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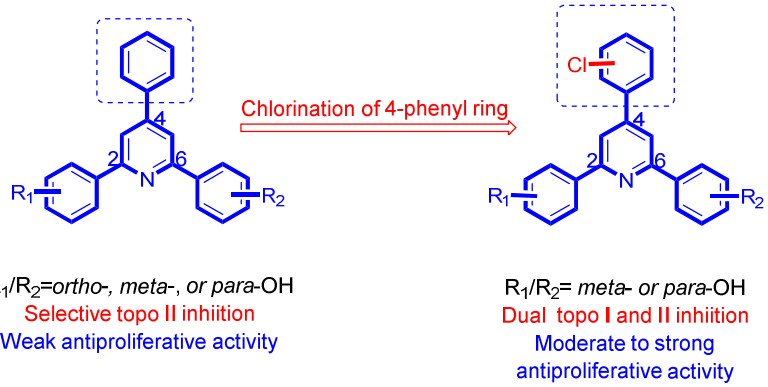
Graphical Abstract



R_1/R_2=*ortho-*, *meta-*, *or para*-OH
Selective topo II inhiition
Weak antiproliferative activity

R_1/R_2= *meta-* or *para*-OH
Dual topo I and II inhiition
Moderate to strong
antiproliferative activity

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Abstract

With the aim to develop novel antiproliferative agents, a new series of eighteen dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines were systematically designed, prepared, and investigated for their topoisomerase (topo) I and IIα inhibitory properties and antiproliferative effect in three different human cancer cell lines (HCT15, T47D, and HeLa). Compounds **22-30** which possess a *meta*- or *para*-phenol on 2-, or 6-position of central pyridine ring showed significant dual topo I and topo IIα inhibitory activities with strong antiproliferative activities against all the tested human cancer cell lines. However, compounds **13-21** which possess an *ortho*-phenol on 2-, or 6-position of central pyridine ring did not show significant topo I and topo IIα inhibitory activities but displayed moderate antiproliferative activities against all the tested human cancer cell lines. Compound **23** exhibited the highest antiproliferative potency as much as 348.5 and 105 times compared to etoposide and camptothecin, respectively, in T47D cancer cell line. The structure-activity relationship study revealed that the *para* position of a hydroxyl group at 2-and 6-phenyl ring and chlorine atom at the *para* position of 4-phenyl ring of the central pyridine exhibited the most significant topo I and topo IIα inhibition, which might indicate introduction of the

chlorine atom at the phenyl ring of 4-pyridine have an important role as dual inhibitors of topo I and topo IIα. Compound **30** which showed the most potent dual topo I and topo IIα inhibition with strong antiproliferative activity in T47D cell line was selected to perform further study on the mechanism of action, which revealed that compound **30** functions as a potent DNA non-intercalative catalytic topo I and IIα dual inhibitor.

Keywords: Antiproliferative agents; Dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines; Dual inhibitors of topo I and topo IIα; Antiproliferative activity; DNA non-intercalative catalytic inhibitor

1. Introduction

Although there has been a significant amount of development in the anticancer therapy, DNA still remains to be the most important target for the cancer treatment [1]. Antiproliferative compounds show their activity by interacting directly with DNA either by forming covalent complexes or by damaging the function of DNA-processing enzymes, thereby initiating the cell cycle arrest and apoptosis. Topoisomerase (topo) inhibitors have a significant part in impairing the function of DNA processing enzymes [2]. Topos solve the topological problems of the DNA double helix that occur during the metabolic processes of transcription, replication, recombination, repair, and chromatin assembly [3, 4]. Therefore, topos have become a popular target in the antiproliferative therapy because of their greater over-expression in the tumor cells [5].

Based on the molecular mechanism of cleavage, topos are categorized as topo I and topo II [6]. Topo I breaks a single-strand of DNA double helix by the trans-esterification reactions and re-ligates DNA strands during the cell cycle process to maintain genomic integrity [7-9]. Topo II works by making a double-strand break in an ATP-dependent reaction. It is mainly involved in the DNA complex processes such as recombination, splitting of daughter chromosomes, and condensation of chromosomes [10]. The topo-directed agents have different mechanism of actions: Topo poisons stabilize the cleavage complex of DNA and topo to restrict DNA breaking-rejoining mechanism of the enzyme, whereas catalytic inhibitors impair either one or more steps within the catalytic cycle of topo [10-12].

Recently, the camptothecin analogs, the target of which is topo I, have been widely used for the treatment of colon cancer [13], whereas drugs such as etoposide, teniposide, doxorubicin, and mitoxantrone which specifically target topo II are usually used for the treatment of various malignancies [14]. However, these topo inhibitors have several problems such as dose limiting side effects, chemical instability, and drug-efflux mediated resistance [15, 16]. Due to these limitations, there is a continuous need for the progressive development and innovation of novel topo I as well as topo II inhibitors. Topo I and topo II have shown to be good targets, so dual inhibition of topo I and topo II can synergistically enhance their antiproliferative property as well as reduce drug resistance. Therefore, development of a sole unique compound which can mutually obstruct topo I and topo II at the same time, will have the combinational advantages of the individual inhibitor and thus can result in an increased antiproliferative property [4]. Recently, dual topo I and topo II inhibitors have received a lot of interest in the development of anticancer drugs because of their greater antiproliferative effects and lower side effects [4, 17, 18]. Therefore, with the aim to search for and develop potent dual topoisomerase inhibitors, our research group has previously synthesized a series of 2,4,6-triaryl pyridine derivatives and discovered that several compounds exhibited strong dual inhibitory activities of topo I and II with significant antiproliferative activities against different types of human cancer cell lines [19-23]. Likewise, various drugs and compounds such as acridine DACA [24], indenoquinolinone TAS-103 [25], benzopyridoindole intoplicine [26], tafluposide (F-11782) [27], and pyrazoloacridine NSC 366140 [28] also have been reported as topo I and II dual inhibitors [17, 20].

From various studies, it has been found that most phenolic and polyphenolic derivatives show antioxidant [29], anti-inflammatory [30], anti-angiogenic, and antiproliferative properties [31,

32]. Most bioflavonoids show antiproliferative property because of their function as topo II poison [33-35]. Epigallocatechin gallate, a polyphenolic compound, which is isolated from green tea is one of the potent topo II poisons [35]. Camptothecin, a very popular topo I inhibitor which exhibits a potent antiproliferative property and also possesses a hydroxyl group in its structure [36, 37]. Additionally, as reported from previous studies in our research group, mono and dihydroxylated 2,4,6-triarylpyridines justified the necessity of the hydroxyl group in order to show their greater topo inhibition and antiproliferative properties. Structure-activity relationship (SAR) studies demonstrated that the inhibition of both topo I and II and antiproliferative properties can be enhanced either by the replacement or by the addition of hydroxyl group on the phenyl ring [38, 39]. Moreover, selective topo II inhibition and antiproliferative properties were observed in the compounds that had hydroxyl moiety at the *meta* or *para* position on 2-phenyl ring of the central pyridine [39]. Additionally, a bulky group like halogen atoms tends to occupy most of the deeper pockets as well as the reacting regions of the molecular targets thereby inducing either antagonistic or agonistic activity. In recent years, much research has been performed by introducing chlorine atom into structures because of its capacity to enhance potency and bioavailability, to increase brain blood barrier penetrability, and also to enhance chemical and metabolic stability through increasing lipophilicity and ~~to~~ acting as a hydrogen bond acceptor [40]. Further studies of terpyridine derivatives with the chlorine substituents have displayed stronger topo I and II inhibitory properties and antiproliferative activities than those with the non-substituted ones [21, 41]. From these various reports exhibiting the importance of the phenolic group together with the introduction of the chlorine atom has inspired us to integrate these functional groups into 2,4,6-triaryl pyridine compounds to synthesize dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines as shown in Figure 1.

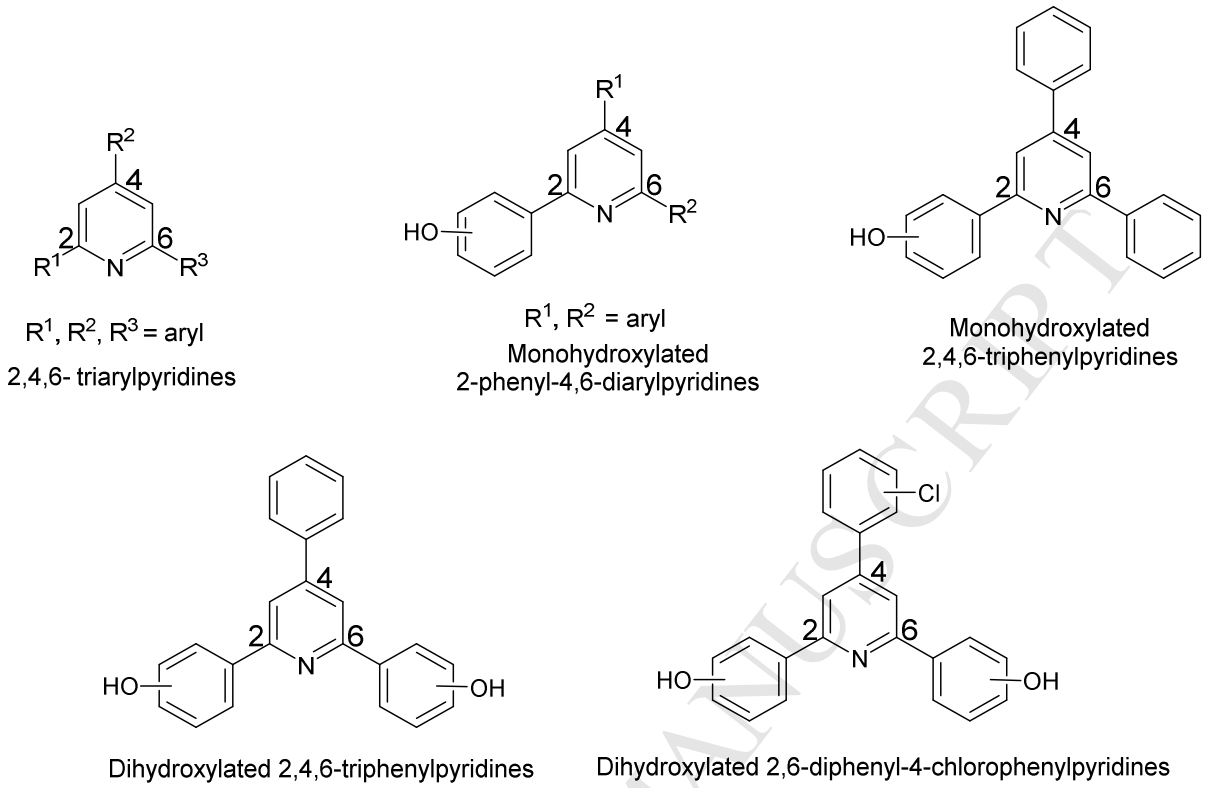


Figure 1. Structures of 2,4,6-triarylpyridines, monohydroxylated 2-phenyl-4,6-diarylpyridines, mono- and dihydroxylated 2,4,6-triphenylpyridines, and dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines

