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2,4,6-Trisubstituted pyridines: Synthesis, topoisomerase I and II inhibitory activity, cytotoxicity, and structure–activity relationship

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Abstract—Designed and synthesized were a series of pyridines substituted at 2, 4, and 6 positions with various 5- or 6-memberd heteroaromatics as antitumor agents. They were evaluated their topoisomerase I and II inhibitory activities along with cytotoxicities against several human cancer cell lines. Among the prepared compounds, **10**–**20** showed significant topoisomerase I or II inhibitory activities, and **21**–**26** showed considerable cytotoxicities against several human cancer cell lines. Structure–activity relationship study indicates that 4′-pyridine at 6-position of central pyridine plays a key role in biological activity.

1. Introduction

Topoisomerases are nuclear enzymes that play crucial roles in DNA metabolism such as replication, transcription, recombination, repair, chromatin assembly, and chromosome segregation.[1–3] Topoisomerases I (Topo I) are monomeric and catalyze an ATP-independent relaxation of DNA supercoils by transiently breaking and religating single-stranded DNA, whereas topoisomerases II (Topo II) are dimeric and relax supercoiled DNA through catalysis of a transient breakage of double-stranded DNA in an ATP-dependent manner.[1,4] Due to the crucial role of topoisomerases in the maintenance and replication of DNA during proliferation, cells become highly vulnerable when these functions are lost.[5] Consequently, Topo I and II have been attractive targets for design of antitumor agents.[6]

α-Terpyridine has been precious due to its ability to form metal complexes[7] and as DNA binding agents since its discovery in 1932.[8] Our research group has previously reported that terpyridine derivatives showed strong cytotoxicities against several human cancer cell lines and considerable Topo I inhibitory activities.[9,10] Some of us also reported that terthiophene derivatives, bioisosteres of terpyridine, showed considerable PKC inhibitory activities and antitumor cytotoxicities against several human cancer cell lines.[11] Although Topo I inhibitory activity and cytotoxicity of terpyridine derivatives have been reported, the effects of terpyridine derivatives on Topo I and II, and cytotoxicity have not been studied systematically.

In an extension to our work, we studied the synthesis of a series of 2,4,6-trisubstituted pyridine derivatives for the evaluation of Topo I and II inhibitory activities and cytotoxicities. In order to study the structure–activity relationship as a whole, eight different heteroaromatics including phenyl were taken as $\mathbf{R}^1$ (**a**–**h**) and $\mathbf{R}^2$ (**a**–**d**) systematically to prepare compounds **7**, **8**, and **9** (Fig. 1 and Scheme 1).

2. Results and discussion

We have previously reported the synthesis of various 2,4,6-trisubstituted pyridine derivatives.[9,10] A similar synthetic sequence was employed to prepare the new 2,4,6-trisubstituted pyridine derivatives. Scheme 1 illustrates the synthetic method for the preparation of pyridine derivatives. At first, we prepared thirty-two intermediates (**3**) in 60.3–97.5% yield by the condensation of eight different aryl aldehydes (**1a**–**h**) with four

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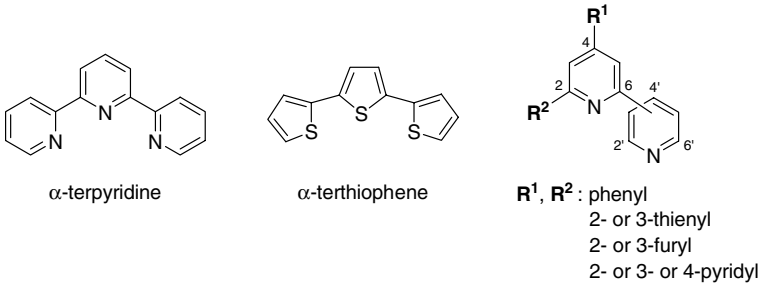
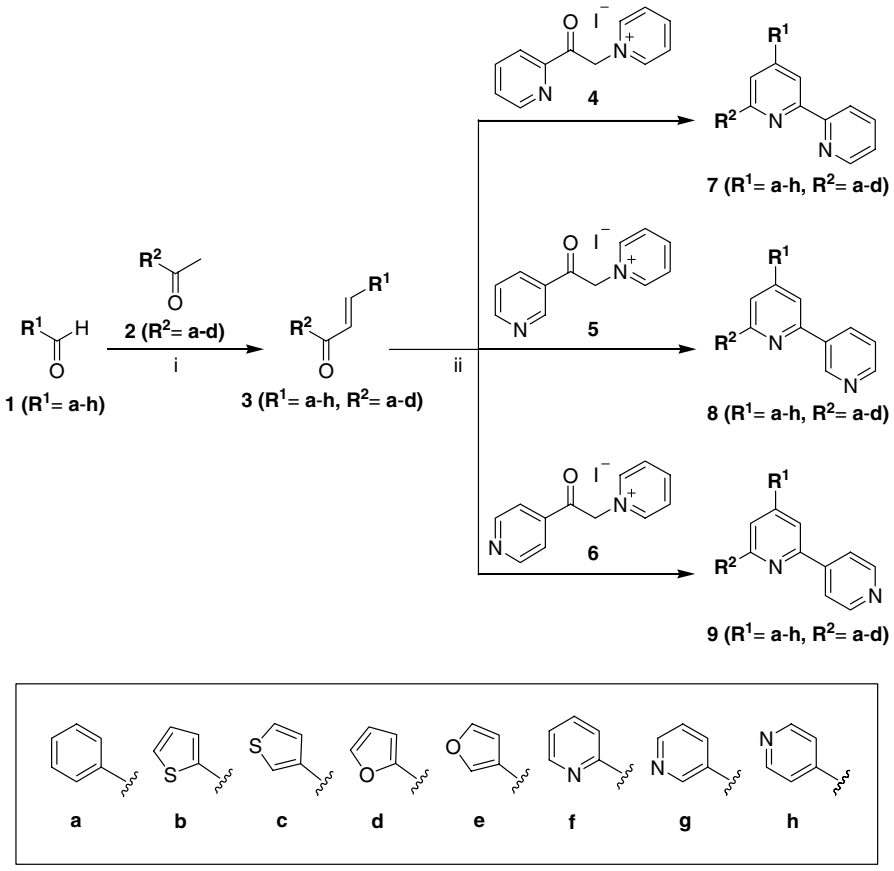


Figure 1. Structures of α-terpyridine, α-terthiophene, and 2,4,6-trisubstituted pyridines.



Scheme 1. General synthetic scheme of 2,4,6-trisubstituted pyridines. Reagents and conditions: (i) aryl acetyl ketones **2a**–**d** (1.0 eq), KOH (1.1 eq), MeOH/H_2O (5:1), 0 °C, 3 h, 60.3–97.5%; (ii) **4**, **5**, or **6** (1.2 eq), NH_4OAc (10.0 eq), MeOH, 20–80 °C, 12–24 h, 20.5–92.5%.

different aryl methyl ketones (**2a**–**d**), respectively. Other three key intermediates, 1-(2-oxo-2-pyridin-2-ylethyl)pyridinium iodide (**4**), 1-(2-oxo-2-pyridin-3-ylethyl)pyridinium iodide (**5**), and 1-(2-oxo-2-pyridin-4-ylethyl)pyridinium iodide (**6**), were prepared in quantitative yield from 2-, 3-, or 4-acetyl pyridine with iodine in pyridine (scheme not shown). Using modified Kröhnke synthesis,[12] ninety-six final products **7** (R^1 = **a**–**h**, R^2 = **a**–**d**), **8** (R^1 = **a**–**h**, R^2 = **a**–**d**), and **9** (R^1 = **a**–**h**, R^2 = **a**–**d**) were synthesized by the condensation of thirty two intermediates **3** with **4**, **5**, and **6**, respectively, in 20.5–92.5% yield. We noted that the yield dramatically dropped when phenyl moiety was placed in 2-position of the central pyridine.

