

Synthesis, Topoisomerase I and II Inhibitory Activities, and Cytotoxicity of 4,6-Diaryl-2,4'-bipyridine Derivatives

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Over the past few years, there has been considerable interest in DNA topoisomerases, as they were shown to be the cellular targets for several anticancer drugs.¹ DNA topoisomerases are ubiquitous enzymes that relieve the torsional stress in the DNA helix that is generated as a result of replication, transcription, and other nuclear processes.² They are classified as topoisomerase I (topo I) and topoisomerase II (topo II). The topo I transiently break DNA strands one at a time, and the topo II cut a pair of strands in a DNA double helix.³ Topoisomerases are essential enzymes, which once inhibited, induce great damage to DNA of tumor cells.⁴

Terpyridine molecule can act as tridentate ligand and form stable complexes by chelating a broad variety of transition metal ions. The numerous reports on DNA binding property and antitumor activity of terpyridine complexes have attracted multiple researchers.⁵ α -Terpyridines are the bioisosteres of α -terthiophene which possess protein kinase C (PKC) inhibitory activity.⁶ Our research group reported that terpyridine derivatives showed a strong cytotoxicity against several human cancer cell lines, and considerable topo I and II inhibitory activity.⁷ 4'-Pyridine at 2-position of central pyridine was found to be important to display topo I and topo II inhibitory activities as well as cytotoxicity.^{7d} In addition, it was observed that substitution with CH₃ or Cl enhanced topo I or topo II inhibitory activity.^{7e,g,h} In this study, we designed and synthesized fifteen compounds substituted by 4'-pyridine at 2 position and various 5- and 6-membered heteroaromatics containing CH₃ or Cl group at 4 and 6 positions of central pyridine, and evaluated for topo I and II inhibitory activity, and cytotoxicity against several human cancer cell lines (Figure 1).

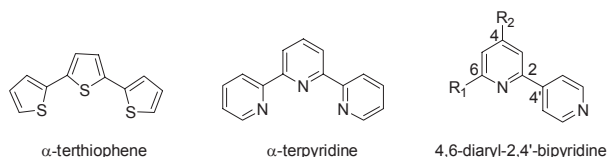


Figure 1. Structures of α -terthiophene, α -terpyridine and 4,6-diaryl-2,4'-bipyridine.

Experimental Section

Compounds used as starting materials and reagents were obtained from Aldrich Chemical Co., Junsei or other chemical companies, and utilized without further purification. HPLC

grade acetonitrile (ACN) and methanol were purchased from Burdick and Jackson, USA. Thin-layer chromatography (TLC) and column chromatography (CC) were performed with Kieselgel 60 F₂₅₄ (Merck) and silica gel (Kieselgel 60, 230 - 400 mesh, Merck) respectively. Since all the compounds prepared contain aromatic ring, they were visualized and detected on TLC plates with UV light (short wave, long wave or both). NMR spectra were recorded on a Bruker AMX 250 (250 MHz, FT) for ¹H NMR and 62.5 MHz for ¹³C NMR, and chemical shifts were calibrated according to TMS. Chemical shifts (δ) were recorded in ppm and coupling constants (J) in hertz (Hz). Melting points were determined in open capillary tubes on electrothermal 1A 9100 digital melting point apparatus and were uncorrected.

HPLC and ESI LC/MS analyses were performed using the same methods which were previously reported.⁷ⁱ

General method for the preparation of 3. Aryl ketone was added to the solution of 85% KOH (1.2 eq) in MeOH/H₂O (5:1) at 0 °C. After complete dissolution, aryl aldehyde was added slowly. The mixture was then stirred for 3 h at 0 °C. Precipitate was formed in most of the cases which was then filtered, washed with cold MeOH, and dried to yield 31.2 - 98.3% as a solid. In those reactions where no precipitate occurred, the reaction mixtures were extracted with ethyl acetate and washed with water. It was then further purified by either recrystallization or column chromatography.

General method for the preparation of 4. A mixture of 4-acetyl pyridine, iodine (1.2 eq) and pyridine was refluxed at 140 °C for 3 h. Precipitate occurred during reaction which was cooled to room temperature. Then it was filtered and washed with cold pyridine to afford 4 in quantitative yield.

