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2-Thienyl-4-furyl-6-aryl pyridine derivatives: Synthesis, topoisomerase I and II inhibitory activity, cytotoxicity, and structure–activity relationship study

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ABSTRACT

Designed and synthesized 60 2-thienyl-4-furyl-6-aryl pyridine derivatives were evaluated for their topoisomerase I and II inhibitory activities at 20 μM and 100 μM and cytotoxicity against several human cancer cell lines. Compounds **8**, **9**, **11**–**29** showed significant topoisomerase II inhibitory activity and compounds **10** and **11** showed significant topoisomerase I inhibitory activity. Most of the compounds (**7**–**21**) possessing 2-(5-chlorothiophen-2-yl)-4-(furan-3-yl) moiety showed higher or similar cytotoxicity against HCT15 cell line as compared to standards. Most of the selected compounds displayed moderate cytotoxicity against MCF-7, HeLa, DU145, and K562 cell lines. Structure–activity relationship study revealed that 2-(5-chlorothiophen-2-yl)-4-(furan-3-yl) moiety has an important role in displaying biological activities.

1. Introduction

DNA topoisomerases, first discovered in 1971 by Wang (the omega protein, ω, from *Escherichia coli*),[1] are nuclear enzymes that alter the topology of DNA by transiently breaking one or two strands of DNA, passing a single or double-stranded DNA through the break and again resealing the breaks. They are involved in a number of vital cellular processes like replication, transcription, recombination, repair, chromatin assembly, and chromosome segregation.[2] Topoisomerases have been basically classified into type I and II depending on their mechanism of action, making either single- or double-stranded breaks, respectively.[3] Because of the crucial role of topoisomerase for the maintenance and replication of DNA during proliferation, cells become highly vulnerable when these functions are lost,[4] so they are now viewed as important therapeutic targets for cancer chemotherapy.[5,6]

2,2′:6′,2″-Terpyridine, bioisostere of α-terthiophene, was first discovered in 1932 and has been precious due to its ability to form metal complexes[7] and as RNA/DNA binding agents[8] (Fig. 1). Since the terthiophene derivatives show considerable PKC inhibitory activity and cytotoxicity against human cancer cell lines,[9] a rationale evolved in our research group that terpyridine derivatives as bioisosteres of terthiophene may have cytotoxicity against human cancer cell lines and topoisomerase inhibitory activity. Recently, our research group has reported that terpyridine derivatives show a strong cytotoxicity against several human cancer cell lines and considerable topoisomerase I (topo I) and II (topo II) inhibitory activities.[10] From previous structure–activity relationship studies, we found 2-thienyl-4-furyl-pyridine skeleton exhibited strong topo I and II inhibitory activities.[10b,g] Further exploration on this skeleton prompted us to design and synthesize a series of 2-thienyl-4-furyl-pyridine skeletons containing compounds with substituents like chlorine or methyl at different aryl moieties as shown in Figure 1. They were evaluated for topo I and II inhibitory activities and cytotoxicity against several human cancer cell lines. In order to study the structure–activity relationship, 60 compounds in six different series were synthesized systematically as shown in Scheme 1 and Figure 2.

2. Results and discussion

2.1. Chemistry

Synthesis of various 2,4,6-trisubstituted pyridine derivatives have already been reported by our research group.[10] Our strategy to synthesize 2,4,6-trisubstituted pyridine derivatives was based on previously proposed methods[11,12] as shown in Scheme 1. At first, using the Claisen–Schmidt KOH catalyzed condensation reaction,[11] we synthesized six propenone intermediates (**3**) in 65.7–94.2% yield by the condensation of two aryl acetyls (**1a**, **b**) with

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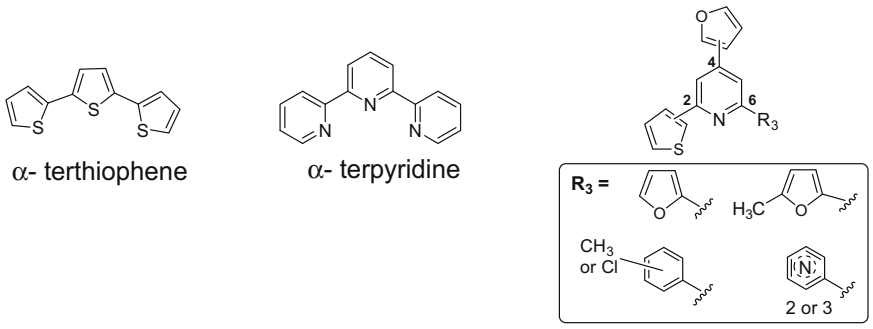
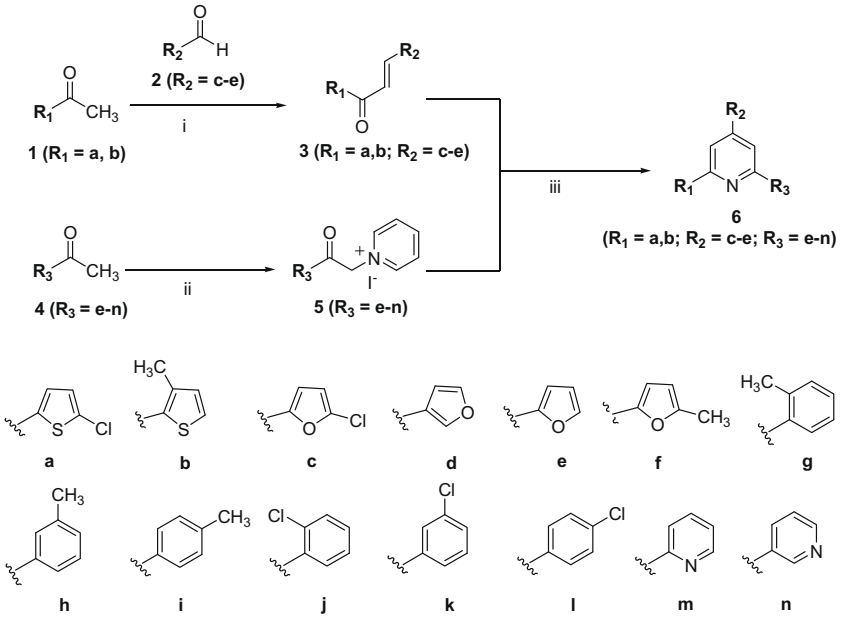


Figure 1. Structure of α-terthiophene, α-terpyridine and 2-thienyl-4-furyl-6-aryl pyridine derivatives.



Scheme 1. General synthetic scheme of 2,4,6-trisubstituted pyridines. Reagents and conditions: (i) aryl aldehyde **2c–e** (1.0 equiv), KOH (1.2 equiv), MeOH/H_2O (5:1), 10 min to 3 h, 0 °C, 65.7–94.2%; (ii) pyridine, iodine (1.0 equiv), 3 h, 140 °C, 42.2–99.4%; (iii) **3** (1.0 equiv), **5** (1.0 equiv), NH_4OAc (10.0 equiv), glacial AcOH, 16–22 h, 80–95 °C, 20.6–71.6%.

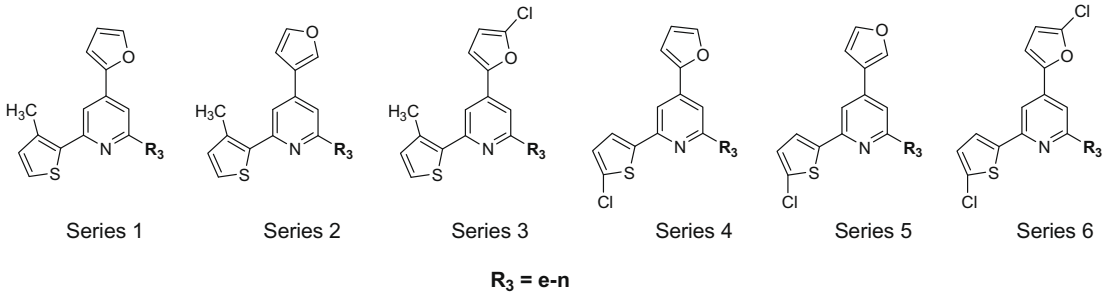


Figure 2. Strategy and design of six different series of 2-thienyl-4-furyl-6-aryl pyridine derivatives.

three different aryl aldehydes (**2c**–**e**). Secondly, 10 pyridinium iodide salts (**5**) were prepared in 42.2–99.4% yield by refluxing 10 different aryl acetyls (**4e**–**n**) with iodine in pyridine. Finally, on the basis of modified Kröhnke synthesis method,[12] 60 2-thienyl-4-furyl-6-aryl pyridine derivatives (**6**) were synthesized by reacting 6 propenone intermediates (**3**) with 10 different pyridinium iodide salts (**5**) in 20.6–71.6% yield. It was noticed that the yield of compounds having substituents Cl or CH_3 at *ortho* position of phenyl ring was lower than those having substituents at *meta* and *para* position of phenyl ring. That is likely due to the steric hindrance by substituents when placed at ortho position.

Sixty 2-thienyl-4-furyl-6-aryl pyridine derivatives were synthesized systematically in six different series as shown in Figure 2.

2.2. Topoisomerase I and II inhibitory activity

Sixty 2-thienyl-4-furyl-6-aryl pyridine derivatives were prepared and evaluated for their topo I and II inhibitory activities.

The structure of selected compounds was displayed in Figure 3. The rest of the compounds were excluded since they did not show considerable activities.