From the preliminary study, compounds **10** (**9**, R^1 = **b**, R^2 = **d**), **11** (**7**, R^1 = **c**, R^2 = **b**), **12** (**9**, R^1 = **c**, R^2 = **b**), **13** (**9**, R^1 = **c**, R^2 = **d**), **14** (**9**, R^1 = **g**, R^2 = **d**), **15** (**9**, R^1 = **g**, R^2 = **b**), **16** (**9**, R^1 = **h**, R^2 = **c**), **17** (**9**, R^1 = **e**, R^2 = **b**), **18** (**9**, R^1 = **e**, R^2 = **c**), **19** (**7**, R^1 = **e**, R^2 = **d**), and **20** (**9**, R^1 = **e**, R^2 = **d**) were selected for the further evaluation of Topo I and II inhibitory activities, and compounds **21** (**9**, R^1, R^2 = **b**), **22** (**9**, R^1 = **b**, R^2 = **c**), **23** (**9**, R^1, R^2 = **c**), **24** (**9**, R^1 = **d**, R^2 = **b**), **25** (**8**, R^1 = **c**, R^2 = **b**), and **26** (**8**, R^1 = **d**, R^2 = **b**) were selected for the evaluation of cytotoxicities. The structures of the selected compounds are shown in Figure 2. The rest of the compounds were excluded since they did not display considerable activities.

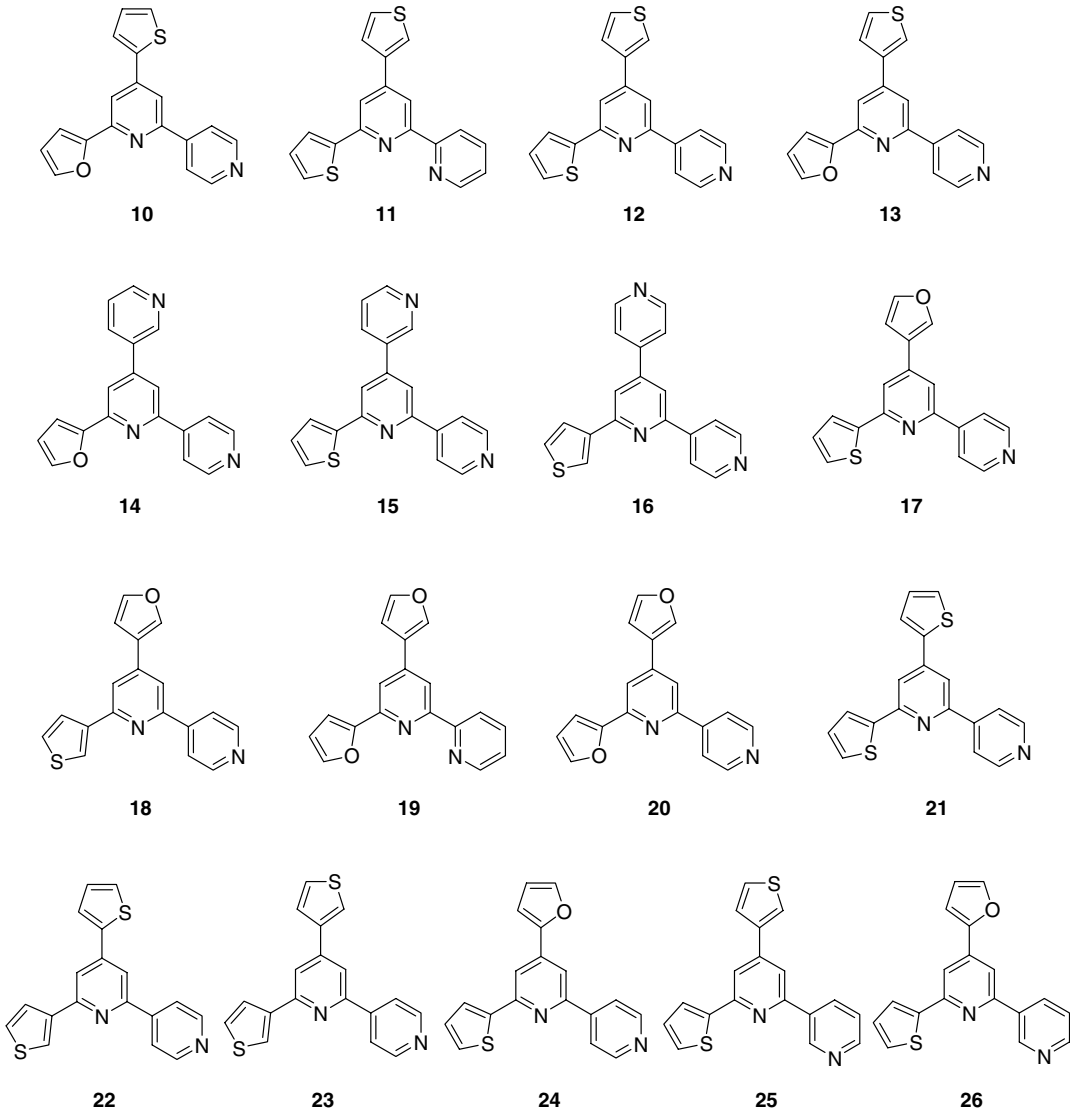


Figure 2. Structures of the selected compounds bearing considerable biological activities.

2.1. Topo I and II inhibitory activity of compounds 10–20

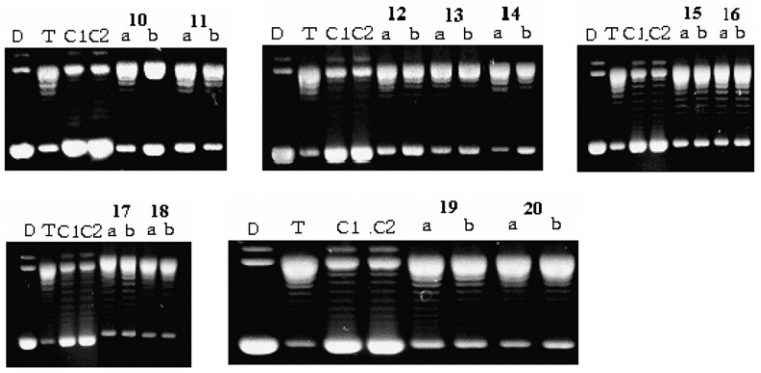
Figure 3 shows the conversion of supercoiled plasmid DNA by calf thymus Topo I in the presence of compounds **10** to **20** with camptothecin, a well-known Topo I inhibitor, as a positive control. Compounds **10**, **11**, **12**, and **14** showed significant inhibitory activities at 20 μM and 100 μM concentration and the rest of the indicated compounds showed moderate inhibitory activities when compared to camptothecin. Figure 4 shows human Topo II inhibitory activities of 2,4,6-trisubstituted pyridine derivatives (**10**–**20**) with etoposide as a positive control. Compounds **13**, **15**–**20** showed significant inhibitory effects on human DNA Topo II at 20 μM and 100 μM concentration and the rest of the indicated compounds displayed weak effects when compared to etoposide. It is interesting to note that the compounds which significantly inhibited Topo I displayed weak inhibitory activities on Topo II, whereas the compounds which significantly inhibited Topo II displayed moderate inhibitory activities on Topo I.

