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2,6-Dithienyl-4-furyl pyridines: Synthesis, topoisomerase I and II inhibition, cytotoxicity, structure–activity relationship, and docking study

Arjun Basnet [a], Pritam Thapa [a], Radha Karki [a], Hoyoung Choi [a], Jae Hun Choi [a], Minho Yun [a], Byeong-Seon Jeong [a], Yurngdong Jahng [a], Younghwa Na [b], Won-Jea Cho [c], Youngjoo Kwon [d], Chong-Soon Lee [e], Eung-Seok Lee [a,*]

[a] College of Pharmacy, Yeungnam University, Kyongsan 712-749, Republic of Korea
[b] College of Pharmacy, Catholic University of Daegu, Kyongsan 712-702, Republic of Korea
[c] College of Pharmacy, Chonnam National University, Kwangju 500-757, Republic of Korea
[d] College of Pharmacy, Pharmacy & Division of Life & Pharmaceutical Sciences, Ewha Womans University, Seoul 120-750, Republic of Korea
[e] Department of Biochemistry, Yeungnam University, Kyongsan 712-749, Republic of Korea

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ABSTRACT

For the development of novel antitumor agents, 2,6-dithienyl-4-furyl pyridine derivatives were prepared and evaluated for their topoisomerase I and II inhibitory activity as well as cytotoxicity against several human cancer cell lines. Among the 21 prepared compounds, compound **24** exhibited strong topoisomerase I inhibitory activity. In addition, a docking study with topoisomerase I and compound **24** was performed.

Topoisomerases, generally classified as type I and II, are critical cellular enzymes necessary for cell proliferation by solving topological hurdles in the process of DNA replication.[1–3] Topoisomerase I mediates the breaking and rejoining of single strand of DNA duplex to relax the supercoiled condition of chromosomes. On the other hand, topoisomerase II produces the relaxation of DNA double helices by scissoring and religating the two strands.[1,4] Due to the critical role of these enzymes for the cell proliferative process, topoisomerases have been one of the major targets in the anticancer drug development area.[5,6]

Due to its ability to form metal complexes[7] and its status as a DNA binding agent, α-terpyridine has been precious 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 topoisomerase I inhibitory activity.[9,10] Some of us also reported that terthiophene derivatives, bioisosteres of terpyridine, showed considerable protein kinase C (PKC) inhibitory activity and antitumor cytotoxicities against several human cancer cell lines.[11]

From the results of the previous studies, it was revealed that the 2-thienyl-4-furylpyridine skeleton (compound **6** in Fig. 2) exhibited strong topoisomerase I inhibitory activity (Fig. 1).[10] Such previous studies prompted us to design 2,6-dithienyl-4-furyl pyridine derivatives as topoisomerase I or II inhibitors in our endeavor to develop novel anticancer agents. Since 2,6-dithienyl-4-furyl pyridines possess 2-thienyl-4-furylpyridine skeleton, we expected that substituents at the 6-position on the central pyridine ring as thienyl group may affect biological activities such as topoisomerase I or II as well as cytotoxicity. It would be very interesting to observe the difference of biological activities by systematic substitution on the 6-position of 2-thienyl-4-furyl pyridine skeletons with thienyl group and to evaluate cytotoxicity and topoisomerase I and II inhibitory activities. In addition, we anticipated that we may obtain valuable information on the correlation between positions of thienyl or furyl groups employed in 2-thienyl-4-furyl pyridine skeletons and biological activities such as cytotoxicity and topoisomerase I or II inhibitory activity, or on the effect of branching on thienyl or furyl groups such as methyl or chloride. In connection with previous studies, we designed and prepared 21 2,6-dithienyl-4-furyl pyridine derivatives as terpyridine or terthiophene bioisosteres employing 2-thienyl-4-furyl pyridine skeletons, and

* Corresponding author. Tel.: +82 53 810 2827; fax: +82 53 810 4654.
E-mail address: eslee@yu.ac.kr (E.-S. Lee).