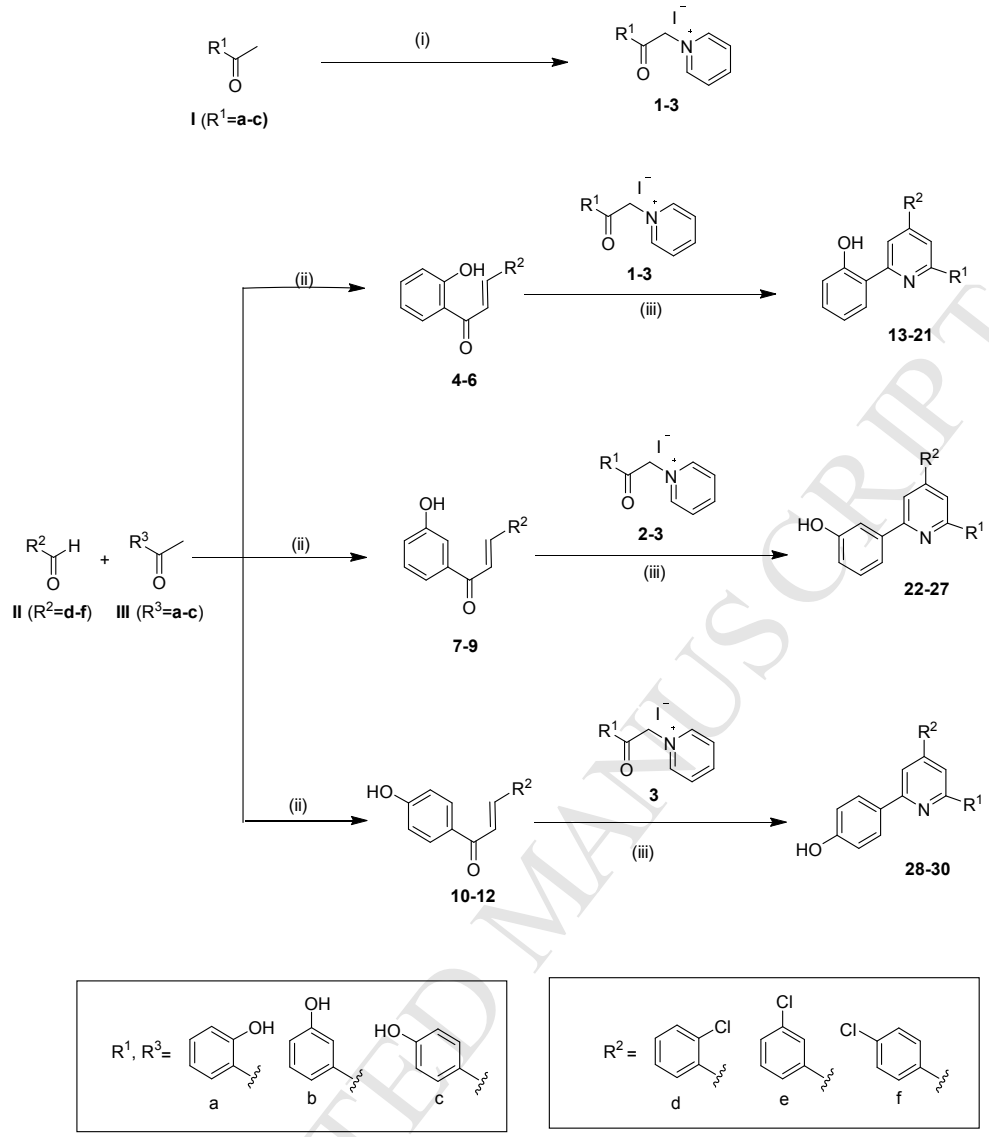
In this study, we have systematically designed and prepared eighteen dihydroxylated 2,6-diphenyl-4-chlorophenylpyridine derivatives. A hydroxyl group at 2- and 6-phenyl ring together with the chlorine atom at 4-phenyl ring attached to the central pyridine was prepared for investigating their topo I and IIα inhibitory property and antiproliferative activity against three different types of human cancer cell lines. Subsequently, *ortho*, *meta*, and *para*

substitution of the hydroxyl and chlorine atom would give us an evidence to further verify the effect of these positions on topo inhibitory activity and antiproliferative activity.

2. Results and discussion

2.1. Synthetic Chemistry

According to the method reported by W. Zecher and F. Kröhnke in 1961, dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines were prepared [42]. Synthesis of compounds involved three basic methods as outlined in scheme 1. At first, aryl methyl ketone **I** (R^1=**a-c**) was refluxed in pyridine in the presence of iodine for 3 h at 140 ^oC to synthesize aryl pyridinium iodide salts **1-3** to yield 65.4-99.5% by using Ortoleva-King reaction [43]. In parallel, aryl aldehyde **II** (R^2=**d-f**) and aryl methyl ketone **III** (R^3=**a-c**) were reacted in methanol in the presence of NaOH to synthesize propenone intermediates **4-12** to yield 57.2-83.1% by using Claisen-Schmidt condensation reaction [44, 45]. Finally, aryl pyridinium iodide salts **1-3** and propenone intermediates **4-12** were refluxed either in glacial acetic acid or methanol for 2-36 h at 90-100 ^oC in the presence of ammonium acetate (NH_4OAc) to give dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines (**13-30**) in 11.4-63.8% yield by using modified Kröhnke pyridine synthesis**.** The compounds **13-30** were prepared in nine separate series as shown in Figure 2. Structure of previously synthesized dihydroxylated 2,4,6-triphenylpyridines (**A-F)** which were reported earlier [39] and newly synthesized dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines (**13-30**) are shown in Figure 3, and their physical properties are shown in Table S1 (Supplementary Data).



Scheme 1. Schematic representation for synthesis of pyridinium iodide salts **1-3**, propenone intermediates **4-12** and 2,6-diphenyl-6-chlorophenylpyridines **13-30**. Reagents and conditions: (i) Iodine (1.0 equivalent), pyridine, 3 h, 140 ^{o}C, 90.1- 99.8% yield, (ii) aq. NaOH (3.0 equivalent), MeOH, 1-3 h, room temperature, 90.1- 99.8% yield, (iii) NH_4OAc (10.0 equivalent), glacial acetic acid or methanol, 12-36 h, 95-100 ^{o}C, 22.3-67.5% yield.

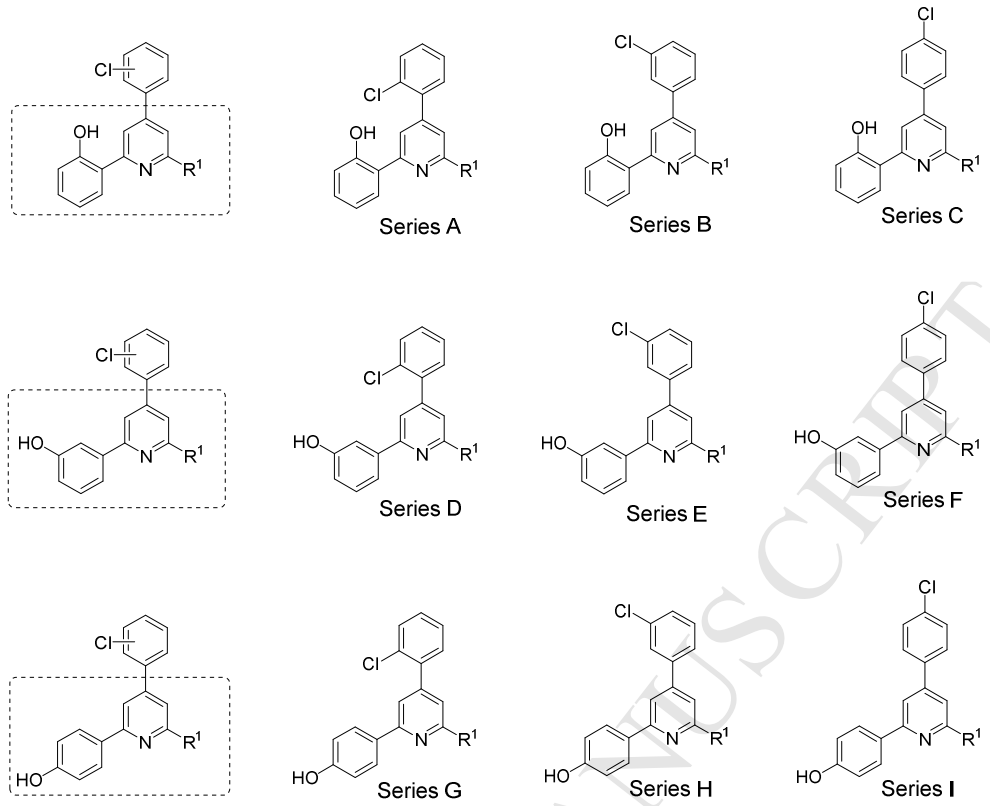
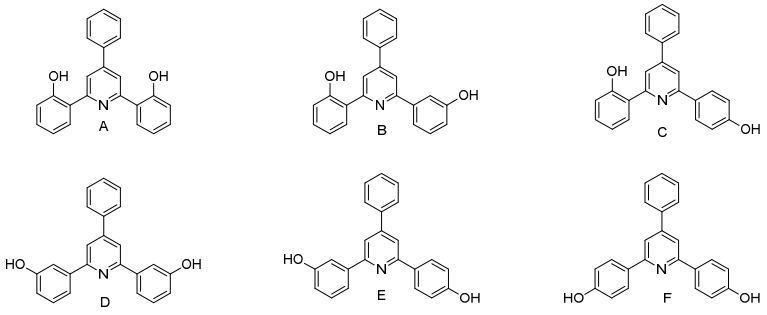
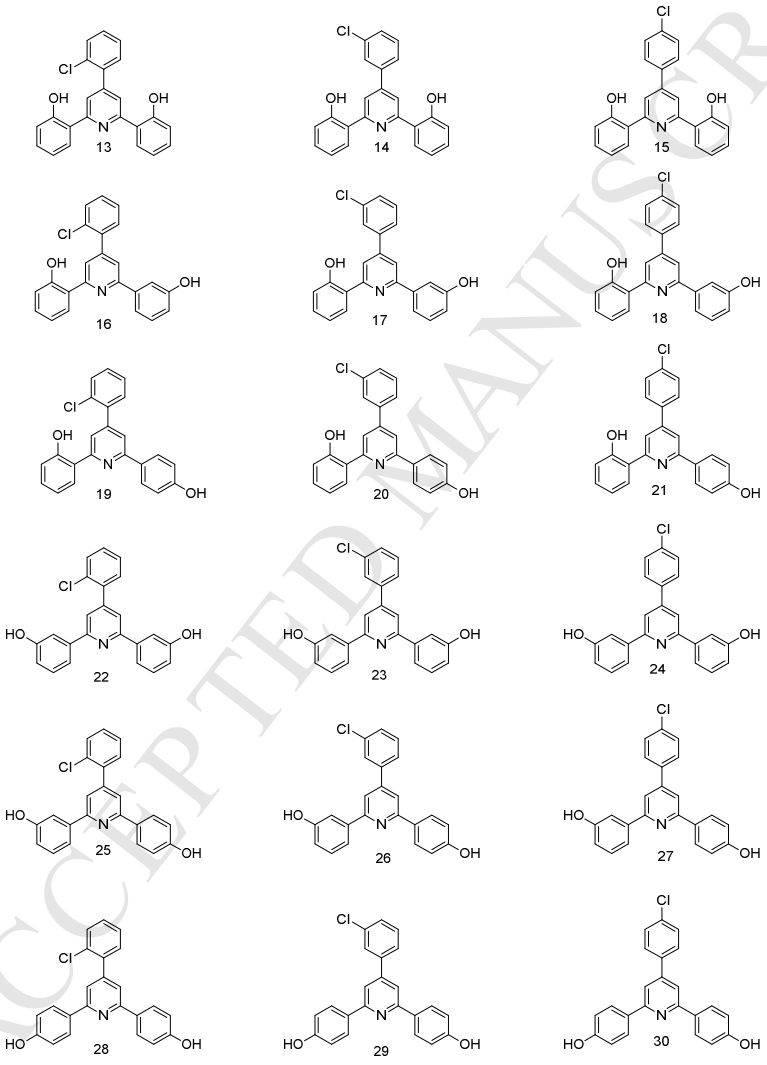


Figure 2. Strategic design of dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines



a) Previously synthesized dihydroxylated 2,4,6-triphenylpyridines



b) Newly synthesized dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines

Figure 3. Structures of a) previously synthesized dihydroxylated 2,4,6-triphenylpyridines, and b) newly synthesized dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines

2. 2. Topo I and IIα inhibitory property

The inhibition on transformation of supercoiled pBR322 DNA to the relaxed form was examined and analyzed in the presence of the prepared dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines (**13-30**) to measure their inhibitory activity of topo I and IIα. Camptothecin and etoposide, selective topo I and II inhibitors, respectively, were used as positive controls. The inhibitory properties were measured at 100 µM and 20 µM concentration.

Figure 4 and Table 1 illustrates the topo I inhibitory property of prepared compounds **13-30**. Among the synthesized compounds, compounds **18**, **23**, **24**, **26-30** and compounds **18**, **19**, **24**, **25**, **27**, **30** have shown significant topo I inhibitory property at 100 µM and 20 µM, respectively, which were stronger or comparable to the positive control, camptothecin. Compounds **27** and **30** showed the most potent topo I inhibitory property compared to camptothecin at both 100 µM and 20 µM in which compound **27** displayed 70.6% and 28.2% inhibition, and compound **30** displayed 82.7% and 23.7% inhibition, while camptothecin displayed 64.4% and 21.6% inhibition at 100 µM and 20 µM, respectively. Compounds **19**, **22**, and **23** exhibited moderate topo I inhibition (35.0%, 44.2%, and 42.3%, respectively) at 100 µM. Similarly, compound **24** exhibited moderate topo I inhibition (13.2%) at 20 µM.

