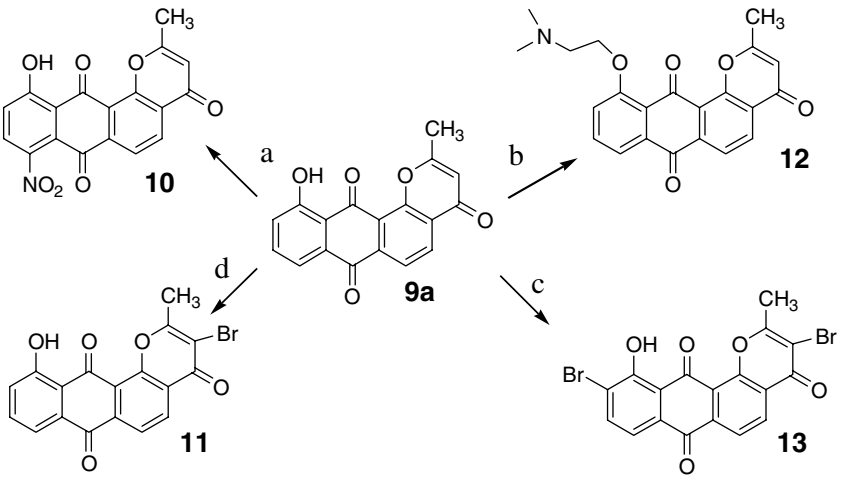


Figure 1. Topoisomerase I-mediated DNA cleavage by SN38 and different topopyrones. Samples were reacted with 1, 10, and 50 μM drug at 37 °C for 30 min. Reaction was then stopped by adding 0.5% SDS, 0.3 mg/ml of proteinase K and incubating for 45 min at 42 °C before loading on a denaturing 8% polyacrylamide gel. Experiments were performed three times and a representative image is reported. C, Control DNA; T, reaction without drug; M, purine markers.

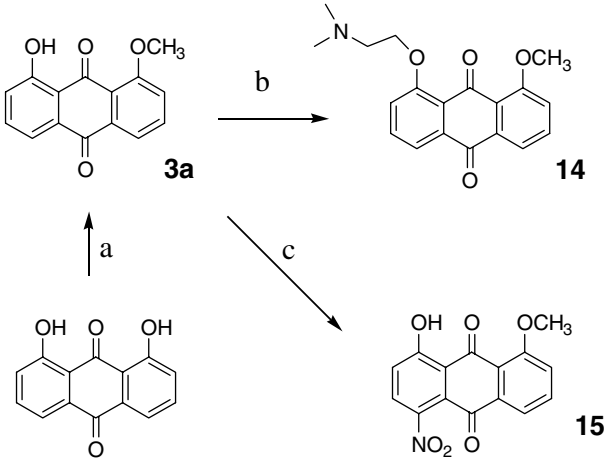


Scheme 2. Reagents and conditions: (a) $Cu(NO_3)2{\cdot}3H_2O$, CF_3COOH, rt, 5 days, 20%; (b) $(Me)_2N(CH_2)_2Cl{\cdot}HCl$, K_2CO_3, tetraglyme, 120 °C, 3 h, 27%; (c) Br_2, CH_3CONH_2, CH_2Cl_2, AcOH, reflux, 6 h, 83%; (d) NBS, $pTsOH{\cdot}H_2O$, MeOH,reflux, 3 h, 40%.

Thus, it appears evident that addition of the 4-pyrone moiety to the anthraquinone ring is determinant for the poisoning activity of topo I. The capability to inhibit the enzymatic activity of purified topo I was evaluated using the DNA relaxation assay.[16] Compounds **9a** and **13**, the most active analogues in reducing cell growth, were analyzed. Neither compound was active in inhibiting the enzymatic activity (Fig. 2).

These data lead us to suggest that the biological activity of topopyrone C derivatives is similar to that of other planar polycyclic compounds that show stabilization of the binary DNA–topo I complex, (e.g., indenoisoquinolines, benzo[*c*][1,7] and [1,8]phenanthrolines, benzo[*i*]phenanthridines, and other analogues of protoberberine alkaloids), but with cytotoxicity of **a** lower order of magnitude than CPT. The weak correlation between cell growth inhibition and topo I inhibition by the tested compounds suggests that differences in cellular uptake, subcellular distribution, and additional biological targets may contribute to determine antitumor activity. Such observations suggest that optimal drug efficacy can be achieved from a balance between cellular pharmacokinetics and intrinsic ability to induce DNA cleavage.