2.2. Cytotoxicity of compounds 21–26

Cytotoxicities of compounds **21**–**26** were evaluated on four different human cancer cell lines A549 (human kidney carcinoma), SK-OV-3 (human ovary adenocarcinoma), SK-MEL-2 (human malignant melanoma), and HCT15 (human colon adenocarcinoma). For the evaluation of antitumor activities, cytotoxicities were expressed in μM using etoposide as a positive control. Table 1 indicates that the prepared compounds **21**–**26** show considerable cytotoxicities against several human cancer cell lines especially on HCT15 when compared to etoposide. As in the previous study on terpyridine derivatives,[9,10] strong Topo I and II inhibitors have not displayed strong cytotoxicities and vice versa.

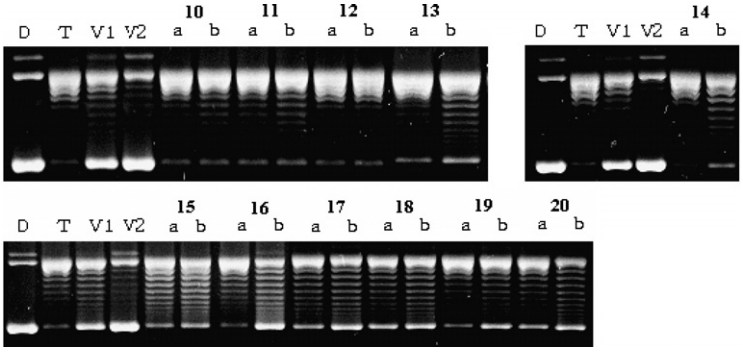
3. Conclusions

We have designed and prepared a series of ninety-six 2,4,6-trisubstituted pyridine derivatives by efficient



Lane D: pBR322 DNA only
Lane T: pBR322 DNA + Topo I
Lane C1: pBR322 DNA + Topo I + Camptothecin 20 μM
Lane C2: pBR322 DNA + Topo I + Camptothecin 100 μM
Lane 10–20 Lane a: pBR322 DNA + Topo I + Compound 20 μM
Lane 10–20 Lane b: pBR322 DNA + Topo I + Compound 100 μM

Figure 3. Calf thymus Topo I inhibitory effects of compounds **10**–**20**.



Lane D: pBR322 DNA only
Lane T: pBR322 DNA + Topo II
Lane V1: pBR322 DNA + Topo II + Etoposide 20 μM
Lane V2: pBR322 DNA + Topo II + Etoposide 100 μM
Lane 10 - 20 Lane a: pBR322 DNA + Topo II + Compound 20 μM
Lane 10 - 20 Lane b: pBR322 DNA + Topo II + Compound 100 μM

Figure 4. Human Topo II inhibitory effects of compounds **10**–**20**.

Table 1. Antitumor cytotoxicity of the prepared compounds **21**–**26**

Compound	IC_{50} (μM)			
	A549	SK-OV-3	SK-MEL-2	HCT15
21	4.16	5.75	3.68	1.72
22	5.69	14.52	8.94	3.79
23	27.83	9.86	9.76	2.54
24	10.94	8.69	5.43	4.18
25	2.73	7.49	5.61	4.06
26	14.51	5.90	1.95	5.87
Etoposide	0.32	1.72	0.30	2.43

synthetic route, and evaluated their Topo I and II inhibitory activity and antitumor cytotoxicity. A structure–activity relationship study of the 2,4,6-trisubstituted pyridine derivatives for Topo I and II inhibitory activities indicates that 2,4′- and 2,2′-bipyridyl moieties with combination of **b**, **c**, **d**, **e**, **g**, and **h** moieties were important to display Topo I and II inhibitory activities. For cytotoxicities, 2,4′- and 2,3′-bipyridyl moieties with combination of **b**, **c**, and **d** moieties were important. There were no direct correlations between antitumor cytotoxicity and topoisomerase inhibitory activity. This study may provide valuable information to the researchers working on the development of antitumor agents.

4. Experimental

Compounds used as starting materials and reagents were purchased from Aldrich Chemical Co., Sigma, Fluka, Junsei and used without further purification. Thin-layer chromatography (TLC) and column chromatography (CC) were performed with Kieselgel $60F_{254}$

(Merck) and silica gel (Kieselgel 60, 230–400 mesh, Merck), respectively. Since all the compounds prepared contain aromatic rings, compounds 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 TMS (tetramethylsilane) was used as an internal standard. 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. 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 1 μl injection volume on a Waters XTerra® 3.5 μm reverse-phase C_{18} column (2.1 × 100 mm) with a gradient elution from 5% to 95% of B in A for 20 min followed by 95% B in A for 10 min at a flow rate of 200 μL/min, where mobile phase A was 100% distilled water with 50 mM ammonium formate and mobile phase B was 100% acetonitrile. MS ionization conditions were: Sheath gas flow rate: 70 arb, aux gas flow rate: 20 arb, I spray voltage: 4.5 KV, capillary temp.: 215 °C, capillary voltage: 21 V, tube lens offset: 10 V.

4.1. General method for the preparation of 3

Aryl aldehyde was added to the solution of 85% KOH (1.2 eq) in MeOH (50 mL) and H_2O (10 mL) at 0 °C. After dissolution, aryl methyl ketone (1.0 eq) was added over a period of 10 min. The mixture was then stirred for 3 h at 0 °C. A solid product precipitated was filtered, washed with cold MeOH, and dried to yield 60.3–97.5%. Utilizing the same procedure, thirty-two compounds were synthesized.

4.2. General method for preparation of 4–6

A mixture of acetyl pyridine, iodine (1.2 eq) and pyridine 60 mL was refluxed for 3 h. The solid product precipitated was cooled to 0 °C and filtered. The product was washed with cold pyridine and dried to afford **4–6** in quantitative yield.

4.2.1. 1-[2-Oxo-(2-pyridin-2-yl-)ethyl]pyridinium iodide (4). Grayish black solid; mp >300 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.00 (d, J = 6.4 Hz, 2H, pyridinium H-2, H-6), 8.86 (dd, J = 4.8, 0.9 Hz, 1H, pyridine H-6), 8.72 (dt, J = 7.8, 1.2 Hz, 1H, pyridinium H-4), 8.27 (t, J = 6.6 Hz, 2H, pyridinium H-3, H-5), 8.13–8.02 (m, 2H, pyridine H-3, H-4), 7.83 (ddd, J = 7.2, 4.8, 1.5 Hz, 1H, pyridine H-5), 6.50 (s, 2H, –CO–CH_2–).