On the other hand, as shown in Figure 5 and Table 1, compounds **22-30** except **28** (45.7% inhibition) and compounds **19, 21-30** except **22** and **23** (19.3% and 15.5% inhibition) exhibited significant inhibition of topo IIα at the concentration of 100 µM and 20 µM, respectively, which were stronger or comparable to the positive control, etoposide. Among them, compound **30** showed the strongest inhibition of topo IIα at both 100 µM and 20 µM

(100% and 57%, respectively) whereas the positive control etoposide displayed 67.2% and 22.8% inhibition, respectively, at 100 µM and 20 µM. Similarly, compounds **22**, **25**, **27**, and **29** displayed 71.6%, 68.5%, 69.2 %, and 82.1% inhibition, respectively, at 100 µM whereas compounds **24-29** displayed 31.0%, 30.4%, 55.0%, 49.0%, 44.5%, and 45.5%, respectively, at 20 µM.

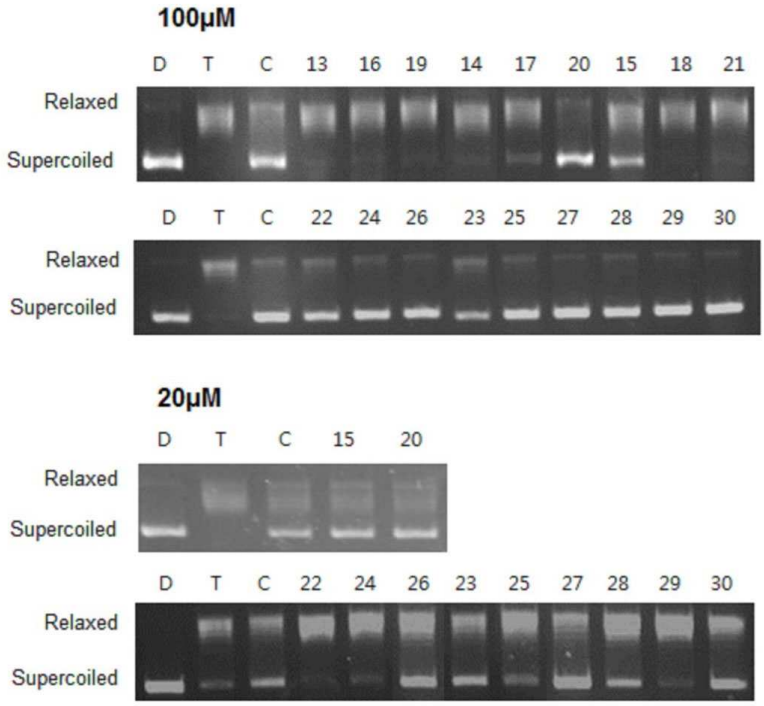


Figure 4. Human DNA topo I inhibitory activity of prepared compounds **13-30**. (A) Lane D: pBR322 only. (B) Lane T: pBR322 + Topo I. (C) Lane C: pBR322 + Topo I + Camptothecin. (D) Lane 13-30(100 µM): pBR322 + Topo I + compounds **13-30**. (E) Lane 18, 19, 22-30 (20 µM): pBR322 + Topo I + compounds **18, 19, 22-30**.

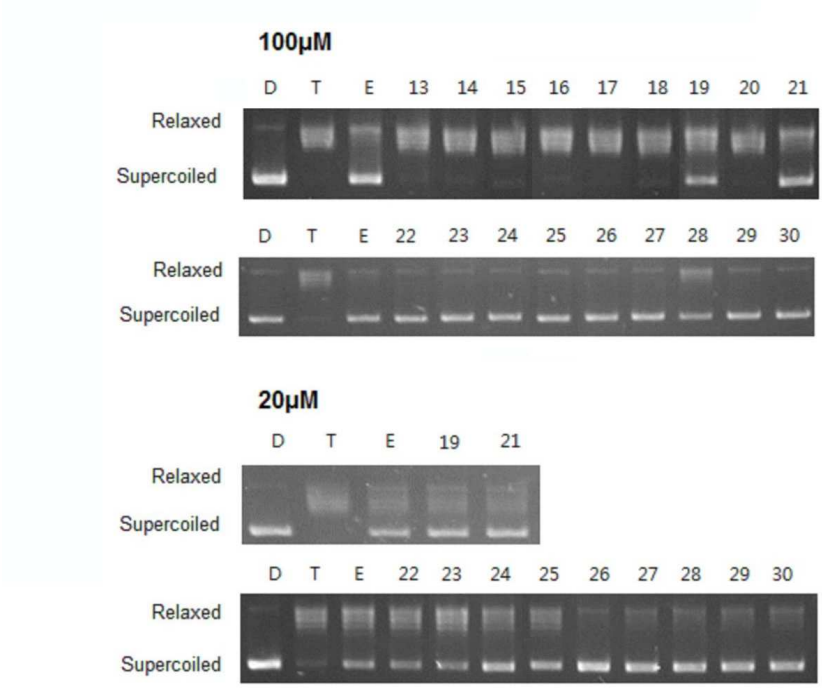


Figure 5. Human DNA topo IIα inhibitory activity of compounds **13-30**. (A) Lane D: pBR322 only. (B) Lane T: pBR322 + Topo IIα. (C) Lane C: pBR322 + Topo IIα + Etoposide (D) Lane 13-30 (100 μM): pBR322 + Topo IIα + compounds **13-30**. (E) Lane 19, 21-30 (20 μM): pBR322 + Topo IIα + compounds **19, 21-30**.

Table 1. Topoisomerase I and II inhibitory properties and antiproliferative activities of compounds **13-30**

Compounds	Topo I (% Inhibition)		Topo II (% Inhibition)		IC_{50} ** (μM)		
	100 μM	20 μM	100 μM	20 μM	HCT15	T47D	HeLa
Adriamycin					1.82±0.02/ 1.28±0.07[d]	0.25±0.02/ 3.69 ± 0.13[d]	1.20±0.03/ 1.45 ± 0.01[d]
Etoposide			69.7	32.7/23.5[a]	5.29±0.28/ 1.33±0.10[d]	6.97±0.05/ 3.25 ± 0.04[d]	9.10±0.20/ 3.20 ± 0.57[d]
Camptothecin	63.0	42.1[b]/21.6[c]			11.96±0.81/ 0.26±0.04[d]	2.10±0.01/ 3.91 ± 0.66[d]	0.06±0.02/ 1.01 ± 0.01[d]
A	0	NT	42.7	12.1	>50	37.3 ± 0.34	14.8 ± 0.05
B	11.0	NT	92.2	52.1	39.90 ± 1.49	>50	>50
C	0	NT	100.0	82.3	7.50 ± 1.13	1.40 ± 0.06	3.90 ± 0.44
D	67.2	NT	86.5	49.0	18.30 ± 0.61	12.70 ± 1.30	13.0 ± 0.08
E	0	NT	96.8	31.0	30.52 ± 0.66	4.99 ± 0.78	3.46 ± 0.15
F	0.3	NT	76.8	33.7	3.00 ± 0.50	6.70 ± 0.78	3.30 ± 0.44
13	7.2	NT	3.0	NT	3.88±0.03	1.22±0.02	3.53±0.01
14	5.0	NT	NA	NT	>50	>50	>50
15	0.8	NT	NA	NT	>50	>50	>50
16	2.2	NT	2.5	NT	4.19±0.12	1.62±0.03	5.24±0.01
17	9.0	NT	2.1	NT	2.21±0.07	0.83±0.01	5.51±0.07
18	70.0	41.0	NA	NT	4.70±0.05	1.01±0.01	8.17±0.06
19	35.0	41.6	34.8	32.7	5.56±0.36	10.81±0.98	18.82±0.13

20	NA	NT	NA	NT	1.67±0.13	>50	>50
21	8.6	NT	38.5	30.9	>50	>50	>50
22	44.2	0	71.6	19.3	3.50±0.02	0.07±0.01	6.34±0.05
23	61.4	0	63.7	15.5	3.44±0.13	0.02±0.01	2.63±0.08
24	68.2	18.4	65.2	31.0	5.97±0.09	0.15±0.01	5.47±0.01
25	42.3	18.3	68.5	30.4	3.80±0.19	0.79±0.01	2.47±0.03
26	62.3	1.5	63.6	55.0	4.27±0.05	0.07±0.01	0.07±0.01
27	70.6	28.2	69.2	49.0	5.35±0.04	0.84±0.01	0.58±0.13
28	66.9	13.2	45.7	44.5	5.08±0.07	0.78±0.22	0.69±0.01
29	79.1	0	82.1	45.5	4.18±0.02	1.65±0.01	1.51±0.01
30	82.7	23.7	100.0	57.0	4.89±0.04	0.93±0.01	5.06±0.01

[**] Each data represents mean ± S.D. from three different experiments performed in triplicate.
NT: Not Tested, NA: Not Active, [a]Control value for compounds **B**, **C**, **E** and **22-30**; [b]Control value for compounds **13-21**
[c]Control value for compounds **22-30**; [d]Control value for compounds **A-F** against HCT15, T47D and HeLa cell lines
HCT15: human colorectal adenocarcinoma; T47D: human breast ductal carcinoma; HeLa: human cervix adenocarcinoma; Adriamycin: positive control for antiproliferative activity; Etoposide: positive control for topo II and antiproliferative activity; Camptothecin: positive control for topo I and antiproliferative activity

2. 3. Antiproliferative activity

Antiproliferative activities of all the prepared compounds were examined against three human cancer cell lines; human breast ductal carcinoma cell line (T47D), human colorectal adenocarcinoma cell line (HCT15), and human cervix adenocarcinoma cell line (HeLa). Camptothecin, etoposide, and adriamycin were used as positive controls. The inhibitory properties (IC_{50} values) of the synthesized dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines (**13-30**) against three different human cancer cell lines are shown in Table 1. Compared to positive controls, many of the prepared compounds exhibited strong antiproliferative properties at low micromolar ranges against all the tested human cancer cell lines. Especially, compounds **22-30** displayed submicromolar antiproliferative property in T47D cell line. Compounds possessing stronger or comparable topo I and/or topo II inhibitory properties also showed significant antiproliferative activities against all the tested human cancer cell lines. Particularly, compounds **22-24**, and **26** displayed stronger antiproliferative activities against T47D cell line as compared to all three positive controls whereas compounds **13**, **16-18**, **25**, and **27-30** displayed stronger antiproliferative activities compared to the two positive controls, etoposide and camptothecin against T47D cell line. In HCT15 cell line, only compound **20** displayed strong antiproliferative activity compared to all the three positive controls whereas compounds **13**, **16-18**, **22**, **23**, **25**, **26**, and **28-30** showed strong antiproliferative activity compared to two positive controls, camptothecin and etoposide. Likewise, in HeLa cell line, compounds **26-29** showed stronger antiproliferative activity compared to two positive controls, adriamycin and etoposide whereas compounds **13**, **16-18**, **22-25**, **29**, and **30** showed stronger antiproliferative activity against single positive control, etoposide. Compound **30** exhibiting the highest topo I and topo IIα inhibitory activity and stronger antiproliferative activity in HCT15 and T47D cancer cell lines compared to

camptothecin and etoposide was selected for further study to determine the mode of action.

2.4. Compound 30 functions as a DNA non-intercalative topo IIα catalytic inhibitor

Compound **30** which displayed the most strong topo IIα inhibitory property at both 100 µM and 20 µM was selected to determine the mode of action. Several experiments were accomplished such as cleavage complex stabilization assay, band depletion assay, and EtBr displacement assay for investigating the mode of action of compound **30**. In cleavage complex stabilization assay, etoposide, a popular topo II poison, produced the linear DNA, while compound **30** did not produce any linear DNA up to 500 µM as shown in Figure 6A, which indicated compound **30** did not act as a topo IIα poison. Similarly, in band depletion assay, etoposide treatment depleted the free topo IIα due to its stabilizing action on the covalent topo IIα-DNA complex whereas compound **30** treatment showed an intense band of free topo IIα as shown in Figure 6B. These results from cleavage complex stabilization and band depletion assay propose that compound **30** acts as a topo IIα catalytic inhibitor. Further, fluorescence titrimetric method of EtBr-ctDNA complex was performed to check the DNA intercalative activity of compound **30**. The EtBr-ctDNA complex emission intensity was recorded from 500-700 nm with excitation at 471 nm. As displayed in Figure 6C, the intense fluorescence emission of EtBr was detected because of intercalation of EtBr into the base pairs of DNA. Addition of m-AMSA, known as DNA intercalative topo II poison, caused a progressive reduction in the fluorescence emission intensity of EtBr as increased the concentration of m-AMSA. It reflected that the intercalated EtBr was displaced with m-AMSA and then the free EtBr could not generate the fluorescence emission as much as the bound EtBr. However, the addition of compound **30** did not affect the fluorescence intensity

of EtBr-ctDNA complex. This result suggests that the compound **30** acts as a DNA non-intercalator.

