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Synthesis, topoisomerase I and II inhibitory activity, cytotoxicity, and structure–activity relationship study of hydroxylated 2,4-diphenyl-6-aryl pyridines

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ABSTRACT

A new series of 2,4-diphenyl-6-aryl pyridines containing hydroxyl group(s) at the *ortho*, *meta*, or *para* position of the phenyl ring were synthesized, and evaluated for topoisomerase I and II inhibitory activity and cytotoxicity against several human cancer cell lines for the development of novel anticancer agents. Structure–activity relationship study revealed that the substitution of hydroxyl group(s) increased topoisomerase I and II inhibitory activity in the order of *meta* > *para* > *ortho* position. Substitution of hydroxyl group on the *para* position showed better cytotoxicity.

1. Introduction

DNA topoisomerases have been established as molecular targets of anticancer drugs.[1,2] They are ubiquitous enzymes that solve the topological problems associated with DNA replication, transcription, recombination, and other nuclear processes by introducing temporary single- or double-strand breaks in the DNA. In addition, these enzymes fine-tune the steady-state level of DNA supercoiling both to facilitate protein interactions with the DNA and to prevent excessive supercoiling that is deleterious.[3,4] DNA topoisomerases fall into two categories, topoisomerase I (topo I) and topoisomerase II (topo II). For topo I, the DNA strands are transiently broken one at a time. For topo II, a pair of strands in a DNA double helix is transiently broken in concert by a dimeric enzyme molecule.[5] In the course of several years, different agents which are able to inhibit the topo-DNA cleavable complex have been found and developed.[6]

Terpyridines can act as tridentate ligand and form stable complexes by chelating a broad variety of transition metal ions. Platinum(II) complexes of terpyridine derivatives have been well studied and evaluated for their antitumor potential.[7,8] In clinical applications and biochemistry, a wide range of potential uses for functionalized terpyridines have been found, ranging from colorimetric metal determination to DNA binding agents[9,10] and antitumor research.[8] These numerous reports on DNA binding property and antitumor activity of terpyridine complexes have attracted many researchers. In addition, α-terpyridines are the bioisosteres of α-terthiophenes, which possess protein kinase C (PKC) inhibitory activities (Fig. 1).[11]

Our research group has previously reported that terpyridine derivatives show strong cytotoxicity against several human cancer cell lines and considerable topo I and II inhibitory activity.[12–19] Among prepared compounds, methyl or chloro substituted terpyridine derivatives have shown strong topoisomerase I and II inhibitory activities and cytotoxicity compared to the non-substituted ones.[17–19] These results suggested that the substitution of other functional group on aromatic rings may increase topoisomerase I and II inhibitory activities and cytotoxicity.

Many natural and synthetic derivatives bearing the hydroxyl group or phenol have exhibited a wide spectrum of biological activities with potential for application as pharmaceutical drugs. Numerous polyphenolic phytochemicals such as resveratrol, curcumin, flavonoids, epigallocatechin gallate, and chalcones have been well studied and reported to interfere with specific stages of the carcinogenic process.[20–23] Camptothecin, a well-known topo I inhibitor, also contains the hydroxyl group. Redinbo et al. and Fan

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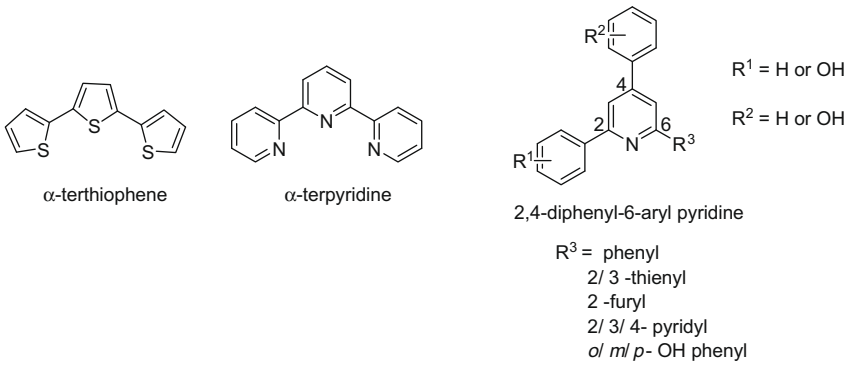


Figure 1. Structures of α-terthiophene, α-terpyridine, and 2,4-diphenyl-6-aryl pyridine derivatives.

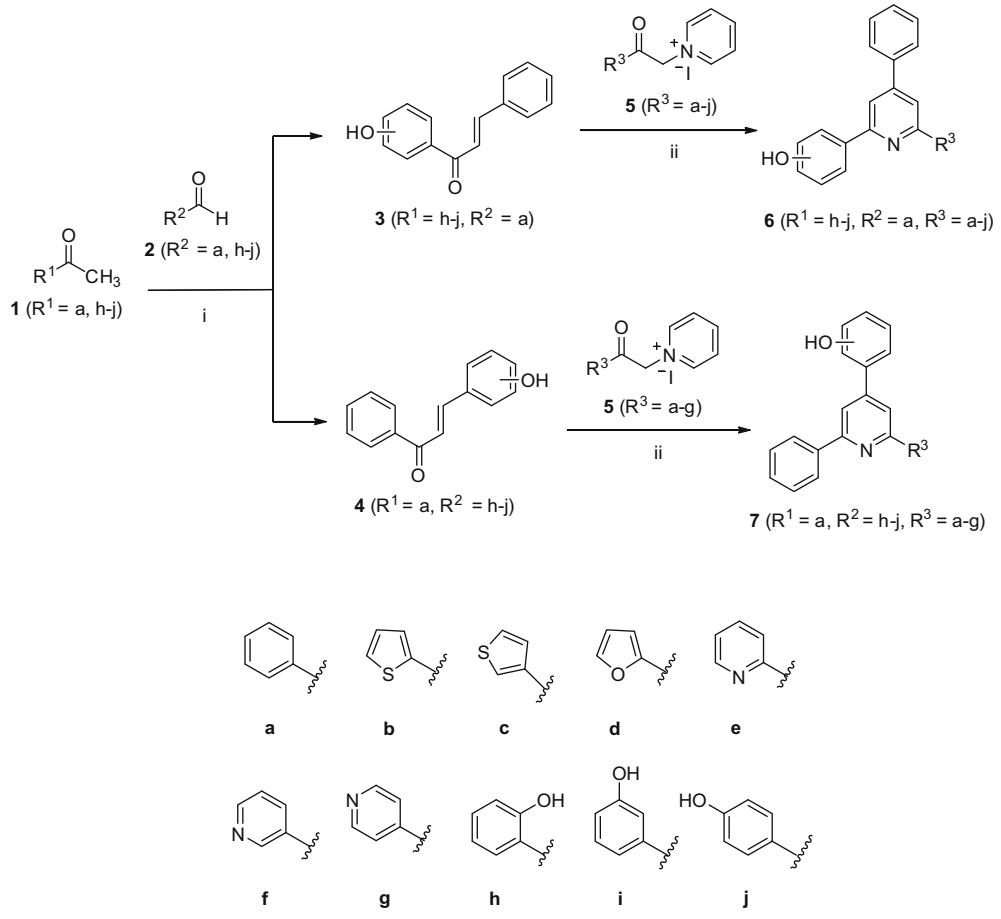
et al. have described the role of the 20-OH group of camptothecin in the topo I-DNA binding models as they participate in a donor hydrogen bond.[24,25] These reports on the importance of the hydroxyl group motivated us to synthesize terpyridine compounds with hydroxyl moiety.

In this study, we incorporated the hydroxyl group on phenyl moiety to investigate the effect on topoisomerase I and II inhibitory activities and cytotoxicity. We have systematically designed and synthesized 45 2,4-diphenyl-6-aryl pyridine derivatives containing hydroxyl groups at different positions (*ortho*, *meta*, or *para*) of the phenyl ring attached to the central pyridine (Scheme 1 and Fig. 2). They were then evaluated for topoisomerase I and II inhibitory activity and cytotoxicity against several human cancer cell lines. From the results of the biological activity of the prepared compounds, the structure–activity relationship was established. In addition, three dihydroxylated terpyridine compounds (**15, 23, and 31**) were synthesized and evaluated for biological activity to observe the effect of increase in the number of hydroxyl groups.

2. Results and discussion

2.1. Chemistry

Synthesis of 2,4-diphenyl-6-aryl pyridine derivatives **8–52** involved three steps as summarized in Scheme 1. Six different hydroxylated chalcones were synthesized as intermediates using



Scheme 1. General synthetic scheme of 2,4-diphenyl-6-aryl pyridine derivatives. Reagents and conditions: (i) aryl aldehydes (1.0 equiv), KOH (50%), MeOH/H_2O (5:1), 2–3 h, room temperature, 60.7–94.1% yield; (ii) NH_4OAc (10.0 equiv), glacial AcOH, 12–24 h, 80–100 °C, 21.4–89.3% yield.

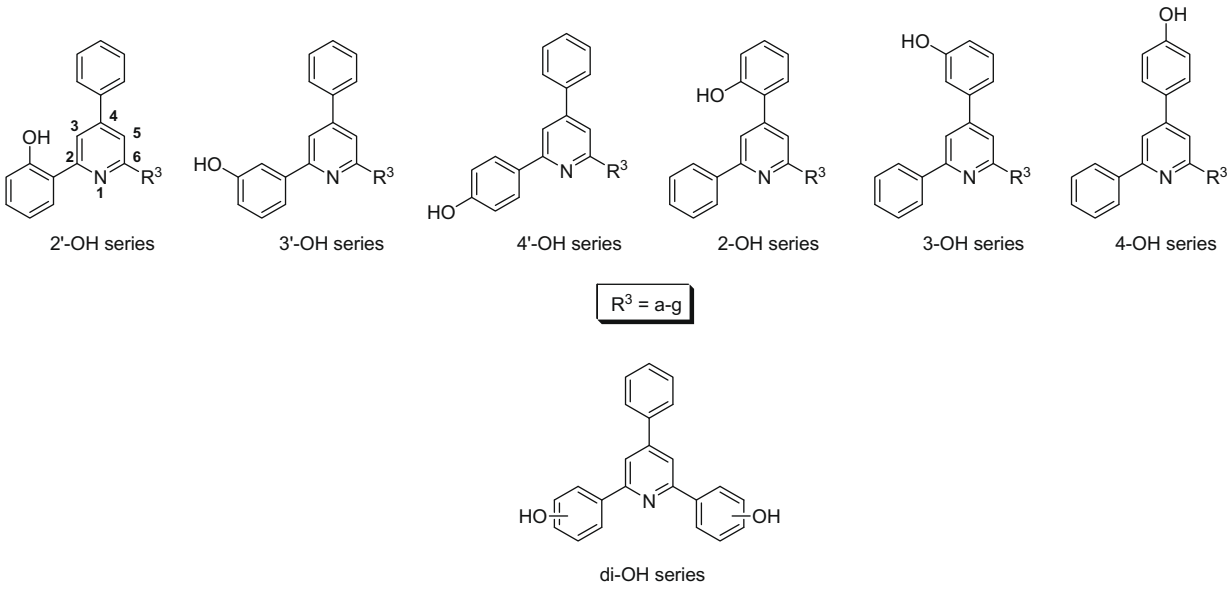


Figure 2. Strategy for design of hydroxylated 2,4-diphenyl-6-aryl pyridines.

