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Synthesis, Biological Activity and Comparative Analysis of DNA Binding Affinities and Human DNA Topoisomerase I Inhibitory Activities of Novel 12-Alkoxy-benzo[*c*]phenanthridinium Salts

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Abstract—New antitumor 12-alkoxy-benzo[*c*]phenanthridinium derivatives were obtained in high yields through multistep syntheses. Analysis of DNA binding and human DNA topoisomerase I inhibitory activities demonstrates that new compounds, combining 2, 6, and 12 substitutions, interact strongly with DNA and exhibit important topoisomerase I inhibition. The cytotoxicities against solid tumor cell lines are also determined and compared with those for fagaronine and ethoxidine.

Benzo[*c*]phenanthridines (BZPs) are naturally occurring alkaloids which have demonstrated numerous biological activities.[1] Among these structures, nitidine **1** and fagaronine **2** have solicited much interest as anticancer drugs. Preclinical studies were conducted at the US National Cancer Institute, but these compounds were not considered to be practical anticancer drugs.[2] Many synthetic approaches to benzo[*c*]phenanthridinic alkaloids leading to natural products have been reported, but relatively few studies of synthetic analogues have been described.[3] BZPs have also demonstrated binding affinities to DNA, have been presumed to be intercalators and have been shown to be inhibitors of DNA topoisomerases I (top1) and II (top2),[4] the well known targets for clinically important and emerging antitumor drugs.

A recent study reported the impact of 12-alkoxy modification on the in vitro antileukemic activity of *N*-methyl-12-alkoxy-benzo[*c*]phenanthridinium salts.[5] Among the isolated molecules, the ones which had shorter aliphatic and non-functionalized 12-alkoxy-chains gave the best biological results in different in vitro antileukemic activity assays. Of these derivatives, ethoxidine **3** ($R^{12}=CH_2CH_3$, $X=CH_3SO_3^-$) demonstrated high cytotoxic activity on the P388 and K562 cancer cell lines and also had the ability to induce differentiation in K562 human cells.

Compounds **2** and **3** ($R^{12}=CH_2CH_3$, $X=CH_3SO_3^-$) were proven to be DNA intercalators[4c,6] with, for the latter, the 12-ethoxy appendage penetrating into the minor groove.[6] Top1 inhibitors are grouped into two classes: top1 poisons and top1 suppressors. The best characterized top1 poisons are camptothecins (CPTs) which kill cells by trapping cleavage complexes between top1 and DNA.[7] The typical DNA binders, including DNA intercalators and minor groove binders can suppress top1-linked DNA breaks due to prevention of enzyme's access to DNA and/or distortion of the DNA structure. The selectivity of the existing top1 suppressors remains questionable.[7] Subsequent investigations showed that **2** induces DNA cleavage by top1 in a typical CPT-dependent manner[4c] and that the 2-OH group greatly contributed to the biological activity as a hydrogen bond acceptor.[6b] On another hand, **3** ($R^{12}=CH_2CH_3$, $X=CH_3SO_3^-$) suppresses both top1-specific and CPT-dependent cleavage, with AT-sequence

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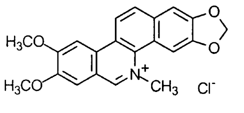
specificity of effect.[6b] Thus, **3** ($R^{12} = CH_2CH_3$, $X = CH_3SO_3^-$) and **2** demonstrate different mechanisms of action towards top1, respectively, suppression and poisoning of the enzyme. Moreover, **3** ($R^{12} = CH_2CH_3$, $X = CH_3SO_3^-$) inhibites DNA relaxation at a concentration 10-fold lower than fagaronine.[6a] These results make **3** ($R^{12} = CH_2CH_3$, $X = CH_3SO_3^-$) potentially attractive for further development of top1 inhibitors.