General method for the preparation of 5. A mixture of propenone intermediate 3 ($R_1 = \text{a-c, e, f}$, $R_2 = \text{a-e, g-i}$), pyridinium iodide salt 4 and anhydrous ammonium acetate in glacial acetic acid were heated at 80 - 100 °C for 12 - 24 h. The reaction mixture was then extracted with ethyl acetate, washed with water and brine solution. The organic layer was dried with magnesium sulfate and filtered. The filtrate was evaporated at reduced pressure, which was then purified by silica gel column chromatography with the gradient elution of ethyl acetate/*n*-hexane to afford solid compounds 5 ($R_1 = \text{a-c, e, f}$, $R_2 = \text{a-e, g-i}$) in 21.1% - 46.4% yield.

Synthesis of 4-(furan-2-yl)-6-(3-methyl-thiophen-2-yl)-2,4'-bipyridine (15): The procedure described above was employed

with **3** ($R_1 = e$, $R_2 = c$), anhydrous ammonium acetate, **4**, and glacial AcOH to yield light yellow solid compound (26.5%). R_f (ethyl acetate/*n*-hexane 1:1, v/v): 0.34; % purity by HPLC: 100%; mp 149.0 - 149.6 °C, LC MS/MS: retention time: 11.52 min; $[MH]^+$: 319.30. 1H NMR (250 MHz, $CDCl_3$) δ 8.72 (dd, $J = 4.6, 1.6$ Hz, 1H, 2-pyridine H-2', H-6'), 8.01 (dd, $J = 4.6, 1.6$ Hz, 1H, 2-pyridine H-3', H-5'), 7.87 (s, 1H, pyridine H-3), 7.79 (s, 1H, pyridine H-5), 7.58 (dd, $J = 1.6, 0.5$ Hz, 1H, 4-furan H-5), 7.29 (d, $J = 5.0$ Hz, 1H, 6-thiophene H-5), 6.96 (m, 2H, 4-furan H-3, 6-thiophene H-4), 6.56 (dd, $J = 3.4, 1.7$ Hz, 1H, 4-furan H-4), 2.62 (s, 3H, 6-thiophene 3- CH_3). ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.38, 154.20, 151.18, 150.36, 146.06, 143.98, 139.12, 137.48, 136.37, 132.35, 125.82, 121.02, 155.15, 112.22, 109.05, 16.58.

Synthesis of 4-(5-methylfuran-2-yl)-6-(thiophen-2-yl)-2,4'-bipyridine (16): The procedure described above was employed with **3** ($R_1 = a$, $R_2 = g$), anhydrous ammonium acetate, **4**, and glacial AcOH to yield light yellow crystal (27.3%). R_f (ethyl acetate/*n*-hexane 1:1, v/v): 0.15; % purity by HPLC: 100%; mp 177.6 - 1178.3 °C, LC MS/MS: retention time: 12.04 min; $[MH]^+$: 319.26. 1H NMR (250 MHz, $CDCl_3$) δ 8.74 (dd, $J = 4.6, 1.5$ Hz, 2H, 2-pyridine H-2', H-6'), 8.03 (dd, $J = 4.6, 1.5$ Hz, 2H, 2-pyridine H-3', H-5'), 7.81 (s, 2H, pyridine H-3, H-5), 7.72 (dd, $J = 3.6, 0.8$ Hz, 1H, 6-thiophene H-3), 7.43 (dd, $J = 5.0, 0.8$ Hz, 1H, 6-thiophene H-5), 7.15 (dd, $J = 5.0, 3.7$ Hz, 1H, 6-thiophene H-4), 6.88 (d, $J = 3.3$ Hz, 1H, 4-furan H-3), 6.16 (dd, $J = 3.2, 0.7$ Hz, 1H, 4-furan H-4), 2.42 (s, 3H, 4-furan 5- CH_3). ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.47, 154.35, 153.08, 150.38, 149.41, 146.02, 144.84, 139.48, 128.01, 124.96, 121.05, 112.49, 112.08, 110.36, 108.58, 13.91.