the Clasien–Schmidt condensation reaction,[26] which was not necessary to protect hydroxyl groups. Aryl ketone **1** (R^1 = **a**, **h**–**j**) was added to the solution of 50% KOH in MeOH/H_2O (5:1) followed by aryl aldehydes **2** (R^2 = **a**, **h**–**j**) to obtain compounds **3** (R^1 = **h**–**j**, R^2 = **a**) and **4** (R^1 = **a**, R^2 = **h**–**j**) in 60.7–94.1% yield. In the second step, 10 pyridinium iodide salts **5** (R^3 = **a**–**j**) were synthesized in a quantitative yield by the treatment of aryl ketone **1** (R^1 = **a**–**j**) with iodine in pyridine. Using modified Kröhnke synthesis,[27,28] final compounds **6** (R^1 = **h**–**j**, R^2 = **a**, R^3 = **a**–**j**) and **7** (R^1 = **a**, R^2 = **h**–**j**, R^3 = **a**–**g**) were synthesized by the reaction of appropriate hydroxylated chalcones **3** or **4** with pyridinium iodide salt **5** in the presence of ammonium acetate and glacial acetic acid in 21.4–89.3% yield. Through all the reactions for the preparation of the final compounds, protection of hydroxyl groups was not required. It was noticed that the yields were relatively lower in compounds having hydroxyl groups at *ortho* position compared to *meta* or *para* substitution, which may be from the higher steric hinderance of compounds bearing substitution on *ortho* position.

Total of 45 compounds were synthesized as shown in Table 1. All the synthesized compounds contained hydroxyl groups at *ortho*, *meta*, or *para* position of either 2- or 4- phenyl moiety, and various aryl groups were attached on the 6-position of central pyridine. Among the 45 compounds, 42 were monohydroxylated with substitution at 2- or 4-phenyl moiety, and three were dihydroxylated with substitution at 2- and 6-phenyl moiety. The substitutions of hydroxyl groups at various positions of the phenyl ring allowed us to investigate the effect of the position of hydroxyl on biological activity. In addition, we are able to observe the correlation between increase in the number of hydroxyl groups and biological activity from dihydroxylated compounds.

2.2. Topoisomerase I and II inhibitory activity

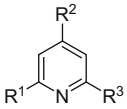
The conversion of supercoiled plasmid DNA to relaxed DNA by topo I and II was examined in the presence of synthesized 2,4-diphenyl-6-aryl pyridines (**8–52**). Camptothecin and etoposide, well-known topo I and II inhibitors, respectively, were used as positive controls. The structures of selected compounds having significant biological activity are displayed in Figure 3.

The effect of synthesized compounds on human DNA topo IIα were observed in the relaxation assay using supercoiled pBR322 plasmid DNA in the presence of ATP. As shown in Figure 4 and Table 2, compounds **16**–**19**, **23**, **31**, **39**–**43**, and **45** exhibited significant topo II inhibition at both 100 and 20 μM concentrations, which showed stronger or similar inhibitory activity than etoposide. Compounds **15**, **24**–**27**, **35**, **44**, and **49** showed considerable topo II inhibitory activity, although weaker than etoposide. Structure–activity relationship study revealed that most of the compounds from 3-OH series (**39**–**45**) or 3′-OH series (**16**–**19**) showed significant topo II inhibitory activity (Fig. 2). The activity was independent of the aryl group (–R^3 in Fig. 2) at 6-position of central pyridine. Several compounds from 4′-OH series (**24**–**27**) and 4-OH series (**49**) possessed moderate topo II inhibitory activity, which is weaker than etoposide. All the compounds from 2′-OH and 2-OH series did not show significant topo II inhibitory activity. These structure–activity relationship results provide valuable information that the substitution of hydroxyl groups on the phenyl ring increase the topo II inhibitory activity of compounds in the order of *meta* > *para* > *ortho* position.

Compound **23** showed the strongest topo II inhibitory activity, 86.5% and 49.0% at 100 μM and 20 μM, respectively, which is stronger than that of etoposide (68.2% and 30.4%, respectively). Similarly, compound **31** had 76.8% and 33.7% topo II inhibition at 100 μM and 20 μM, respectively. It is interesting to note that both compounds possessed dihydroxyl groups at *meta* or *para* position of 2- and 6- phenyl on central pyridine. In addition, *ortho* dihydroxylated compound **15** showed moderate topo II inhibitory activity (42.7% and 12.1% at 100 μM and 20 μM, respectively), which is relatively higher than that of *ortho* hydroxylated compounds (2′-OH and 2-OH series), which display no significant topo II inhibitory activity. These results might indicate that increase in number of hydroxyl groups increase topo II inhibitory activity in the order of substitution at *meta* > *para* > *ortho* position.

Figure 5 and Table 2 illustrate the topo I inhibitory activity of selected compounds. Compounds **22** and **24** showed significant topo I inhibitory activity, which was similar to the activity of camptothecin. Compounds **17**, **18**, **23**, **29**, **42**, **45**, and **52** displayed moderate topo I inhibitory activity. Most of the compounds having topo I inhibitory activity were from 3′-OH (**17**, **18**, and **22**) and 3-OH (**42** and **45**) series. Several compounds from 4′-OH (**24** and **29**) or 4-OH series (**52**) also displayed significant topo I inhibitory activity. None of the compounds from 2-OH or 2′-OH series had topo I inhibitory activity.

Table 1
Prepared compounds with yield, purity by HPLC, and melting point



Entry	R^1	R^2	R^3	Yield (%)	Purity (%)	Mp (°C)
8	2′-OH phenyl	Phenyl	Phenyl	57.1	99.3	168.8–169.6
9	2′-OH phenyl	Phenyl	2-Thienyl	30.4	99.3	142.3–143.1
10	2′-OH phenyl	Phenyl	3-Thienyl	56.5	100.0	134.0–134.9
11	2′-OH phenyl	Phenyl	2-Furyl	45.0	100.0	138.2–139.1
12	2′-OH phenyl	Phenyl	2-Pyridyl	56.4	98.7	177.9–178.8
13	2′-OH phenyl	Phenyl	3-Pyridyl	57.6	100.0	169.2–170.3
14	2′ OH phenyl	Phenyl	4-Pyridyl	46.2	100.0	189.2–190.3
15	2′-OH phenyl	Phenyl	2′-OH phenyl	37.7	97.7	200.1–200.7
16	3′-OH phenyl	Phenyl	Phenyl	83.8	100.0	180.0–180.7
17	3′-OH phenyl	Phenyl	2-Thienyl	88.1	100.0	127.2–127.9
18	3′-OH phenyl	Phenyl	3-Thienyl	76.8	98.0	102.1–103.0
19	3′-OH phenyl	Phenyl	2-Furyl	89.3	98.1	168.1–168.9
20	3′-OH phenyl	Phenyl	2-Pyridyl	58.1	98.6	182.1–182.9
21	3′-OH phenyl	Phenyl	3-Pyridyl	64.6	99.0	218.0–218.7
22	3′-OH phenyl	Phenyl	4-Pyridyl	64.9	98.7	278.0–278.6
23	3′-OH phenyl	Phenyl	3′-OH phenyl	58.1	97.6	284.5–285.3
24	4′-OH phenyl	Phenyl	Phenyl	51.0	100.0	194.5–195.5
25	4′-OH phenyl	Phenyl	2-Thienyl	53.2	100.0	164.2–165.0
26	4′-OH phenyl	Phenyl	3-Thienyl	71.4	100.0	156.9–157.5
27	4′-OH phenyl	Phenyl	2-Furyl	58.1	100.0	184.5–185.6
28	4′-OH phenyl	Phenyl	2-Pyridyl	56.8	100.0	259.1–259.7
29	4′-OH phenyl	Phenyl	3-Pyridyl	75.8	100.0	237.7–238.4
30	4′-OH phenyl	Phenyl	4-Pyridyl	72.7	100.0	306.0–306.5
31	4′-OH phenyl	Phenyl	4′-OH phenyl	31.1	95.9	194.5–195.2
32	Phenyl	2-OH phenyl	Phenyl	21.4	100.0	178.1–178.9
33	Phenyl	2-OH phenyl	2-Thienyl	29.6	99.2	146.1–146.8
34	Phenyl	2-OH phenyl	3-Thienyl	23.2	95.4	121.0–121.7
35	Phenyl	2-OH phenyl	2-Furyl	40.4	95.3	162.3–163.0
36	Phenyl	2-OH phenyl	2-Pyridyl	28.7	98.0	227.6–228.2
37	Phenyl	2-OH phenyl	3-Pyridyl	43.5	100.0	210.0–210.7
38	Phenyl	2-OH phenyl	4-Pyridyl	40.9	97.0	242.1–242.9
39	Phenyl	3-OH phenyl	Phenyl	76.5	100.0	215.8–216.6
40	Phenyl	3-OH phenyl	2-Thienyl	82.3	100.0	172.8–173.5
41	Phenyl	3-OH phenyl	3-Thienyl	79.0	96.2	200.1–200.5
42	Phenyl	3-OH phenyl	2-Furyl	77.5	96.3	214.2–214.8
43	Phenyl	3-OH phenyl	2-Pyridyl	40.4	98.3	198.2–199.0
44	Phenyl	3-OH phenyl	3-Pyridyl	57.4	99.2	210.1–210.8
45	Phenyl	3-OH phenyl	4-Pyridyl	48.0	95.7	208.1–208.8
46	Phenyl	4-OH phenyl	Phenyl	76.9	100.0	230.1–230.7
47	Phenyl	4-OH phenyl	2-Thienyl	70.7	100.0	200.0–200.5
48	Phenyl	4-OH phenyl	3-Thienyl	70.5	97.1	218.1–218.9
49	Phenyl	4-OH phenyl	2-Furyl	80.4	98.0	212.0–212.8
50	Phenyl	4-OH phenyl	2-Pyridyl	41.2	98.6	276.9–277.8
51	Phenyl	4-OH phenyl	3-Pyridyl	68.8	99.4	257.6–258.2
52	Phenyl	4-OH phenyl	4-Pyridyl	62.8	97.7	325.7–326.6

Compound **23**, dihydroxylated at *meta* position of 2- and 6-phenyl on central pyridine, had significant topo I inhibition, 67.2% at 100 μM along with strong topo II inhibitory activity. Several compounds (**17**, **18**, **42**, and **45**) which possessed dual topo I and II inhibitory activity belonged to 3′-OH and 3-OH series. These results indicate that hydroxyl substitution at 3- or 3′-position of phenyl ring on 2,4-diphenyl-6-aryl pyridines might be required in order to show significant topo inhibitory activity.