Figure 4 and Table 1 depict the human DNA topo II inhibitory activities of 2-thienyl-4-furyl-6-aryl pyridine derivatives (**7**–**29**) with etoposide as a positive control. Compounds **8**, **9**, **11**–**29** showed significant topo II inhibitory activity at 20 μM and 100 μM whereas compounds **7** and **10** showed weaker inhibitory activity as compared to etoposide. Similarly, Figure 5 and Table 1 depict human topo I inhibitory activities of 2-thienyl-4-furyl-6-aryl pyridine derivatives (**7**–**29**) with camptothecin as a positive control. Most of all compounds were devoid of topo I inhibitory activity. However, compound **10** has shown significant inhibitory activity at 100 μM and compound **11** has shown considerable inhibitory activity at both 20 μM and 100 μM compared to camptothecin.

From the structure–activity relationship study, most of the compounds with significant topo II inhibitory activity, which have stronger inhibitory activity than etoposide, possess 2-(5-chlorothiophen-2-yl)-4-(furan-3-yl) moiety (series 5 in Fig. 2) in common. It was also noticed that there is not much variation in activity within a series.

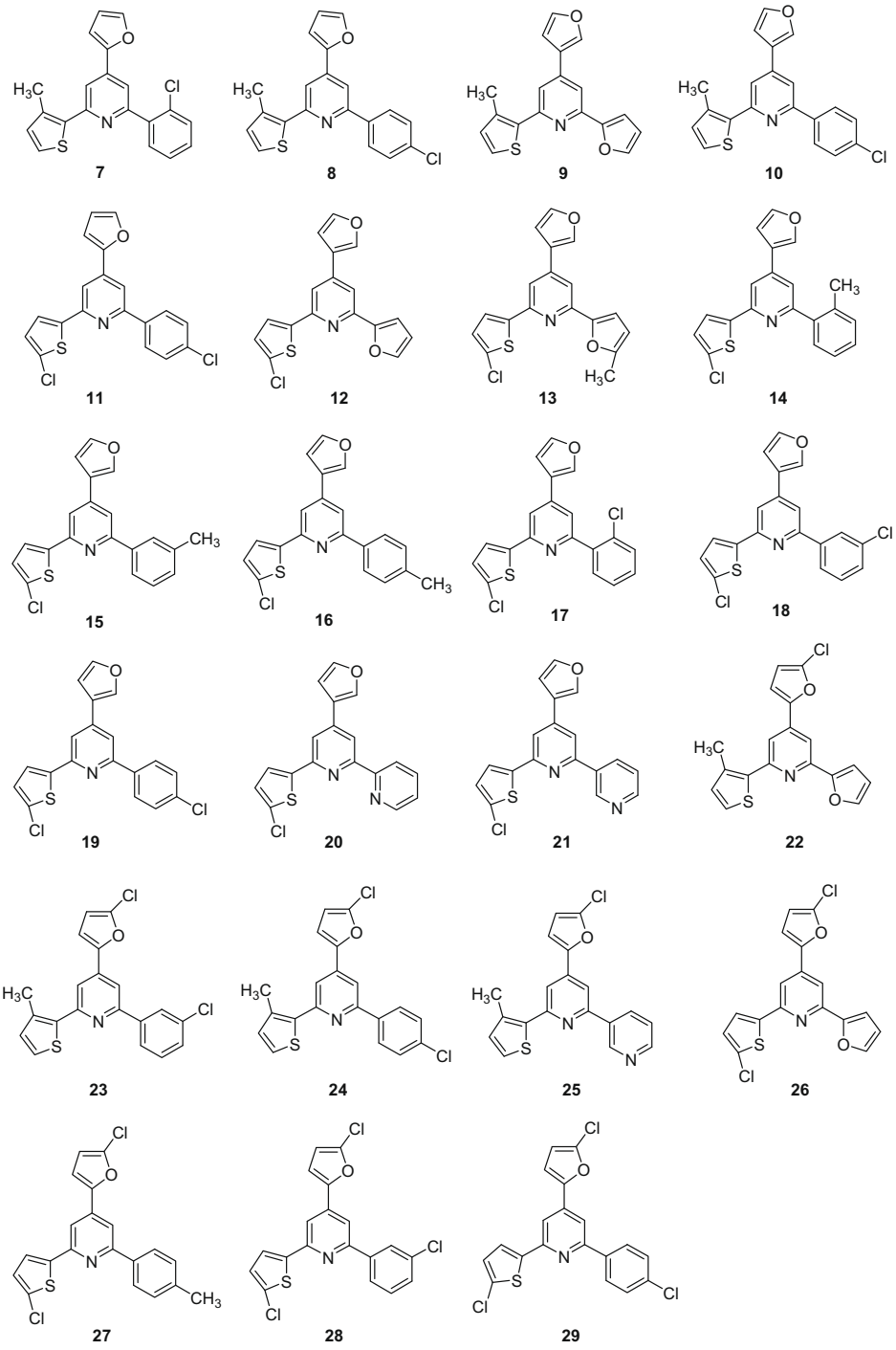
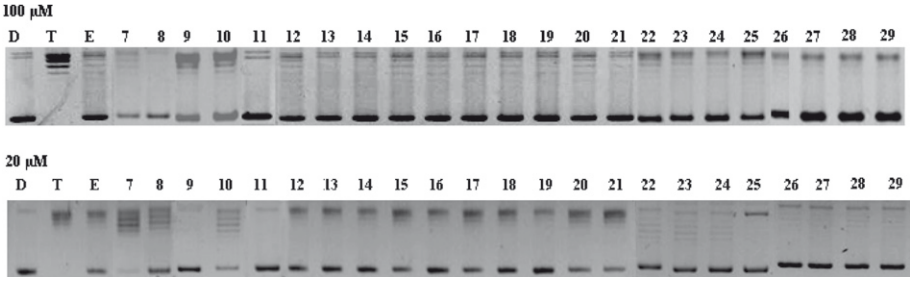


Figure 3. Structures of selected compounds possessing significant biological activities.



Lane D: pBR322 DNA only

Lane T: pBR322 DNA + Topo II

Lane E: pBR322 DNA + Topo II + Etoposide

Lane 7 – 29: pBR322 DNA + Topo II + Compound **7** - **29**

Figure 4. Human DNA topoisomerase IIα inhibitory activity of compounds **7**–**29**.

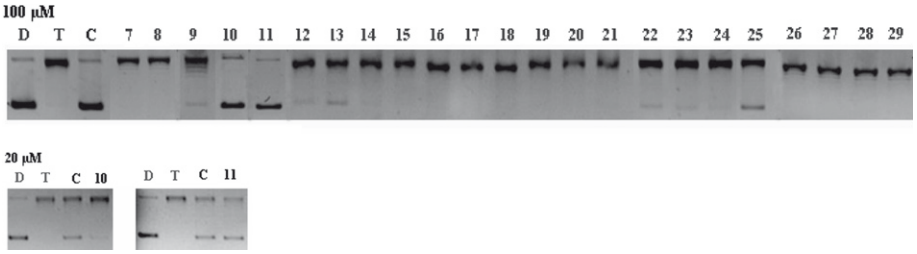
Table 1
Inhibitory effects of compounds **7–29** on topoisomerase I and II (% inhibition ratio of relaxation) and their IC_{50} values against MCF-7, HeLa, DU145, HCT15, K562 cell lines

Compounds	% Inhibition				[a]IC_{50} (μM)				
	Topo II		Topo I		[b]MCF-7	[c]HeLa	[d]DU145	[e]HCT15	[f]K562
	100 μM	20 μM	100 μM	20 μM					
7	33.2	3.5	2.0	NA	14.21 ± 3.31	24.02 ± 2.95	29.87 ± 1.58	0.54 ± 0.05	25.41 ± 0.17
8	56.3	17.8	1.0	NA	0.89 ± 0.32	24.49 ± 1.46	26.68 ± 4.37	0.71 ± 0.07	20.69 ± 5.81
9	59.9	36.4	5.4	NA	18.25 ± 1.91	30.5 ± 2.2	30.44 ± 2.72	0.64 ± 0.02	23.30 ± 1.23
10	33.8	8.1	85.9	10.6	20.33 ± 0.24	17.43 ± 2.52	20.09 ± 0.78	2.99 ± 2.75	19.60 ± 3.97
11	53.3	48.4	53.1	42.9	1.52 ± 0.58	16.98 ± 3.08	14.75 ± 4.29	1.08 ± 0.27	10.45 ± 1.15
12	49.1	48.6	9.6	NA	4.74 ± 1.27	22.69 ± 4.96	17.23 ± 2.28	1.28 ± 0.23	16.17 ± 1.93
13	60.5	54.0	13.2	NA	1.90 ± 0.44	26.52 ± 1.16	33.44 ± 3.48	0.79 ± 0.10	12.66 ± 4.18
14	60.7	49.2	4.6	NA	1.43 ± 0.10	18.96 ± 3.49	18.51 ± 3.72	0.99 ± 0.21	12.29 ± 0.45
15	50.2	37.6	2.6	NA	3.99 ± 0.77	23.63 ± 1.88	20.34 ± 2.68	0.88 ± 0.06	13.71 ± 1.65
16	50.3	53.6	0.0	NA	5.34 ± 1.49	28.71 ± 3.35	25.32 ± 2.37	1.17 ± 0.27	17.86 ± 4.91
17	49.5	37.6	0.0	NA	7.36 ± 1.37	19.01 ± 0.85	16.41 ± 0.66	1.10 ± 0.17	15.76 ± 2.75
18	54.0	50.6	2.1	NA	2.92 ± 0.79	23.66 ± 1.83	22.96 ± 1.80	0.95 ± 0.18	22.05 ± 1.19
19	62.7	56.9	2.3	NA	2.60 ± 0.55	16.51 ± 1.47	29.47 ± 3.15	0.64 ± 0.02	19.60 ± 4.20
20	49.6	38.9	3.1	NA	3.36 ± 0.37	21.56 ± 2.5	20.52 ± 2.43	1.27 ± 0.29	19.96 ± 2.84
21	60.8	40.9	2.0	NA	3.48 ± 0.54	19.99 ± 2.39	18.49 ± 2.38	0.72 ± 0.06	18.69 ± 2.83
22	61.1	30.7	7.8	NA	5.57 ± 0.42	22.22 ± 2.17	27.31 ± 2.79	26.31 ± 0.07	10.59 ± 2.13
23	51.5	35.7	5.9	NA	18.83 ± 2.31	24.83 ± 2.4	20.52 ± 1.18	26.43 ± 4.63	13.36 ± 2.01
24	59.8	40.4	5.1	NA	15.28 ± 7.87	29.32 ± 1.45	21.93 ± 3.30	30.34 ± 2.85	15.58 ± 2.72
25	57.2	25.5	21.4	NA	5.81 ± 0.98	>50	30.81 ± 5.55	>50	12.33 ± 0.67
26	53.8	39.9	23.1	NA	16.85 ± 0.70	>50	27.67 ± 2.20	>50	22.48 ± 0.78
27	58.6	41.1	14.2	NA	3.04 ± 1.02	>50	>50	>50	21.4 ± 0.53
28	53.8	40.7	13.3	NA	15.09 ± 0.00	25.17 ± 0.91	21.57 ± 0.40	>50	19.61 ± 2.85
29	59.8	42.7	2.2	NA	12.55 ± 2.33	25.08 ± 0.46	22.98 ± 0.66	25.27 ± 5.17	19.32 ± 3.21
[g]Camptothecin (C)			79.0	41.2	0.06 ± 0.02	0.63 ± 0.05	0.66 ± 0.01	0.76 ± 0.08	0.08 ± 0.00
[h]Etoposide (E)	54.9	28.9			1.89 ± 0.34	1.29 ± 0.16	1.30 ± 0.20	1.09 ± 0.04	2.41 ± 0.70
[i]Adriamycin					2.10 ± 0.75	1.13 ± 0.27	1.50 ± 0.37	2.12 ± 0.05	2.50 ± 0.48