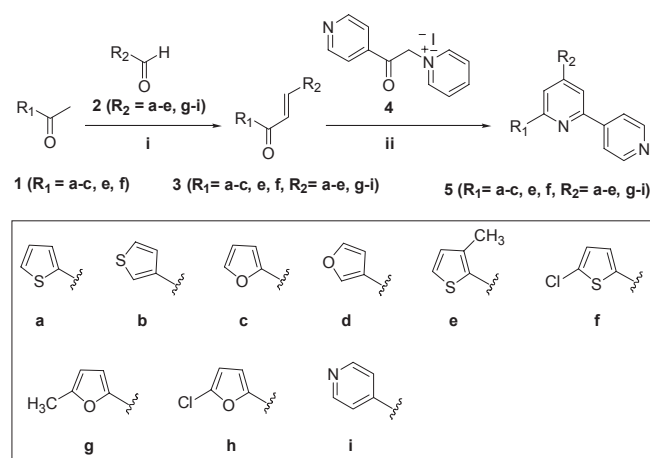
Synthesis of 4-(5-chlorofuran-2-yl)-6-(thiophen-2-yl)-2,4'-bipyridine (17): The procedure described above was employed with **3** ($R_1 = a$, $R_2 = h$), anhydrous ammonium acetate, **4**, and glacial AcOH to yield brown solid (24.9%). R_f (ethyl acetate/*n*-hexane 1:1, v/v): 0.36; % purity by HPLC: 100%; mp 196.3 - 197.0 °C, LC MS/MS: retention time: 12.41 min; $[MH]^+$: 339.21. 1H NMR (250 MHz, $CDCl_3$) δ 8.75 (dd, $J = 4.5, 1.6$ Hz, 2H, 2-pyridine H-2', H-6'), 8.01 (dd, $J = 4.5, 1.6$ Hz, 2H, 2-pyridine H-3', H-5'), 7.79 (s, 2H, pyridine H-3, H-5), 7.72 (dd, $J = 3.7, 1.0$ Hz, 1H, 6-thiophene H-3), 7.45 (dd, $J = 5.0, 1.0$ Hz, 1H, 6-thiophene H-5), 7.15 (dd, $J = 5.0, 3.7$ Hz, 1H, 6-thiophene H-4), 6.96 (d, $J = 3.5$ Hz, 1H, 4-furan H-3), 6.36 (d, $J = 3.5$ Hz, 1H, 4-furan H-4). ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.63, 153.32, 150.52, 150.44, 145.68, 144.46, 138.52, 138.28, 128.33, 128.10, 125.24, 121.02, 112.34, 112.03, 111.10, 109.01.

Synthesis of 6-(5-chlorothiophen-2-yl)-4-(furan-2-yl)-2,4'-bipyridine (18): The procedure described above was employed with **3** ($R_1 = f$, $R_2 = c$), anhydrous ammonium acetate, **4**, and glacial AcOH to yield greenish solid (23.2%). R_f (ethyl acetate/*n*-hexane 2:1, v/v): 0.40; % purity by HPLC: 97.64%; mp 196.6 - 197.2 °C, LC MS/MS: retention time: 12.88 min; $[MH]^+$: 339.25. 1H NMR (250 MHz, $CDCl_3$) δ 8.74 (dd, $J = 4.5, 1.6$ Hz, 2H, 2-pyridine H-2', H-6'), 7.98 (dd, $J = 4.5, 1.6$ Hz, 2H, 2-pyridine H-3', H-5'), 7.86 (d, $J = 1.2$ Hz, 1H, pyridine H-3), 7.78 (d, $J = 1.2$ Hz, 1H, pyridine H-5), 7.59 (d, $J = 1.6$ Hz, 1H, 4-furan H-5), 7.45 (d, $J = 3.9$ Hz, 1H, 6-thiophene H-3), 6.98 (d, $J = 3.4$ Hz, 1H, 4-furan H-3), 6.95 (d, $J = 3.9$ Hz, 1H, 6-thiophene H-4), 6.57 (dd, $J = 3.4, 1.8$ Hz, 1H, 4-furan H-4). ^{13}C

NMR (62.5 MHz, $CDCl_3$) δ 154.56, 152.45, 150.98, 150.47, 145.58, 144.13, 143.36, 139.43, 133.15, 127.22, 124.00, 120.95, 113.12, 112.32, 111.81, 109.32.