2.3. Cytotoxicity

For the evaluation of cytotoxicity, five different human cancer cell lines were utilized: MDA-MB231 (human breast adenocarcinoma cell line), HeLa (human cervix tumor cell line), DU145 (human prostate tumor cell line), HCT15 (human colorectal adenocarcinoma cell line), and HL60 (human myeloid leukemic tumor cell line). Adriamycin, camptothecin, and etoposide were used as positive controls. Most of the compounds displayed moderate cytotoxicity against those cell lines in relatively lower micromolar concentrations when compared to the standard. The IC_{50} values (μM) of the prepared compounds against those cell lines are shown in Table 2. Among the compounds which showed significant topo I or II inhibitory activity, compounds **24**–**27**, **29**, **31**, **35**, **49**, and **52** showed significant cytotoxicity in most of the cell lines, which displayed similar or slightly weaker cytotoxicity than positive controls. It is evident that all of these compounds are from 4′-OH and 4-OH series except **35** which is from 2-OH series. Compounds from 2′-OH, 2-OH, 3′-OH, or 3-OH series did not show significant cytotoxicity. It is observed that compounds from 3′-OH or 3-OH series are potent topoisomerase inhibitors but less cytotoxic to cancer cell lines. Compounds of 4′-OH and 4-OH series have shown positive correlations between topoisomerase inhibition and cytotoxicity.

3. Conclusion

We systematically designed and synthesized 45 hydroxylated 2,4-diphenyl-6-aryl pyridines by efficient synthetic routes, and

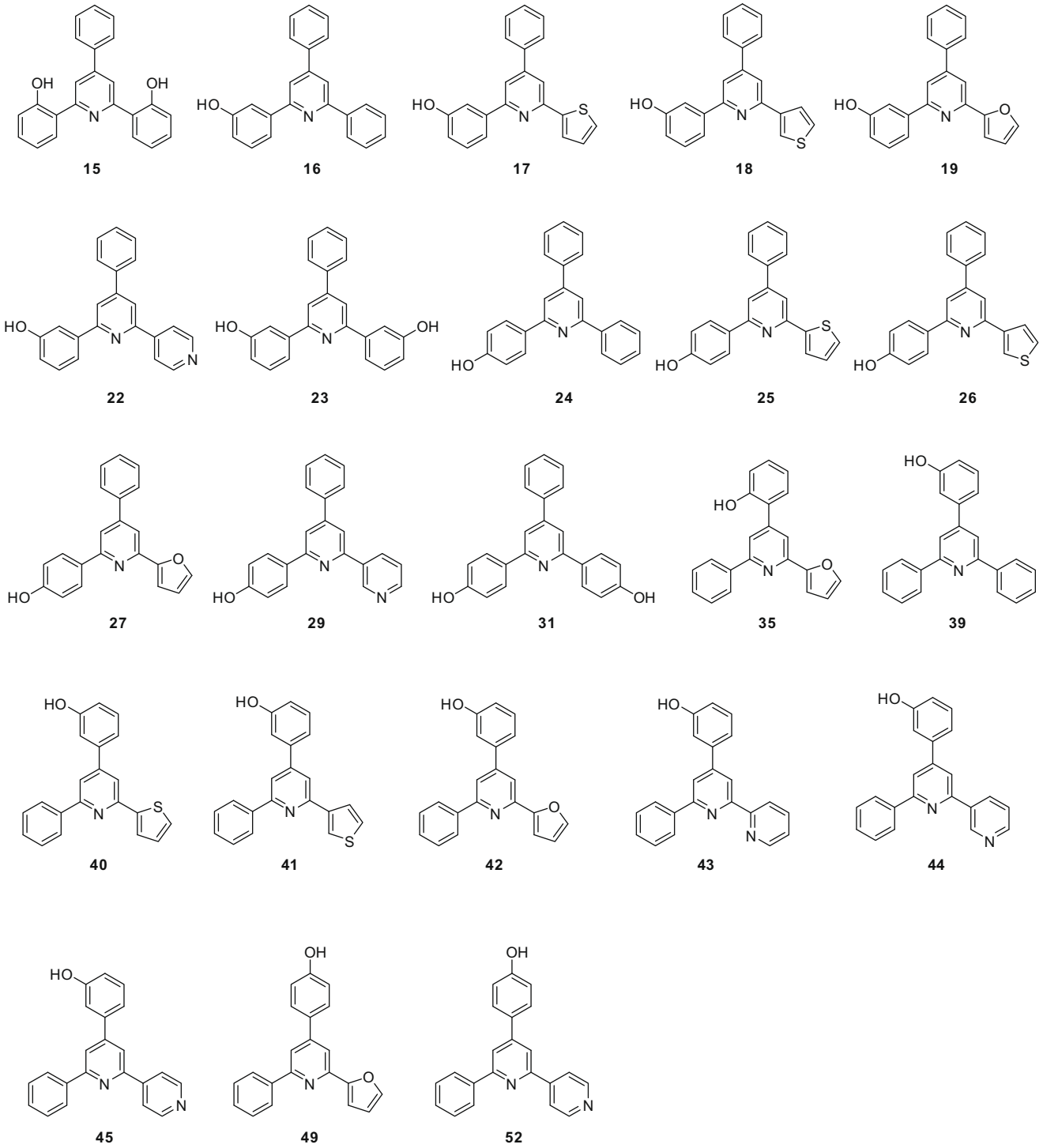
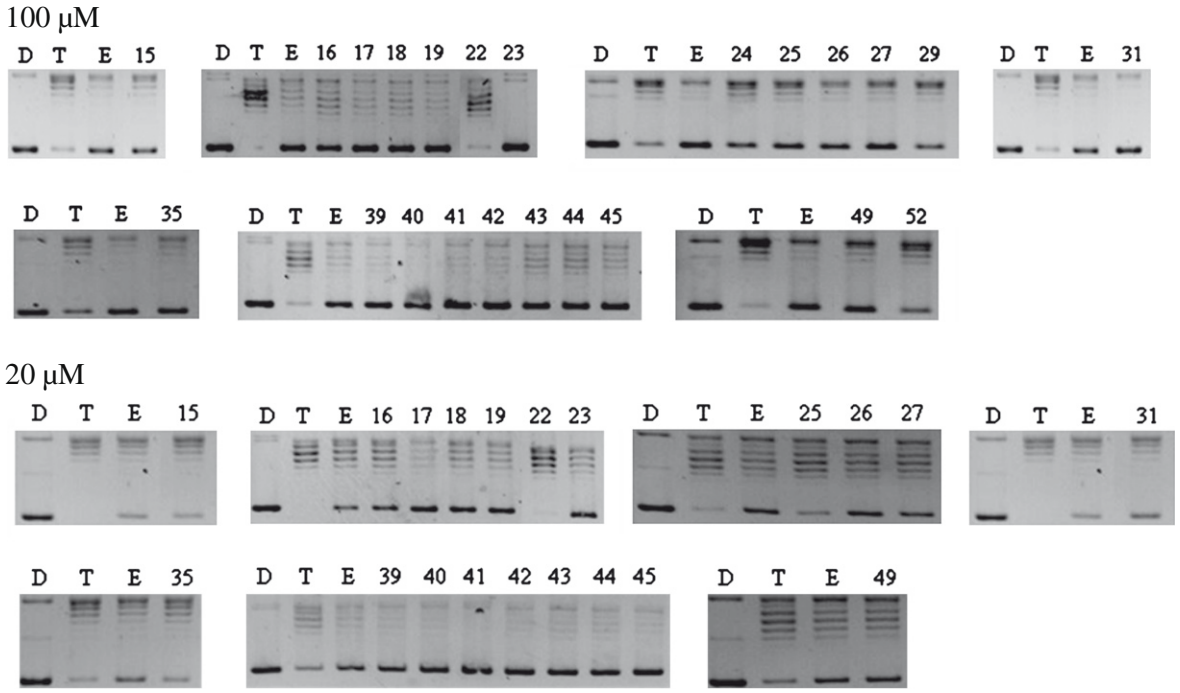


Figure 3. Structure of the selected compounds possessing significant biological activity.

were evaluated these for topo I and II inhibitory activity and cytotoxicity against several human cancer cell lines. Most of the compounds (**16**–**19**, **23**, **31**, and **39**–**43**, **45**) showed potent topo II inhibitory activity. Compounds **22** and **24** displayed significant topo I inhibitory activity. The structure–activity relationship study revealed that the substitution of hydroxyl groups on the phenyl ring increase the topo I and II inhibitory activity of compounds in the order of substitution at *meta* > *para* > *ortho* position. The *para*-substitution on phenyl ring (4′-OH or 4-OH series) showed relatively stronger cytotoxicity against several human cancer cell lines. It was observed that the increase in the number of hydroxyl groups increased the topo I and II inhibitory activity. This study may provide valuable information to researchers working on the development of antitumor agents.

4. Experimental

Compounds used as starting materials and reagents were obtained from Aldrich Chemical Co., Junsei, or other chemical compa-



Lane D: pBR322 DNA only

Lane T: pBR322 DNA + Topo II

Lane E: pBR322 DNA + Topo II + Etoposide

Lane **15-52** (100 μM): pBR322 DNA + Topo II + Compound **15-52**

Lane **15-49** (20 μM): pBR322 DNA + Topo II + Compound **15-49**

Figure 4. Human DNA topoisomerase IIα inhibitory activity of the selected compounds.

nies, 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 F_{254} (Merck) and silica gel (Kieselgel 60, 230–400 mesh, Merck), respectively. Since all the compounds prepared contained aromatic rings, 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 ^{1}H NMR and 62.5 MHz for ^{13}C 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 analyses were performed using two Shimadzu LC-10AT pumps gradient-controlled HPLC system equipped with Shimadzu system controller (SCL-10A VP) and photo diode array detector (SPD-M10A VP) utilizing Shimadzu Class VP program. Sample volume of 10 μL was injected in Waters X-Terra® 5 μM reverse-phase C_{18} column (4.6 × 250 mm) with a gradient elution of 60–100% of B in A for 15 min followed by 100–60% of B in A for 15 min at a flow rate of 1.0 mL/min at 254 nm UV detection, where mobile phase A was double distilled water with 20 mM ammonium formate (AF) and B was 90% ACN in water with 20 mM AF. Purity of compound is described as percent (%).