4.2.2. 1-[2-Oxo-(2-pyridin-3-yl-)ethyl]pyridinium iodide (5). Brown solid; mp 180.2 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.23 (br, 1H, pyridine H-2), 8.99 (d, J = 6.5 Hz, 2H, pyridinium H-2, H-6), 8.92 (dd, J = 4.8, 1.5 Hz, 1H, pyridine H-6), 8.76 (dt, J = 7.8, 1.2 Hz, 1H, pyridinium H-4), 8.42 (dd d, J = 8.0, 1.5, 0.6 Hz, 1H, pyridine H-4) 8.30 (t, J = 6.6 Hz, 2H, pyridinium H-3, H-5), 7.72 (dd, J = 7.7, 4.8 Hz, 1H, pyridine H-5), 6.48 (s, 2H, –CO–CH_2–).

4.2.3. 1-[2-Oxo-(2-pyridin-4-yl-)ethyl]pyridinium iodide (6). Brown solid; mp 130.0 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.98 (d, J = 6.4 Hz, 2H, pyridinium H-2, H-6), 8.95 (dd d, J = 4.8, 1.4, 0.9 Hz, 2H, pyridine H-2, H-6), 8.76 (dt, J = 7.8, 1.1 Hz, 1H, pyridinium H-4), 8.30 (t, J = 6.7 Hz, 2H, pyridinium H-3, H-5), 7.95 (dd d, J = 4.8, 1.4, 0.9 Hz, 2H, pyridine H-3, H-5), 6.45 (s, 2H, –CO–CH_2–).

4.3. General method for the preparation of 10–26

A mixture of **3** ($\mathbf{R^1}$ = **a**–**h**, $\mathbf{R^2}$ = **a**–**d**), dry ammonium acetate and **4**, **5**, or **6** in dry MeOH was heated to 80 °C for 12–24 h under nitrogen gas. The solvent was removed by evaporation under reduced pressure, and the residue was diluted with ethyl acetate (100 mL), washed with water (75 mL × 2) and saturated NaCl solution (50 mL). The organic layer was dried with magnesium sulfate and filtered. The filtrate was evaporated at reduced pressure, which was purified by silica gel column chromatography with a gradient elution of ethyl acetate/*n*-hexane to afford a white solid in 20.5–92.5% yield. Utilizing the same procedure, ninety-six compounds were synthesized.

4.3.1. 6-(Furan-2-yl)-4-(thiophene-2-yl)-[2,4′]-bipyridyl (10). The same procedure described at 4.3 was employed with **3** ($\mathbf{R^1}$ = **b**, $\mathbf{R^2}$ = **d**) (1.02 g, 5.0 mmol), anhydrous ammonium acetate (3.85 g, 50.0 mmol), **6** (1.63 g, 5.0 mmol), and dry MeOH (30 mL) to yield a white solid (335 mg, 22.0%). R_f (ethyl acetate/*n*-hexane 2:1): 0.3; mp 140.5 °C; LC MS/MS: retention time: 18.07 min; $[MH]^+$: 305.2; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.77 (dd, J = 4.6, 1.6 Hz, 2H, 2-pyridine H-2′, H-6′), 8.01(dd, J = 4.6, 1.6 Hz, 2H, 2-pyridine H-3′, H-5′), 7.93 (d, J = 1.2 Hz, 1H, pyridine H-3), 7.82 (d, J = 1.2 Hz, 1H, pyridine H-5), 7.48 (dd, J = 3.7, 0.9 Hz, 1H, 4-thiophene H-5), 7.60 (dd, J = 1.7, 0.9 Hz, 1H, 6-furan H-5), 7.48 (dd, J = 5.0, 0.9 Hz, 1H, 4-thiophene H-3), 7.24 (d, J = 3.4 Hz, 1H, 6-furan H-3), 7.19 (dd, J = 5.0, 3.7 Hz, 1H, 4-thiophene H-4), 6.60 (dd, J = 3.4, 1.8 Hz, 1H, 6-furan H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 155.04, 153.41, 150.40, 150.23, 146.13, 143.61, 143.29, 141.06, 128.51, 127.46, 125.73, 121.17, 115.25, 114.55, 112.23, 109.61.

4.3.2. 4-(Thiophene-3-yl)-6-(thiophene-2-yl)-[2,2′]-bipyridyl (11). The same procedure described at 4.3 was employed with **3** ($\mathbf{R^1}$ = **c**, $\mathbf{R^2}$ = **b**) (1.10 g, 5.0 mmol), anhydrous ammonium acetate (3.85 g, 50.0 mmol), **4** (1.63 g, 5.0 mmol), and dry MeOH (30 mL) to yield a white solid (360 mg, 22.5%). R_f (ethyl acetate/*n*-hexane 2:1): 0.5; mp 156.2 °C; LC MS/MS: retention time: 20.12 min; $[MH]^+$: 321.2; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.71 (dd d, J = 4.7, 1.6, 0.7 Hz, 1H, 2-pyridine H-6′), 8.61 (d, J = 8.0 Hz, 1H, 2-pyridine H-3′), 8.53 (d, J = 1.5 Hz, 1H, pyridine H-3), 7.87 (td, J = 7.7, 1.8 Hz, 1H, 2-pyridine H-4′), 7.86 (d, J = 1.5 Hz, 1H, pyridine H-5), 7.84 (dd, J = 2.8, 1.2 Hz, 1H, 4-thiophene H-2), 7.73 (dd, J = 3.7, 1.0 Hz, 1H, 6-thiophene H-5), 7.61 (dd, J = 5.0, 1.2 Hz, 1H, 4-thiophene H-5), 7.47 (dd, J = 5.0, 2.9 Hz, 1H, 4-thiophene H-4), 7.44 (dd, J = 5.1, 1.0 Hz, 1H, 6-thiophene H-3), 7.35 (dd d, J = 7.5, 4.8, 1.2 Hz, 1H, 2-pyridine H-5′), 7.16 (dd, J = 5.0, 3.7 Hz, 1H, 6-thiophene H-4); ^{13}C NMR

(62.5 MHz, $CDCl_3$) δ 156.16, 155.80, 152.45, 148.97, 146.39, 145.24, 144.50, 140.83, 139.80, 136.96, 128.02, 127.64, 126.85, 126.07, 124.57, 123.93, 123.41, 121.52, 116.44, 115.75.

4.3.3. 4-(Thiophene-3-yl)-6-(thiophene-2-yl)-[2,4′]-bipyridyl (12). The same procedure described at 4.3 was employed with **3** ($\mathbf{R}^1$ = **c**, $\mathbf{R}^2$ = **b**) (1.10 g, 5.0 mmol) anhydrous ammonium acetate (3.85 g, 50.0 mmol), **6** (1.63 g, 5.0 mmol), and dry MeOH (30 mL) to yield a white solid (328 mg, 20.5%). R_f (ethyl acetate/*n*-hexane 2:1): 0.3, mp 154.4 °C; LC MS/MS: retention time: 18.52 min; $[MH]^+$: 321.3; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.76 (dd, J = 4.6, 1.6 Hz, 2H, 2-pyridine H-2′, H-6′), 8.03(dd, J = 4.6, 1.6 Hz, 2H, 2-pyridine H-3′, H-5′), 7.82 (br, 2H, pyridine H-3, H-5), 7.76 (dd, J = 2.6, 1.6 Hz, 1H, 4-thiophene H-2), 7.72 (dd, J = 3.7, 1.0 Hz, 1H, 6-thiophene H-5), 7.53 (dd, J = 5.0, 1.6 Hz, 1H, 4-thiophene H-5), 7.50 (dd, J = 5.0, 2.7 Hz, 1 H, 4-thiophene H-4), 7.45 (dd, J = 5.0, 1.0 Hz, 1H, 6-thiophene H-3), 7.16 (dd, J = 5.0, 3.7 Hz, 1H, 6-thiophene H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.57, 153.28, 150.36, 145.99, 144.74, 144.68, 139.39, 128.14, 128.06, 127.34, 127.04, 126.30, 125.80, 125.16, 125.00, 124.19, 123.48, 121.24, 121.08, 116.20, 115.89.