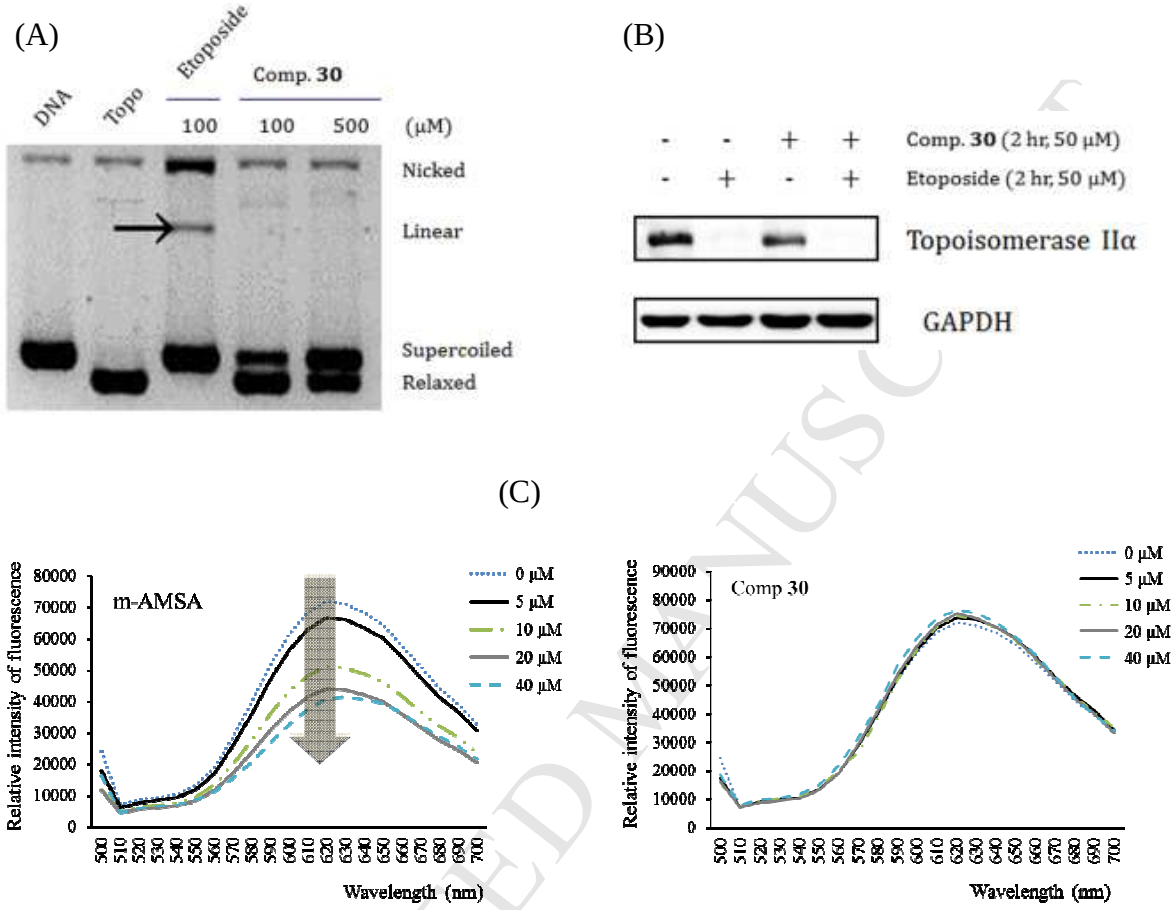


Figure 6. DNA non-intercalative topo IIα catalytic activity of compound **30**. (A) Cleavage complex assay. (B) Band depletion assay in T47D cells. (C) EtBr displacement assay.

2.5. Compound 30 induced apoptosis with very low DNA damage in T47D cells

Since compound **30** functioned as a topo IIα catalytic inhibitor, the degree of the induction of DNA damage by compound **30** was further analyzed. In general, topo II poison such as etoposide produces serious DNA damage by stabilizing the cleavage complex, resulting in

blocking the re-ligation of DNA double strand breaks [46]. Comet assay was used to examine whether or not compound **30** and etoposide caused the aggregation of broken DNA strand in T47D cancer cells. Comet assay is a simple but an effective way for evaluating the DNA toxicity caused by the buildup of broken DNA strands in cells. The greater the percentile of comet (tail) formation, more is the breakage of DNA strand [47]. Figure 7 indicated that compound **30** (11.6 ± 2.4% at 5 µM and 12.5 ± 4.2% at 10 µM) did not induce comet tail as compared to etoposide (62.7 ± 9.2% at 5 µM and 66.3 ± 6.9% at 10 µM). In other words, compound **30** caused less DNA toxicity when compared to etoposide in T47D cells.

In other to determine whether the T47D cell death by compound **30** was due to apoptosis, we further examined the expression levels of proteins involved in apoptosis, such as poly ADP-ribose polymerase (PARP), Bax (pro-apoptotic protein), and Bcl-2 (anti-apoptotic protein), in T47D cells through western blot analysis. As shown in Figure 8, the amount of cleaved PARP-is directly proportional to the concentration of treated compound **30**. In addition, the expression level of Bcl-2 decreased and that of Bax increased by the increase of compound **30** treatment amount. Increase of PARP cleavage, reduction of anti-apoptotic Bcl-2, and increase of pro-apoptotic Bax by compound **30** treatment were reflected as characteristics of induction of apoptosis [48, 49].

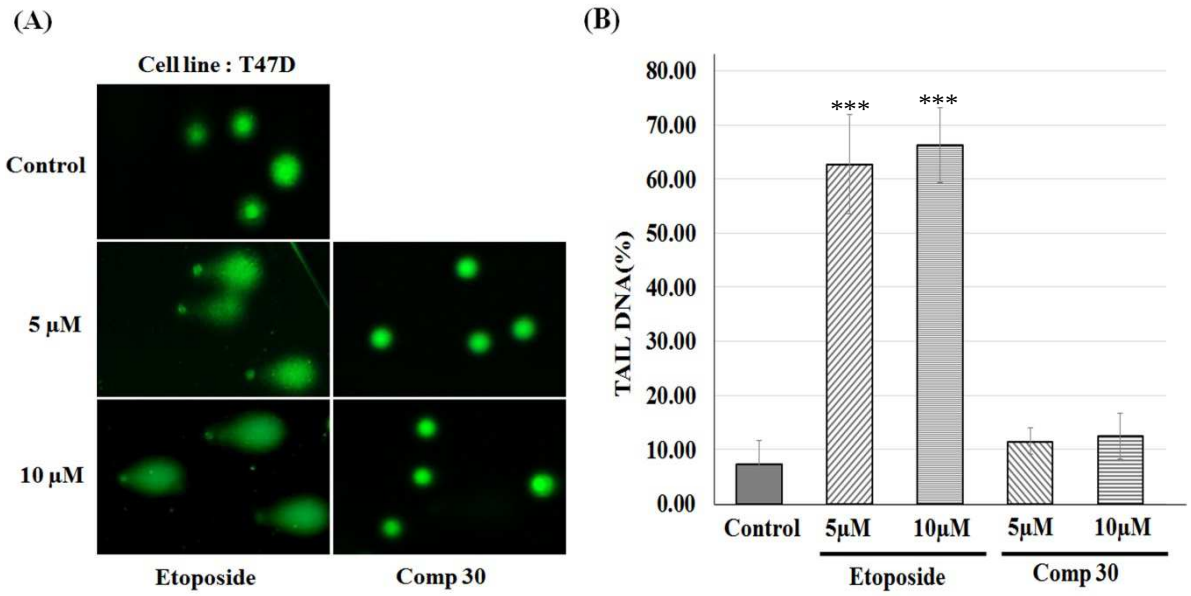


Figure 7. Comet assay for assessment of DNA damage. (A) The images of control (non-treated), etoposide (topo poison) and compound **30** treated T47D cells showing comet formation. (B) Graphical representation of the selected comet tail lengths of untreated- and treated-T47D cells using Komet 5.0 software. Columns and error bars indicate mean ± SD (n ¼ 50). ***P < 0.001, for significant differences from the vehicle control.

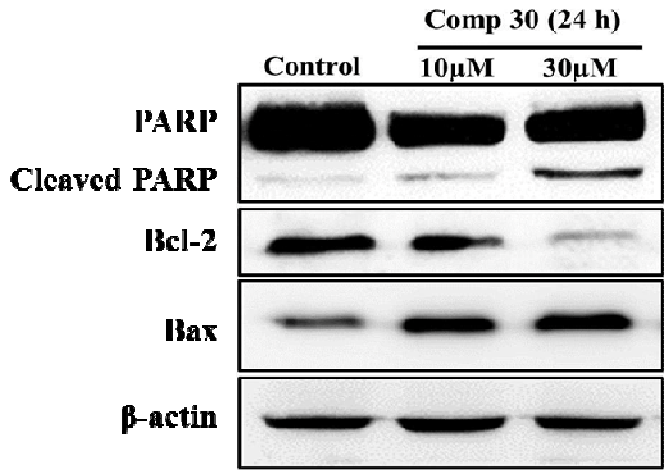


Figure 8. Representative western blots of apoptosis-related proteins including cleaved PARP, Bcl-2 and Bax after T47D cells were treated with compound **30** for 24 h.

2.6. SAR study

SAR study was performed in accordance with the results of topo I and topo IIα inhibitory property and antiproliferative activity of the prepared compounds **13-30** and previously synthesized non-chlorinated compounds **A-F**. As shown in Table 1, it was revealed that most compounds **13-21**, containing a hydroxyl moiety at the *ortho*-position of 2-, or 6-phenyl ring displayed either a very weak or no topo I and/or topo IIα inhibitory property at both 100 μM and 20 μM. On the other hand, compounds **22-30** which possess hydroxyl moiety either at *meta*- or *para*-position of 2-, or 6-phenyl ring showed significantly strong to moderate topo I and/or topo IIα inhibitory activities at 100 μM and/or 20 μM. These results indicated that the *meta*- or *para*-hydroxyl group of 2-, or 6-phenyl ring rather than *ortho*-hydroxyl group is crucial for topo I and topo IIα inhibition. Furthermore, most compounds **22-30** have an increasing order of topo I and topo IIα inhibitory activities with respect to the *ortho*-, *meta*-, or *para*-chlorophenyl substituents, respectively. This revealed that the *para* position of the

chlorine at the 4-phenyl ring is important for exhibiting the most significant topo I and topo IIα inhibition, as displayed in Figure 9.

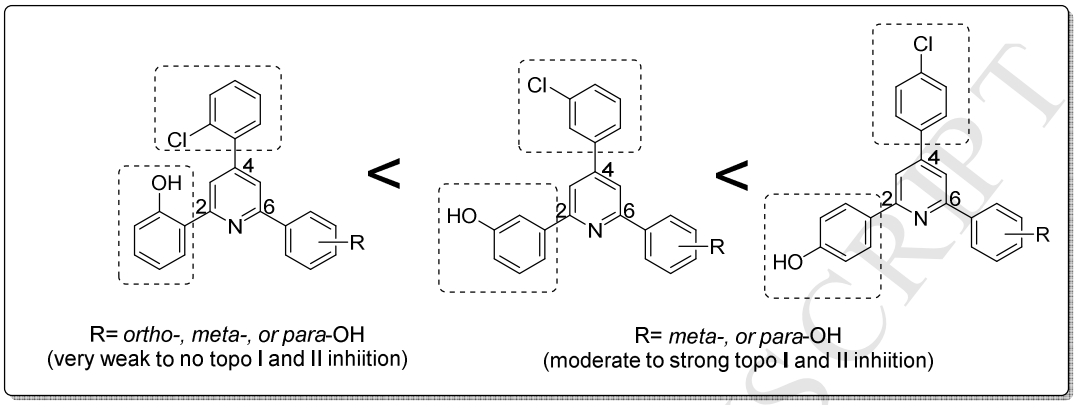


Figure 9. SAR study showing the favorable substitution order on dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines for topo I and IIα inhibition.