ESI LC/MS analyses were performed with a Finnigan LCQ Advantage® LC/MS/MS spectrometry utilizing Xcalibur® program. For ESI LC/MS, LC was performed with a 2 μL injection volume on Waters Atlantis® T3 reverse-phase C_{18} column (2.1 × 50 mm, 3 μM). The mobile phase consisted of 100% distilled water (A) and 100% ACN (B). A gradient program was used with a flow rate of 200 μL/min. The initial composition was 10% B and programmed linearly to 90% B after 5 min and finally 10% B after 15 min. MS ionization conditions were: Sheath gas flow rate: 70 arb, aux gas flow rate: 20 arb, I spray voltage: 4.5 KV, capillary temperature 215 °C, column temperature 40 °C, capillary voltage: 21 V, tube lens offset: 10 V. Retention time was given in minutes.

4.1. General method for the preparation of 3 and 4

Aryl ketone **1** (R^1 = **a**, **h**–**j**) was added to the solution of 50% KOH in MeOH/H_2O (5:1). After complete dissolution, aryl aldehyde **2** (R^2 = **a**, **h**–**j**) was added slowly. The mixture was then stirred for 2–3 h at room temperature. Precipitate was formed in most of the cases which was then filtered, washed with cold MeOH, and dried to yield 60.70–94.11% solid compound. In those reactions where no precipitate occured, the reaction mixtures were extracted with ethyl acetate and washed with water and brine. They were then further purified by either recrystallization or column

Table 2
Topoisomerase I and II inhibitory activity and cytotoxicity of the selected compounds

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	68.2	30.4			0.70 ± 0.06	1.40 ± 0.15	0.50 ± 0.03	1.10 ± 0.02	1.00 ± 0.01
Camptothecin			68.8	40.3	0.30 ± 0.01	0.40 ± 0.13	0.40 ± 0.16	0.50 ± 0.06	0.10 ± 0.00
Adriamycin					0.40 ± 0.02	1.00 ± 0.07	1.00 ± 0.14	1.20 ± 0.02	0.70 ± 0.01
15	42.7	12.1	0.0	NA	37.3 ± 0.34	14.8 ± 0.05	8.0 ± 0.32	>50	22.4 ± 2.07
16	48.4	32.0	12.9	0.0	19.29 ± 2.87	16.07 ± 1.75	17.95 ± 0.46	9.95 ± 0.46	11.47 ± 0.86
17	53.8	43.5	51.2	5.8	18.06 ± 2.78	16.96 ± 1.9	18.92 ± 1.09	10.85 ± 0.7	14.12 ± 0.48
18	51.8	37.2	75.8	6.8	12.06 ± 0.43	13.82 ± 0.37	11.7 ± 0.58	7.66 ± 0.53	10.3 ± 1.14
19	51.6	38.6	10.2	0.0	13.68 ± 0.2	9.43 ± 0.75	14.41 ± 0.95	13.47 ± 0.58	9.91 ± 1.65
22	3.1	1.8	70.4	25.4	10.39 ± 1.83	28.13 ± 2.55	19.21 ± 1.34	27.44 ± 6.25	23.88 ± 0.86
23	86.5	49.0	67.2	10.5	12.78 ± 1.3	13.04 ± 0.08	24.93 ± 0.78	18.39 ± 0.61	16.44 ± 0.75
24	27.6	NA	52.9	43.6	1.30 ± 0.10	1.70 ± 0.01	3.00 ± 0.42	3.70 ± 0.66	1.40 ± 0.01
25	35.4	1.3	4.9	NA	2.40 ± 0.54	1.70 ± 0.10	3.70 ± 0.46	12.50 ± 2.18	1.70 ± 0.01
26	38.3	8.3	20.4	NA	5.70 ± 1.12	17.30 ± 0.19	8.50 ± 0.50	1.10 ± 0.08	3.60 ± 0.03
27	35.2	10.2	18.6	NA	1.40 ± 0.07	10.90 ± 0.14	3.20 ± 0.22	3.50 ± 0.57	3.30 ± 0.07
29	18.6	NA	44.0	0.9	2.20 ± 0.12	13.60 ± 0.46	8.40 ± 0.61	15.10 ± 1.45	4.10 ± 0.13
31	76.8	33.7	0.3	NA	6.70 ± 0.78	3.30 ± 0.44	22.0 ± 0.63	3.00 ± 0.50	5.70 ± 0.84
35	31.2	3.1	1.5	NA	4.60 ± 0.50	9.70 ± 0.15	4.90 ± 0.35	2.50 ± 0.20	1.90 ± 0.01
39	68.2	32.0	11.5	0.0	19.50 ± 0.45	15.05 ± 2.08	17.89 ± 0.55	17.95 ± 2.34	13.49 ± 3.24
40	57.5	34.7	9.1	2.0	17.73 ± 1.68	15.32 ± 0.34	19.68 ± 0.31	14.12 ± 2.83	18.92 ± 2.22
41	52.6	30.8	6.9	3.2	18.97 ± 2.97	14.90 ± 1.03	11.70 ± 0.58	24.56 ± 2.64	11.99 ± 1.45
42	47.5	31.1	48.8	3.5	20.81 ± 1.18	10.92 ± 5.15	34.91 ± 3.82	13.86 ± 1.4	24.03 ± 1.49
43	48.1	21.3	10.8	2.8	18.73 ± 2.65	29.84 ± 2.43	15.51 ± 0.64	20.91 ± 1.71	15.13 ± 1.87
44	37.1	21.3	8.1	1.5	22.77 ± 1.93	17.52 ± 1.9	18.93 ± 1.64	18.39 ± 2.53	12.63 ± 1.39
45	42.8	32.7	60.8	0.9	12.18 ± 1.41	14.95 ± 1.37	18.14 ± 4.58	18.71 ± 1.0	12.67 ± 1.32
49	30.2	10.3	5.0	NA	1.00 ± 0.21	4.50 ± 0.12	8.60 ± 0.60	5.10 ± 0.82	13.70 ± 0.26
52	8.4	NA	57.5	5.8	2.10 ± 0.05	1.60 ± 0.05	2.20 ± 0.09	11.70 ± 1.07	1.40 ± 0.00

NA: not applicable.
Etoposide: positive control for topo II and cytotoxicity.
Camptothecin: positive control for topo I and cytotoxicity.
Adriamycin: positive control for cytotoxicity.
MDA-MB231: human breast adenocarcinoma.
HeLa: human cervix tumor.
DU145: human prostate tumor.
HCT 15: human colorectal adenocarcinoma.
HL60: human myeloid leukemia.
[a] Each data represents mean ± S.D. from three different experiments performed in triplicate.

chromatography. Six different hydroxylated chalcones were synthesized as intermediates following the same method.

4.1.1. Synthesis of 1-(2-hydroxyphenyl)-3-phenylpropenone (3a)

The procedure described in Section 4.1 was employed with aryl ketone **1** (R^1 = **h**) (2.40 mL, 20.00 mmol) and aryl aldehyde **2** (R^2 = **a**) (2.02 mL, 20.00 mmol) to yield a yellow solid compound (2.72 g, 60.7%).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.46; mp 97.1–97.8 °C.

^{1}H NMR (250 MHz, $CDCl_3$) δ 12.84 (s, 1H, 1-phenyl 2-OH), 7.96 (d, *J* = 15.5 Hz, 1H, CO–C=C*H*), 7.95 (dd, *J* = 8.1, 1.6 Hz, 1H, 1-phenyl 6-H), 7.70 (d, *J* = 15.5 Hz, 1H, CO–C*H*=C), 7.70–7.66 (m, 2H, 3-phenyl H-2, H-6), 7.51 (dt, *J* = 8.5, 1.6 Hz, 1H, 1-phenyl 4-H), 7.46–7.43 (m, 3H, 3-phenyl H-3, H-4, H-5), 7.05 (dd, *J* = 8.5, 1.0 Hz, 1H, 1-phenyl 3-H), 6.95 (dt, *J* = 8.1, 1.0 Hz, 1H, 1-phenyl 5-H).

4.1.2. Synthesis of 1-(3-hydroxyphenyl)-3-phenylpropenone (3b)

The procedure described in Section 4.1 was employed with aryl ketone **1** (R^1 = **i**) (4.08 g, 30.00 mmol) and aryl aldehyde **2** (R^2 = **a**) (3.03 mL, 30.00 mmol) to yield a light yellow solid compound (5.90 g, 87.7%).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.35; mp 120.2–120.6 °C.

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.86 (d, *J* = 15.7 Hz, 1H, CO–C=C*H*), 7.66–7.62 (m, 3H, 3-phenyl H-2, H-6, 1-phenyl H-2), 7.60 (d, *J* = 7.8 Hz, 1H, 1-phenyl H-6), 7.55 (d, *J* = 15.7 Hz, 1H, CO–C*H*=C), 7.44–7.41 (m, 3H, 3-phenyl H-3, H-4, H-5), 7.38 (t, *J* = 7.8 Hz, 1H, 1-phenyl H-5), 7.15 (dd, *J* = 8.0, 2.4 Hz, 1H, 1-phenyl H-4), 6.59 (br, 1H, 1-phenyl 3-OH).

4.1.3. Synthesis of 1-(4-hydroxyphenyl)-3-phenylpropenone (3c)

The procedure described in Section 4.1 was employed with aryl ketone **1** (R^1 = **j**) (2.72 g, 20.00 mmol) and aryl aldehyde **2** (R^2 = **a**) (2.02 mL, 20.00 mmol) to yield a light yellow solid compound (3.85 g, 86.1%).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.40; mp 180.0–180.7 °C.