One aim of the present work is to take into account the 12-*O*-ethyl and 2-hydroxy substitutions which play major roles in the biological activities. We thus report the synthesis of new 12-ethoxy-fagaronine derivatives. We also report their cytotoxicities against human solid tumor cell lines and make a comparative analysis of DNA binding affinities and top1 inhibitory activities of these BZPs. The contribution of 6-position substitution of new 2,3,7,8-tetraoxygenated-BZPs was recently evaluated on cytotoxicities and reduction-resistance properties.[8] We have thus synthesized 6-methyl-ethoxidine to measure the impact of the 6-substitution on DNA binding and top1 inhibition.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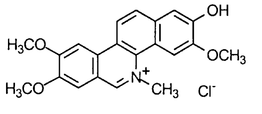
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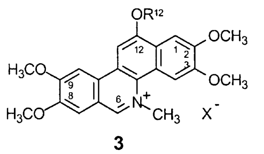
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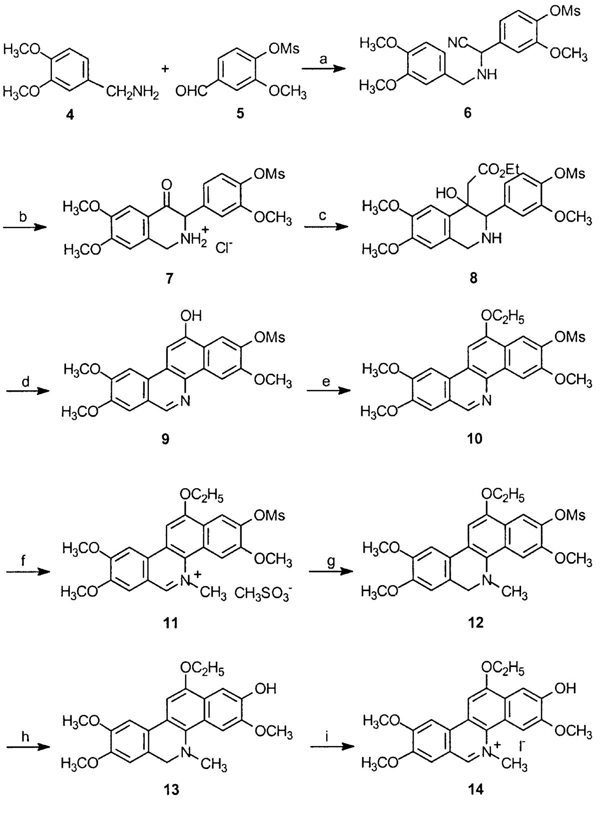
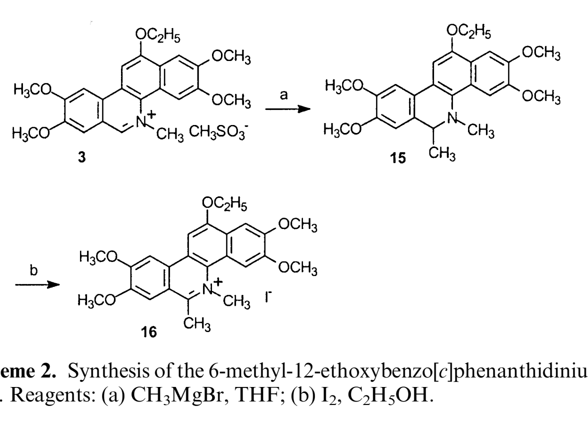
Ethoxidine : $R^{12} = CH_2CH_3$, $X^- = CH_3SO_3^-$



Scheme 1. Synthesis of the novel 12-ethoxy-benzo[*c*]phenanthidinium salts. Reagents: (a) KCN, H_2O/C_2H_5OH, rt; (b) HF, brine 15 °C; (c) $BrCH_2CO_2Et$, Zn, dioxane/DMM; (d) H_2SO_4; (e) K_2CO_3, acetone, $(C_2H_5O)_2SO_2$; (f) $CH_3SO_3CH_3$, 180 °C; (g) $NaBH_4$, CH_3OH; (h) KOH (5 N), CH_3OH; (i) I_2, C_2H_5OH.

Chemistry

Harsh acidic conditions were required for the formation of the B and C rings during the synthesis of 12-alkoxy BZPs.[5] In a previous study, **2** was prepared through deethylation of a phenolic ether protecting group using concentrated sulfuric acid.[9] The major drawbacks in this type of deprotection are controlling the reaction time and the need for laborious purification steps to separate related polar by-products. To avoid this, we chose the methanesulfonate phenolic protection of vanillin in the early steps of our synthesis, which was easily carried out using methanesulfonyl chloride in pyridine to give **5**[10] (Scheme 1). The amino nitrile **6** was obtained under Strecker reaction conditions in 86% yield. A nearly quantitative hydrogen fluoride cyclization of **6** at room temperature, led to the ketone **7**. The Reformatski reaction, which introduced the two-carbon synthon required for the ensuing cyclisation, yielded the amine **8**. Sulfuric acidic cyclisation led to the BZP **9**, which was thereafter *O*-ethylated in the 12-position using diethylsulfate and sodium carbonate in dried acetone giving **10**, and then *N*-methylated in the presence of methyl methanesulfonate at 180 °C to yield the 12-ethoxy-2-methanesulfonoxy-3,8,9-trimethoxybenzo[*c*]-phenanthridinium salt **11**. The target compound, 12-ethoxyfagaronine **14**, was obtained following the reduction of the iminium bond in **11** with sodium borohydride in methanol to give the tertiary amine **12**, sulfonyl ester hydrolysis of **12** using 5 N potassium hydroxide to yield the 2-hydroxyBZP **13** and oxidation of **13** with iodine in ethanol to **14**. The overall yield of this multistep synthesis was 20%. In a similar second synthesis, the 6-methyl group was introduced into **3** ($R^{12} = CH_2CH_3$, $X = CH_3SO_3^-$) using methyl magnesium bromide in THF. Subsequent oxidation gave 6-methyl-ethoxidine iodide in 75% yield (Scheme 2).