NA: not applicable.
[a] Each data represents mean ± S.D. from three different experiments performed in triplicate.
[b] MCF-7: human breast adenocarcinoma.
[c] HeLa: human cervix tumor.
[d] DU145: human prostate tumor.
[e] HCT15: human colorectal adenocarcinoma.
[f] K562: chronic myelogenous leukemia.
[g] Camptothecin; positive control for topo I and cytotoxicity.
[h] Etoposide: positive control for topo II and cytotoxicity.
[i] Adriamycin: positive control for cytotoxicity.

2.3. Cytotoxicity

Synthesized compounds were evaluated for cytotoxicity on five different human cancer cell lines: human breast adenocarcinoma cell line (MCF-7), human cervix tumor cell line (HeLa), human prostate tumor cell line (DU145), human colorectal adenocarcinoma cell line (HCT15), and chronic myelogenous leukemia cell line (K562). Inhibitory activities (IC_{50}) were presented as micromolar concentrations of the compounds as shown in Table 1. It was found that compounds **7**–**21** displayed stronger or similar cytotoxicity against HCT15 cell line compared to positive controls. And several compounds exhibit similar or moderate cytotoxicity



Lane D: pBR322 DNA only

Lane T: pBR322 DNA + Topo I

Lane C: pBR322 DNA + Topo I + Camptothecin

Lane 7 – 29 (100μM): pBR322 DNA + Topo I + Compound **7** – **29**

Lane 10 and 11 (20 μM): pBR322 DNA + Topo I + Compound **10** and **11**

Figure 5. Human DNA topoisomerase I inhibitory activity of compounds **7**–**29**.

against the MCF-7 cell line. Most of the compounds exhibit moderate cytotoxicity against HeLa, DU145, and K562 cell lines but weaker than those of positive controls.

3. Conclusion

We have designed and synthesized 60 2-thienyl-4-furyl-6-aryl pyridine derivatives systematically in a total of six series by efficient synthetic routes and evaluated them for topo I and II inhibitory activities and antitumor cytotoxicity. Structure–activity relationship study revealed that 2-(5-chlorothiophen-2-yl)-4-(furan-3-yl) moiety was important to display topo II inhibitory activity. As almost all of the compounds were devoid of topo I inhibitory activity, it was difficult to infer the structure–activity relationship. However, two compounds (**10** and **11**), which showed considerable topo I inhibitory activity, have 4-chlorophenyl in common. That indicates, to some extent, that 4-chlorophenyl might have an important role in displaying topo I inhibitory activity. For cytotoxicity, 4-(furan-2 or 3-yl)-6-(3-methylthiophen-2-yl) and 2-(5-chlorothiophen-2-yl)-4-(furan-3-yl) moieties with combination of **j**, **l**, **e**, and **l**, **n**, respectively, were important.

4. Experimental

Compounds used as starting materials and reagents were obtained from Aldrich Chemicals Co., or Junsei, and utilized without further purification. HPLC grade acetonitrile (ACN) and methanol were purchased from Burdick and Jackson, USA. Thin-layer chromatography (TLC) and column chromatography (CC) were performed with Kieselgel 60 F_{254} (Merck) and silica gel (Kieselgel 60, 230–400 mesh, Merck) respectively. Since all the compounds prepared contained aromatic rings, they were visualized and detected on TLC plates with UV light (short wave, long wave or both). NMR spectra were recorded on a Bruker AMX 250 (250 MHz, FT) for ^{1}H NMR and 62.5 MHz for ^{13}C NMR, and chemical shifts were calibrated to TMS (tetramethylsilane). Chemical shifts (δ) were recorded in ppm and coupling constants (J) in hertz (Hz). Melting points were determined in open capillary tubes on electrothermal 1A 9100 digital melting point apparatus and were uncorrected.

HPLC analyses were performed using two Shimadzu LC-10AT pumps gradient-controlled HPLC system equipped with Shimadzu system controller (SCL-10A VP) and photo diode array detector (SPD-M10A VP) utilizing Shimadzu Class VP program. Sample volume of 10 μL, was run in Waters X-Terra® 5 μM reverse-phase C_{18} column (4.6 × 250 mm) with a gradient elution of 80–100% of B in A for 10 min followed by 100–80% of B in A for 20 min at a flow rate of 1.0 mL/min at 254 nm UV detection, in which mobile phase A was doubly distilled water with 20 mM ammonium formate (AF) and B was 90% ACN in water with 20 mM AF. Purity of compound is described as percent (%).

ESI LC/MS analyses were performed with a Finnigan LCQ Advantage® LC/MS/MS spectrometry utilizing Xcalibur® program. For ESI LC/MS, LC was performed with 8 μL injection volume on a Waters X-Terra® 3.5 μm reverse-phase C_{18} column (2.1 × 100 mm) with a gradient elution: (A) from 70% to 90% of B in A for 6 min followed by 90% to 70% of B in A for 1 min, and 70% of B in A for 8 min (B) from 70% to 90% of B in A for 6 min followed by 90% to 70% of B in A for 1 min, and 70% to 30% of B in A for 8 min (C) from 80% to 95% of B in A for 5 min followed by 95% to 80% of B in A for 1 min, and 80% of B in A for 9 min at a flow rate of 200 μL/min, in which mobile phase A was 100% distilled water with 20 mM AF and mobile phase B was 100% ACN. MS ionization conditions were: Sheath gas flow rate: 40 arb, aux gas flow rate: 0 arb, I spray voltage: 5.3 KV, capillary temp.: 275 °C, capillary voltage: 27 V, tube lens offset: 45 V. Retention time is given in minutes.

4.1. General method for preparation of 3

To an ice cold solution of 85% KOH (1.2 equiv) in methanol (10 mL) and H_2O (2 mL) aryl acetyl **1** (1.0 equiv) was added. After dissolution, aryl aldehyde **2** (1.0 equiv) was added slowly. The reaction mixture was then stirred for 10 min to 3 h at 0 °C. Precipitate formed was filtered, washed with cold MeOH and dried to get solid compound **3** in 65.7–94.2% yield. Following the same procedure, 6 compounds were synthesized.

4.1.1. 3-Furan-2-yl-1-(3-methyl-thiophen-2-yl)-propenone 3 (R_1 = b, R_2 = e)

The same procedure described in Section 4.1 was employed with **1** (R_1 = b) and **2** (R_2 = e) to yield yellow solid 65.70%.

R_f (ethyl acetate/*n*-hexane 1:5 v/v): 0.35; mp 69.6 °C.

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.50 (d, J = 14.88 Hz, 1H, –CO–C=C*H*–), 7.47 (br, 1H, 3-furan H-5), 7.43 (d, J = 4.92 Hz, 1H, 1-thiophene H-5), 7.20 (d, J = 15.01 Hz, 1H, –CO–C*H*=C–), 6.96 (d, J = 4.88 Hz, 1H, 1-thiophene H-4), 6.67 (d, J = 3.26 Hz, 1H, 3-furan H-3), 6.46 (dd, J = 3.19, 1.71 Hz, 1H, 3-furan H-4), 2.60 (s, 3H, 1-thiophene 3-CH_3).

4.1.2. 3-Furan-3-yl-1-(3-methyl-thiophen-2-yl)-propenone 3 (R_1 = b, R_2 = d)

The same procedure described in Section 4.1 was employed with **1** (R_1 = b) and **2** (R_2 = d) to yield creamy white solid 92.85%.

R_f (ethyl acetate/*n*-hexane 1:5 v/v): 0.35; mp 68.6 °C.