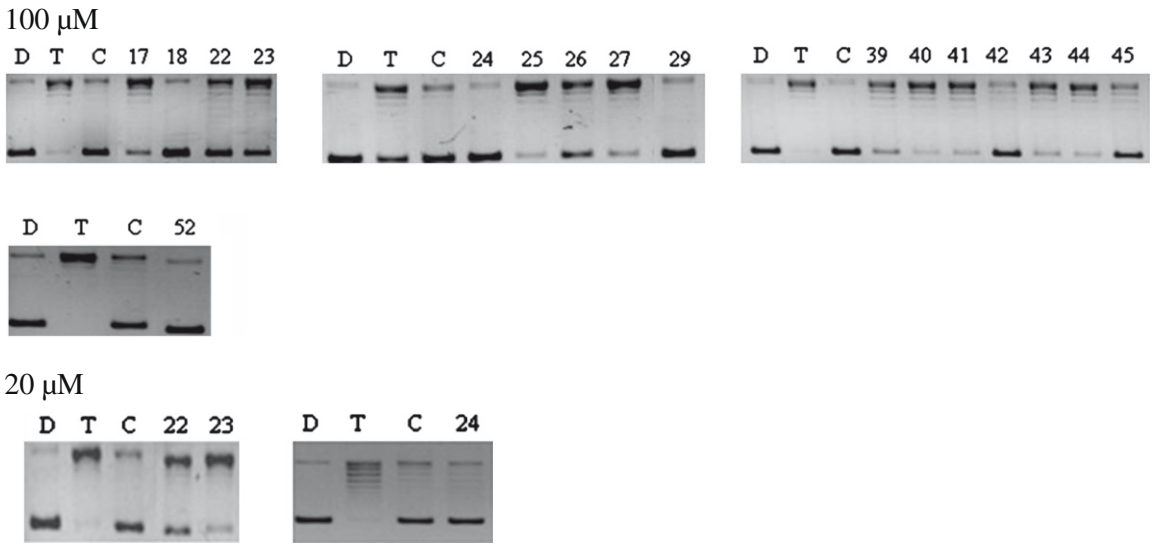
^{1}H NMR (250 MHz, $CDCl_3$) δ 8.02 (d, *J* = 8.4 Hz, 2H, 1-phenyl H-2, H-6), 7.84 (d, *J* = 15.6 Hz, 1H, CO–C=C*H*), 7.66–7.63 (m, 2H, 3-phenyl H-2, H-6), 7.58 (d, *J* = 15.6 Hz, 1H, CO–C*H*=C), 7.43–7.40 (m, 3H, 3-phenyl H-3, H-4, H-5), 6.96 (d, *J* = 8.4 Hz, 2H, 1-phenyl H-3, H-5), 6.38 (br, 1H, 1-phenyl 4-OH).

4.1.4. Synthesis of 3-(2-hydroxyphenyl)-1-phenylpropenone (4a)

The procedure described in Section 4.1 was employed with aryl ketone **1** (R^1 = **a**) (2.33 mL, 20.00 mmol) and aryl aldehyde **2** (R^2 = **h**) (2.09 mL, 20.00 mL) to yield a greenish yellow solid compound (3.95 g, 88.2%).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.37; mp 149.9–150.6 °C.

^{1}H NMR (250 MHz, DMSO) δ 10.31 (br, 1H, 3-phenyl 2-OH), 8.10–8.06 (m, 2H, 1-phenyl H-2, H-6), 8.07 (d, *J* = 15.8 Hz, 1H,



Lane D: pBR322 DNA only

Lane T: pBR322 DNA + Topo I

Lane C: pBR322 DNA + Topo I + Camptothecin

Lane **17-52** (100 μM): pBR322 DNA + Topo I + Compound **17-52**

Lane **22-24** (20 μM): pBR322 DNA + Topo I + Compound **22-24**

Figure 5. Human DNA topoisomerase I inhibitory activity of the selected compounds.

CO–C═C*H*), 7.87 (d, *J* = 15.8 Hz, 1H, CO–C*H*═C), 7.85 (dd, *J* = 7.3, 1.4 Hz, 1H, 3-phenyl H-6), 7.68–7.52 (m, 3H, 1-phenyl H-3, H-4, H-5), 7.26 (dt, *J* = 8.2, 1.5 Hz, 1H, 3- phenyl H-4), 6.94 (dd, *J* = 8.2, 0.8 Hz, 1H, 3-phenyl H-3), 6.89 (t, *J* = 7.2 Hz, 1H, 3-phenyl H-5).

4.1.5. Synthesis of 3-(3-hydroxyphenyl)-1-phenylpropenone (4b)

The procedure described in Section 4.1 was employed with aryl ketone **1** (R^1 = **a**) (1.16 mL, 10.00 mmol) and aryl aldehyde **2** (R^2 = **i**) (1.22 g, 10.00 mmol) to yield a light yellow solid compound (2.11 g, 94.1%).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.35; mp 170.1–170.9 °C.

^{1}H NMR (250 MHz, DMSO) δ 9.67 (br, 1H, 3-phenyl 3-OH), 8.15–8.11 (m, 2H, 1-phenyl H-2, H-6), 7.87 (d, *J* = 15.6 Hz, 1H, CO–C═C*H*), 7.69–7.53 (m, 3H, 1-phenyl H-3, H-4, H-5), 7.67 (d, *J* = 15.6 Hz, 1H, CO–C*H*═C), 7.33–7.24 (m, 2H, 3-phenyl H-5, H-6), 7.22 (br, 1H, 3-phenyl H-2), 6.87 (ddd, *J* = 7.7, 2.1, 1.0 Hz, 1H, 3-phenyl H-4).

4.1.6. Synthesis of 3-(4-hydroxyphenyl)-1-phenylpropenone (4c)

The procedure described in Section 4.1 was employed with aryl ketone **1** (R^1 = **a**) (2.33 mL, 20.00 mmol) and aryl aldehyde **2** (R^2 = **j**) (2.44 g, 20.00 mmol) to yield a yellow solid compound (3.78 g, 84.5%).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.34; mp 174.2–175.0 °C.

^{1}H NMR (250 MHz, DMSO) δ 10.14 (br, 1H, 3-phenyl 4-OH), 8.12–8.08 (m, 2H, 1-phenyl H-2, H-6), 7.75 (d, *J* = 8.6 Hz, 2H, 3-phenyl H-2, H-6), 7.70 (d, *J* = 15.4 Hz, 1H, CO–C═C*H*), 7.69–7.51 (m, 4H, 1-phenyl H-3, H-4, H-5, CO-C*H*═C), 6.84 (d, *J* = 8.6 Hz, 2H, 3-phenyl H-3, H-5).

4.2. General method for the preparation of 5

A mixture of aryl ketone (R^1 = **a–j**), iodine (1.2 equiv) and pyridine was refluxed at 140 °C for 3 h. Precipitate occurred during reaction, and the mixture was cooled to room temperature. Then it was filtered and washed with cold pyridine to afford **5** (R^3 = **a–j**) in quantitative yield. Ten different pyridinium iodide salts were synthesized by this method.

4.3. General method for the preparation of 6 and 7

A mixture of propenone intermediate **3** (R^1 = **h–j,** R^2 = **a**), or **4** (R^1 = **a,** R^2 = **h–j**), pyridinium iodide salt **5** (R^3 = **a–j**) 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, and washed with water and brine. 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 **6** (R^1 = **h–j,** R^2 = **a,** R^3 = **a–j**) and **7** (R^1 = **a,** R^2 = **h–j,** R^3 = **a–g**) in 21.4–89.3% yield. Forty-five 2,4-diphenyl-6-aryl pyridine compounds were synthesized by this method.

4.3.1. Synthesis of 2,2′-(4-phenylpyridine-2,6-diyl)diphenol (15)

The procedure described in Section 4.3 was employed with **3a** (0.67 g, 3.00 mmol), anhydrous ammonium acetate (2.31 g, 30.00 mmol), **5** (R^3 = **h**) (1.02 g, 3.00 mmol), and glacial AcOH (3 mL) to yield a yellow solid (0.38 g, 1.13 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.42; LC/MS/MS: retention time: 7.79 min; $[MH]^+$: 340.27.

^{1}H NMR (250 MHz, DMSO) δ 12.31 (s, 2H, 2-phenyl 2-OH, 6-phenyl 2′-OH), 8.20 (s, 2H, pyridine H-3, H-5), 7.98–7.95 (m, 4H, 2-phenyl H-6, 6-phenyl H-6, 4-phenyl H-2, H-6), 7.60–7.52 (m, 3H, 4-phenyl H-3, H-4, H-5), 7.32 (t, J = 8.2 Hz, 2H, 2-phenyl H-4, 6-phenyl H-4), 6.99–6.93 (m, 4H, 2-phenyl H-3, H-5, 6-phenyl H-3, H-5).

^{13}C NMR (62.5 MHz, DMSO) δ 157.51, 155.59, 149.91, 137.76, 131.23, 129.77, 129.44, 128.95, 127.64, 122.26, 119.43, 117.90, 117.51.

4.3.2. Synthesis of 3-(4,6-diphenylpyridin-2-yl)phenol (16)

The procedure described in Section 4.3 was employed with **3b** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **a**) (0.65 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a white solid (0.54 g, 1.67 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.44; LC/MS/MS: retention time: 8.29 min; $[MH]^+$: 324.27.

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.18–8.15 (m, 2H, 6-phenyl H-2, H-6), 7.88 (d, J = 1.1 Hz, 1H, pyridine H-5), 7.86 (d, J = 1.1 Hz, 1H, pyridine H-3), 7.75–7.72 (m, 3H, 2-phenyl H-2, 4-phenyl H-2, H-6), 7.70 (d, J = 8.0 Hz, 1H, 2-phenyl H-6), 7.57–7.43 (m, 6H, 6-phenyl H-3, H-4, H-5, 4-phenyl H-3, H-4, H-5), 7.36 (t, J = 8.0 Hz, 1H, 2-phenyl H-5), 6.92 (dd, J = 8.0, 2.5 Hz, 1H, 2-phenyl H-4), 5.56 (br, 1H, 2-phenyl 3-OH).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 157.56, 157.00, 156.05, 150.23, 141.08, 139.44, 138.85, 129.92, 129.11, 129.08, 129.03, 128.70, 127.16, 119.41, 117.49, 117.31, 116.13, 114.11.

4.3.3. Synthesis of 3-(4-phenyl-6-(thiophen-2-yl)pyridin-2-yl)-phenol (17)

The procedure described in Section 4.3 was employed with **3b** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **b**) (0.66 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a white solid (0.58 g, 1.76 mmol).

R_f (ethyl acetate/*n*-hexane 1:3, v/v): 0.34; LC/MS/MS: retention time: 8.05 min; $[MH]^+$: 330.24.

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.77 (s, 2H, pyridine H-3, H-5), 7.72–7.70 (m, 3H, 2-phenyl H-2, 4-phenyl H-2, H-6), 7.70 (d, J = 4.4 Hz, 1H, 6-thiophene H-3), 7.68 (d, J = 8.4 Hz, 1H, 2-phenyl H-6), 7.56–7.47 (m, 3H, 4-phenyl H-3, H-4, H-5), 7.42 (d, J = 4.9 Hz, 1H, 6-thiophene H-5), 7.35 (t, J = 8.0 Hz, 1H, 2-phenyl H-5), 7.15 (dd, J = 4.9, 3.7 Hz, 1H, 6-thiophene H-4), 6.93 (dd, J = 8.0, 2.5 Hz, 1H, 2-phenyl H-4), 5.46 (br, 1H, 2-phenyl 3-OH).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 156.79, 156.06, 152.66, 150.22, 145.16, 140.54, 138.62, 129.93, 129.11, 127.98, 127.73, 127.10, 124.73, 119.30, 117.03, 116.24, 115.60, 114.03.