4.3.4. 6-(Furan-2-yl)-4-(thiophene-3-yl)-[2,4′]-bipyridyl (13). The same procedure described at 4.3 was employed with **3** ($\mathbf{R}^1$ = **c**, $\mathbf{R}^2$ = **d**) (1.02 g, 5.0 mmol), anhydrous ammonium acetate (3.85 g, 50.0 mmol), **6** (1.63 g, 5.0 mmol), and dry MeOH (30 mL) to yield a white solid (400 mg, 26.8%). R_f (ethyl acetate/*n*-hexane 2:1): 0.3; mp 120.6 °C; LC MS/MS: retention time: 17.50 min; $[MH]^+$: 305.3; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.75 (dd, J = 4.6, 1.6 Hz, 2H, 2-pyridine H-2′, H-6′), 8.00 (dd, J = 4.6, 1.6 Hz, 2H, 2-pyridine H-3′, H-5′), 7.91 (d, J = 1.4 Hz, 1H, pyridine H-3), 7.80 (d, J = 1.4 Hz, 1H, pyridine H-5), 7.77 (dd, J = 2.8, 1.4 Hz, 1H, 4-thiophene H-2), 7.57 (dd, J = 1.7, 0.6 Hz, 1H, 6-furan H-5), 7.54 (dd, J = 5.0, 1.4 Hz, 1H, 4-thiophene H-5), 7.48 (dd, J = 5.0, 2.9 Hz, 1H, 4-thiophene H-4), 7.24 (dd, J = 3.4, 0.5 Hz, 1H, 6-furan H-3), 6.58 (dd, J = 3.4, 1.8 Hz, 1H, 6-furan H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.87, 153.54, 151.42, 150.30, 150.17, 146.27, 144.56, 143.48, 140.65, 139.38, 127.22, 126.27, 126.01, 125.84, 125.76, 123.48, 122.60, 121.57, 121.16, 120.69, 116.13, 115.53, 113.03, 112.18, 111.57, 109.42.

4.3.5. 6′-(Furan-2-yl)-[3,4′;2′,4″]-terpyridine (14). The same procedure described at 4.3 was employed with **3** ($\mathbf{R}^1$ = **g**, $\mathbf{R}^2$ = **d**) (0.99 g, 5.0 mmol), anhydrous ammonium acetate (3.85 g, 50.0 mmol), **6** (1.63 g, 5.0 mmol), and dry MeOH (30 mL) to yield a white solid (900 mg, 60.0%). R_f (ethyl acetate/*n*-hexane 2:1): 0.19; mp 184.3 °C; LC MS/MS: retention time: 13.82 min; $[MH]^+$: 300.3; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.01 (d, J = 2.2 Hz, 1H, pyridine H-2), 8.77 (dd, J = 4.6, 1.6 Hz, 2H, pyridine H-2″, H-6″), 8.74 (dd, J = 4.8, 1.6 Hz, 1H, pyridine H-6), 8.06 (dt, J = 7.9, 1.9 Hz, 1H, pyridine H-4), 8.03 (dd, J = 4.8, 1.6 Hz, 2H, pyridine H-3″, H-5″), 7.95 (d, J = 1.4 Hz, 1H, pyridine H-3′), 7.84 (d, J = 1.4 Hz, 1H, pyridine H-5′), 7.60 (dd, J = 1.8, 0.7 Hz, 1H, 6′-furan H-5), 7.49 (ddd, J = 8.0, 4.8, 0.7 Hz, 1H, pyridine H-5), 7.29 (d, J = 3.4 Hz, 1H, 6′-furan H-3), 6.61 (dd, J = 3.4, 1.8 Hz, 1H, 6′-furan H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 155.64, 153.74, 150.86, 148.57, 147.72, 146.40, 144.22, 134.89, 134.37, 124.29, 121.61, 117.23, 116.74, 112.74, 110.34.

4.3.6. 6′-(Thiophene-2-yl)-[3,4′;2′,4″]-terpyridine (15). The same procedure described at 4.3 was employed with **3** ($\mathbf{R}^1$ = **g**, $\mathbf{R}^2$ = **b**) (0.54 g, 2.5 mmol), anhydrous ammonium acetate (1.93 g, 25.0 mmol), **6** (0.82 g, 2.5 mmol), and dry MeOH (20 mL) to yield a white solid (320 mg, 40.4%). R_f (ethyl acetate/*n*-hexane 3:1): 0.21; mp 199.8 °C; LC MS/MS: retention time: 15.04 min; $[MH]^+$: 316.3; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.99 (br, 1H, pyridine H-2), 8.76 (br, 2H, pyridine H-2″, H-6″), 8.76 (br, 1H, pyridine H-6), 8.05 (br, 2H, pyridine H-3″, H-5″), 8.00 (dt, J = 7.8, 1.8 Hz, 1H, pyridine H-4), 7.84 (d, J = 1.2 Hz, 1H, pyridine H-3′), 7.83 (d, J = 1.2 Hz, 1H, pyridine H-5′), 7.76 (dd, J = 3.7, 1.0 Hz, 1H, 6′-thiophene H-5), 7.51 (br, 1H, pyridine H-5), 7.48 (dd, J = 5.0, 1.0 Hz, 1H, 6′-thiophene H-3), 7.18 (dd, J = 5.0, 3.7 Hz, 1H, 6′-thiophene H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.88, 153.53, 150.46, 148.15, 147.43, 145.62, 144.80, 144.31, 140.90, 134.47, 133.98, 133.30, 128.54, 128.18, 125.87, 125.36, 123.89, 121.31, 116.81, 116.65.

4.3.7. 6′-(Thiophen-2-yl)-[4,2′;4′,4″]-terpyridine (16). The same procedure described at 4.3 was employed with **3** ($\mathbf{R}^1$ = **h**, $\mathbf{R}^2$ = **c**) (1.08 g, 5.0 mmol), anhydrous ammonium acetate (3.86 g, 50.0 mmol), **6** (1.63 g, 5.0 mmol), and dry MeOH (30 mL) to yield a white solid (1.26 g, 79.6%). R_f (ethyl acetate/*n*-hexane 3:1): 0.12, mp 239.5 °C; LC MS/MS: retention time: 14.95 min; $[MH]^+$: 316.3; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.81 (dd, J = 4.5, 1.6 Hz, 2H, pyridine H-2, H-6), 8.79 (dd, J = 4.5, 1.6 Hz, 2H, pyridine H-2″, H-6″), 8.05 (dd, J = 4.5, 1.6 Hz, 2H, pyridine H-3, H-5), 7.85 (br, 2H, pyridine H-3′, H-5′), 7.76 (dd, J = 3.7, 1.0 Hz, 1H, 6′-thiophene H-5), 7.62 (dd, J = 4.5, 1.6 Hz, 2H, pyridine H-3″, H-5″), 7.49 (dd, J = 5.0, 1.0 Hz, 1H, 6′-thiophene H-3), 7.18 (dd, J = 5.0, 3.7 Hz, 1H, 6′-thiophene H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 155.03, 153.67, 150.77, 150.51, 147.87, 145.63, 145.49, 144.18, 128.65, 128.21, 125.45, 121.51, 121.05, 116.59, 116.49.