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.73 (br, 1H, 3-furan H-2), 7.69 (d, J = 15.46 Hz, 1H, –CO–C=C*H*–), 7.46 (br, 1H, 3-furan H-5), 7.45 (d, J = 5.04 Hz, 1H, 1-thiophene H-5), 7.06 (d, J = 15.20 Hz, 1H, –CO–C*H* =C–), 6.99 (d, J = 4.93 Hz, 1H, 1-thiophene H-4), 6.69 (d, J = 1.06 Hz, 1H, 3-furan H-4).

4.1.3. 3-(5-Chlorofuran-2-yl)-1-(3-methyl-thiophen-2-yl)-propenone 3 (R_1 = b, R_2 = c)

The same procedure described in Section 4.1 was employed with **1** (R_1 = b) and **2** (R_2 = c) to yield light yellow solid 92.43%.

R_f (ethyl acetate/*n*-hexane 1:5 v/v): 0.34; mp 95.6 °C.

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.47 (d, J = 4.98 Hz, 1H, 1-thiophene H-5), 7.43 (d, J = 15.10 Hz, 1H, –CO–C=C*H*–), 7.20 (d, J = 15.07 Hz, 1H, –CO–C*H*=C–), 6.99 (d, J = 4.96 Hz, 1H, 1-thiophene H-4), 6.67 (d, J = 3.45 Hz, 1H, 3-furan H-3), 6.30 (d, J = 3.45 Hz, 1H, 3-furan H-4).

4.1.4. 1-(5-Chlorothiophen-2-yl)-3-(furan-2-yl)-propenone 3 (R_1 = a, R_2 = e)

The same procedure described in Section 4.1 was employed with **1** (R_1 = a) and **2** (R_2 = e) to yield light yellow solid 94.21%.

R_f (ethyl acetate/*n*-hexane 1:5 v/v): 0.36; mp 100.3 °C.

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.61 (d, J = 4.09 Hz, 1H, 1-thiophene H-3), 7.56 (d, J = 15.33 Hz, 1H, –CO–C=C*H*–), 7.52 (d, J = 1.41 Hz, 1H, 3-furan H-5), 7.20 (d, J = 15.27 Hz, 1H, –CO–C*H*=C–), 6.98 (d, J = 4.05 Hz, 1H, 1-thiophene H-4), 6.71 (d, J = 3.40 Hz, 1H, 3-furan H-3), 6.50 (dd, J = 3.42, 1.80 Hz, 1H, 3-furan H-4).

4.1.5. 1-(5-Chlorothiophen-2-yl)-3-(furan-3-yl)-propenone 3 (R_1 = a, R_2 = d)

The same procedure described in Section 4.1 was employed with **1** (R_1 = a) and **2** (R_2 = d) to yield white solid 85.56%.

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.28; mp 124.5 °C.

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.75 (d, J = 0.39 Hz, 1H, 3-furan H-2), 7.74 (d, J = 15.34 Hz, –CO–C=C*H*–), 7.61 (d, J = 4.07 Hz, 1H, 1-thiophene H-3), 7.48 (br, 1H, 3-furan H-5), 7.03 (d, J = 14.94 Hz, 1H, –CO–C*H*=C–), 6.99 (d, J = 3.79 Hz, 1H, 1-thiophene H-4), 6.69 (dd, J = 1.24, 0.43 Hz, 1H, 3-furan H-4).

4.1.6. 3-(5-Chlorofuran-2-yl)-1-(5-chlorothiophen-2-yl)-propenone 3 (R_1 = a, R_2 = c)

The same procedure described in Section 4.1 was employed with **1** (R_1 = a) and **2** (R_2 = c) to yield yellow solid 93.99%.

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.29; mp 109.4 °C.

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.65 (d, J = 4.08 Hz, 1H, 1-thiophene H-3), 7.46 (d, J = 15.18 Hz, 1H, –CO–C=C*H*–), 7.19 (d, J = 15.18 Hz, 1H, –CO–C*H*=C–), 7.00 (d, J = 4.07 Hz, 1H, 1-thiophene H-4), 6.70 (d, J = 3.47 Hz, 1H, 3-furan H-3), 6.32 (d, J = 3.47 Hz, 1H, 3-furan H-4).

4.2. General method for preparation of 5

A mixture of aryl acetyl, iodine (1.0 equiv) and pyridine (60 mL) was refluxed at 140 °C for 3 h. The reaction mixture was cooled to 0 °C, precipitate formed was filtered, washed with cold pyridine and dried to get solid compound **5** in 42.2–99.4% yield. Following the same procedure, 10 compounds were synthesized.

4.3. General method for preparation of 6

A mixture of **3** (R_1 = a, b, R_2 = c–e), **5** (R_3 = e–n) and dry ammonium acetate in glacial acetic acid was heated to 80–95 °C for 16–22 h under nitrogen gas. The solvent was evaporated and the residue extracted with ethyl acetate (80 mL), washed with water (50 mL × 3), saturated NaCl solution (30 mL) and dried over $MgSO_4$. After filtration, the filtrate was concentrated and purified by silica gel column chromatography with a gradient elution of ethyl acetate/*n*-hexane to afford a white solid compound **6** in 20.6–71.6% yield. Following the same procedure, 60 compounds were synthesized.

4.3.1. 2-(2-Chlorophenyl)-4-(furan-2-yl)-6-(3-methylthiophen-2-yl) pyridine (7)

The same procedure described in Section 4.3 was employed with **3** (R_1 = b, R_2 = e) (0.76 g, 3.50 mmol), dry ammonium acetate (2.69 g, 35.00 mmol), **5** (R_3 = j) (1.25 g, 3.50 mmol) and glacial acetic acid (8.00 mL) to yield a white solid (284 mg, 23.10%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.31; mp 89.5 °C, purity: 99.2%.

LC/MS/MS (condition A): retention time: 6.74 min; [MH^+]: 352.2 (100%), [MH+2]: 354.2 (38%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.77 (d, J = 1.32 Hz, 1H, pyridine H-3), 7.76 (d, J = 1.34 Hz, 1H, pyridine H-5), 7.73–7.69 (m, 1H, 2-phenyl H-6), 7.56 (dd, J = 1.72, 0.60 Hz, 1H, 4-furan H-5), 7.49–7.46 (m, 1H, 2-phenyl H-3), 7.40–7.32 (m, 2H, 2-phenyl H-4, H-5), 7.27 (d, J = 5.04 Hz, 1H, 6-thiophene H-5), 6.94 (d, J = 5.01 Hz, 1H, 6-thiophene H-4), 6.92 (dd, J = 3.38, 0.63 Hz, 1H, 4-furan H-3), 6.54 (dd, J = 3.44, 1.79 Hz, 1H, 4-furan H-4), 2.59 (s, 3H, 6-thiophene 3-CH_3).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 156.91, 153.92, 151.61, 143.82, 139.08, 138.00, 137.76, 135.95, 132.31, 132.07, 131.80, 130.18, 129.58, 126.95, 125.48, 116.66, 114.26, 112.12, 108.80, 16.28.

4.3.2. 2-(4-Chlorophenyl)-4-(furan-2-yl)-6-(3-methylthiophen-2-yl) pyridine (8)

The same procedure described in Section 4.3 was employed with **3** (R_1 = b, R_2 = e) (0.43 g, 2.00 mmol), dry ammonium acetate (1.54 g, 20.00 mmol), **5** (R_3 = l) (0.72 g, 2.00 mmol) and glacial acetic acid (5.00 mL) to yield a white solid (250 mg, 35.58%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.34; mp 97.6 °C, purity: 99.4%.

LC/MS/MS (condition A): retention time: 8.62 min; [MH^+]: 352.2 (100%), [MH+2]: 354.2 (38%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.09 (d, J = 8.55 Hz, 2H, 2-phenyl H-2, H-6), 7.80 (d, J = 1.01 Hz, Hz, pyridine H-3), 7.73 (d, J = 0.93 Hz, 1H, pyridine H-5), 7.57 (d, J = 1.69 Hz, 1H, 4-furan H-5), 7.46 (d, J = 8.53 Hz, 2H, 2-phenyl H-3, H-5), 7.30 (d, J = 5.05 Hz, 1H, 6-thiophene H-5), 6.96 (d, J = 5.40 Hz, 1H, 6-thiophene H-4), 6.95 (d, J = 3.13 Hz, 1H, 4-furan H-3), 6.55 (dd, J = 3.43, 1.79 Hz, 1H, 4-furan H-4), 2.62 (s, 3H, 6-thiophene 3-CH_3).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 156.02, 153.90, 151.65, 143.79, 138.97, 137.96, 137.53, 136.03, 135.25, 132.25, 128.85, 128.24, 125.56, 114.04, 112.14, 111.69, 108.70, 16.52.

4.3.3. 2-(Furan-2-yl)-4-(furan-3-yl)-6-(3-methylthiophen-2-yl) pyridine (9)

The same procedure described in Section 4.3 was employed with **3** (R_1 = b, R_2 = d) (0.39 g, 1.80 mmol), dry ammonium acetate (1.38 g, 18.00 mmol), **5** (R_3 = e) (0.56 g, 1.80 mmol) and glacial acetic acid (2.00 mL) to yield a white solid (396 mg, 71.60%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.37; mp 94.6 °C, purity: 99.2%.