Pharmacology. The topoisomerase I inhibitory activity was carried out as following.⁸ The prepared compounds were dissolved in DMSO at 20 mM as stock solution. The activity of DNA topoisomerase I was determined by assessing the relaxation of supercoiled DNA pBR322. The mixture of 100 ng of plasmid pBR322 DNA and 0.2 units of calf thymus DNA topoisomerase I (Fermentas, USA) was incubated without and with the prepared compounds at 37 °C for 30 minutes in the relaxation buffer (35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM spermidine, 0.01% bovine serum albumin). The reaction in the final volume of 10 μ L was terminated by adding 2.5 μ L of the stop solution containing 10% SDS, 0.2% bromophenol blue, 0.2% xylene cyanol and 30% glycerol. DNA samples were then electrophoresed on a 1% agarose gel at 15 V for 7 h with a running buffer of TAE. Gels were stained for 30 min in an aqueous solution of ethidium bromide (0.5 μ g/mL). DNA bands were visualized by transillumination with UV light and were quantitated using AlphaImagerTM (Alpha Innotech Corporation).

DNA topoisomerase II inhibitory activity of compounds were measured as follows.⁹ The mixture of 200 ng of supercoiled pBR322 plasmid DNA and 2 units of human DNA topoisomerase II α (Amersham, USA) was incubated without and with the prepared compounds in the assay buffer (10 mM Tris-HCl (pH 7.9) containing 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, and 15 μ g/mL bovine serum albumin) for 30 min at 30 °C. The reaction in a final volume of 20 μ L was terminated by the addition of 3 μ L of 7 mM EDTA. Reaction products were analyzed on 1% agarose gel at 25 V for 4 h with a running buffer of TAE. Gels were stained for 30 min in an aqueous solution of ethidium bromide (0.5 μ g/mL). DNA bands were visualized by transillumination with UV light and supercoiled DNA was quantitated using AlphaImagerTM (Alpha Innotech Corporation).



Scheme 1. General synthetic scheme of 4,6-diaryl-2,4'-bipyridine. Reagents and conditions: (i) aryl aldehydes **2** (**a-e**, **g-i**), (1.0 eq), KOH (1.2 eq), MeOH/ H_2O (5:1), 0 °C, 3 h, 31.2 - 98.3%; (ii) **3** ($R_1 = a-c, e, f, R_2 = a-e, g-i$) (1.0 eq), **4** (1.0 eq), NH_4OAc (10.0 eq), AcOH, 80 - 100 °C, 12 - 24 h, 21.1 - 46.4% yield.

Cancer cells were cultured according to the supplier's instructions. Cells were seeded in 96-well plates at a density of $2 \sim 4 \times 10^4$ cells per well and incubated for overnight in 0.1 mL of media supplied with 10% Fetal Bovine Serum (Hyclone, USA) in 5% CO₂ incubator at 37 °C. On day 2, culture medium in each well was exchanged with 0.1 mL aliquots of medium containing graded concentrations of compounds. On day 4, each well was added with 5 μ L of the cell counting kit-8 solution (Dojindo, Japan) then incubated for additional 4 h under the same condition. The absorbance of each well was determined by an Automatic Elisa Reader System (Bio-Rad 3550) at 450 nm wavelength. For determination of the IC₅₀ values, the absorbance readings at 450 nm were fitted to the four-parameter logistic equation. Adriamycin, etoposide, and camptothecin were purchased from Sigma and used as positive controls.

Results and Discussion

Synthetic chemistry. Synthetic method for the preparation of 4,6-diaryl-2,4'-bipyridine derivatives **6-20** are summarized in Scheme 1. Aryl acetyl ketones **1 (a-e, f)** were treated with aryl aldehydes **2 (a-e, g-i)** in the presence of KOH in methanol/water (5:1), to afford propenone intermediates **3 (R₁ = a-c, e, f, R₂ = a-e, g-i)** in 31.2 - 98.3% yield. Pyridinium iodide salt **4** was synthesized in a quantitative yield by the treatment of 4-acetyl pyridine with iodine in pyridine. Using modified Kröhnke synthesis,¹⁰ final compounds **5 (R₁ = a-c, e, f, R₂ = a-e, g-i)** were synthesized by the reaction of propenone intermediate **3** with pyridinium iodide salt **4** in the presence of ammonium acetate and acetic acid in 21.1 - 46.4% yield. Figure 2 shows structure of prepared compounds.