Scheme 2. Synthesis of the 6-methyl-12-ethoxybenzo[*c*]phenanthidinium salt. Reagents: (a) CH_3MgBr, THF; (b) I_2, C_2H_5OH.

Affinities of DNA Binding

Stock solutions (10 mM) of **2** and its derivatives were prepared in DMSO and diluted to the desired concentration immediately before use. Concentrations of calf thymus (CT) DNA and the double-stranded poly(dA-dT)poly(dA-dT) and poly(dG-dC)poly(dG-dC) polymers were determined by using molar extinction coefficients of 13,200, 13,900 and 13,200 M^{-1} cm^{-1}, respectively. UV–vis spectra were recorded with a JASCO V-530 UV–vis scanning spectrophotometer. DNA binding constants were calculated as described.[6b]

Activities of top1-DNA Relaxation Inhibition

Recombinant human top1 was purified from insect cells using a two-step procedure as described.[11,12] Specific activity of top1 used was 1.8×10^6 units/mg, where one unit of activity is an amount of enzyme yielding 100% of relaxation of 300 ng of supercoiled pGEM7Z(f+) plasmid DNA in 30 min at 37 °C. The DNA relaxation assay was carried out as described.[12] Inhibition of the top1-mediated plasmid DNA relaxation was tested when the aliquots of drug solutions were added to the reaction mixture.

Cytotoxicities

HT-29, A-549, MCF-7 and CaOv3 human solid tumor cell lines were examined in cell growth inhibition assays using the microtiter tetrazolium test.[13] Plating densities were chosen such that exponential growth was maintened throughout the subsequent 4 days cultured period. Drugs were added 24 h after plating in nine concentrations. After cells had grown for 72 h in the presence of drug, 20 μL of 2.5 mg/mL solution of dimethylthiazodiphenyltetrazolium bromide (Sigma) in PBS was added. After 3 h of incubation, the liquid content of each well was aspirated and the purple formazan precipitate dissolved in 200 μL dimethylsulfoxide. Absorbances were measured at 570 nm (BioRad-750 microplate reader). IC_{50} values were determined from dose/inhibition curves.

We initially evaluated the binding constants of our BZPs within calf thymus DNA, poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC) complexes (Table 1). Data show that **2** has the highest CT DNA binding constants but no base preference of intercalation. On the other hand, **3** ($R^{12}=CH_2CH_3$, $X=CH_3SO_3^-$) has a lower CT DNA affinity, but exhibits clear AT-specificity of intercalation: binding with poly(dA-dT)·poly(dA-dT) is found to be 25-fold higher than with poly(dG-dC)·poly(dG-dC). Compounds **11** and **14** showed slightly less affinity for CT DNA compared to **2** and were similar to **3** ($R^{12}=CH_2CH_3$, $X=CH_3SO_3^-$). As far as sequence specificity is concerned, **11** is found to be similar to **2** (no sequence specificity), whereas **14** is more similar to **3** ($R^{12}=CH_2CH_3$, $X=CH_3SO_3^-$), demonstrating pronounced AT specificity. Both compounds demonstrate lower affinity to poly(dA-dT)·poly(dA-dT) relative to **3** ($R^{12}=CH_2CH_3$, $X=CH_3SO_3^-$) (50- and 8.3-fold less, respectively). On the other hand, the sequence-specificity of 6-methylethoxidine **16** was found to be comparable to that for **3** ($R^{12}=CH_2CH_3$, $X=CH_3SO_3^-$), but with 2- and 3-fold less affinities toward poly(dG-dC)·poly(dG-dC) and poly(dA-dT)×poly(dA-dT), respectively.

Table 1. DNA binding affinities of fagaronine and BZP derivatives

Compound	K, CT DNA, M^{-1}	K, poly(GC), M^{-1}	K, poly(AT), M^{-1}
2	10^6	1.1×10^6	10^6
3	2.5×10^5	10^5	2.5×10^6
14	2.5×10^5	6×10^4	3×10^5
11	2.5×10^5	5.3×10^4	5×10^4
16	7×10^5	5×10^4	7×10^5

The inhibition of top1-mediated plasmid DNA relaxation by the same derivatives was also determined. Each compound was found to be active at nearly the same minimal inhibitory concentration (MIC: 0.75 μM), corresponding to the lowest concentration of drug able to inhibit top1-mediated DNA relaxation (Fig. 1).