LC/MS/MS (condition B): retention time: 4.91 min; [MH^+]: 308.2 (100%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.95 (dd, J = 1.36, 0.89 Hz, 1H, 4-furan H-2), 7.65 (d, J = 1.36 Hz, 1H, pyridine H-3), 7.55 (t, J = 1.66 Hz, 1H, 4-furan H-5), 7.54 (dd, J = 1.65, 0.84 Hz, 1H, 2-furan H-5), 7.48 (d, J = 1.35 Hz, 1H, pyridine H-5), 7.28 (d, J = 5.05 Hz, 1H, 6-thiophene H-5), 7.17 (dd, J = 3.36, 0.68 Hz, 1H, 2-furan H-3), 6.95

(d, J = 5.05 Hz, 1H, 6-thiophene H-4), 6.82 (dd, J = 1.85, 0.85 Hz, 1H, 4-furan H-4), 6.55 (dd, J = 3.38, 1.76 Hz, 1H, 2-furan H-4), 2.60 (s, 3H, 6-thiophene 3-CH_3).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 153.92, 153.81, 149.48, 144.28, 143.19, 141.18, 140.43, 137.40, 136.17, 132.16, 125.29, 124.62, 116.43, 112.55, 112.11, 109.17, 108.45, 16.28.

4.3.4. 2-(4-Chlorophenyl)-4-(furan-3-yl)-6-(3-methylthiophen-2-yl) pyridine (10)

The same procedure described in Section 4.3 was employed with **3** (R_1 = b, R_2 = d) (0.39 g, 1.80 mmol), dry ammonium acetate (1.38 g, 18.00 mmol), **5** (R_3 = l) (0.64 g, 1.80 mmol) and glacial acetic acid (2.00 mL) to yield a white solid (450 mg, 71.11%).

R_f (ethyl acetate/n-hexane 1:10 v/v): 0.35; mp 89.30 °C, purity: 99.2%.

LC/MS/MS (condition B): retention time: 8.69 min; [MH^+]: 352.2 (100%), [MH+2]: 354.2 (38%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.06 (d, J = 8.53 Hz, 2H, 2-phenyl H-2, H-6), 7.94 (br, 1H, 4-furan H-2), 7.63 (d, J = 1.16 Hz, 1H, pyridine H-3), 7.56 (d, J = 1.45 Hz, 1H, pyridine H-5), 7.55 (t, J = 2.01 Hz, 1H, 4-furan H-5), 7.45 (d, J = 8.54 Hz, 2H, 2-phenyl H-3, H-5), 7.30 (d, J = 5.06 Hz, 1H, 6-thiophene H-5), 6.96 (d, J = 5.06 Hz, 1H, 6-thiophene H-4), 6.81 (dd, J = 1.81, 0.81 Hz, 1H, 4-furan H-4), 2.62 (s, 3H, 6-thiophene 3-CH_3).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 156.09, 153.95, 144.38, 141.50, 140.33, 137.83, 137.53, 135.98, 135.22, 132.24, 128.85, 128.23, 125.52, 124.70, 116.76, 114.35, 108.46, 16.48.

4.3.5. 2-(4-Chlorophenyl)-6-(5-chlorothiophen-2-yl)-4-(furan-2-yl) pyridine (11)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = e) (0.47 g, 2.00 mmol), dry ammonium acetate (1.54 g, 20.00 mmol), **5** (R_3 = l) (0.72 g, 2.00 mmol) and glacial acetic acid (5.00 mL) to yield a white solid (320 mg, 43.03%).

R_f (ethyl acetate/n-hexane 1:10 v/v): 0.25; mp 187.4 °C, purity: 97.8%.

LC/MS/MS (condition C): retention time: 7.23 min; [M^+]: 372.2 (100%), [M+2]: 374.2 (71%), [M+4]: 376.2 (15%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.04 (d, J = 8.62 Hz, 2H, 2-phenyl H-2, H-6), 7.77 (d, J = 1.18 Hz, 1H, pyridine H-3), 7.70 (d, J = 1.18 Hz, 1H, pyridine H-5), 7.57 (d, J = 1.40 Hz, 1H, 4-furan H-5), 7.44 (d, J = 8.61 Hz, 2H, 2-phenyl H-3, H-5), 7.42 (d, J = 3.99 Hz, 1H, 6-thiophene H-3), 6.94 (d, J = 3.49 Hz, 1H, 4-furan H-3), 6.93 (d, J = 3.98 Hz, 1H, 6-thiophene H-4), 6.55 (dd, J = 3.43, 1.79 Hz, 1H, 4-furan H-4).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 155.97, 152.00, 151.26, 143.87, 143.76, 139.13, 136.92, 135.41, 132.73, 128.86, 128.14, 127.14, 123.63, 112.48, 112.21, 110.49, 108.95.

4.3.6. 2-(5-Chlorothiophen-2-yl)-6-(furan-2-yl)-4-(furan-3-yl) pyridine (12)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = d) (0.35 g, 1.50 mmol), dry ammonium acetate (1.15 g, 15.00 mmol), **5** (R_3 = e) (0.47 g, 1.50 mmol) and glacial acetic acid (5.00 mL) to yield a white solid (298 mg, 60.71%).

R_f (ethyl acetate/n-hexane 1:10 v/v): 0.30; mp 105.0 °C, purity: 100%.

LC/MS/MS (condition B): retention time: 7.67 min; [MH^+]: 328.1 (100%), [MH+2]: 330.1 (38%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.96 (dd, J = 1.26, 0.97 Hz, 1H, 4-furan H-2), 7.63 (d, J = 1.36 Hz, 1H, pyridine H-5), 7.56 (t, J = 1.90 Hz, 1H, 4-furan H-5), 7.55 (br, 1H, 6-furan H-5), 7.47 (d, J = 1.38 Hz, 1H, pyridine H-3), 7.40 (d, J = 3.96 Hz, 1H, 2-thiophene H-3), 7.17 (dd, J = 3.38, 0.70 Hz, 1H, 6-furan H-3), 6.93 (d, J = 3.96 Hz, 1H, 2-thiophene H-4), 6.82 (dd, J = 1.88, 0.88 Hz, 1H, 4-furan H-4), 6.56 (dd, J = 3.39, 1.77 Hz, 1H, 6-furan H-4).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 153.38, 151.99, 149.52, 144.35, 143.53, 143.35, 141.47, 140.53, 132.58, 127.07, 124.38, 123.58, 113.42, 112.67, 112.16, 109.40, 108.37.

4.3.7. 2-(5-Chlorothiophen-2-yl)-4-(furan-3-yl)-6-(5-methylfuran-2-yl) pyridine (13)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = d) (0.35 g, 1.50 mmol), dry ammonium acetate (1.15 g, 15.00 mmol), **5** (R_3 = f) (0.49 g, 1.50 mmol) and glacial acetic acid (5.00 mL) to yield a white solid (180 mg, 35.20%).

R_f (ethyl acetate/n-hexane 1:10 v/v): 0.35; mp 166.9 °C, purity: 100%.

LC/MS/MS (condition B): retention time: 8.52 min; [MH^+]: 342.1 (100%), [MH+2]: 344.1 (38%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.95 (dd, J = 1.22, 0.90 Hz, 1H, 4-furan H-2), 7.57 (d, J = 1.32 Hz, 1H, pyridine H-5), 7.55 (t, J = 1.60 Hz, 1H, 4-furan H-5), 7.42 (d, J = 1.35 Hz, 1H, pyridine H-3), 7.39 (d, J = 3.96 Hz, 1H, 2-thiophene H-3), 7.06 (d, J = 3.21 Hz, 1H, 6-furan H-3), 6.92 (d, J = 3.96 Hz, 1H, 2-thiophene H-4), 6.82 (dd, J = 1.82, 0.82 Hz, 1H, 4-furan H-4), 6.15 (dd, J = 3.24, 0.93 Hz, 1H, 6-furan H-4), 2.42 (s, 3H, 6-furan 5-CH_3).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 153.64, 151.88, 151.75, 149.68, 144.25, 143.70, 141.32, 140.49, 132.41, 127.02, 124.46, 123.46, 112.89, 112.17, 110.57, 108.46, 108.43, 13.94.

4.3.8. 2-(5-Chlorothiophen-2-yl)-4-(furan-3-yl)-6-o-tolyl pyridine (14)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = d) (0.35 g, 1.50 mmol), dry ammonium acetate (1.15 g, 15.00 mmol), **5** (R_3 = g) (0.50 g, 1.50 mmol) and glacial acetic acid (5.00 mL) to yield a white solid (114 mg, 21.60%).

R_f (ethyl acetate/n-hexane 1:10 v/v): 0.33; mp 93.3 °C, purity: 99.1%.

LC/MS/MS (condition B): retention time: 8.75 min; [MH^+]: 352.2 (100%), [MH+2]: 354.2 (38%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.93 (br, 1H, 4-furan H-2), 7.57 (d, J = 1.27 Hz, 1H, pyridine H-3), 7.55 (t, J = 1.54 Hz, 1H, 4-furan H-5), 7.49–7.46 (m, 1H, 6-phenyl H-6), 7.42 (d, J = 3.96 Hz, 1H, 2-thiophene H-3), 7.36 (d, J = 1.23 Hz, 1H, pyridine H-5), 7.32–7.28 (m, 3H, 6-phenyl H-3, H-4, H-5), 6.93 (d, J = 3.96 Hz, 1H, 2-thiophene H-4), 6.79 (dd, J = 1.80, 0.84 Hz, 1H, 4-furan H-4), 2.49 (s, 3H, 6-phenyl 2-CH_3).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 160.40, 151.44, 144.40, 143.93, 141.13, 140.38, 139.57, 136.48, 132.48, 131.06, 129.56, 128.55, 127.07, 125.90, 124.44, 123.34, 119.24, 112.37, 108.36, 20.70.