As shown in Table 1, previously synthesized representative compounds **A-F** displayed significant topo IIα inhibitory property with the exception of compound **A** whereas these compounds showed very weak or no topo I inhibitory activity with the exception of compound **D** compared to the positive controls, etoposide and camptothecin, respectively. Interestingly, substitution of the chlorine atom in the phenyl ring of 4-pyridine with the *ortho*-hydroxyl group on 2-, or 6-phenyl ring of the previously synthesized compounds **A-F** did not show considerable topo I and topo IIα inhibitory properties (compounds **13-21**). However, substitution of the chlorine atom in the phenyl ring of 4-pyridine with the *meta*- or *para*-hydroxyl group on 2-, or 6-phenyl ring of the previously synthesized compounds **A-F** displayed both topo I and topo IIα inhibitory properties in compounds **22-30**. These results

indicated that the addition of the chlorine atom in the phenyl ring of 4-pyridine in case of *meta*- or *para*-hydroxyl group on 2-, or 6-phenyl ring is essential for acting as a dual inhibitors of topo I and topo IIα by disrupting the topo IIα selectivity of compounds **A-F**.

For the comparative potency of topo inhibition study, Table 2 illustrates the relative topo I and IIα inhibition potencies of newly synthesized compounds **13-30** and previously synthesized compounds **A-F** compared to positive controls, camptothecin and etoposide.

Since, most of the compounds **13-21**, and their corresponding non-chlorinated compounds **A-C** displayed either very weak or no topo I inhibitory property at both100 µM and 20 µM, it was difficult to correlate their relative topo I inhibition study. However, the relative topo IIα inhibition potency of compounds **A-C** were significantly higher compared to compounds **13-30**. This signifies that at least one *ortho*-phenol moiety at either 2-, or 6-position and chloro-phenyl moiety at 4-position of central pyridine is responsible for neither topo I nor topo II α inhibitory potency whereas removal of chlorine atom from the 4-phenyl ring significantly enhances only the topo IIα inhibitory potency without having any effect on the topo I inhibitory potency. On the other hand, among the newly synthesized compounds **22-30**, the relative topo I inhibitory potency of compounds **25-30** were significantly higher compared to their corresponding non-chlorinated compounds **E-F** whereas compounds **23** and **24** showed similar relative potency like that of corresponding compound **D.** This signifies that the *meta-or para*-phenol moiety at 2-position, *para*-phenol moiety at 6-position and chloro-phenyl moiety at 4-position of the central pyridine is responsible for their better topo I inhibitory potency than that of corresponding compound **E-F.** In contrast, the relative topo IIα inhibition potency of these newly synthesized compounds **22-30** displayed mixed results compared with their corresponding non-chlorinated compounds **D-F.** Compounds **22**, **23**, and **24** that possess *meta*-phenol moiety at both 2- and 6-position with chloro-phenyl moiety at 4-position of the

central pyridine, displayed relatively low potency for inhibition of topo IIα at both 100 µM and 20 µM than that of corresponding non-chlorinated compound **D**. However, it was observed that compounds **25**, **26**, **27** and their corresponding non-chlorinated compounds **E** showed relatively high potency at 20 µM**.** This signifies that the *meta*-phenol moiety at 2-position, *para*-phenol moiety at 6-position and chloro-phenyl moiety at 4-position of the central pyridine is responsible for their higher topo IIα inhibitory potency than that of corresponding compound **E** at 20 µM**.** In case of compounds **28**, **29**, **30** and their corresponding non-chlorinated compound **F,** compounds **29** and **30** showed relatively higher topo IIα inhibition potency at both 100 µM and 20 µM**.** However, compound **28** displayed low potency at 100 µM but showed higher relative potency at 20 µM compared to corresponding non-chlorinated compounds **F.** This signifies that the *para*-phenol moiety at 2- and 6-position and chloro-phenyl moiety at 4-position of the central pyridine is responsible for their higher topo IIα inhibitory potency than the corresponding compound **E.**

Table 2. Relative topo I and topo II potencies of newly synthesized compounds **13-30** and previously synthesized compounds **A-F** compared to positive controls, camptothecin and etoposide.

Relative potency* for % inhibition compared to positive controls (Topo II: Etoposide; Topo I: Camptothecin)										
Compounds	Topo I		Compounds	Topo I		Compounds	Topo II		Compounds	Topo II	
	100 µM	20 µM		100 µM	20µM		100 µM	20 µM		100 µM	20 µM
			13	0.11	NT				**13**	N	NT
A	NA	NT	**14**	N	NT	**A**	0.61	0.37	**14**	NA	NT
			15	N	NT				**15**	NA	NT
			16	N	NT				**16**	N	NT
B	0.17	NT	**17**	0.14	NT	B	1.32	2.22	**17**	N	NT
			18	1.11	0.97				**18**	NA	NT
			19	0.56	0.99				**19**	0.47	1.00
C	NA	NT	**20**	NA	NT	**C**	1.43	3.50	**20**	NA	NT
			21	0.14	NT				**21**	0.55	0.94
			22	0.70	NA				**22**	1.03	0.82
D	1.07	NT	**23**	0.97	NA	**D**	1.24	1.50	**23**	0.91	0.66

			24	1.08	0.85				**24**	0.94	1.32
			25	0.67	0.85				**25**	0.98	1.29
E	NA	NT	**26**	0.99	N	**E**	1.39	1.32	**26**	0.91	2.34
			27	1.12	1.31				**27**	0.99	2.08
			28	1.06	0.61				**28**	0.66	1.89
F	N	NT	**29**	1.26	NA	**F**	1.10	1.03	**29**	1.18	1.94
			30	1.31	1.10				**30**	1.43	2.43

* Relative potency: % inhibition of compounds / % inhibition of positive control, NT: Not Tested, NA: Not Active, N: Value less than 0.1
Results of compounds **A-F** are reported earlier [39]

Table 3. Relative antiproliferative potencies of newly synthesized compounds **13-30** and previously synthesized compounds **A-F** compared to positive controls, etoposide and camptothecin, against HCT15, HeLa, and T47D cancer cell lines.

	Relative potency* for IC_{50} (μM) compared to positive controls Etoposide(ETP); Camptothecin(CPT)											
Compounds	HCT15		T47D		HeLa		Compounds	HCT15		T47D		HeLa	
	ETP	CPT	ETP	CPT	ETP	CPT		ETP	CPT	ETP	CPT	ETP	CPT
A	<0.03	<0.01	0.09	0.10	0.22	0.07	**13**	1.36	3.08	5.71	1.72	2.58	0.02
							14	<0.11	<0.24	<0.14	<0.04	<0.18	<0.01
							15	<0.11	<0.24	<0.14	<0.04	<0.18	<0.01
B	0.03	0.01	<0.07	<0.08	<0.06	<0.02	**16**	1.26	2.85	4.30	1.30	1.74	0.01
							17	2.39	5.41	8.40	2.53	1.65	0.01
							18	1.13	2.54	6.90	2.08	1.11	0.01
							19	0.95	2.15	0.64	0.19	0.48	0.01

C	0.18	0.04	2.32	2.79	0.82	0.26	**20**	3.17	7.16	<0.14	<0.04	<0.18	<0.01
							21	<0.11	<0.24	<0.14	<0.04	<0.18	<0.01
							22	1.51	3.42	99.57	30.00	1.44	0.01
D	0.07	0.01	0.26	0.31	0.25	0.08	**23**	1.54	3.48	348.50	105.00	3.46	0.02
							24	0.89	2.00	46.47	14.00	1.66	0.01
							25	1.39	3.15	8.82	2.66	3.68	0.02
E	0.04	0.01	0.65	0.78	0.92	0.29	**26**	1.24	2.80	99.57	30.00	130.00	0.86
							27	0.99	2.24	8.30	2.50	15.69	0.10
							28	1.04	2.35	8.94	2.69	13.19	0.09
F	0.44	0.09	0.49	0.58	0.97	0.31	**29**	1.27	2.86	4.22	1.27	6.03	0.04
							30	1.08	2.45	7.49	2.26	1.80	0.01

* Relative potency: IC_{50} (µM) of positive control / IC_{50} (µM) of compounds, results of compounds **A-H** are reported earlier [39]
'<' represents the value of those compounds whose IC_{50} is greater than 50µM

As shown in Table 1, since most compounds displayed strong antiproliferative activities, it is difficult to draw the positive correlation between antiproliferative activity and topo inhibitory property. However, compounds **22-30** that possess *meta- or para*-hydroxyl group in the phenyl ring of 2-pyridine, showed relevant positive relationships between topo inhibitory property and antiproliferative activity. Table 3 illustrates the relative antiproliferative potencies of the newly synthesized compounds **13-30** and the previously prepared compounds **A-F** compared to the positive controls, etoposide and camptothecin, against HCT15, HeLa, and T47D cancer cell lines. In general, compared with non-chlorinated compounds **A-F**, the corresponding chlorinated compounds significantly increased the antiproliferative potencies against HCT15, HeLa, and T47D cancer cell lines. Interestingly, compound **23** has shown the highest relative antiproliferative potency as much as 348.5 and 105 times compared to etoposide and camptothecin, respectively, in T47D human cancer cell line. Similarly, compounds **22** and **26** have shown 100 and 30 times more potent antiproliferative effect than etoposide and camptothecin, respectively, against T47D cell lines. In addition, compound **26** displayed 130 times more potent antiproliferative effect in HeLa cell lines compared to etoposide. These results may suggest that the introduction of chlorine moiety at 4-phenyl ring, a hydroxyl moiety at the *meta* position of 2-phenyl ring, and a hydroxyl moiety at the *meta* or the *para* position of 6-phenyl ring in dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines was important for antiproliferative activity.

3. Conclusion

Eighteen dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines were systematically designed and prepared by well-defined synthetic methods. The prepared compounds were evaluated for

their topo I as well as topo IIα inhibitory properties and antiproliferative activity against three different human cancer cell lines. SAR study indicated that the introduction of a hydroxyl moiety at the *meta- or para*-position of 2-phenyl ring of the central pyridine is necessary for dysplaying their potent dual topo I and topo IIα inhibitory properties. In addition, SAR study revealed that the *para* position of a hydroxyl group at 2-and 6-phenyl ring and chlorine atom at the *para* position of 4-phenyl ring of central pyridine exhibited the most significant topo I and topo IIα inhibitory property. Interestingly, the substitution of a chlorine atom in the 4-phenyl ring of central pyridine may indicate its importance as a dual topo I and topo IIα inhibitor. Compound **30** showed the most potent dual topo I and topo IIα inhibition with strong antiproliferative activity in T47D cell line. Based on direct evidences obtained from cleavage complex assay (linear truncated DNA was not induced by compound **30**), band depletion assay (compound **30** did not induce topo II depletion) and EtBr displacement assay (compound **30** did not affect the fluorescence emission intensity of the intercalated EtBr), compound **30** obviously worked as non-intercalative topo IIα catalytic inhibitor. Comet tail assay we performed is a typical method to detect DNA damage caused by double strand breaks as well as single strand breaks [50, 51]. If compound **30** worked as topo I poison, then compound **30** treatment induced comet tail. But it almost did not make comet tail. In conclusion, it can be reasonably concluded that compound **30** functions as a non-intercalative catalytic topo I and IIα dual inhibitor.

4. Experimental

Commercially available starting materials and reagents were purchased from Sigma-Aldrich Chemical Co., TCI Chemicals, Alfa-Aesar, and Junsei and used 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. All the prepared compounds contained aromatic ring, so 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 analysis were performed using an HPLC system that consisted of a pump (LC-20AD), an autoinjector (SIL-20-A), a UV-visible detector (SPD-20A), and communications bus module (CBM-20A) from Shimadzu Scientific Instruments (Kyoto Japan). A Waters COSMOSIL 5C18-MS-II column (5µm, 4.6 x 250 mm) was used with a gradient solvent system from 90:10 to 95:05 for 20 min with 100% methanol: doubly distilled water, at a flow rate of 0.8 mL/min at 254 nm UV detection. The purity of compound was described as percentage (%), and retention time was given in minutes (min).

ESI-MS analysis was performed with Advion expression compact mass spectrometry utilizing Advion Data Express™ program and was recorded on an electrospray ionization mass spectrometer as the value m/z. MS ionization conditions were: Polarity: Positive, Ionization spray voltage: 3.5 KV, capillary temperature: 250°C, capillary voltage: 180 V.

*4. 1. General method for the preparation of **1-3***

A mixture of aryl methyl ketone **I** (R^1= **a-c**) (1.0 equiv.) and iodine (1.2 equiv.) in pyridine

(15 equiv.) was refluxed at 140 °C for 3 h. After cooling the mixture to room temperature, the precipitate formed, which was filtered and washed with cold pyridine followed by drying overnight to afford **1-3** in 65.4-99.5% yield. Compounds were used without further purification.