4.3.4. Synthesis of 3-(4-phenyl-6-(thiophen-3-yl)pyridin-2-yl)-phenol (18)

The procedure described in Section 4.3 was employed with **3b** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **c**) (0.66 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a white solid (0.50 g, 1.53 mmol).

R_f (ethyl acetate/*n*-hexane 1:3, v/v): 0.34; LC/MS/MS: retention time: 8.01 min; $[MH]^+$: 330.24.

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.06 (dd, J = 3.0, 1.2 Hz, 1H, 6-thiophene H-2), 7.81 (dd, J = 5.5, 1.2 Hz, 1H, 6-thiophene H-4), 7.79 (d, J = 1.3, 1H, pyridine H-3), 7.75 (d, J = 1.3 Hz, 1H, pyridine H-5), 7.73–7.69 (m, 3H, 2-phenyl H-2, 4-phenyl H-2, H-6), 7.67 (d, J = 7.8 Hz, 1H, 2-phenyl H-6), 7.56–7.47 (m, 3H, 4-phenyl H-3, H-4, H-5), 7.43 (dd, J = 5.0, 3.0 Hz, 1H, 6-thiophene H-5), 7.32 (t, J = 8.0 Hz, 1H, 2-phenyl H-5), 6.93 (ddd, J = 8.0, 2.5, 0.7 Hz, 1H, 2-phenyl H-4), 5.92 (br, 1H, 2-phenyl 3-OH)

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 157.07, 156.16, 153.63, 150.19, 142.28, 140.96, 138.76, 129.88, 129.09, 129.02, 127.10, 126.46, 126.21, 123.91, 119.30, 117.18, 117.08, 116.16, 114.11.

4.3.5. Synthesis of 3-(6-(furan-2-yl)-4-phenylpyridin-2-yl)-phenol (19)

The procedure described in Section 4.3 was employed with **3b** (0.33 g, 1.50 mmol), anhydrous ammonium acetate (1.15 g, 15.00 mmol), **5** (R^3 = **d**) (0.47 g, 1.50 mmol), and glacial AcOH (2 mL) to yield a white solid (0.41 g, 1.33 mmol).

R_f (ethyl acetate/*n*-hexane 1:3, v/v): 0.31; LC/MS/MS: retention time: 7.77 min; $[MH]^+$: 314.24.

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.88 (d, J = 1.3 Hz, 1H, pyridine H-3), 7.77 (d, J = 1.3 Hz, 1H, pyridine H-5), 7.76–7.72 (m, 2H, 4-phenyl H-2, H-6), 7.70 (t, J = 2.0 Hz, 1H, 2-phenyl H-2), 7.60 (d, J = 7.8 Hz, 1H, 2-phenyl H-6), 7.55–7.47 (m, 4H, 4-phenyl H-3, H-4, H-5, 6-furan H-5), 7.34 (t, J = 7.9 Hz, 1H, 2-phenyl H-5), 7.23 (d, J = 3.3 Hz, 1H, 6-furan H-3), 6.91 (dd, J = 8.0, 2.5 Hz, 1H, 2-phenyl H-4), 6.56 (dd, J = 3.3, 1.7 Hz, 1H, 6-furan H-4), 6.12 (br, 1H, 2-phenyl 3-OH).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 157.45, 156.27, 153.74, 150.08, 149.63, 143.29, 140.79, 138.48, 129.91, 129.12, 129.08, 127.08, 119.26, 117.29, 116.28, 115.33, 114.26, 112.13, 109.14.

4.3.6. Synthesis of 3-(4-phenyl-2,4′-bipyridin-6-yl)phenol (22)

The procedure described in Section 4.3 was employed with **3b** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **g**) (0.65 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a white solid (0.42 g, 1.29 mmol).

R_f (ethyl acetate/*n*-hexane 1:1, v/v): 0.30; LC/MS/MS: retention time: 6.90 min; $[MH]^+$: 325.29.

^{1}H NMR (250 MHz, DMSO) δ 9.61 (br, 1H, 6-phenyl 3-OH), 8.76 (d, J = 4.3 Hz, 2H, 2-pyridine H-2′, H-6′), 8.33 (d, J = 1.1 Hz, 1H, pyridine H-3), 8.29 (dd, J = 4.3, 1.4 Hz, 2H, 2-pyridine H-3′, H-5′), 8.21 (d, J = 1.1 Hz, 1H, pyridine H-5), 8.06–8.02 (m, 2H, 4-phenyl H-2, H-6), 7.75 (s, 1H, 6-phenyl H-2), 7.72 (d, J = 8.4 Hz, 1H, 6-phenyl H-6), 7.61–7.51 (m, 3H, 4-phenyl H-3, H-4, H-5), 7.33 (t, J = 7.8 Hz, 1H, 6-phenyl H-5), 6.91 (dd, J = 8.1, 1.7 Hz, 1H, 6-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO) δ 158.08, 157.15, 154.08, 150.59, 150.09, 145.95, 140.01, 137.56, 130.08, 129.77, 129.39, 127.70, 121.37, 118.40, 118.06, 117.69, 116.75, 114.00.

4.3.7. Synthesis of 3,3′-(4-phenylpyridine-2,6-diyl)diphenol (23)

The procedure described in Section 4.3 was employed with **3b** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **i**) (0.68 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a white solid (0.37 g, 1.16 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.35; LC/MS/MS: retention time: 7.10 min; $[MH]^+$: 340.27.

^{1}H NMR (250 MHz, DMSO) δ 9.57 (br, 2H, 2-phenyl 3-OH, 6-phenyl 3′-OH), 8.06 (s, 2H, pyridine H-3, H-5), 8.00–7.98 (m, 2H, 4-phenyl H-2, H-6), 7.73–7.68 (m, 4H, 2-phenyl H-2, H-6, 6-phenyl H-2, H-6), 7.58–7.50 (m, 3H, 4-phenyl H-3, H-4, H-5), 7.32 (t, J = 7.9 Hz, 2H, 2-phenyl H-5, 6-phenyl H-5), 6.89 (dd, J = 8.0, 1.7 Hz, 2H, 2-phenyl H-4, 6-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO) δ 158.01, 156.65, 149.62, 140.46, 138.02, 129.96, 129.51, 129.36, 127.56, 117.94, 116.79, 116.47, 113.96.

4.3.8. Synthesis of 4-(4,6-diphenylpyridin-2-yl)phenol (24)

The procedure described in Section 4.3 was employed with **3c** (0.56 g, 2.50 mmol), anhydrous ammonium acetate (1.92 g, 25.00 mmol), **5** (R^3 = **a**) (0.81 g, 2.50 mmol), and glacial AcOH (3 mL) to yield a white solid (0.41 g, 1.27 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.40; LC/MS/MS: retention time: 8.03 min; $[MH]^+$: 324.27.

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.19–8.17 (m, 2H, 6-phenyl H-2, H-6), 8.12 (d, J = 8.6 Hz, 2H, 2-phenyl H-2, H-6), 7.83 (s, 1H, pyridine H-5), 7.81 (s, 1H, pyridine H-3), 7.75–7.72 (m, 2H, 4-phenyl H-2, H-6), 7.56–7.41 (m, 6H, 6-phenyl H-3, H-4, H-5, 4-phenyl H-3, H-4, H-

5), 6.96 (d, J = 8.6 Hz, 2H, 2-phenyl H-3, H-5), 5.24 (br, 1H, 2-phenyl 4-OH).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 157.38, 157.09, 156.61, 150.13, 139.64, 139.13, 132.37, 129.07, 128.98, 128.92, 128.67, 128.65, 127.15, 127.11, 116.58, 116.38, 115.56.

4.3.9. Synthesis of 4-(4-phenyl-6-(thiophen-2-yl)pyridin-2-yl)-phenol (25)

The procedure described in Section 4.3 was employed with **3c** (0.56 g, 2.50 mmol), anhydrous ammonium acetate (1.92 g, 25.00 mmol), **5** (R^3 = **b**) (0.82 g, 2.50 mmol), and glacial AcOH (3 mL) to yield a light yellow solid (0.43 g, 1.32 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.40; LC/MS/MS: retention time: 8.03 min; $[MH]^+$: 330.26.

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.09 (d, J = 8.3 Hz, 2H, 2-phenyl H-2, H-6), 7.72 (s, 2H, pyridine H-3, H-5), 7.72–7.70 (m, 3H, 4-phenyl H-2, H-6, 6-thiophene H-3), 7.55–7.46 (m, 3H, 4-phenyl H-3, H-4, H-5), 7.42 (d, J = 5.0 Hz, 1H, 6-thiophene H-5), 7.15 (t, J = 4.3, 1H, 6-thiophene H-4), 6.96 (d, J = 8.3 Hz, 2H, 2-phenyl H-3, H-5), 5.22 (br, 1H, 2-phenyl 4-OH).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 156.88, 156.70, 152.56, 150.11, 145.51, 138.90, 131.86, 129.07, 128.99, 128.58, 127.93, 127.56, 127.10, 124.53, 116.06, 115.57, 114.72.

4.3.10. Synthesis of 4-(4-phenyl-6-(thiophen-3-yl)pyridin-2-yl)-phenol (26)

The procedure described in Section 4.3 was employed with **3c** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **c**) (0.66 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a white solid (0.47 g, 1.42 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.44; LC/MS/MS: retention time: 8.00 min; $[MH]^+$: 330.25.

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.09–8.05 (m, 3H, 2-phenyl H-2, H-6, 6-thiophene H-2), 7.83 (dd, J = 5.0, 1.2 Hz, 1H, 6-thiophene H-4), 7.75–7.71 (m, 2H, 4-phenyl H-2, H-6), 7.74 (s, 1H, pyridine H-3), 7.70 (s, 1H, pyridine H-5), 7.56–7.46 (m, 3H, 4-phenyl H-3, H-4, H-5), 7.43 (dd, J = 5.0, 3.0 Hz, 1H, 6-thiophene H-5), 6.95 (dd, J = 8.7, 2.0 Hz, 2H, 2-phenyl H-3, H-5), 5.11 (br, 1H, 2-phenyl 4-OH).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 157.07, 156.57, 153.53, 150.12, 142.56, 139.06, 132.32, 129.07, 128.93, 128.62, 127.12, 126.48, 126.11, 123.75, 116.33, 116.12, 115.55.