4.3.8. 4-(Furan-3-yl)-6-(thiophene-2-yl)-[2,4′]-bipyridyl (17). The same procedure described at 4.3 was employed with **3** ($\mathbf{R}^1$ = **e**, $\mathbf{R}^2$ = **b**) (0.71 g, 3.5 mmol), anhydrous ammonium acetate (2.70 g, 35.0 mmol), **6** (1.14 g, 3.5 mmol), and dry MeOH (20 mL) to yield a white solid (250 mg, 23.6%). R_f (ethyl acetate/*n*-hexane 1:2): 0.12; mp 161.9 °C; LC MS/MS: retention time: 21.47 min; $[MH]^+$: 305.2; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.75 (dd, J = 4.5, 1.6 Hz, 2H, 2-pyridine H-2′, H-6′), 8.03 (dd, J = 4.5, 1.6 Hz, 2H, 2-pyridine H-3′, H-5′), 7.99 (br, 1H, 4-furan H-2), 7.73 (d, J = 1.3 Hz, 1H, pyridine H-3), 7.71 (d, J = 1.3 Hz, 1H, pyridine H-5), 7.70 (dd, J = 3.7, 1.0 Hz, 1H, 6-thiophene H-5), 7.59 (t, J = 1.7 Hz, 1H, 4-furan H-5), 7.46 (dd, J = 5.0, 1.0 Hz, 1H, 6-thiophene H-3), 7.16 (dd, J = 5.0, 3.7 Hz, 1H, 6-thiophene H-4), 6.84 (dd, J = 1.8, 0.8 Hz, 1H, 4-furan H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.48, 153.24, 150.54, 150.25, 146.00, 144.57, 144.51, 141.96, 140.53,

128.16, 128.06, 126.88, 125.01, 124.25, 122.84, 121.10, 120.40, 115.60, 115.28, 108.34, 107.63.

4.3.9. 4-(Furan-3-yl)-6-(thiophene-3-yl)-[2,4′]-bipyridyl (18). The same procedure described at 4.3 was employed with **3** (**R**^{1} = **e**, **R**^{2} = **c**) (0.71 g, 3.5 mmol), anhydrous ammonium acetate (2.70 g, 35.0 mmol), **6** (1.14 g, 3.5 mmol), and dry MeOH (20 mL) to yield a white solid (400 mg, 37.8%). R_f (ethyl acetate/*n*-hexane 1:2): 0.12; mp 160.5 °C; LC MS/MS: retention time: 21.28 min; $[MH]^+$: 305.2; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.76 (dd, J = 4.6, 1.5 Hz, 2H, 2-pyridine H-2′, H-6′), 8.06 (dd, J = 3.0, 1.1 Hz, 1H, 6-thiophene H-3), 8.03 (dd, J = 4.6, 1.5 Hz, 2H, 2-pyridine H-3′, H-5′), 7.99 (br, 1H, 4-furan H-2), 7.80 (dd, J = 5.0, 1.1 Hz, 1H, 6-thiophene H-5), 7.73 (d, J = 1.1 Hz, 1H, pyridine H-3), 7.71 (d, J = 1.1 Hz, 1H, pyridine H-5), 7.59 (t, J = 1.6 Hz, 1H, 4-furan H-5), 7.45 (dd, J = 5.0, 3.0 Hz, 1H, 6-thiophene H-4), 6.84 (dd, J = 1.7, 0.8 Hz, 1H, 4-furan H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.73, 154.14, 150.36, 146.38, 144.51, 141.95, 141.84, 140.45, 126.45, 126.28, 124.42, 124.21, 121.15, 116.85, 115.64, 108.37.

4.3.10. 6-(Furan-2-yl)-4-(furan-3-yl)-[2,2′]-bipyridyl (19). The same procedure described at 4.3 was employed with **3** (**R**^{1} = **e**, **R**^{2} = **d**) (0.66 g, 3.5 mmol), anhydrous ammonium acetate (2.70 g, 35.0 mmol), **4** (1.14 g, 3.5 mmol), and dry MeOH (30 mL) to yield a white solid (0.80 g, 79.1%). R_f (ethyl acetate/*n*-hexane 1:2): 0.28; mp 61.5 °C; LC MS/MS: retention time: 21.89 min; $[MH]^+$: 289.3; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.70 (dd d, J = 4.8, 1.6, 0.8 Hz, 1H, 2-pyridine H-6′), 8.57 (d, J = 8.0 Hz, 1H, 2-pyridine H-3′), 8.40 (d, J = 1.4 Hz, 1H, pyridine H-3), 8.05 (br, 1H, 4-furan H-2), 7.86 (td, J = 7.7, 1.7 Hz, 1H, 2-pyridine H-4′), 7.81 (d, J = 1.4, 1H, pyridine H-5), 7.57 (br, 1H, 6-furan H-5), 7.55 (t, J = 1.7 Hz, 1H, 4-furan H-5), 7.34 (dd d, J = 7.4, 4.8, 0.8 Hz, 1H, 2-pyridine H-5′), 7.22 (d, J = 3.3 Hz, 1H, 6-furan H-3), 6.92 (dd, J = 1.7, 0.8 Hz, 1H, 4-furan H-4), 6.58 (dd, J = 3.3, 1.7 Hz, 1H, 6-furan H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 156.26, 155.90, 153.89, 149.33, 148.96, 144.16, 143.22, 141.63, 140.75, 136.91, 124.62, 123.90, 121.42, 115.77, 114.91, 112.08, 108.84, 108.54.

4.3.11. 6-(Furan-2-yl)-4-(furan-3-yl)-[2,4′]-bipyridyl (20). The same procedure described at 4.3 was employed with **3** (**R**^{1} = **e**, **R**^{2} = **d**) (0.66 g, 3.5 mmol), anhydrous ammonium acetate (2.70 g, 35.0 mmol), **6** (1.14 g, 3.5 mmol), and dry MeOH (30 mL) to yield a white solid (320 mg, 31.6%). R_f (ethyl acetate/*n*-hexane 1:2): 0.12; mp 80.0 °C; LC MS/MS: retention time: 20.31 min; $[MH]^+$: 289.2; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.75 (dd, J = 4.6, 1.5 Hz, 2H, 2-pyridine H-2′, H-6′), 8.02 (br, 1H, 4-furan H-2), 8.00 (dd, J = 4.6, 1.5 Hz, 2H, 2-pyridine H-3′, H-5′), 7.82 (d, J = 1.2 Hz, 1H, pyridine H-3), 7.71 (d, J = 1.2 Hz, 1H, pyridine H-5), 7.58 (br, 1H, 6-furan H-5), 7.57 (t, J = 1.7 Hz, 1H, 4-furan H-5), 7.23 (d, J = 3.3 Hz, 1H, 6-furan H-3), 6.86 (dd, J = 1.7, 0.8 Hz, 1H, 4-furan H-4), 6.59 (dd, J = 3.3, 1.7 Hz, 1H, 6-furan H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.78, 153.45, 150.11, 146.44, 144.73, 144.49, 143.53, 143.46, 141.89, 140.62, 138.22, 124.29, 121.25, 115.62, 115.00, 113.22, 112.22, 111.63, 109.47, 109.32, 108.32.