*4. 2. General method for the preparation of **4-12***

Aryl methyl ketones **III** (R^3=**a-c**) (1.0 equiv) and aryl aldehyde **II** (R^2=**d-f**) (1.0 equiv) were dissolved in methanol. After complete dissolution, aqueous NaOH (3.0 equiv) was added slowly to the reaction mixture at room temperature. The reaction mixture was stirred for 2-36 h. The reaction was terminated by the addition of 4M HCL solution. The mixture was extracted with EtOAc, washed with H_2O, saturated aqueous NaCl solution, dried over MgSO4, and filtered. It was further purified either by recrystallization or by silica gel column chromatography using EtOAc and *n*-hexane as eluents to obtain compounds **4-12** in 56.6-83.1% yield.

*4. 3. General method for the preparation of **13-30***

Hydroxylated 2,6-diphenyl-4-chlorophenylpyridines **13-30** were synthesized according to the Kröhnke synthesis method [25]. Anhydrous ammonium acetate (10.0 equiv.) was mixed with glacial acetic acid or methanol followed by addition of propenone intermediates (1.0 equiv.) **4-12** (R^3= **a-c,** R^2= **d-f**) and pyridinium iodide salts (1.5 equiv.) **1-3** (R^1=**a-c**). The mixture was then refluxed at 90-100 °C for 2-36 h. The reaction mixture was 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 purified with

silica gel column chromatography with the gradient elution of ethyl acetate/*n*-hexane to afford solid compounds **13-30** in 11.4-63.8% yield.

*4.3.1. Synthesis of 2,2'-(4-(2-chlorophenyl)pyridine-2,6-diyl)diphenol (**13**)*

The compound was synthesized as described in section **4.3** with **4** (R^2= **d**) (0.52 g, 2.00 mmol, 1.00 equiv), **1** (R^1= **a**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and glacial acetic acid (6.00 mL) to yield 239 mg (32.0%, 0.64 mmol) as a yellow solid.

TLC (ethyl acetate / *n*-hexane = 1:4) R_f = 0.24; Mp: 201.5-202.5 ^oC; HPLC: Retention time: 11.12 min, purity: 97.2%; ESI LC/MS: *m/z* Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 374.1.

^{1}H NMR (250 MHz, DMSO-d_6) δ 12.29 (br, 1H, 2-phenyl 2-OH), 8.00 (s, 2H, pyridine H-3, H-5), 7.87 (d, *J* = 7.30 Hz, 2H, 2-phenyl H-6, 6- phenyl H-6), 7.66-7.61 (m, 2H, 4-phenyl H-3, H-6), 7.53-7.49 (m, 2H, 4-phenyl H-4, H-5), 7.31 (t, *J* = 7.20 Hz, 2H, 2-phenyl H-4, 6-phenyl H-4), 6.99-6.62 (m, 4H, 2-phenyl H-3, H-5, 6-phenyl H-3, H-5).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 157.59 (2C), 155.26 (2C), 148.97, 137.80, 131.82, 131.57 (2C), 131.46, 130.99, 130.50, 128.97 (2C), 128.27, 122.12 (2C), 120.80 (2C), 119.77 (2C), 117.72 (2C).

4.3.2. Synthesis of 2,2'-(4-(3-chlorophenyl)pyridine-2,6-diyl)diphenol (**14**)

The compound was synthesized as described in section **4.3** with **5** (R^2= **e**) (0.52 g, 2.00 mmol, 1.00 equiv), **1** (R^1= **a**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 mL) to yield 365 mg (48.8%, 0.98 mmol) as a pale yellow solid.

TLC (ethyl acetate / *n*-hexane = 1:4) R_f = 0.27; Mp: 223.6-224.2 ^{o}C; HPLC: Retention time: 9.18 min, purity: 98.6%; ESI LC/MS: *m/z* Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 373.9.

^{1}H NMR (250 MHz, DMSO-d_6) δ 12.30 (s, 2H, 2-phenyl 2-OH, 6-phenyl 2-OH), 8.24 (s, 2H, pyridine H-3, H-5), 8.12 (s, 1H, 4-phenyl H-2), 8.01-7.93 (m, 3H, 2-phenyl H-6, 6-phenyl H-6, 4-phenyl H-6), 7.59 (d, *J* = 4.90 Hz, 2H, 4-phenyl H-4, H-5), 7.33 (d, *J* = 8.30 Hz, 2H, 2-phenyl H-4, 6-phenyl H-4), 6.70-6.94 (m, 4H, 2-phenyl H-3, H-5, 6-phenyl H-3, H-5).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 157.53 (2C), 155.76 (2C), 148.40, 139.91, 134.32, 131.33 (2C), 131.25, 129.60, 129.13 (2C), 127.54, 126.43, 122.21 (2C), 119.42 (2C), 118.02 (2C), 117.52 (2C).

4.3.3. Synthesis of 2,2'-(4-(4-chlorophenyl)pyridine-2,6-diyl)diphenol (**15**)

The compound was synthesized as described in section **4.3** with **6** (R^2= **f**) (0.52 g, 2.00 mmol, 1.00 equiv), **1** (R^1= **a**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 mL) to yield 324.6 mg (43.4%, 0.87 mmol) as a yellow solid.

TLC (ethyl acetate / *n*-hexane = 1:4) R_f = 0.30; Mp: 248.8-249.6 ^{o}C; HPLC: Retention time: 8.72 min, purity: 98.4%; ESI LC/MS: m/z Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 374.2.

^{1}H NMR (250 MHz, DMSO-d_6) δ 12.32 (s, 2H, 2-phenyl 2-OH, 6-phenyl 2-OH), 8.20 (s, 2H, pyridine H-3, H-5), 8.01 (d, *J* = 8.50 Hz, 4-phenyl H-2, H-6), 7.95 (d, *J* = 7.90 Hz, 2H, 2-phenyl H-6, 6-phenyl H-6), 7.62 (d, *J* = 8.45 Hz, 2H, 4-phenyl H-3, H-5), 7.32 (t, *J* = 8.70 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-d_6) δ 157.60 (2C), 155.82 (2C), 148.71, 136.63, 134.85, 131.45 (2C), 129.54 (2C), 129.11 (2C), 122.30 (2C), 117.88 (2C), 117.63 (2C).

*4.3.4. Synthesis of 2-(4-(2-chlorophenyl)-6-(3-hydroxyphenyl)pyridin-2-yl)phenol (**16**)*

The compound was synthesized as described in section **4.3** with **4** (R^2= **d**) (0.52 g, 2.00 mmol, 1.00 equiv), **2** (R^1= **b**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and glacial acetic acid (6.00 mL) to yield 180 mg (24.1%, 0.48 mmol) as a pale yellow solid.

TLC (ethyl acetate / *n*-hexane = 1:4) R_f = 0.24; Mp: 199.6-200.5 ^oC; HPLC: Retention time: 7.91 min, purity: 98.5%; ESI LC/MS: *m/z* Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 373.8.

^{1}H NMR (250 MHz, DMSO-d_6) δ 14.16 (s, 1H, 2-phenyl 2-OH), 9.88 (s, 1H, 6-phenyl 3-OH), 8.17 (s, 1H, pyridine H-3), 8.10 (d, *J* = 6.80 Hz, 1H, 2-phenyl H-6), 7.86 (s, pyridine H-5), 7.67-7.63 (m, 2H, 4-phenyl H-3, H-6), 7.55-7.50 (m, 2H, 4-phenyl H-4, H-5), 7.46-7.30 (m, 4H, 2-phenyl H-4, 6-phenyl H-2, H-5, H-6), 6.98-6.91 (m, 3H, 2-phenyl H-3, H-5, 6-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 159.42, 158.56, 157.28, 154.55, 150.02, 139.23, 137.75, 132.28, 131.95, 131.60, 131.21, 130.99, 130.60, 128.39, 128.14, 120.25, 119.75, 119.71, 119.53, 118.35, 118.09, 117.53, 113.79.

4.3.5. Synthesis of 2-(4-(3-chlorophenyl)-6-(3-hydroxyphenyl)pyridin-2-yl)phenol (**17**)

The compound was synthesized as described in section **4.3** with **5** (R^2= **e**) (0.52 g, 2.00 mmol, 1.00 equiv), **2** (R^1= **b**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00

equiv) and methanol (14.00 mL) to yield 150.6 mg (20.1%, 0.40 mmol) as a yellow solid.

TLC (ethyl acetate / *n*-hexane = 1:4) R_f = 0.27, Mp: 213.2-214.0 ^oC; HPLC: Retention time: 9.51 min, purity: 96.4%; ESI LC/MS: *m/z* Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 374.2

^{1}H NMR (250 MHz, DMSO-d_6) δ 14.42 (s, 1H, 2-phenyl 2-OH), 9.82 (S, 1H, 6-phenyl 3-OH), 8.44 (s, 1H, pyridine H-3), 8.32 (d, *J* = 7.40 Hz, 2-phenyl H-6), 8.20 (s, 1H, 4-phenyl H-2), 8.15 (s, 1H, pyridine H-5), 8.05-8.03 (m, 1H, 4-phenyl H-6), 7.63-7.54 (t, 3H, 4-phenyl H-4, H-5, 6-phenyl H-6), 7.49 (s, 1H, 6-phenyl H-2), 7.41-7.32 (m, 2H, 2-phenyl H-4, 6-phenyl H-5), 6.99-6.93 (m, 3H, 2-phenyl H-3, H-5, 6-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 159.42, 158.56, 157.28, 154.55, 150.02, 139.23, 137.75, 132.28, 131.95, 131.60, 131.21, 130.99, 130.60, 128.39, 128.14, 120.25, 119.75, 119.71, 119.53, 118.35, 118.09, 117.53, 113.79.

4.3.6. Synthesis of 2-(4-(4-chlorophenyl)-6-(3-hydroxyphenyl)pyridin-2-yl)phenol (**18**)

The compound was synthesized as described in section **4.3** with **6** (R^2= **f**) (0.52 g, 2.00 mmol, 1.00 equiv), **2** (R^1= **b**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 mL) to yield 85 mg (11.4%, 0.23 mmol) as a yellow solid.

TLC (ethyl acetate / *n*-hexane = 1:4) R_f = 0.30; Mp: 239.6-240.2 ^oC; HPLC: Retention time: 9.12 min, purity: 98.9%; ESI LC/MS: m/z Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 374.2.

^{1}H NMR (250 MHz, DMSO-d_6) δ 14.42 (s, 1H, 2-phenyl 2-OH), 9.89 (s, 1H, 6-phenyl 3-OH), 8.40 (s, 1H, pyridine H-3), 8.27 (d, *J* = 7.60 Hz, 1H, 2-phenyl H-6), 8.10 (d, *J* = 8.20 Hz, 2H, 4-phenyl H-2, H-6), 8.10 (s, 1H, pyridine H-5), 7.62 (d, *J* = 8.40 Hz, 2H, 4-phenyl H-3, H-5), 7.52 (d, *J* = 7.70 Hz, 1H, 6-phenyl H-6), 7.46 (s, 1H, 6-phenyl H-2), 7.41-7.32 (m, 2H, 6-

phenyl H-4, 6-phenyl H-5), 6.99-6.92 (m, 3H, 2-phenyl H-3, H-5, 6-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 159.52, 158.43, 157.79, 155.09, 149.59, 139.28, 136.34, 135.06, 132.08, 130.70, 129.85 (2C), 129.49 (2C), 128.14, 119.37 (2C), 118.20, 118.10, 117.30 (2C), 116.42, 113.83.

4.3.7. Synthesis of 2-(4-(2-chlorophenyl)-6-(4-hydroxyphenyl)pyridin-2-yl)phenol (**19**)

The compound was synthesized as described in section **4.3** with **4** (R^2= **d**) (0.52 g, 2.00 mmol, 1.00 equiv), **3** (R^1= **c**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 ml) to yield 477 mg (63.8%, 1.28 mmol) as a pale yellow solid.

TLC (ethyl acetate / *n*-hexane = 1:3) R_f = 0.30; Mp: 253.8-254.7 ^{o}C; HPLC: Retention time: 9.0 min, purity: 95.4%; ESI LC/MS: *m/z* Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 374.1.

^{1}H NMR (250 MHz, DMSO-d_6) δ 14.46 (s, 1H, 2-phenyl 2-OH), 10.03 (s, 1H, 6-phenyl 4-OH), 8.12-8.10 (m, 2H, 2-phenyl H-6, pyridine H-3), 7.89 (d, *J* = 8.70 Hz, 2H, 6-phenyl H-2, H-6), 7.86 (s, 1H, pyridine H-5), 7.67-7.61 (m, 2H, 4-phenyl H-4, H-5), 7.55-7.50 (m, 2H, 4-phenyl H-4, H-5), 7.33 (t, *J* = 7.20 Hz, 2-phenyl H-4), 6.96-6.89 (m, 4H, 6-phenyl H-3, H-5, 2-phenyl H-3, H-5).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 159.57, 159.41, 156.87, 154.29, 149.73, 137.83, 131.87, 131.72, 131.44, 130.84, 130.32, 128.51(2C), 128.32, 128.06, 127.81, 119.32, 118.99, 118.29, 118.11, 116.33.