BZPs are potent inhibitors of the growth of leukemia tumor cells[14] and fagaronine and ethoxidine have both demonstrated this type of activity.[6] In this work, we have evaluated the cytotoxicity of our molecules against various solid cancer cell lines (Table 2). Compounds **3** ($R^{12}=CH_2CH_3$, $X=CH_3SO_3^-$) and **2** were found to be cytotoxic towards all cell lines with better activities for the former except in the case of the CaOv3 ovarian cell line. Compounds **11** and **14** exhibited weaker activities in all in vitro tests. Finally, poor activities were found with **16**, in which 6-position substitution is known to reduce cytotoxicity.

In conclusion, the synthetic approach developed here to prepare new 2,12-functionalized benzo[*c*]phenanthridine derivatives in association with their biochemical and biological evaluation is providing interesting clues in the step-by-step comprehension of the mechanism of action of this class of cytotoxic agents.

In terms of affinity for DNA, our results demonstrate the importance of the 2-substitution in this series. Modifications in **11** and **14** slightly decrease affinities to DNA: the introduction of a bulky polar substituent in the 2-position in **11** resulted in no selectivity effect related to **2** behavior towards complexes. On the other

Table 2. Comparative cytotoxicities of BZP derivatives

	IC_{50}, μM			
Compound	A-549	CaOv3	HT-29	MCF-7
2	0.1	0.5	9	4
3	0.08	0.5[a]	2	2
14	0.5	na	5[a]	na
11	0.5	na	1[a]	na
16	10[a]	10[a]	9[a]	1[a]

na, not active.

[a] Only IC_{30} attainable.

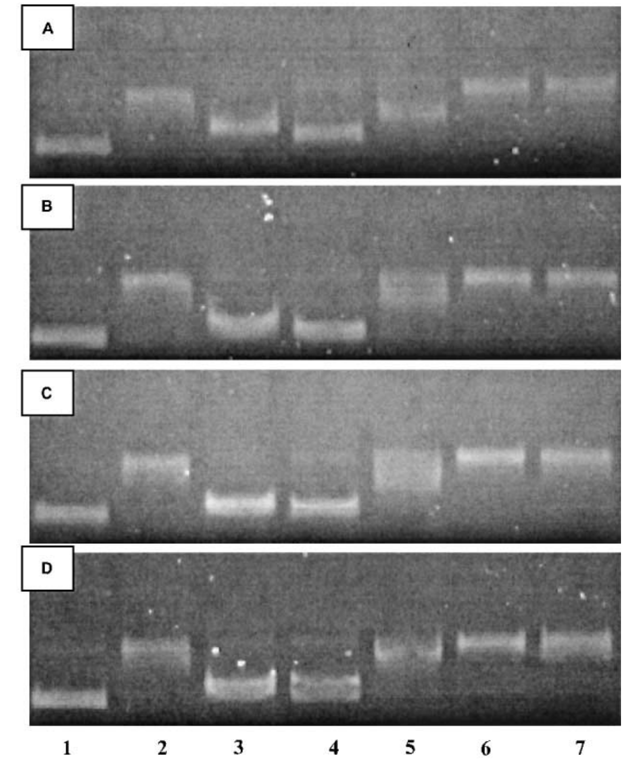


Figure 1. Inhibition of the topoisomerase I-mediated plasmid DNA relaxation by new BZP derivatives. DNA plasmid pGEM-7Zf(+) (lane 1) incubated with DNA topoisomerase I (lane 2). Topoisomerase I-mediated plasmid DNA relaxation observed in the presence of fagaronine **1** (panel A), derivative **14** (panel B), derivative **11** (panel C), derivative **16** (panel D) at 12 μM (lane 3), 3 μM (lane 4), 0.75 μM (lane 5), 0.1875 μM (lane 6), 0.0469 μM (lane 7).

hand, selectivity with AT is observed with **14**. Hence, a smaller group in this position induces a different effect, more similar to that for **3** ($R^{12} = CH_2CH_3$, $X = CH_3SO_3^-$). The results from both types of modifications indicate that the nature of the 2-position substituent is of great significance for DNA interaction. Moreover, a structural combination of the stronger DNA binder **3** ($R^{12} = CH_2CH_3$, $X = CH_3SO_3^-$) with the weaker DNA binder **2** leads to decrease of DNA-binding potency, giving additional proof that each molecule interacts with nucleic acids in a particular manner.

The good DNA binding and top1 inhibition effects, in conjunction with the poor in vitro cytotoxic activities, may indicate that **11**, **14** and **16** experience major problems in reaching their cellular targets. Further studies are in progress aimed at understanding the mechanisms involved in penetration of the cytotoxic agents of this class through cellular membranes. New BZPs are also being synthesized to establish the exact nature of substituents required in this structure for optimal activity. Site-specific DNA cleavage studies using top1 will also help to determine the mode of action of each molecule and will further explore the antitumor potential of this class of compound.

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