4.3.12. 4,6-(Dithiophen-2-yl)-[2,4′]-bipyridyl (21). The same procedure described at 4.3 was employed with **3** (**R**^{1}, **R**^{2} = **b**) (1.10 g, 5.0 mmol), anhydrous ammonium acetate (3.85 g, 50.0 mmol), **6** (1.63 g, 5.0 mmol), and dry MeOH (50 mL) to yield a white solid (347 mg, 21.7%). R_f (ethyl acetate/*n*-hexane 2:1): 0.3; mp 147.6 °C; LC MS/MS: retention time: 18.12 min; $[MH]^+$: 321.2; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.77 (dd, J = 4.6, 1.6 Hz, 2H, 2-pyridine H-2′, H-6′), 8.03 (dd, J = 4.5, 1.6 Hz, 2H, 2-pyridine H-3′, H-5′), 7.84 (d, J = 1.3 Hz, 1H, pyridine H-3), 7.82 (d, J = 1.3 Hz, 1H, pyridine H-5), 7.73 (dd, J = 3.7, 1.0 Hz, 1H, 6-thiophene H-5), 7.63 (dd, J = 3.7, 1.0 Hz, 1H, 4-thiophene H-5), 7.49 (dd, J = 5.0, 1.0 Hz, 1H, 6-thiophene H-3), 7.45 (dd, J = 5.0, 1.0 Hz, 1H, 4-thiophene H-3), 7.20 (dd, J = 5.0, 3.7 Hz, 1H, 6-thiophene H-4), 7.17 (dd, J = 5.0, 3.7 Hz, 1H, 4-thiophene H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.72, 153.37, 150.42, 145.83, 144.52, 143.38, 140.99, 128.53, 128.28, 128.09, 127.47, 125.70, 125.15, 121.08, 115.26, 114.89.

4.3.13. 4-(Thiophene-2-yl)-6-(thiophene-3-yl)-[2,4′]-bipyridyl (22). The same procedure described at 4.3 was employed with **3** (**R**^{1} = **b**, **R**^{2} = **c**) (1.10 g, 5.0 mmol), anhydrous ammonium acetate (3.85 g, 50.0 mmol), **6** (1.63 g, 5.0 mmol), and dry MeOH (30 mL) to yield a white solid (0.40 g, 25.0%). R_f (ethyl acetate/*n*-hexane 2:1): 0.3, mp 165.8 °C; LC MS/MS: retention time:18.87 min; $[MH]^+$: 321.2; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.76 (dd, J = 4.6, 1.6 Hz, 2 H, 2-pyridine H-2′, H-6′), 8.07 (dd, J = 3.0, 1.2 Hz, 1H, 6-thiophene H-2), 8.03(dd, J = 4.6, 1.6 Hz, 2H, 2-pyridine H-3′, H-5′), 7.84 (d, J = 1.4 Hz, 1H, pyridine H-3), 7.81 (d, J = 1.4 Hz, 1H, pyridine H-5), 7.80 (dd, J = 5.0, 1.2 Hz, 1H, 6-thiophene H-5), 7.61 (dd, J = 3.7, 1.0 Hz, 1H, 4-thiophene H-5), 7.48 (dd, J = 5.0, 1.0 Hz, 1H, 4-thiophene H-3), 7.45 (dd, J = 5.0, 3.0 Hz, 1H, 6-thiophene H-4), 7.19 (dd, J = 5.0, 3.7 Hz, 1H, 4-thiophene H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.82, 154.21, 150.34, 146.25, 143.33, 141.73, 141.14, 138.78, 136.97, 128.50, 127.35, 126.43, 126.29, 125.56, 124.33, 121.14, 117.66, 116.45, 115.24.

4.3.14. 4, 6-(Dithiophen-2-yl)-[2,4′]-bipyridyl (23). The same procedure described at 4.3 was employed with **3** (**R**^{1}, **R**^{2} = **c**) (1.10 g, 5.0 mmol), anhydrous ammonium acetate (3.85 g, 50.0 mmol), **6** (1.63 g, 5.0 mmol), and dry MeOH (30 mL) to yield a white solid (0.50 g, 31.3%). R_f (ethyl acetate/*n*-hexane 2:1): 0.3; mp 194.9 °C; LC MS/MS: retention time: 18.30 min; $[MH]^+$: 321.3; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.76 (dd, J = 4.6, 1.6 Hz, 2H, 2-pyridine H-2′, H-6′), 8.08 (dd, J = 3.0, 1.2 Hz, 1H, 6-thiophene H-2), 8.05(dd, J = 4.6, 1.6 Hz, 2H, 2-pyridine H-3′, H-5′), 7.86 (d, J = 1.2 Hz, 1H, pyridine H-3), 7.83 (d, J = 1.2 Hz, 1H, pyridine H-5), 7.81 (dd, J = 5.0, 1.2 Hz, 1H, 6-thiophene H-5), 7.45 (dd, J = 2.7, 1.5 Hz, 1H, 4-thiophene H-2), 7.54 (dd, J = 5.0, 1.5 Hz, 1H, 4-thiophene H-5), 7.52 (dd, J = 5.0, 2.8 Hz, 1H, 4-thiophene H-4), 7.45 (dd, J = 5.0, 3.0 Hz, 1H, 6-thiophene H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.81, 154.22, 150.39, 149.00, 146.45, 144.81, 141.94, 139.62, 127.33, 126.44, 126.32, 125.84, 124.20, 123.36, 121.17, 117.49, 116.26.

4.3.15. 4-(Furan-2-yl)-6-(thiophene-2-yl)-[2,4′]-bipyridyl (24). The same procedure described at 4.3 was employed with **3** ($\mathbf{R}^1$ = **d**, $\mathbf{R}^2$ = **b**) (1.02 g, 5.0 mmol), anhydrous ammonium acetate (3.85 g, 50.0 mmol), **6** (1.63 g, 5.0 mmol), and dry MeOH (30 mL) to yield a white solid (360 mg, 24.1%). R_f (ethyl acetate/*n*-hexane 2:1): 0.28; mp 156.5 °C; LC MS/MS: retention time: 18.32 min; $[MH]^+$: 305.2; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.76 (dd, J = 4.6, 1.6 Hz, 2H, 2-pyridine H-2′, H-6′), 8.03 (dd, J = 4.6, 1.6 Hz, 2H, 2-pyridine H-3′, H-5′), 7.87 (s, 2H, pyridine H-3, H-5), 7.72 (dd, J = 3.7, 1.0 Hz, 1H, 6-thiophene H-5), 7.60 (dd, J = 1.6, 0.5 Hz, 1H, 4-furan H-5), 7.45 (dd, J = 5.0, 1.0 Hz, 1H, 6-thiophene H-3), 7.15 (dd, J = 5.0, 3.7 Hz, 1H, 6-thiophene H-4), 7.99 (d, J = 3.4 Hz, 1H, 4-furan H-3), 6.58 (dd, J = 3.4, 1.8 Hz, 1H, 4-furan H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.42, 153.20, 151.09, 150.31, 145.91, 144.65, 143.98, 139.28, 128.13, 128.05, 125.07, 121.03, 112.91, 112.60, 112.25, 109.15.