Topo I and II inhibitory activity of compounds 6-20. The

conversion of supercoiled plasmid DNA to relaxed DNA by topo I and II was examined in the presence of prepared 4,6-diaryl-2,4'-bipyridine derivatives **6-20**. Camptothecin and etoposide, well-known topo I and II inhibitors, respectively, were used as positive controls. All the compounds, except **16**, were devoid of topo I inhibitory activity at both 20 μ M and 100 μ M concentrations. Compound **16** had considerable topo I inhibitory activity as shown in Figure 3. The effect of prepared compounds on human DNA topo II α were observed in the relaxation assays using supercoiled pBR322 plasmid DNA in the presence of ATP. The reaction products were analyzed by electrophoretic mobility and developed in ethidium bromide in the presence of UV light. As shown in Figure 4, compounds **13-20** exhibited significant topo II α inhibitory activity. Compounds **6-12** did not show considerable topo II inhibitory activity compared to other compounds. Table 1 indicates the percentage inhibition of topo I and II with compounds **13-20** at concentrations 20 μ M and 100 μ M.

Cytotoxicity. Compounds **6-14** did not show considerable cytotoxicity but compounds **15-20** showed significant cytotoxicity. The IC₅₀ values of 4,6-diaryl-2,4'-bipyridine derivatives **15-20** against those cell lines are shown in Table 1. Most of the compounds showed significant cytotoxicity, generally less than IC₅₀ value of 10 μ M. Compounds **15-18, 20** show the most significant cytotoxicity against HCT15 with IC₅₀ value of 2 - 3 μ M. It supports the idea that 4'-pyridine at 2-position of central pyridine with combination of 2-thienyl, 3-thienyl or 2-furyl moieties are crucial in displaying cytotoxicity, especially on HCT15, as reported previously.^{7d}

In conclusion, we have designed and synthesized fifteen compounds by efficient synthetic routes and evaluated them for topo I and II inhibitory activity along with cytotoxicity against

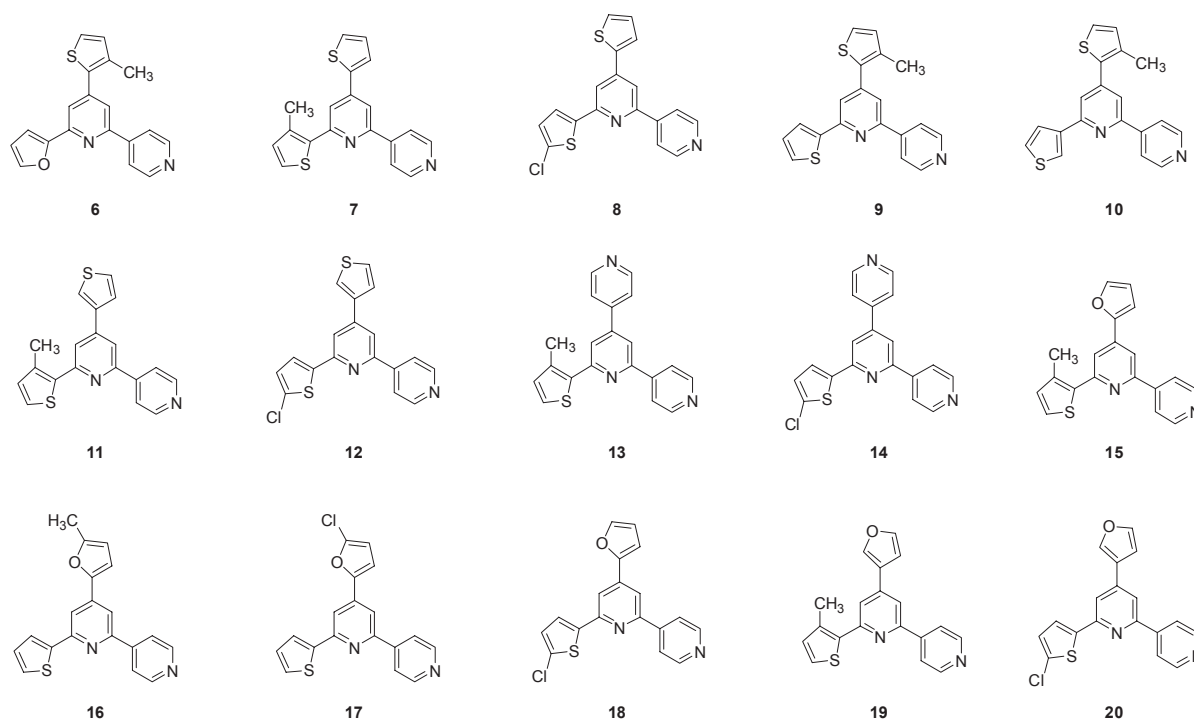
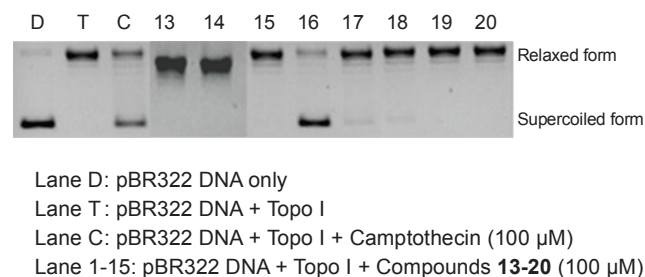
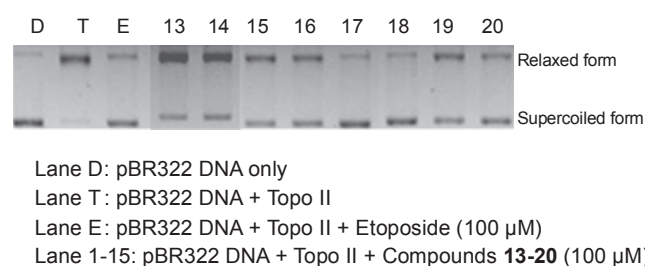