In conclusion, the first series of structurally simple analogues of natural topopyrone C were synthesized and tested for their biological activity. The available results support that some structural features are fundamental for the topo I inhibitory effect, such as the pyrone ring and a polar group in position 11, whereas the presence of hydroxy groups in positions 5 and 9 is detrimental for activity. Interestingly, most of the new analogues showed a stronger cytotoxic activity than the parent compound. Therefore, a new lead structure was found and further profiling of selected compounds will be reported in due course.



Scheme 3. Reagents and conditions: (a) Na_2CO_3, $TsOCH_3$, MW, 15 min, 81%; (b) tetraglyme, K_2CO_3 2-(dimethylamino)ethylchloride hydrochloride, 5 h, 120 °C, 40%; (c) TFA, $CuNO_3{\cdot}3H_2O$, 90 min, rt, 30%.

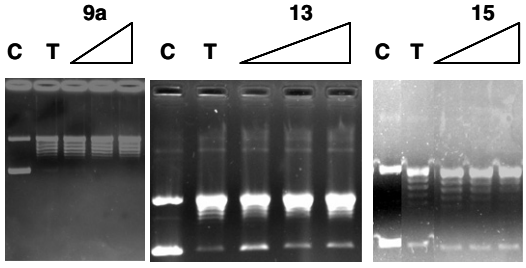
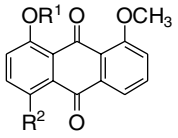


Figure 2. Topoisomerase I-mediated DNA plasmid relaxation in presence of **9a**, **13**, and **15**. Samples were reacted with 1, 10, and 50 μM compound at 37 °C for 30 min. Reaction was then stopped by adding 0.5% SDS, 0.3 mg/ml of proteinase K and incubating for 45 min at 42 °C before loading on 0.8% agarose gel. C, Control DNA; T, reaction without drug.

Table 2. In vitro cytotoxic activity of anthraquinones on H460 cell lines



Compound	R^1	R^2	IC_{50}[a] (μM)
Topotecan			1.18
3a	H	H	>100
14	$(CH_2)_2N(Me)_2$	H	>100
15	H	NO_2	160

[a] The IC_{50} values are means of three inhibition assays.

Acknowledgment

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Supplementary data

Experimental procedures and characterization data for all compounds. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.12.055.

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13. Human non-small cell lung cancer NCI-H460 cells were cultured in RPMI 1640 containing 10% fetal calf serum. Cytotoxicity was assessed by growth inhibition assay after 1 h drug exposure. Cells in the logarithmic phase of growth were harvested and seeded in duplicates into 6-well plates. Twenty-four hours after seeding, cells were exposed to the drug, harvested 72 h, and counted with a Coulter counter. IC_{50} is defined as the inhibitory drug concentration causing a 50% decrease of cell growth over that of untreated control.
14. The cytotoxic activity of compound **8a** on human A 431 cells has been recently reported.
15. A 3′-end labeled gel purified 751-bp BamHI–EcoRI fragment of SV40 DNA was used for the cleavage assay. SV40 plasmid was first linearized with BamHI enzyme and then 3′-labeled by using DNA polymerase I large (Klenow) fragment (Invitrogen, Paisley, UK) in the presence of dGTP and α^{32}P ddATP. The labeled DNA was then restricted with EcoRI enzyme and the corresponding 751-bp was purified on agarose gel. Topoisomerase I DNA cleavage reactions (20,000 cpm/sample) were performed in 20 μl of 10 mM Tris–HCl (pH 7.6), 150 mM KCl, 5 mM $MgCl_2$, 15 μg/ml BSA, 0.1 mM dithiothreitol, and 640 ng of human recombinant enzyme (full length topoisomerase I) for 30 min at 37 °C. Reactions were stopped by adding 0.5% SDS and 0.3 mg/ml of proteinase K for 45 min at 42 °C. After precipitation, DNA was resuspended in denaturing buffer (80% formamide, 10 mM NaOH,

0.01 M EDTA, and 1 mg/ml dyes) before loading on a denaturing 8% polyacrylamide gel in TBE buffer.
16. DNA relaxation activity was assayed with 200 ng of supercoiled pBR322 DNA in 20 mM Tris–HCl (pH 7.6), 0.1 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 μg/ml BSA, 0.1 mM EDTA, 150 mM KCl in the presence of purified protein and with 1, 10, and 50 μM of compound. Samples were incubated for 30 min at 37 °C and then stopped by adding 0.5% SDS and 0.3 mg/ml proteinase K at 42 °C for 45 min. Plasmid relaxation was analyzed on 0.8% agarose gel followed by ethidium bromide staining.