4.3.8. Synthesis of 2-(4-(3-chlorophenyl)-6-(4-hydroxyphenyl)pyridin-2-yl)phenol (**20**)

The compound was synthesized as described in section **4.3** with **5** (R^2= **e**) (0.52 g, 2.00 mmol, 1.00 equiv), **3** (R^1= **c**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00

equiv) and glacial acetic acid (6.00 mL) to yield 197 mg (26.4%, 0.53 mmol) as a yellow solid.

TLC (ethyl acetate / *n*-hexane = 2:5) R_f = 0.26, Mp: 273.8-274.5 ^{o}C; HPLC: Retention time: 10.91 min, purity: 96.1%; ESI LC/MS: m/z Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 373.9.

^{1}H NMR (250 MHz, DMSO-d_6) δ 14.65 (s, 1H, 2-phenyl 2-OH), 10.0 (s, 1H, 6-phenyl 3-OH), 8.36 (s, 1H, pyridine H-3), 8.31 (d, *J* = 8.30 Hz, 2-phenyl H-6), 8.20 (s, 1H, 4-phenyl H-2), 8.13 (s, 1H, pyridine H-5), 8.05-8.02 (m, 1H, 4-phenyl H-6), 7.98 (d, *J* = 8.40 Hz, 2H, 6-phenyl H-2, H-6), 7.59 (d, *J* = 3.90 Hz, 2H, 4-phenyl H-4, H-5), 7.34 (t, *J* = 7.80 Hz, 2-phenyl H-4), 6.97-6.92 (m, 4H, 6-phenyl H-3, H-5, 2-phenyl H-3, H-5).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 159.54 (2C), 157.56, 154.89, 149.13, 139.72, 134.28, 131.82, 131.11, 129.68, 128.64(2C), 128.41, 128.04, 127.64, 126.59, 119.22, 119.09, 118.03, 116.28, 116.18 (2C), 115.34.

4.3.9. Synthesis of 2-(4-(4-chlorophenyl)-6-(4-hydroxyphenyl)pyridin-2-yl)phenol (**21**)

The compound was synthesized as described in section **4.3** with **6** (R^2= **f**) (0.52 g, 2.00 mmol, 1.00 equiv), **3** (R^1= **c**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 mL) to yield 224 mg (30.0%, 0.60 mmol) as a yellow solid.

TLC (ethyl acetate / *n*-hexane = 2:5) R_f = 0.27; Mp: 313.3-313.9 ^{o}C; HPLC: Retention time: 10.32 min, purity: 95.7%; ESI LC/MS: m/z Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 373.9.

^{1}H NMR (250 MHz, DMSO-d_6) δ 14.66 (s, 1H, 2- phenyl 2-OH), 10.09 (br, 1H, 6-phenyl 4-OH), 8.30 (s, 1H, pyridine H-3), 8.24 (d, *J* = 8.10 Hz, 1H, 2-phenyl H-6), 8.08 (d, 3H, 4-phenyl H-2, H-6, pyridine H-5), 7.94 (d, *J* = 8.60 Hz, 2H, 6-phenyl H-2, H-6), 7.61 (d, *J* =

8.40 Hz, 2H, 4-phenyl H-3, H-5), 7.33 (t, *J* = 8.20 Hz, 1H, 2-phenyl H-4), 6.97-6.92 (m, 4H, 6-phenyl H-3, H-5, 2-phenyl H-3, H-5).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 159.63 (2C), 157.65, 155.02, 149.48, 136.49, 134.97, 131.98, 129.78 (2C), 129.46 (2C), 128.72 (2c), 128.59, 127.99, 119.32 (2C), 118.20, 116.36 (2C), 116.17, 115.20.

4.3.10. Synthesis of 3,3'-(4-(2-chlorophenyl)pyridine-2,6-diyl)diphenol (**22**)

The compound was synthesized as described in section **4.3** with **7** (R^2= **d**) (0.52 g, 2.00 mmol, 1.00 equiv), **2** (R^1= **b**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 mL) to yield 246 mg (32.9%, 0.66 mmol) as a white solid.

TLC (ethyl acetate / *n*-hexane = 2:5) R_f = 0.23; Mp: 235.8-236.6 ^{o}C; HPLC: Retention time: 6.04 min, purity: 99.6%; ESI LC/MS: m/z Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 373.8.

^{1}H NMR (250 MHz, DMSO-d_6) δ 9.64 (s, 2H, 2- phenyl 3-OH, 6-phenyl H-3), 7.85 (s, 2H, 6-pyridine H-3, H-5), 7.68-7.59 (m, 6H, 4-phenyl H-3, H-6, 2-phenyl H-2, H-6, 6-phenyl H-2, H-6), 7.51-7.47 (m, 2H, 2-phenyl H-4, H-5), 7.30 (t, *J* = 7.80 Hz, 2H, 2-phenyl H-5, 6-phenyl H-5), 6.85 (dd, *J* = 8.00, 1.8 Hz, 2H, 2-phenyl H-4, 6-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 158.08 (2C), 156.04 (2C), 148.51, 140.18 (2C), 137.95, 131.76, 131.42, 130.64, 130.33, 130.11 (2C), 128.08, 119.58 (2C), 117.85 (2C), 116.64 (2C), 113.85 (2C).

4.3.11. Synthesis of 3, 3'-(4-(3-chlorophenyl) pyridine-2,6-diyl)diphenol (**23**)

The compound was synthesized as described in section **4.3** with **8** (R^2= **e**) (0.52 g, 2.00 mmol,

1.00 equiv), **2** (R^1= **b**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 mL) to yield 230 mg (30.8%, 0.61 mmol) as an off white solid.

TLC (ethyl acetate / *n*-hexane = 2:5) R_f = 0.23; Mp: 241.8-242.6 ^oC; HPLC: Retention time: 7.07 min, purity: 95.4%; ESI LC/MS: m/z Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 374.1.

^{1}H NMR (250 MHz, DMSO-d_6) δ 9.62 (s, 2H, 2- phenyl 3-OH, 6-phenyl 3-OH), 8.11 (d, 3H, 4-phenyl H-2, pyridine H-3, H-5), 7.98-7.96 (m, 1H, 4-phenyl H-6), 7.74-7.71 (d, 4H, 2-phenyl H-2, H-6, 6-phenyl H-2, H-6), 7.61-7.55 (m, 2H, 4-phenyl H-4, H-5), 7.33 (t, *J* = 8.00 Hz, 2H, 2-phenyl H-5, 6-phenyl H-5), 6.88 (d, *J* = 7.90 Hz, 2H, 2-phenyl H-4, 6-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 158.02 (2C), 156.84 (2C), 148.20, 140.37 (2C), 140.20, 134.30, 131.17, 129.99 (2C), 129.34, 127.40, 126.34, 118.10 (2C), 116.89 (2C), 116.62 (2C), 114.07 (2C).

4.3.12. Synthesis of 3,3'-(4-(4-chlorophenyl)pyridine-2,6-diyl)diphenol (**24**)

The compound was synthesized as described in section **4.3** with **9** (R^2= **f**) (0.52 g, 2.00 mmol, 1.00 equiv), **2** (R^1= **b**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 mL) to yield 278.3mg (37.2%, 0.74mmol) as a white solid.

TLC (ethyl acetate / *n*-hexane = 2:5) R_f = 0.23; Mp: 245.3-245.9 ^oC; HPLC: Retention time: 6.76 min, purity: 99.8%; ESI LC/MS: m/z Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 374.4.

^{1}H NMR (250 MHz, DMSO-d_6) δ 9.74 (br, 2H, 2- phenyl 3-OH, 6-phenyl 3-OH), 8.06 (s, 2H, pyridine H-3, H-5), 8.02 (d, *J* = 8.60 Hz, 2H, 4-phenyl H-2, H-6), 7.70-7.67 (d, 4H, 2-phenyl H-2, H-6, 6-phenyl H-2, H-6), 7.59 (d, *J* = 8.50 Hz, 2H, 4-phenyl H-3, H-5), 7.33 (t, *J* = 8.20 Hz, 2-phenyl H-5, 6-phenyl H-5), 6.87 (d, *J* =8.00 Hz, 2H, 2-phenyl H-4, 6-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 158.52 (2C), 157.35 (2C), 148.95, 140.91 (2C), 137.32, 135.02, 130.66 (2C), 129.98 (2C), 129.93 (2C), 118.60 (2C), 114.51 (2C).

4.3.13. Synthesis of 3-(4-(2-chlorophenyl)-6-(4-hydroxyphenyl) pyridin-2-yl)phenol (**25**)

The compound was synthesized as described in section **4.3** with **7** (R^2= **d**) (0.52 g, 2.00 mmol, 1.00 equiv), **3** (R^1= **c**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 mL) to yield 123mg (16.5%, 0.33 mmol) as an off white solid.

TLC (ethyl acetate / *n*-hexane = 1:2) R_f = 0.28; Mp: 223.6-224.5 ^oC; HPLC: Retention time: 6.08 min, purity: 98.5%; ESI LC/MS: m/z Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 374.2.

^{1}H NMR (600 MHz, DMSO-d_6) δ 9.80 (s, 1H, 6- phenyl 4-OH), 9.58 (s, 1H, 6-phenyl H-5), 8.11 (d, *J* = 8.60 Hz, 2H, 6-phenyl H-2, H-6), 7.82 (s, 1H, pyridine H-3), 7.76 (s, 1H, pyridine H-5), 7.70 (s, 1H, 2-phenyl H-2), 7.66 – 7.58 (m, 3H, 2-phenyl H-6, 4-phenyl H-3, H-6), 7.52-7.49 (m, 2H, 4-phenyl H-4, H-5), 7.31 (t, *J* = 7.90 Hz, 1H, 2-phenyl H-5), 6.92 (d, *J* = 8.60 Hz, 2H, 6-phenyl H-3, H-5), 6.87 (dd, *J* = 8.0, 2.1 Hz, 1H, 2-phenyl H-4).

^{13}C NMR (150 MHz, DMSO-d_6) δ 158.98, 157.97(2C), 156.08, 155.74, 148.34, 140.27, 138.13, 131.58, 131.36, 130.42, 130.20, 129.96, 129.66, 128.44(2C), 127.92, 118.31, 117.70, 116.44, 115.75(2C), 113.74.

4.3.14. Synthesis of 3-(4-(3-chlorophenyl)-6-(4-hydroxyphenyl)pyridin-2-yl)phenol (**26**)

The compound was synthesized as described in section **4.3** with **8** (R^2=**e**) (0.52 g, 2.00 mmol, 1.00 equiv), **3** (R^1= **c**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 mL) to yield 357.2 mg (47.8%, 0.95 mmol) as a white solid.

TLC (ethyl acetate / *n*-hexane = 3:7) R_f = 0.21; Mp: 216.4-217.3 ^oC; HPLC: Retention time:

7.08 min, purity: 96.1%; ESI LC/MS: m/z Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 374.4.

^{1}H NMR (250 MHz, DMSO-d_6) δ 9.85 (br, 1H, 6- phenyl 4-OH), 9.68 (br, 1H, 2-phenyl 3-OH), 8.18 (d, *J* = 8.70 Hz, 2H, 6-phenyl H-2, H-6), 8.12 (s, 1H, 4-phenyl H-2), 8.07 (s, 1H, pyridine H-3), 8.02 (s, 1H, pyridine H-5), 7.99-7.95 (m, 1H, 4-phenyl H-6), 7.73-7.70 (d, 2H, 2-phenyl H-2, H-6), 7.60-7.52 (m, 2H, 4-phenyl H-4, H-5), 7.31 (t, *J* = 7.80 Hz, 1H, 2-pheny;l H-5), 6.91 (d, *J* = 8.70 Hz, 2H, 6-phenyl H-3, H-5), 6.86 (d, *J* = 7.90 Hz, 1H, 2-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 159.10, 158.05 (2C), 156.90, 156.66, 148.09, 140.35, 140.55, 140.42, 134.34, 131.24, 130.06, 129.95, 129.36, 128.78 (2C), 127.43, 118.44, 126.38, 118.11, 116.57, 115.81 (3C), 114.03.

4.3.15. Synthesis of 3-(4-(4-chlorophenyl)-6-(4-hydroxyphenyl)pyridin-2-yl)phenol (**27**)

The compound was synthesized as described in section **4.3** with **9** (R^2= **f**) (0.52 g, 2.00 mmol, 1.00 equiv), **3** (R^1= **c**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 mL) to yield 427.5mg (57.2%, 1.14 mmol) as a white solid.