4.3.9. 2-(5-Chlorothiophen-2-yl)-4-(furan-3-yl)-6-m-tolyl pyridine (15)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = d) (0.35 g, 1.50 mmol), dry ammonium acetate (1.15 g, 15.00 mmol), **5** (R_3 = h) (0.50 g, 1.50 mmol) and glacial acetic acid (5.00 mL) to yield a white solid (133 mg, 25.33%).

R_f (ethyl acetate/n-hexane 1:10 v/v): 0.35; mp 103.7 °C, purity: 100%.

LC/MS/MS (condition C): retention time: 5.59 min; [MH^+]: 352.2 (100%), [MH+2]: 354.2 (38%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.96 (d, J = 0.79 Hz, 1H, 4-furan H-2), 7.90 (s, 1H, 6-phenyl H-2), 7.89 (d, J = 8.09 Hz, 1H, 6-phenyl H-6), 7.65 (d, J = 1.12 Hz, 1H, pyridine H-5), 7.56 (t, J = 1.75 Hz, 1H, 4-furan H-5), 7.54 (d, J = 1.14 Hz, 1H, pyridine H-3), 7.42 (d, J = 3.91 Hz, 1H, 2-thiophene H-3), 7.39 (t, J = 7.48 Hz, 1H, 6-phenyl H-5), 7.26 (d, J = 7.08 Hz, 1H, 6-phenyl H-4), 6.95 (d, J = 3.95 Hz, 1H, 2-thiophene H-4), 6.82 (dd, J = 1.83, 0.87 Hz, 1H, 4-furan H-4), 2.47 (s, 3H, 6-phenyl 3-CH_3).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 157.54, 151.91, 144.35, 144.05, 141.52, 140.37, 138.55, 138.34, 132.50, 130.09, 128.62, 127.57, 127.07, 124.58, 124.06, 123.36, 115.59, 112.87, 108.45, 21.62.

4.3.10. 2-(5-Chlorothiophen-2-yl)-4-(furan-3-yl)-6-p-tolyl pyridine (16)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = d) (0.35 g, 1.50 mmol), dry ammonium acetate (1.15 g, 15.00 mmol), **5** (R_3 = i) (0.50 g, 1.50 mmol) and glacial acetic acid (5.00 mL) to yield a white solid (241 mg, 45.74%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.41; mp 118.4 °C, purity: 100%.

LC/MS/MS (condition C): retention time: 5.67 min; [MH^+]: 352.2 (100%), [MH+2]: 354.2 (38%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.20 Hz, 2H, 6-phenyl H-2, H-6), 7.94 (br, 1H, 4-furan H-2), 7.64 (d, *J* = 1.22 Hz, 1H, pyridine H-5), 7.56 (t, *J* = 1.63 Hz, 1H, 4-furan H-5), 7.52 (d, *J* = 1.23 Hz, 1H, pyridine H-3), 7.41 (d, *J* = 3.95 Hz, 1H, 2-thiophene H-3), 7.30 (d, *J* = 8.03 Hz, 2H, 6-phenyl H-3, H-5), 6.94 (d, *J* = 3.95 Hz, 1H, 2-thiophene H-4), 6.81 (dd, *J* = 1.80, 0.81 Hz, 1H, 4-furan H-4), 2.42 (s, 3H, 6-phenyl 4-CH_3).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 157.33, 151.86, 144.33, 144.15, 141.49, 140.33, 139.39, 135.77, 132.48, 129.43, 127.05, 126.76, 124.62, 123.28, 115.16, 112.63, 108.45, 21.33.

4.3.11. 2-(2-Chlorophenyl)-6-(5-chlorothiophen-2-yl)-4-(furan-3-yl) pyridine (17)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = d) (0.47 g, 2.00 mmol), dry ammonium acetate (1.54 g, 20.00 mmol), **5** (R_3 = j) (0.72 g, 2.00 mmol) and glacial acetic acid (5.00 mL) to yield a white solid (153 mg, 20.64%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.35; mp 160.4 °C, purity: 100%.

LC/MS/MS (condition C): retention time: 4.49 min; [M^+]: 372.2 (100%), [M+2]: 374.1 (71%), [M+4]: 376.1 (15%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.94 (d, *J* = 0.91 Hz, 1H, 4-furan H-2), 7.72–7.68 (m, 1H, 2-phenyl H-6), 7.63 (d, *J* = 1.31 Hz, 1H, pyridine H-3), 7.60 (d, *J* = 1.33 Hz, 1H, pyridine H-5), 7.55 (t, *J* = 1.71 Hz, 1H, 4-furan H-5), 7.52–7.48 (m, 1H, 2-phenyl H-3), 7.43 (d, *J* = 3.94 Hz, 1H, 6-thiophene H-3), 7.40–7.35 (m, 2H, 2-phenyl H-4, H-5), 6.94 (d, *J* = 3.95 Hz, 1H, 6-thiophene H-4), 6.80 (dd, *J* = 1.80, 0.83 Hz, 1H, 4-furan H-4).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 156.95, 152.06, 144.42, 143.53, 140.78, 140.52, 138.54, 132.60, 132.24, 131.75, 130.26, 129.76, 127.10, 127.03, 124.35, 123.57, 120.11, 113.22, 108.40.

4.3.12. 2-(3-Chlorophenyl)-6-(5-chlorothiophen-2-yl)-4-(furan-3-yl) pyridine (18)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = d) (0.35 g, 1.50 mmol), dry ammonium acetate (1.15 g, 15.00 mmol), **5** (R_3 = k) (0.54 g, 1.50 mmol) and glacial acetic acid (5.00 mL) to yield a white solid (225 mg, 40.29%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.35; mp 138.3 °C, purity: 100%.

LC/MS/MS (condition C): retention time: 6.22 min; [M^+]: 372.2 (100%), [M+2]: 374.1 (71%), [M+4]: 376.1 (15%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.08–8.07 (m, 1H, 2-phenyl H-2), 7.99–7.97 (m, 1H, 2-phenyl H-6), 7.96 (dd, *J* = 1.31, 1.06 Hz, 1H, 4-furan H-2), 7.62 (d, *J* = 1.24 Hz, 1H, pyridine H-3), 7.57 (t, *J* = 1.70 Hz, 1H, 4-furan H-5), 7.56 (d, *J* = 1.26 Hz, 1H, pyridine H-5), 7.42 (d, *J* = 3.89 Hz, 1H, 6-thiophene H-3), 7.42–7.40 (m, 2H, 2-phenyl H-4, H-5), 6.95 (d, *J* = 3.96 Hz, 1H, 6-thiophene H-4), 6.81 (dd, *J* = 1.81, 0.83 Hz, 1H, 4-furan H-4).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 155.84, 152.11, 144.47, 143.64, 141.85, 140.49, 140.37, 134.79, 132.82, 129.95, 129.27, 127.14, 127.03, 124.99, 124.38, 123.64, 115.49, 113.43, 108.38.

4.3.13. 2-(4-Chlorophenyl)-6-(5-chlorothiophen-2-yl)-4-(furan-3-yl) pyridine (19)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = d) (0.35 g, 1.50 mmol), dry ammonium acetate (1.15 g, 15.00 mmol), **5** (R_3 = l) (0.54 g, 1.50 mmol) and glacial acetic acid (5.00 mL) to yield a white solid (232 mg, 41.54%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.26; mp 152.6 °C, purity: 100%.

LC/MS/MS (condition C): retention time: 6.23 min; [M^+]: 372.1 (100%), [M+2]: 374.1 (71%), [M+4]: 376.1 (15%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.04 (d, *J* = 8.72 Hz, 2H, 2-phenyl H-2, H-6), 7.95 (dd, *J* = 1.30, 0.98 Hz, 1H, 4-furan H-2), 7.62 (d, *J* = 1.28 Hz, 1H, pyridine H-3), 7.57 (t, *J* = 1.67 Hz, 1H, 4-furan H-5), 7.55 (d, *J* = 1.27 Hz, 1H, pyridine H-5), 7.46 (d, *J* = 8.70 Hz, 2H, 2-phenyl H-3, H-5), 7.42 (d, *J* = 3.97 Hz, 1H, 6-thiophene H-3), 6.94 (d, *J* = 3.97 Hz, 1H, 6-thiophene H-4), 6.81 (dd, *J* = 1.87, 0.88 Hz, 1H, 4-furan H-4).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 156.08, 152.05, 144.44, 143.74, 141.79, 140.43, 136.96, 135.42, 132.75, 128.88, 128.15, 127.13, 124.43, 123.55, 115.19, 113.13, 108.38.

4.3.14. 6-(5-Chlorothiophen-2-yl)-4-(furan-3-yl)-2, 2′-bipyridine (20)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = d) (0.35 g, 1.50 mmol), dry ammonium acetate (1.15 g, 15.00 mmol), **5** (R_3 = m) (0.49 g, 1.50 mmol) and glacial acetic acid (5.00 mL) to yield a white solid (275 mg, 54.11%).

R_f (ethyl acetate/*n*-hexane 1:2 v/v): 0.56; mp 166.7 °C, purity: 100%.

LC/MS/MS (condition B): retention time: 7.67 min; [MH^+]: 339.2 (100%), [MH+2]: 341.2 (38%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.69 (d, *J* = 4.74 Hz, 1H, 2-pyridine H-6′), 8.53 (d, *J* = 7.98 Hz, 1H, 2-pyridine H-3′), 8.40 (d, *J* = 1.34 Hz, 1H, pyridine H-3), 8.03 (br, 1H, 4-furan H-2), 7.86 (dt, *J* = 7.70, 1.73 Hz, 1H, 2-pyridine H-4′), 7.63 (d, *J* = 1.35 Hz, 1H, pyridine H-5), 7.55 (t, *J* = 1.61 Hz, 1H, 4-furan H-5), 7.43 (d, *J* = 3.95 Hz, 1H, 6-thiophene H-3), 7.35 (ddd, *J* = 7.44, 4.80, 0.98 Hz, 1H, 2-pyridine H-5′), 6.95 (d, *J* = 3.94 Hz, 1H, 6-thiophene H-4), 6.88 (dd, *J* = 1.02, 0.80 Hz, 1H, 4-furan H-4).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 156.04, 155.43, 151.57, 148.96, 144.27, 143.85, 141.85, 140.75, 136.99, 132.52, 127.14, 124.45, 124.05, 123.40, 121.46, 116.02, 114.25, 108.48.