Figure 2. Structure of the prepared compounds.

Table 1. Topoisomerase I and II inhibitory activity and cytotoxicity of the prepared compounds **13-20**

Compounds	% Inhibition				IC ₅₀ ^a (μM)				
	Topo II		Topo I		MDA-MB231	HeLa	DU145	HCT15	HL60
	100 μM	20 μM	100 μM	20 μM					
Etoposide	61.7	40.5			0.9 ± 0.06	1.6 ± 0.14	0.6 ± 0.04	0.9 ± 0.11	0.8 ± 0.01
Camptothecin			56.2	43.9	0.3 ± 0.07	0.2 ± 0.07	0.2 ± 0.03	0.5 ± 0.09	0.1 ± 0.00
Adriamycin					0.4 ± 0.07	0.8 ± 0.07	1.0 ± 0.22	1.1 ± 0.08	0.7 ± 0.01
13	29.8	NA	4.3	NA	NA	NA	NA	NA	NA
14	27.3	NA	3.6	NA	NA	NA	NA	NA	NA
15	35.3	6.7	10.6	NA	6.7 ± 0.81	4.5 ± 2.09	6.5 ± 0.61	3.4 ± 0.16	4.7 ± 1.18
16	29.5	0.0	55.8	15.2	7.3 ± 0.74	4.7 ± 1.00	3.6 ± 0.57	3.4 ± 0.33	4.3 ± 0.64
17	42.9	0.0	13.7	NA	28.3 ± 0.77	33.7 ± 5.91	18.2 ± 2.04	2.3 ± 0.08	2.1 ± 0.09
18	44.7	0.0	6.2	NA	18.7 ± 1.80	3.7 ± 0.15	6.4 ± 1.56	2.1 ± 0.08	6.5 ± 0.24
19	26.0	0.0	12.9	NA	11.8 ± 0.74	5.7 ± 0.21	4.6 ± 1.65	18.4 ± 0.09	14.4 ± 0.33
20	30.9	4.7	5.6	NA	11.1 ± 1.26	6.8 ± 0.25	4.5 ± 1.22	2.6 ± 0.53	11.0 ± 0.14

^aEach data point represents mean ± S. D. from three different experiments performed in triplicate. NA: Not applicable. Cell lines used are MDA-MB231, human breast tumor cell line; HeLa, human cervix tumor cell line; DU145, human prostate tumor cell line; HCT15, human colorectal adenocarcinoma cell line; HL60, human myeloid leukemic tumor cell lines.

**Figure 3.** Calf thymus DNA Topo I inhibitory effect of compounds **13-20**.**Figure 4.** Human DNA Topo II α inhibitory effect of compounds **13-20**.

several human cancer cell lines. Although most of the compounds did not display significant topo I inhibitory activity, several compounds (**13-20**) possessed considerable topo II inhibitory activity and cytotoxicity. A structure-activity relationship study of 4,6-diaryl-2,4'-bipyridine derivatives for topo II inhibitory activity indicates that 6-thienyl-4-furyl moiety along with 4'-pyridine at 2 position is crucial in displaying topo II inhibitory activity, and cytotoxicity. Further research in this skeleton is necessary for the development of potent anticancer agents. This study may provide valuable information to researchers working on the development of antitumor agents.

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