TLC (ethyl acetate / n-hexane = 3:7) R_f = 0.21; Mp: 257.8-258.6 ^oC; HPLC: Retention time: 6.80 min, purity: 98.9%; ESI LC/MS: m/z Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 374.2.

^{1}H NMR (250 MHz, DMSO-d_6) δ 9.87 (s, 1H, 3- phenyl 4-OH), 9.65 (s, 1H, 2-phenyl 3-OH), 8.16 (d, *J* = 8.60 Hz, 2H, 6-phenyl H-2, H-6), 8.04-7.99 (t, 4H, pyridine H-3, H-5, 4- phenyl H-2, H-6), 7.72-7.62 (m, 2H, 2-phenyl H-2, H-6), 7.60 (d, *J* = 8.50 Hz, 2H, 4-phenyl H-3, H-5), 7.31 (t, *J* = 7.80 Hz, 1H, 2-phenyl H-5), 6.91 (d, *J* = 8.60 Hz, 2H, 6-phenyl H-3, H-5),

6.86 (d, *J* = 8.40 Hz, 1H, 2-phenyl H-4).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 159.08, 158.06 (2C), 156.89, 156.66, 148.26, 140.59, 137.06, 134.42, 130.08, 129.98, 129.48 (2C), 129.38 (2C), 128.72 (2C), 118.04, 116.55, 115.83 (2C), 115.60, 114.00.

4.3.16. Synthesis of 4,4'-(4-(2-chlorophenyl) pyridine-2,6-diyl)diphenol (**28**)

The compound was synthesized as described in section **4.3** with **10** (R^2= **d**) (0.52 g, 2.00 mmol, 1.00 equiv), **3** (R^1= **c**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 mL) to yield 327mg (43.7%, 0.87 mmol) as a white solid.

TLC (ethyl acetate / *n*-hexane = 1:2) R_f = 0.25; Mp: 261.7-262.5 ^{o}C; HPLC: Retention time: 6.17 min, purity: 99.5%; ESI LC/MS: m/z Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 374.4.

^{1}H NMR (250 MHz, DMSO-d_6) δ 9.80 (s, 2H, 2-phenyl 4-OH, 6-phenyl 4-OH), 8.08 (d, *J* = 8.70 Hz, 4H, 2-phenyl H-2, H-6, 6-phenyl H-2, H-6), 7.72 (s, 2H, pyridine H-3, H-5), 7.65-7.58 (m, 2H, 4- phenyl H-3, H-6), 7.51-7.47 (m, 2H, 4-phenyl H-4, H-5), 6.88 (d, *J* = 8.70 Hz, 4H, 2-phenyl H-3, H-5, 6-phenyl H-3, H-5).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 158.92 (2C), 155.87 (2C), 148.32, 138.42, 131.62, 131.42, 130.42, 130.24, 129.84 (2C), 128.45 (4C), 127.97, 117.25, 115.76 (4C).

4.3.17. Synthesis of 4,4'-(4-(3-chlorophenyl)pyridine-2,6-diyl)diphenol (**29**)

The compound was synthesized as described in section **4.3** with **11** (R^2= **e**) (0.52 g, 2.00 mmol, 1.00 equiv), **3** (R^1= **c**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 mL) to yield 368 mg (49.2%, 0.98 mmol) as a white solid.

TLC (ethyl acetate / *n*-hexane = 1:2) R_f = 0.25; Mp: 262.7-263.5 ^oC; HPLC: Retention time: 7.18 min, purity: 98.2%; ESI LC/MS: m/z Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 374.0.

^{1}H NMR (250 MHz, DMSO-d_6) δ 9.79 (s, 2H, 2-phenyl 4-OH, 6-phenyl 4-OH), 8.18 (d, *J* = 8.60 Hz, 4H, 2-phenyl H-2, H-6, 6-phenyl H-2, H-6), 8.11 (s, 1H, 4-phenyl H-2), 7.99-7.95 (m, 3H, pyridine H-3, H-5, 4-phenyl H-6), 7.57-7.54 (m, 2H, 4-phenyl H-4, H-5), 6.90 (d, *J* = 8.60 Hz, 4H, 2-phenyl H-3, H-5, 6-phenyl H-3, H-5).

^{13}C NMR (62.5 MHz, DMSO-d_6) δ 158.92 (2C), 156.63 (2C), 147.81, 140.53, 134.22, 131.07, 130.01 (2C), 129.16, 128.64 (4C), 127.30, 126.23, 115.66 (4C), 114.56.

4.3.18. Synthesis of 4,4'-(4-(4-chlorophenyl)pyridine-2,6-diyl)diphenol (**30**)

The compound was synthesized as described in section **4.3** with **12**(R^2= **f**) (0.52 g, 2.00 mmol, 1.00 equiv), **3** (R^1= **c**) (1.02 g, 2.00 mmol, 1.50 equiv), NH_4OAc (1.54 g, 20.00 mmol, 10.00 equiv) and methanol (14.00 mL) to yield 423mg (56.6%, 1.13 mmol) as a yellow solid.

TLC (ethyl acetate / *n*-hexane = 1:2) R_f =: 0.25; Mp: 248.9-249.7 ^oC; HPLC: Retention time: 6.87 min, purity: 97.9%; ESI LC/MS: m/z Calcd for $C_{23}H_{17}ClNO_2$ $[MH]^+$ 374.09; found 373.9.

^{1}H NMR (600 MHz, DMSO-d_6) δ 9.76 (s, 2H, 2-phenyl 4-OH, 6-phenyl 4-OH), 8.16 (d, *J* = 8.50 Hz, 4H, 2-phenyl H-2, H-6, 6-phenyl H-2, H-6), 8.01 (d, *J* = 8.10 Hz, 2H, 4-phenyl H-2, H-6), 7.95 (s, 2H, pyridine H-3, H-5), 7.58 (d, *J* = 8.10 Hz, 2H, 4-phenyl H-3, H-5), 6.91 (d, *J* = 8.5 Hz, 4H, 2-phenyl H-3, H-5, 6-phenyl H-3, H-5).

^{13}C NMR (150 MHz, DMSO-d_6) δ 158.85 (2C), 156.59 (2C), 147.93, 137.13, 134.15, 130.03 (2C), 129.23 (2C), 129.16 (2C), 128.51 (4C), 115.64 (4C), 114.31.

4.4. Pharmacology

4.4.1. Assay for DNA topo I and IIα inhibition

Human DNA topo I and topo IIα inhibitory activity of the prepared compounds were determined following the previously reported method [52, 53], with minor modifications. 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 1 unit of recombinant human DNA topo 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. For the topo IIα relaxation assay, the mixture of 200 ng of supercoiled pBR322 plasmid DNA and 1 unit of human DNA topo 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 or 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 supercoiled DNA was quantitated using AlphaImager™ (Alpha Innotech Corporation).

4.4.2. Antiproliferative assay

Cells were cultured according to the supplier's instructions. Cells were seeded in 96-well plates at a density of 2~4 x 10^4 cells per well and incubated for overnight in 0.1 mL of media supplied with 10% fetal bovine serum (FBS, Hyclone, USA) in 5% CO_2 incubator at 37 °C. On day 2, after FBS starvation for 4 h, culture medium in each well was exchanged with 0.1 mL aliquots of medium containing graded concentrations of compounds. After 72 h treatment of compound, 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 used as positive controls were purchased from Sigma.

4.4.3. Cleavage complex stabilization assay

The mixture of 250 ng of supercoiled DNA pBR322 and 3 units of human topo IIα with or without compound at the designated concentrations in the Figure legend was incubated at 37 °C for 30 min in the topo II relaxation assay buffer. The reaction was then terminated by the addition of 2.5 μL of stop solution (5% SDS, 25% ficoll and 0.05% bromophenol blue) followed by treatment of 2 μL of 0.25 mg/mL proteinase K (Sigma, USA) with continuous incubation at 45 °C for 30 min to eliminate the protein. Samples were electrophoresed on a 1% (w/v) agarose gel containing 0.5 μg/mL EtBr at 30 V for 6 h in TAE running buffer. DNA bands on the gel were detected by UV and visualized by AlphaImager™.

4.4.4. Band depletion assay

T47D cells were seeded overnight at a density of 2 x 10^5 cells per well. The cells were treated for 2 h at 37 °C with each of etoposide and compound **30**, and co-treated with etoposide and compound **30** followed by cell harvesting. The cell pellet after centrifugation (4 °C, 3200 rpm, and 3 min) was washed with 1 mL of PBS and then ice-incubated for 1.5 h, followed by lysis with denaturing agent (62.5 mM Tris-HCl (pH 6.8), 1 mM EDTA and 2% SDS) and sonication (10e20 bursts, 2 s). The samples were ice-incubated for 20 min and centrifuged for 20 min (4 °C, 12,000 rpm and 30 min) again. Finally the supernatant of sample was taken to perform Western blot analysis on 10% SDS-PAGE gels.

4.4.5. Competitive EtBr displacement assay

Calf thymus DNA (ctDNA) (30 μM) was mixed with EtBr (20 μM) in the 10 mM tris buffer solution (pH 7.2), followed by incubation for 30 min at room temperature with shaking. Each compound (m-AMSA and compound **30**) was added to the well containing the mixture of EtBr and ctDNA with increased concentrations up to 40 μM as designated in the Figure legend. The DMSO was added for untreated control in the same amount as compound addition and the final DMSO amount was equally 1% for all samples. After addition of compound, the plate was continuously incubated for 30 min at room temperature with shaking. The fluorescence intensity of EtBr was measured by TECAN multimode microplate reader (Swizerland) with excitation at 471 nm. The emission spectrum was collected at 500~700 nm.

4.4.6. Comet Assay

To evaluate DNA damage, comet assay was performed using single-cell gel electrophoresis

with a Trevigen kit (Gaithersburg, USA) according to the method previously reported [50]. Briefly, T47D cells, seeded in a density of 1 x 10^5 cells per well in six-well plates were treated with compound **30** and etoposide for 24 h at the designated concentration in Figure legend and harvested by trypsinization followed by resuspending cells in 1 mL of ice-cold PBS. Then, 8 μL of resuspened cells were mixed with 80 μL of low-melting agarose at 37 °C, spread on slides and solidified in the dark for 40 min at 4 °C. Slides were lysed in ice-cold lysis solution in the dark for 30 min at 4 °C and then submerged in a fresh alkaline solution (pH > 13) at room temperature for 30 min to allow alkaline unwinding. Electrophoresis was performed under alkaline conditions for 20 min for 15 V. Slides were rinsed twice with distilled water, once with 70% ethanol and stained with SYBR Green (Trevigen INC., USA) in a TE buffer for 5 min in the dark at 4 °C. Comet images were obtained using an inverted fluorescence microscope (Zeiss, Axiovert 200) at 10X magnification and percent DNA in tail was analyzed by Komet 5.0 software (Kinetic imaging Ltd, UK). Data were represented both by imaging and graphically by randomly selecting comet lengths of HCT15 cells.

4.4.7. Western blot analysis.

T47D cells were grown on 60 mm tissue culture dishes in a density of 1 x 10^6 cells until reaching 80% confluency. The cells were then treated with compound **30** in concentrations of 10 and 30 μM for 24 h followed by lysis with a lysis buffer solution containing 50 mM Tris HCl, 300 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF and 1% protease inhibitor cocktail. 40 μg of protein per sample was resolved by 12% SDS-PAGE and transferred to a PVDF membrane (Millipore, USA). The membranes were blocked with 5% skim milk in Tris buffered saline containing 0.1% tween 20 (TBST) and

probed with primary antibodies in a dilution ratio of 1:1000 for 2~3 h. The blots were washed, exposed to HRP-conjugated anti-rabbit IgG (Cell Signaling Technology Inc. USA) in a dilution ratio of 1:2000 for 2 h, and detected with ECL western blotting detection reagent (Animal genetics Inc, Korea). All primary antibodies used were purchased from Cell Signaling Technology Inc. (USA). Western blot images were taken by LAS-3000 (Fuji Photo Film Co., Ltd, Japan) and analyzed using Multi-Gauge Software (Fuji Photo Film Co. Ltd., Japan).

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Highlights

- Dihydroxylated 2,6-diphenyl-4-chlorophenylpyridines were designed and synthesized.
- Introduction of chlorine on 4-phenyl ring of central pyridine showed strong dual topo I and IIα inhibitor.
- Compound **30** exhibited the most potent dual topo I and IIα inhibition with strong antiproliferative activity.
- Compound **30** acts as a DNA non-intercalative catalytic topo IIα inhibitor.