4.3.15. 6-(5-Chlorothiophen-2-yl)-4-(furan-3-yl)-2,3′-bipyridine (21)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = d) (0.47 g, 2.00 mmol), dry ammonium acetate (1.54 g, 20.00 mmol), **5** (R_3 = n) (0.65 g, 2.00 mmol) and glacial acetic acid (5.00 mL) to yield a white solid (315 mg, 46.51%).

R_f (ethyl acetate/*n*-hexane 1:2 v/v): 0.28; mp 178.6 °C, purity: 100%.

LC/MS/MS (condition B): retention time: 6.22 min; [MH^+]: 339.2 (100%), [MH+2]: 341.2 (38%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 9.28 (dd, *J* = 2.22, 0.72 Hz, 1H, 2-pyridine H-2′), 8.68 (dd, *J* = 4.80, 1.63 Hz, 1H, 2-pyridine H-6'), 8.43 (td, *J* = 8.02, 1.72 Hz, 1H, 2-pyridine H-4'), 7.97 (dd, *J* = 1.34, 0.96 Hz, 1H, 4-furan H-2), 7.67 (d, *J* = 1.26 Hz, 1H, pyridine H-3), 7.60 (d, *J* = 1.30 Hz, 1H, pyridine H-5), 7.58 (t, *J* = 1.67 Hz, 1H, 4-furan H-5), 7.45 (ddd, *J* = 7.92, 4.75, 0.80 Hz, 1H, 2-pyridine H-5'), 7.44 (d, *J* = 3.97 Hz, 1H, 6-thiophene H-3), 6.96 (d, *J* = 3.97 Hz, 1H, 6-thiophene H-4), 6.83 (dd, *J* = 1.87, 0.90 Hz, 1H, 4-furan H-4).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 154.77, 152.38, 150.18, 148.17, 144.54, 143.44, 141.97, 140.54, 134.43, 134.11, 132.96, 127.18, 124.26, 123.76, 123.59, 115.43, 113.54, 108.34.

4.3.16. 4-(5-Chlorofuran-2-yl)-2-(furan-2-yl)-6-(3-methylthiophen-2-yl) pyridine (22)

The same procedure described in Section 4.3 was employed with **3** (R_1 = b, R_2 = c) (0.25 g, 1.00 mmol), dry ammonium acetate (0.77 g, 10.00 mmol), **5** (R_3 = e) (0.31 g, 1.00 mmol) and glacial acetic acid (2.00 mL) to yield a white solid (152 mg, 44.61%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.38; mp 115.0 °C, purity: 99.6%.

LC/MS/MS (condition B): retention time: 8.76 min; [MH^+]: 342.1 (100%), [MH+2]: 344.1 (38%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.73 (d, J = 1.36 Hz, 1H, pyridine H-3), 7.58 (d, J = 1.35 Hz, 1H, pyridine H-5), 7.55 (dd, J = 1.70, 0.78 Hz, 1H, 2-furan H-5), 7.29 (d, J = 5.05 Hz, 1H, 6-thiophene H-5), 7.17 (dd, J = 3.38, 0.70 Hz, 1H, 2-furan H-3), 6.95 (d, J = 5.10 Hz, 1H, 6-thiophene H-4), 6.93 (d, J = 3.45 Hz, 1H, 4-furan H-3), 6.55 (dd, J = 3.39, 1.76 Hz, 1H, 2-furan H-4), 6.34 (d, J = 3.48 Hz, 1H, 4-furan H-4), 2.61 (s, 3H, 6-thiophene 3-CH_3).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 153.98, 153.65, 151.03, 149.49, 143.32, 138.13, 137.67, 137.27, 136.41, 132.21, 125.45, 113.13, 112.13, 110.69, 109.47, 109.34, 108.84, 16.35.

4.3.17. 4-(5-Chlorofuran-2-yl)-2-(3-chlorophenyl)-6-(3-methylthiophen-2-yl) pyridine (23)

The same procedure described in Section 4.3 was employed with **3** (R_1 = b, R_2 = c) (0.30 g, 1.20 mmol), dry ammonium acetate (0.92 g, 12.00 mmol), **5** (R_3 = k) (0.43 g, 1.20 mmol) and glacial acetic acid (2.00 mL) to yield a white solid (250 mg, 53.95%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.39; mp 114.0 °C, purity: 98.9%.

LC/MS/MS (condition C): retention time: 7.23 min; [M^+]: 386.1 (100%), [M+2]: 388.1 (71%), [M+4]: 390.1 (15%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.13–8.12 (m, 1H, 2-phenyl H-2), 8.04–7.99 (m, 1H, 2-phenyl H-6), 7.75 (d, J = 1.26 Hz, 1H, pyridine H-3), 7.67 (d, J = 1.23 Hz, 1H, pyridine H-5), 7.43–7.40 (m, 2H, 2-phenyl H-4, H-5), 7.31 (d, J = 5.06 Hz, 1H, 6-thiophene H-5), 6.97 (d, J = 5.09 Hz, 1H, 6-thiophene H-4), 6.94 (d, J = 3.49 Hz, 1H, 4-furan H-3), 6.36 (d, J = 3.48 Hz, 1H, 4-furan H-4), 2.63 (s, 3H, 6-thiophene 3-CH_3).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 155.87, 154.03, 150.95, 140.68, 138.27, 137.99, 137.60, 136.27, 134.76, 132.29, 129.93, 129.20, 127.11, 125.73, 125.05, 113.70, 111.39, 110.74, 108.90, 16.53.

4.3.18. 4-(5-Chlorofuran-2-yl)-2-(4-chlorophenyl)-6-(3-methylthiophen-2-yl) pyridine (24)

The same procedure described in Section 4.3 was employed with **3** (R_1 = b, R_2 = c) (0.30 g, 1.20 mmol), dry ammonium acetate (0.92 g, 12.00 mmol), **5** (R_3 = l) (0.43 g, 1.20 mmol) and glacial acetic acid (2.00 mL) to yield a white solid (221 mg, 47.82%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.39; mp 101.8 °C, purity: 99.2%.

LC/MS/MS (condition C): retention time: 7.24 min; [M^+]: 386.1 (100%), [M+2]: 388.1 (71%), [M+4]: 390.1 (15%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.08 (d, J = 8.57 Hz, 2H, 2-phenyl H-2, H-6), 7.74 (d, J = 1.07 Hz, 1H, pyridine H-3), 7.65 (d, J = 1.00 Hz, 1H, pyridine H-5), 7.46 (d, J = 8.56 Hz, 2H, 2-phenyl H-3, H-5), 7.31 (d, J = 5.05 Hz, 1H, 6-thiophene H-5), 6.96 (d, J = 5.07 Hz, 1H, 6-thiophene H-4), 6.93 (d, J = 3.48 Hz, 1H, 4-furan H-3), 6.35 (d, J = 3.46 Hz, 1H, 4-furan H-4), 2.62 (s, 3H, 6-thiophene 3-CH_3).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 156.10, 153.94, 151.02, 138.20, 137.94, 137.68, 137.27, 136.19, 135.34, 132.29, 128.86, 128.22, 125.67, 113.40, 111.09, 110.65, 108.87, 16.54.

4.3.19. 4-(5-Chlorofuran-2-yl)-6-(3-methylthiophen-2-yl)-2,3′-bipyridine (25)

The same procedure described in Section 4.3 was employed with **3** (R_1 = b, R_2 = c) (0.25 g, 1.00 mmol), dry ammonium acetate (0.77 g, 10.00 mmol), **5** (R_3 = n) (0.32 g, 1.00 mmol) and glacial acetic acid (2.00 mL) to yield a white solid (195 mg, 55.26%).

R_f (ethyl acetate/*n*-hexane 1:2 v/v): 0.21; mp 168.0 °C, purity: 98.5%.

LC/MS/MS (condition B): retention time: 6.98 min; [MH^+]: 353.2 (100%), [MH+2]: 355.2 (38%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 9.34 (d, J = 1.58 Hz, 1H, 2-pyridine H-2'), 8.68 (dd, J = 4.77, 1.54 Hz, 1H, 2-pyridine H-6'), 8.43 (td, J = 8.01, 1.75 Hz, 1H, 2-pyridine H-4'), 7.79 (d, J = 1.23 Hz, 1H, pyridine H-3), 7.70 (d, J = 1.18 Hz, 1H, pyridine H-5), 7.43 (ddd, J = 7.98, 4.82, 0.70 Hz, 1H, 2-pyridine H-5'), 7.32 (d, J = 5.04 Hz, 1H, 6-thiophene H-5), 6.97 (d, J = 4.88 Hz, 1H, 6-thiophene H-4), 6.95 (d, J = 3.44 Hz, 1H, 4-furan H-3), 6.37 (d, J = 3.49 Hz, 1H, 4-furan H-4), 2.64 (s, 3H, 6-thiophene 3-CH_3).