4.3.11. Synthesis of 4-(6-(furan-2-yl)4-phenylpyridin-2-yl)-phenol (27)

The procedure described in Section 4.3 was employed with **3c** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **d**) (0.63 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a white solid (0.36 g, 1.16 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.38; LC/MS/MS: retention time: 7.61 min; $[MH]^+$: 314.24.

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.03 (dd, J = 8.7, 2.0 Hz, 2H, 2-phenyl H-2, H-6), 7.83 (d, J = 1.4 Hz, 1H, pyridine H-3), 7.76–7.72 (m, 2H, 4-phenyl H-2, H-6), 7.72 (d, J = 1.4 Hz, 1H, pyridine H-5), 7.55–7.45 (m, 4H, 4-phenyl H-3, H-4, H-5, 6-furan H-5), 7.23 (dd, J = 3.3, 0.5 Hz, 1H, 6-furan H-3), 6.94 (dd, J = 8.7, 2.0 Hz, 2H, 2-phenyl H-3, H-5), 6.57 (dd, J = 3.3, 1.7 Hz, 1H, 6-furan H-4), 5.54 (br, 1H, 2-phenyl 4-OH).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 157.38, 156.76, 154.09, 149.95, 149.56, 143.17, 138.78, 132.06, 129.05, 129.00, 128.66, 127.08, 116.31, 115.61, 114.50, 112.07, 108.94.

4.3.12. Synthesis of 4-(4-phenyl-2,3′-bipyridin-6-yl)phenol (29)

The procedure described in Section 4.3 was employed with **3c** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **f**) (0.65 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a light yellow solid (0.49 g, 1.51 mmol).

R_f (ethyl acetate/*n*-hexane 1:1, v/v): 0.38; LC/MS/MS: retention time: 6.67 min; $[MH]^+$: 325.27.

^{1}H NMR (250 MHz, DMSO) δ 9.79 (s, 1H, 6-phenyl 4-OH), 9.48 (s, 1H, 2-pyridine H-2′), 8.67–8.63 (m, 2H, 2-pyridine H-6′, H-4′), 8.20 (d, J = 8.5 Hz, 2H, 6-phenyl H-2, H-6), 8.19 (s, 1H, pyridine H-3), 8.12 (s, 1H, pyridine H-5), 8.04–8.02 (m, 2H, 4-phenyl H-2, H-6), 7.58–7.50 (m, 4H, 4-phenyl H-3, H-4, H-5, 2-pyridine H-5′), 6.93 (d, J = 8.5 Hz, 2H, 6-phenyl H-3, H-5).

^{13}C NMR (62.5 MHz, DMSO) δ 159.03, 157.11, 154.20, 150.09, 149.70, 148.37, 137.84, 134.54, 134.48, 129.65, 129.50, 129.25, 128.65, 127.53, 123.92, 116.13, 116.05, 115.70.

4.3.13. Synthesis of 4,4′-(4-phenylpyridine-2,6-diyl)diphenol (31)

The procedure described in Section 4.3 was employed with **3c** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **j**) (0.68 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a yellow solid (0.21 g, 0.62 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.24; LC/MS/MS: retention time: 6.72 min; $[MH]^+$: 340.27.

^{1}H NMR (250 MHz, DMSO) δ 9.76 (br, s, 2H, 2-phenyl 4-OH, 6-phenyl 4′-OH), 8.16 (d, J = 8.6 Hz, 4H, 2-phenyl H-2, H-6, 6-phenyl H-2, H-6), 7.98–7.94 (m, 4H, 4-phenyl H-2, H-6, pyridine H-3, H-5), 7.58–7.48 (m, 3H, 4-phenyl H-3, H-4, H-5), 6.91 (d, J = 8.6 Hz, 4H, 2-phenyl H-3, H-5, 6-phenyl H-3, H-5).

^{13}C NMR (62.5 MHz, DMSO) δ 158.80, 156.47, 149.29, 138.36, 130.10, 129.25, 128.49, 127.41, 115.63, 114.48.

4.3.14. Synthesis of 2-(2-(furan-2-yl)-6-phenylpyridin-4-yl)-phenol (35)

The procedure described in Section 4.3 was employed with **4a** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **d**) (0.63 g, 2.00 mmol), and glacial AcOH (2 mL) to yield an orange solid (0.25 g, 0.80 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.44; LC/MS/MS: retention time: 7.45 min; $[MH]^+$: 314.23.

^{1}H NMR (250 MHz, DMSO) δ 9.96 (br, 1H, 4-phenyl 2-OH), 8.19–8.16 (m, 2H, 6-phenyl H-2, H-6), 7.97 (s, 1H, pyridine H-5), 7.91 (s, 1H, pyridine H-3), 7.87 (s, 1H, 2-furan H-5), 7.55–7.45 (m, 4H, 6-phenyl H-3, H-4, H-5, 4-phenyl H-6), 7.32 (t, J = 8.0 Hz, 1H, 4-phenyl H-4), 7.26 (d, J = 3.0 Hz, 1H, 2-furan H-3), 7.03 (d, J = 8.0 Hz, 1H, 4-phenyl H-3), 6.99 (t, J = 7.4 Hz, 1H, 4-phenyl H-5), 6.69 (dd, J = 3.3, 1.7 Hz, 1H, 2-furan H-4).

^{13}C NMR (62.5 MHz, DMSO) δ 156.17, 155.05, 153.63, 148.46, 148.32, 144.40, 138.85, 130.52, 129.41, 128.99, 127.00, 125.11, 119.95, 119.02, 117.32, 116.59, 112.57, 109.29.

4.3.15. Synthesis of 3-(2,6-diphenylpyridin-4-yl)phenol (39)

The procedure described in Section 4.3 was employed with **4b** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **a**) (0.65 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a light yellow solid (0.49 g, 1.52 mmol).

R_f (ethyl acetate/*n*-hexane 1:3, v/v): 0.35; LC/MS/MS: retention time: 8.26 min; $[MH]^+$: 324.27.

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.21–8.17 (m, 4H, 2-phenyl H-2, H-6, 6-phenyl H-2, H-6), 7.86 (d, J = 1.0 Hz, 2H, pyridine H-3, H-5), 7.55–7.42 (m, 6H, 2-phenyl H-3, H-4, H-5, 6-phenyl H-3, H-4, H-5), 7.36 (t, J = 7.8 Hz, 1H, 4-phenyl H-5), 7.32–7.29 (m, 1H, 4-phenyl H-6), 7.20 (br, 1H, 4-phenyl H-2), 6.95 (ddd, J = 7.8, 2.3, 1.2 Hz, 1H, 4-phenyl H-4), 5.38 (br, 1H, 4-phenyl 3-OH).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 157.51, 156.16, 149.75, 140.71, 139.46, 130.36, 129.07, 128.71, 127.11, 119.63, 117.11, 115.89, 114.08.

4.3.16. Synthesis of 3-(2-phenyl-6-(thiophen-2-yl)pyridin-4-yl)-phenol (40)

The procedure described in Section 4.3 was employed with **4b** (0.33 g, 1.50 mmol), anhydrous ammonium acetate (1.15 g, 15.00 mmol), **5** (R^3 = **b**) (0.49 g, 1.50 mmol), and glacial AcOH (2 mL) to yield a white solid (0.40 g, 1.23 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.42; LC/MS/MS: retention time: 8.12 min; $[MH]^+$: 330.24.

^{1}H NMR (250 MHz, DMSO) δ 9.73 (br, 1H, 4-phenyl 3-OH), 8.25–8.22 (m, 2H, 2-phenyl H-2, H-6), 8.10 (s, 1H, pyridine H-3), 8.05 (d, *J* = 3.7 Hz, 1H, 6-thiophene H-3), 8.02 (s, 1H, pyridine H-5), 7.68 (d, *J* = 5.0 Hz, 1H, 6-thiophene H-5), 7.56–7.46 (m, 3H, 2-phenyl H-3, H-4, H-5), 7.42–7.35 (m, 2H, 4-phenyl H-5, H-6), 7.33 (br, 1H, 4-phenyl H-2), 7.20 (dd, *J* = 5.0, 3.7 Hz, 1H, 6-thiophene H-4), 6.93 (d, *J* = 7.5 Hz, 1H, 4-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO) δ 158.24, 156.43, 152.54, 149.99, 145.07, 139.14, 138.47, 130.40, 129.62, 129.04, 128.90, 128.77, 127.04, 126.13, 118.27, 116.56, 116.46, 115.06, 114.31.

4.3.17. Synthesis of 3-(2-phenyl-6-(thiophen-3-yl)pyridin-4-yl)-phenol (41)

The procedure described in Section 4.3 was employed with **4b** (0.33 g, 1.50 mmol), anhydrous ammonium acetate (1.15 g, 15.00 mmol), **5** (R^3 = **c**) (0.49 g, 1.50 mmol), and glacial AcOH (2 mL) to yield a light yellow solid (0.39 g, 1.18 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.42; LC/MS/MS: retention time: 8.11 min; $[MH]^+$: 330.25.

^{1}H NMR (250 MHz, DMSO) δ 9.72 (br, 1H, 4-phenyl 3-OH), 8.44 (dd, *J* = 2.9, 1.1 Hz, 1H, 6-thiophene H-2), 8.30–8.27 (m, 2H, 2-phenyl H-2, H-6), 8.06 (s, 1H, pyridine H-3), 8.03 (s, 1H, pyridine H-5), 7.99 (d, *J* = 5.0 Hz, 1H, 6-thiophene H-4), 7.69 (dd, *J* = 5.0, 3.0 Hz, 1H, 6-thiophene H-5), 7.55–7.46 (m, 3H, 2-phenyl H-3, H-4, H-5), 7.42–7.34 (m, 2H, 4-phenyl H-5, H-6), 7.33 (br, 1H, 4-phenyl H-2), 6.93 (d, *J* = 7.4 Hz, 1H, 4-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO) δ 158.23, 156.53, 153.39, 149.92, 142.35, 139.38, 138.95, 130.38, 129.46, 128.99, 127.15, 127.01, 124.94, 118.26, 116.79, 116.44, 116.30, 114.30.

4.3.18. Synthesis of 3-(2-(furan-2-yl)-6-phenylpyridin-4-yl)-phenol (42)

The procedure described in Section 4.3 was employed with **4b** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **d**) (0.63 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a white solid (0.48 g, 1.55 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.39; LC/MS/MS: retention time: 7.74 min; $[MH]^+$: 314.24.