4.3.16. 4-(Thiophene-3-yl)-6-(thiophene-2-yl)-[2,3′]-bipyridyl (25). The same procedure described at 4.3 was employed with **3** ($\mathbf{R}^1$ = **c**, $\mathbf{R}^2$ = **b**) (1.10 g, 5.0 mmol), anhydrous ammonium acetate (3.85 g, 50.0 mmol), **5** (1.63 g, 5.0 mmol), and dry MeOH (30 mL) to yield a white solid (525 mg, 32.8%). R_f (ethyl acetate/*n*-hexane 2:1): 0.3; mp 179.3 °C; LC MS/MS: retention time: 18.36 min; $[MH]^+$: 321.2; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.34 (d, J = 1.8 Hz, 1H, 2-pyridine H-2′), 8.68 (dd, J = 4.8, 1.5 Hz, 1H, 2-pyridine H-6′), 8.47 (dt, J = 8.0, 1.8 Hz, 1H, 2-pyridine H-4′), 7.81 (d, J = 1.2 Hz, 1H, pyridine H-3), 7.79 (d, J = 1.2 Hz, 1H, pyridine H-5), 7.77 (dd, J = 2.8, 1.5 Hz, 1H, 4-thiophene H-2), 7.72 (dd, J = 3.7, 1.0 Hz, 1H, 6-thiophene H-5), 7.54 (dd, J = 5.0, 1.4 Hz, 1H, 4-thiophene H-5), 7.51 (dd, J = 5.0, 2.8 Hz, 1H, 4-thiophene H-4), 7.47 (ddd, J = 7.4, 4.8, 1.0 Hz, 1H, 2-pyridine H-5′), 7.45 (dd, J = 5.0, 1.0 Hz, 1H, 6-thiophene H-3), 7.16 (dd, J = 5.0, 3.7 Hz, 1H, 6-thiophene H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.83, 153.25, 150.07, 148.32, 144.87, 144.66, 139.56, 134.47, 128.03, 127.27, 125.85, 124.88, 123.57, 123.39, 115.87, 115.01.

4.3.17. 4-(Furan-2-yl)-6-(thiophene-2-yl)-[2,3′]-bipyridyl (26). The same procedure described at 4.3 was employed with **3** ($\mathbf{R}^1$ = **d**, $\mathbf{R}^2$ = **b**) (1.02 g, 5.0 mmol), anhydrous ammonium acetate (3.85 g, 50.0 mmol), **5** (1.63 g, 5.0 mmol), and dry MeOH (30 mL) to yield a white solid (490 mg, 28.5%). R_f (ethyl acetate/*n*-hexane 2:1): 0.3, mp 165.8 °C; LC MS/MS: retention time: 18.08 min; $[MH]^+$: 305.3; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.39 (d, J = 1.5 Hz, 1H, 2-pyridine H-2′), 8.68 (dd, J = 4.7, 1.3 Hz, 1H, 2-pyridine H-6′), 8.47 (dt, J = 8.0, 1.9 Hz, 1H, 2-pyridine H-4′), 7.85 (br, 2H, pyridine H-3, H-5), 7.73 (dd, J = 3.7, 1.0 Hz, 1H, 6-thiophene H-5), 7.61 (dd, J = 1.7, 0.5 Hz, 1H, 4-furan H-5), 7.45 (dd, J = 5.0, 1.0 Hz, 1H, 6-thiophene H-3), 7.43 (dd d, J = 8.0, 4.9, 0.7 Hz, 1H, 2-pyridine H-5′), 7.16 (dd, J = 5.0, 3.7 Hz, 1H, 6-thiophene H-4), 7.00 (d, J = 3.4 Hz, 1H, 4-furan H-3), 6.59 (dd, J = 3.4, 1.7 Hz, 1H, 4-furan H-4); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.70, 153.17, 151.25, 150.05, 148.29, 144.84, 143.92, 139.21, 134.44, 134.39, 128.02, 124.96, 123.55, 112.58, 112.22, 111.76, 109.04.

4.4. Pharmacology

For the evaluation of cytotoxicity, four different human cancer cell lines were utilized: A-549 (human lung carcinoma), SK-OV-3 (human ovary adenocarcinoma), SK-MEL-2 (human malignant melanoma), and HCT15 (human colon adenocarcinoma). All experimental procedures followed up the NCI's protocol[13,14] based on the Sulforhodamine B (SRB) method. In brief, tumor cells were cultured to maintain logarithmic growth by changing the medium 24 h before cytotoxicity assay. On the day of the assay, the cells were harvested by trysinization, counted, diluted in media, and added to 96-well plates. The concentrations of tumor cells used were 5×10^3 (A549, HCT15), 1×10^4 (SK-MEL-2), and 2×10^4 cells/well (SK-OV-3). The cells were then preincubated for 24 h in 5% CO_2 incubator at 37 °C. The compounds dissolved in DMSO were added to the wells in six 3-fold dilutions starting from the highest concentrations, and incubated for 48 h in 5% CO_2 incubator at 37 °C. The final DMSO concentration was <0.5%. At the termination of the incubation, the culture medium in each well was removed, and the cells were fixed with cold 10% trichloroacetic acid (TCA) for 1 h at 4 °C. The microplates were washed, dried, and stained with 0.4% SRB in 1% acetic acid for 30 min at room temperature. The cells were washed again and the bound stain was solubilized with 10 mM Tris base solution (pH 10.5), and the absorbances were measured spectrophotometrically at 520 nm on a microtiter plate reader (Molecular Devices, Sunnyvale, CA). The data were transformed into MS Excel format and survival fractions were calculated by regression analysis (plotting the cell viability versus the concentration of the test compound). The EC_{50} values represent concentrations of the compounds that inhibit 50% of cell growth. All data represent average values for a minimum of three wells.

The topoisomerase I inhibitory activity was carried out as follows:[15] the activity of DNA topoisomerase I was determined by measuring the relaxation of supercoiled DNA pBR322. For measurement of topoisomerase I activity, the reaction mixture was comprised of 35 mM Tris–HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine, 0.01% bovine serum albumin (BSA), 1 μg pBR322, and 2 U DNA topoisomerase. Topoisomerase I inhibitors (prepared compounds) were added to the above component for measuring the inhibition of DNA relaxation. The reaction mixture was incubated at 37 °C for 30 min. The reactions were terminated by adding dye solution comprising 1% SDS, 0.02% bromophenol blue, and 50% glycerol. The mixture was applied to 1% agarose gel and electrophoresed for 1 h with a running buffer of Tris–acetate EDTA. The gel was stained with ethidium bromide.

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