^{13}C NMR (62.5 MHz, $CDCl_3$) 154.79, 154.31, 150.78, 150.13, 148.35, 138.39, 138.06, 137.39, 136.47, 134.39, 132.36, 125.82, 123.54, 113.77, 111.32, 110.89, 108.94, 16.60.

4.3.20. 4-(5-Chlorofuran-2-yl)-2-(5-chlorothiophen-2-yl)-6-(furan-2-yl) pyridine (26)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = c) (0.54 g, 2.00 mmol), dry ammonium acetate (1.54 g, 20.00 mmol), **5** (R_3 = e) (0.63 g, 2.00 mmol) and glacial acetic acid (5.00 mL) to yield a light yellow solid (232 mg, 32.09%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.34; mp 148.5 °C, purity: 100%.

LC/MS/MS (condition B): retention time: 9.30 min; [M^+]: 362.1 (100%), [M+2]: 364.1 (71%), [M+4]: 366.1 (15%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 7.69 (d, J = 1.31 Hz, 1H, pyridine H-3), 7.57 (d, J = 1.34 Hz, 1H, pyridine H-5), 7.55 (dd, J = 1.60, 0.65 Hz, 1H, 6-furan H-5), 7.43 (d, J = 3.97 Hz, 1H, 2-thiophene H-3), 7.16 (dd, J = 3.35, 0.46 Hz, 1H, 6-furan H-3), 6.94 (d, J = 3.99 Hz, 1H, 2-thiophene H-4), 6.93 (d, J = 3.43 Hz, 1H, 4-furan H-3), 6.56 (dd, J = 3.38, 1.75 Hz, 1H, 6-furan H-4), 6.35 (d, J = 3.49 Hz, 1H, 4-furan H-4).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 153.20, 152.08, 150.70, 149.49, 143.46, 143.33, 138.25, 137.83, 132.76, 127.12, 123.86, 112.18, 110.93, 110.23, 109.55, 109.51, 108.90.

4.3.21. 4-(5-Chlorofuran-2-yl)-2-(5-chlorothiophen-2-yl)-6-p-tolyl pyridine (27)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = c) (0.54 g, 2.00 mmol), dry ammonium acetate (1.54 g, 20.00 mmol), **5** (R_3 = i) (0.67 g, 2.00 mmol) and glacial acetic acid (5.00 mL) to yield a white solid (195 mg, 25.26%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.41; mp 157.9 °C, purity: 100%.

LC/MS/MS (condition C): retention time: 7.94 min; [M^+]: 386.1 (100%), [M+2]: 388.1 (71%), [M+4]: 390.1 (15%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.01 (d, J = 8.19 Hz, 2H, 6-phenyl H-2, H-6), 7.72 (d, J = 1.20 Hz, 1H, pyridine H-5), 7.61 (d, J = 1.20 Hz, 1H, pyridine H-3), 7.43 (d, J = 3.97 Hz, 1H, 2-thiophene H-3), 7.30 (d, J = 7.99 Hz, 2H, 6-phenyl H-3, H-5), 6.94 (d, J = 3.97 Hz, 1H, 2-thiophene H-4), 6.92 (d, J = 3.49 Hz, 1H, 4-furan H-3), 6.34 (d, J = 3.48 Hz, 1H, 4-furan H-4), 2.42 (s, 3H, 6-phenyl 4-CH_3).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 157.33, 151.93, 151.00, 143.98, 139.53, 138.11, 137.92, 135.55, 132.66, 129.43, 127.09, 126.75, 123.54, 111.83, 110.61, 109.47, 108.86, 21.34.

4.3.22. 4-(5-Chlorofuran-2-yl)-2-(3-chlorophenyl)-6-(5-chlorothiophen-2-yl) pyridine (28)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = c) (0.27 g, 1.00 mmol), dry ammonium acetate (0.77 g, 10.00 mmol), **5** (R_3 = k) (0.36 g, 1.00 mmol) and glacial acetic acid (5.00 mL) to yield a creamy white solid (102 mg, 25.20%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.39; mp 177.4 °C, purity: 98.4%.

LC/MS/MS (condition C): retention time: 9.05 min; [M^+]: 406.1 (97%), [M+2]: 408.1 (100%), [M+4]: 410.1 (36%), [M+6]: 412.1 (5%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.09 (br, 1H, 2-phenyl H-2), 8.01–7.97 (m, 1H, 2-phenyl H-6), 7.72 (d, J = 1.20 Hz, 1H, pyridine H-3), 7.67 (d, J = 1.20 Hz, 1H, pyridine H-5), 7.46 (d, J = 3.96 Hz, 1H, 6-thiophene H-3), 7.44–7.41 (m, 2H, 2-phenyl H-4, H-5), 6.96 (d, J = 3.61 Hz, 2H, 4-furan H-3, 6-thiophene H-4), 6.35 (d, J = 3.49 Hz, 1H, 4-furan H-4).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 155.91, 152.22, 150.65, 143.45, 140.17, 138.45, 138.21, 134.82, 133.03, 129.97, 129.40, 127.20, 127.03, 125.00, 123.93, 112.22, 110.99, 110.28, 108.98.

4.3.23. 4-(5-Chlorofuran-2-yl)-2-(4-chlorophenyl)-6-(5-chlorothiophen-2-yl) pyridine (29)

The same procedure described in Section 4.3 was employed with **3** (R_1 = a, R_2 = c) (0.32 g, 1.20 mmol), dry ammonium acetate (0.92 g, 12.00 mmol), **5** (R_3 = l) (0.43 g, 1.20 mmol) and glacial acetic acid (5.00 mL) to yield a creamy white solid (133 mg, 27.43%).

R_f (ethyl acetate/*n*-hexane 1:10 v/v): 0.37; mp 182.0 °C, purity: 98.1%.

LC/MS/MS (condition A): retention time: 6.41 min; [M^+]: 406.1 (100%), [M+2]: 408.1 (99%), [M+4]: 410.1 (36%), [M+6]: 412.1 (5%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 8.07 (d, J = 8.70 Hz, 2H, 2-phenyl H-2, H-6), 7.72 (d, J = 1.24 Hz, 1H, pyridine H-3), 7.65 (d, J = 1.25 Hz, 1H, pyridine H-5), 7.46 (d, J = 8.70 Hz, 2H, 2-phenyl H-3, H-5), 7.45 (d, J = 3.93 Hz, 1H, 6-thiophene H-3), 6.95 (d, J = 3.90 Hz, 1H, 6-thiophene H-4), 6.94 (d, J = 3.42 Hz, 1H, 4-furan H-3), 6.36 (d, J = 3.50 Hz, 1H, 4-furan H-4).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ 156.08, 152.10, 150.71, 143.56, 138.36, 138.12, 136.72, 135.55, 132.93, 128.88, 128.14, 127.17, 123.80, 111.85, 110.88, 109.93, 108.94.

4.4. Biological assays

DNA topo I inhibition assay was determined following the method reported by Fukuda et al.[13] with minor modifications. The test compounds were dissolved in DMSO at 20 mM as stock solution. The activity of DNA topo I was determined by assessing the relaxation of supercoiled DNA pBR322. The mixture of 100 ng of plasmid pBR322 DNA and 0.4 units of recombinant human DNA topoisomerase I (TopoGEN INC., USA) was incubated without and with the prepared compounds at 37 °C for 30 min in the relaxation buffer (10 mM Tris–HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin, 1 mM spermidine, 5% glycerol). The reaction in the final volume of 10 μL was terminated by adding 2.5 μL of the stop solution containing 5% sarcosyl, 0.0025% bromophenol blue, and 25% glycerol. DNA samples were then electrophoresed on a 1% agarose gel at 15 V for 7 h with a running buffer of TAE. Gels were stained for 30 min in an aqueous solution of ethidium bromide (0.5 μg/mL). DNA bands were visualized by transillumination with UV light and were quantitated using AlphaImager™ (Alpha Innotech Corporation).

DNA topo II inhibitory activity of compounds was measured as follows.[14] The mixture of 200 ng of supercoiled pBR322 plasmid DNA and 1 units of human DNA topoisomerase IIα (Usb Corp., USA) was incubated without and with the prepared compounds in the assay buffer (10 mM Tris–HCl (pH 7.9) containing 50 mM NaCl, 50 mM KCl, 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 a 1% agarose gel at 25 V for 4 h with a running buffer of TAE. Gels were stained for 30 min in an aqueous solution of ethidium bromide (0.5 μg/mL). DNA bands were visualized by transillumination with UV light and supercoiled DNA was quantitated using AlphaImager™ (Alpha Innotech Corporation).

For the evaluation of cytotoxicity, five different cancer cell lines were used: human breast adenocarcinoma cell line (MCF-7), human cervix tumor cell line (HeLa), human prostate tumor cell line (DU145), human colorectal adenocarcinoma cell line (HCT15) and chronic myelogenous leukemia cell line (K562). Experiments were performed as described previously.[14] Cancer cells were cultured according to the supplier's instructions. Cells were seeded in 96-well plates at a density of 2–4 $\times$ 10^4 cells per well and incubated for overnight in 0.1 mL of media supplied with 10% Fetal Bovine Serum (Hyclone, USA) in 5% CO_2 incubator at 37 °C. On day 2, culture medium in each well was exchanged with 0.1 mL aliquots of medium containing graded concentrations of compounds. On day 4, each well was added with 5 μL of the cell counting kit-8 solution (Dojindo, Japan) then incubated for additional 4 h under the same condition. The absorbance of each well was determined by an Automatic Elisa Reader System (Bio-Rad 3550) with a 450 nm wavelength. For determination of the IC_{50} values, the absorbance readings at 450 nm were fitted to the four-parameter logistic equation. The compounds adriamycin, etoposide, and camptothecin were purchased from Sigma and used as positive controls.

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