^{1}H NMR (250 MHz, DMSO) δ 9.72 (br, 1H, 4-phenyl 3-OH), 8.28–8.24 (m, 2H, 6-phenyl H-2, H-6), 8.04 (d, *J* = 1.3 Hz, 1H, pyridine H-5), 7.90 (dd, *J* = 1.6, 0.7 Hz, 1H, 2-furan H-5), 7.87 (d, *J* = 1.3 Hz, 1H, pyridine H-3), 7.56–7.47 (m, 3H, 6-phenyl H-3, H-4, H-5), 7.37–7.34 (m, 2H, 4-phenyl H-5, H-6), 7.33 (dd, *J* = 3.4, 0.6 Hz, 1H, 2-furan H-3), 7.29 (br, 1H, 4-phenyl H-2), 6.93 (m, 1H, 4-phenyl H-4), 6.71 (dd, *J* = 3.4, 1.7 Hz, 1H, 2-furan H-4).

^{13}C NMR (62.5 MHz, DMSO) δ 158.27, 156.92, 153.40, 149.75, 149.26, 144.61, 139.04, 138.60, 130.51, 129.60, 128.97, 127.17, 118.09, 116.62, 116.51, 114.56, 114.03, 112.64, 109.76.

4.3.19. Synthesis of 3-(6-phenyl-2,2′-bipyridin-4-yl)phenol (43)

The procedure described in Section 4.3 was employed with **4b** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **e**) (0.65 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a white solid (0.26 g, 0.80 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.39; LC/MS/MS: retention time: 7.66 min; $[MH]^+$: 325.30.

^{1}H NMR (250 MHz, DMSO) δ 9.75 (br, 1H, 4-phenyl 3-OH), 8.74 (d, *J* = 4.7 Hz, 1H, 2-pyridine H-6′), 8.63 (d, *J* = 8.0, 1H, 2-pyridine H-3′), 8.56 (s, 1H, pyridine H-3), 8.35–8.32 (m, 2H, 6-phenyl H-2, H-6), 8.22 (s, 1H, pyridine H-5), 8.04 (dt, *J* = 7.7, 1.5 Hz, 1H, 2-pyridine H-4′), 7.58–7.48 (m, 4H, 6-phenyl H-3, H-4, H-5, 2-pyridine H-5′), 7.41–7.36 (m, 2H, 4-phenyl H-5, H-6), 7.32 (br, 1H, 4-phenyl H-2), 6.93 (d, *J* = 6.7 Hz, 1H, 4-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO) δ 158.34, 156.68, 155.85, 155.39, 149.86, 149.59, 139.18, 138.74, 137.73, 130.63, 129.62, 129.05, 127.23, 124.72, 121.09, 118.23, 118.05, 116.65, 113.92.

4.3.20. Synthesis of 3-(6-phenyl-2,3′-bipyridin-4-yl)phenol (44)

The procedure described in Section 4.3 was employed with **4b** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **f**) (0.65 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a white solid (0.37 g, 1.14 mmol).

R_f (ethyl acetate/*n*-hexane 1:1, v/v): 0.38; LC/MS/MS: retention time: 6.95 min; $[MH]^+$: 325.29.

^{1}H NMR (250 MHz, DMSO) δ 9.74 (br, 1H, 4-phenyl 3-OH), 9.48 (s, 1H, 2-pyridine H-2′), 8.68–8.65 (m, 2H, 2-pyridine H-6′, H-4′), 8.33–8.30 (m, 2H, 6-phenyl H-2, H-6), 8.22 (s, 1H, pyridine H-3), 8.16 (s, 1H, pyridine H-5), 7.58–7.51 (m, 3H, 6-phenyl H-3, H-4, H-5), 7.47 (t, *J* = 8.3 Hz, 2H, 2-pyridine H-5′, 4-phenyl H-5), 7.38–7.32 (m, 2H, 4-phenyl H-2, H-6), 6.94 (d, *J* = 8.0 Hz, 1H, 4-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO) δ 158.25, 156.98, 154.50, 150.25, 148.46, 139.17, 138.78, 134.64, 134.44, 130.39, 129.62, 129.04, 127.24, 124.04, 118.39, 117.39, 117.28, 116.57, 114.42.

4.3.21. Synthesis of 3-(6-phenyl-2,4′-bipyridin-4-yl)phenol (45)

The procedure described in Section 4.3 was employed with **4b** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **g**) (0.65 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a white solid (0.31, 0.96 mmol).

R_f (ethyl acetate/*n*-hexane 1:1, v/v): 0.38; LC/MS/MS: retention time: 6.94 min; $[MH]^+$: 325.30.

^{1}H NMR (250 MHz, DMSO) δ 9.75 (br, 1H, 4-phenyl 3-OH), 8.75 (d, *J* = 5.0 Hz, 2H, 2-pyridine H-2′, H-6′), 8.34–8.28 (m, 4H, 2-pyridine H-3′, H-5′, 6-phenyl H-2, H-6), 8.28 (s, 1H, pyridine H-3), 8.22 (s, 1H, pyridine H-5), 7.57–7.48 (m, 3H, 6-phenyl H-3, H-4, H-5), 7.44–7.33 (m, 3H, 4-phenyl H-2, H-5, H-6), 6.94 (d, *J* = 6.9 Hz, 1H, 4-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO) δ 158.27, 157.06, 154.15, 150.59, 150.41, 145.91, 139.02, 138.64, 130.42, 129.73, 129.07, 127.28, 121.39, 118.42, 117.69, 116.65, 114.45.

4.3.22. Synthesis of 4-(2-(furan-2-yl)-6-phenylpyridin-4-yl)-phenol (49)

The procedure described in Section 4.3 was employed with **4c** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g, 20.00 mmol), **5** (R^3 = **d**) (0.63 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a light orange solid (0.50 g, 0.96 mmol).

R_f (ethyl acetate/*n*-hexane 1:2, v/v): 0.42; LC/MS/MS: retention time: 7.44 min; $[MH]^+$: 314.24.

^{1}H NMR (250 MHz, DMSO) δ 9.90 (br, 1H, 4-phenyl 4-OH), 8.26–8.24 (m, 2H, 6-phenyl H-2, H-6), 8.04 (s, 1H, pyridine H-5), 7.88 (s, 2H, 2-furan H-5, pyridine H-3), 7.86 (d, *J* = 8.6 Hz, 2H, 4-phenyl H-2, H-6), 7.55–7.43 (m, 3H, 6-phenyl H-3, H-4, H-5), 7.30 (d, *J* = 3.2 Hz, 1H, 2-furan H-3), 6.94 (d, *J* = 8.6 Hz, 2H, 4-phenyl H-3, H-5), 6.70 (dd, *J* = 3.2, 1.7 Hz, 1H, 2-furan H-4).

^{13}C NMR (62.5 MHz, DMSO) δ 159.14, 156.78, 153.58, 149.33, 149.18, 144.41, 138.78, 129.45, 128.89, 128.69, 127.97, 127.12, 116.15, 115.61, 113.68, 112.56, 109.51.

4.3.23. Synthesis of 4-(6-phenyl-2,4′-bipyridin-4-yl)phenol (52)

The procedure described in Section 4.3 was employed with **4c** (0.44 g, 2.00 mmol), anhydrous ammonium acetate (1.54 g,

20.00 mmol), **5** (R^3 = **g**) (0.65 g, 2.00 mmol), and glacial AcOH (2 mL) to yield a creamy white solid (0.40 g, 1.25 mmol).

R_f (ethyl acetate/*n*-hexane 1:1, v/v): 0.22; LC/MS/MS: retention time: 6.66 min; $[MH]^+$: 325.29.

^{1}H NMR (250 MHz, DMSO) δ 9.92 (br, 1H, 4-phenyl 4-OH), 8.75 (dd, *J* = 4.7, 1.4 Hz, 2H, 2-pyridine H-2′, H-6′), 8.34–8.27 (m, 4H, 6-phenyl H-2, H-6, 2-pyridine H-3′, H-5′), 8.27 (s, 1H, pyridine H-3), 8.23 (s, 1H, pyridine H-5), 7.96 (d, *J* = 8.6 Hz, 2H, 4-phenyl H-2, H-6), 7.57–7.48 (m, 3H, 6-phenyl H-3, H-4, H-5), 6.95 (d, *J* = 8.6 Hz, 2H, 4-phenyl H-3, H-5).

^{13}C NMR (62.5 MHz, DMSO) δ 159.24, 156.96, 154.02, 150.53, 149.93, 146.09, 138.81, 129.59, 129.01, 127.90, 127.22, 121.34, 117.38, 116.67, 116.10.

4.4. Pharmacology

4.4.1. Assay for DNA topoisomerase I inhibition in vitro

DNA topo I inhibition assay was determined following the method reported by Fukuda et al.[29] with minor modifications. The test compounds were dissolved in DMSO at 20 mM as stock solution. The activity of DNA topo I was determined by assessing the relaxation of supercoiled DNA pBR322. The mixture of 100 ng of plasmid pBR322 DNA and 0.4 units of recombinant human DNA topoisomerase I (TopoGEN INC., USA) was incubated without and with the prepared compounds at 37 °C for 30 min in the relaxation buffer (10 mM Tris–HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin, 1 mM spermidine, 5% glycerol). The reaction in the final volume of 10 μL was terminated by adding 2.5 μL of the stop solution containing 5% sarcosyl, 0.0025% bromophenol blue, and 25% 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 μg/mL). DNA bands were visualized by transillumination with UV light and were quantitated using AlphaImager™ (Alpha Innotech Corporation).

4.4.2. Assay for DNA topoisomerase II inhibition in vitro

DNA topo II inhibitory activity of compounds were measured as follows.[30] The mixture of 200 ng of supercoiled pBR322 plasmid DNA and 1 unit of human DNA topoisomerase IIα (Usb Corp., 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 μg/mL bovine serum albumin) for 30 min at 30 °C. The reaction in a final volume of 20 μL was terminated by the addition of 3 μL 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 μg/mL). DNA bands were visualized by transillumination with UV light and supercoiled DNA was quantitated using AlphaImager™ (Alpha Innotech Corporation).

4.4.3. Cytotoxicity assay

Five different cancer cell lines were used: MDA-MB231 (human breast adenocarcinoma cell line), HeLa (human cervix tumor cell line), DU145 (human prostate tumor cell line), HCT15 (human colorectal adenocarcinoma cell line), and HL60 (human myeloid leukemic tumor cell line), and assay was carried out according to previous method.[29] Cancer cells were cultured according to the supplier's instructions. Cells were seeded in 96-well plates at a density of 2–4 × 10^4 cells per well and incubated overnight in 0.1 mL of media supplied with 10% Fetal Bovine Serum (Hyclone, USA) in 5% CO_2 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 μL 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 IC_{50} 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.

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