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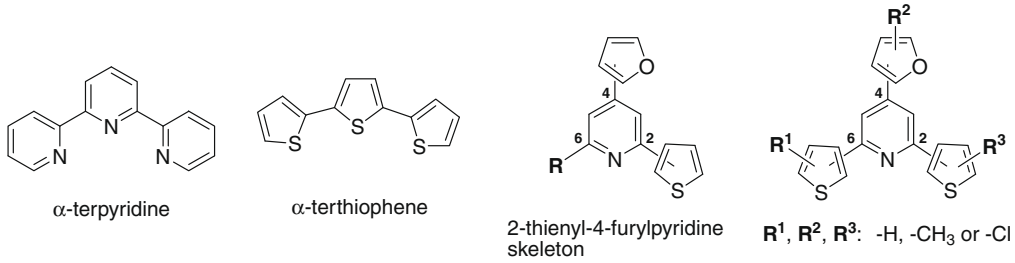


Figure 1. Structure of α-terpyridine, α-terthiophene and 2-thienyl-4-furylpyridine skeleton.

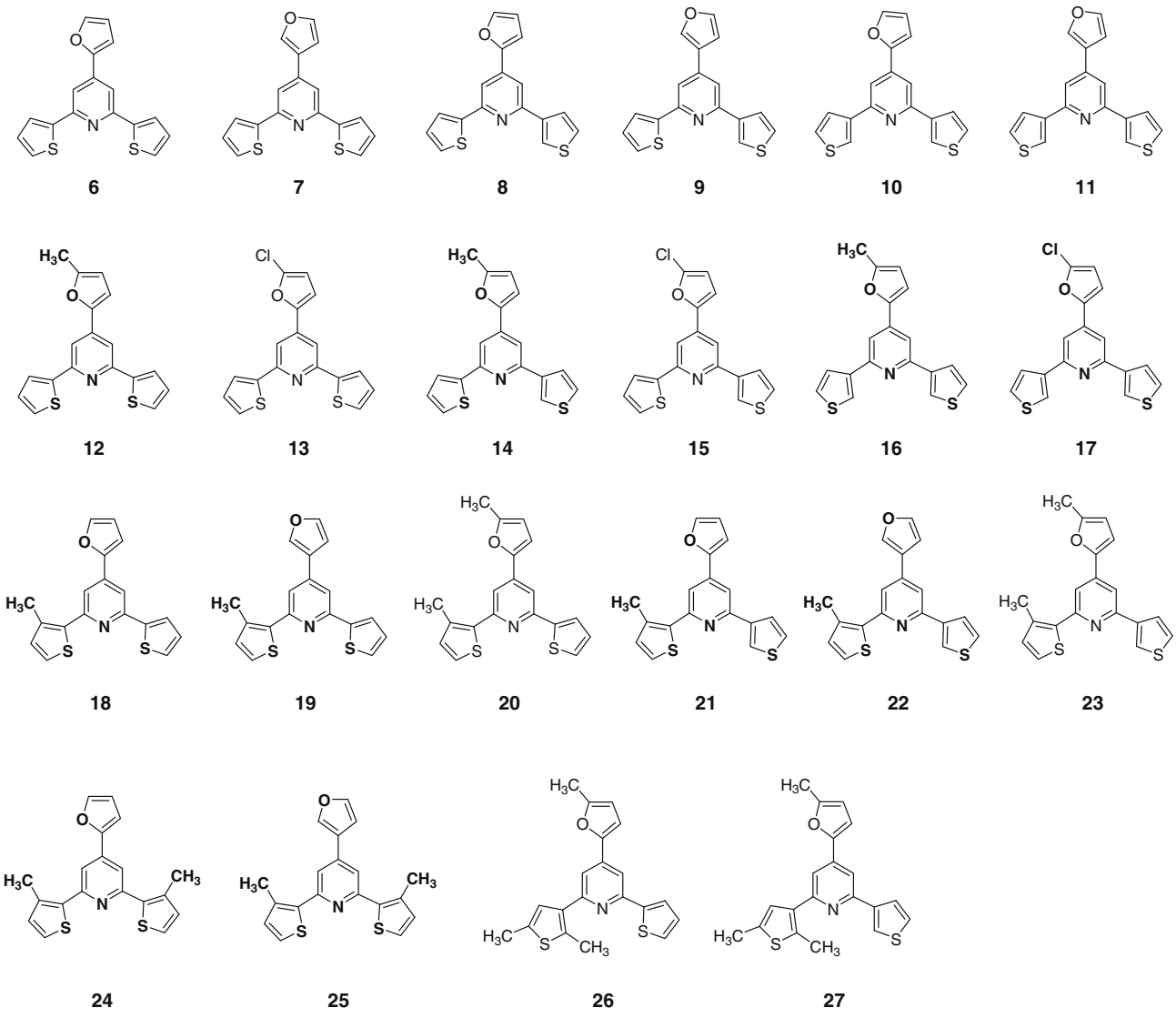
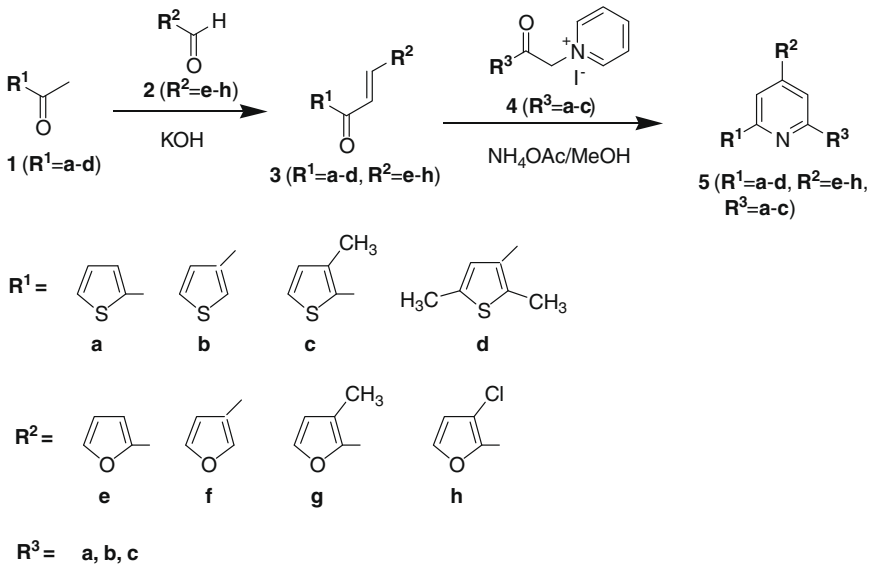


Figure 2. The prepared compounds.

evaluated them for their topoisomerase I and II inhibitory activity and cytotoxicity against several human cancer cell lines, as well as their structure–activity relationship. In addition, we performed a docking study with topoisomerase I and the prepared compound **24** exhibited strong topoisomerase I inhibition.

For the design, pyridine moiety was utilized as a basic skeleton, and thienyl derivatives were attached to the 2,6-position, and furyl derivatives to the 4 position of the pyridine structure (Fig. 1).

Synthetic methods for the preparation of 2,6-dithienyl-4-furyl pyridines (**6**–**27**) are summarized in Scheme 1. Acetylthiophenes **1a–d** were treated with furancarboxaldehydes **2e–h** in the presence of KOH in methanol–water (5:1) to afford intermediates **3** in a 27.1–89.6% yields. Using modified Kröhnke synthesis,[12] 2,6-dithienyl-4-furyl pyridines were prepared by treatment of **3** with 1-(2-oxo-2-thieny-ethyl)pyridinium iodide (**4a–c**) in the presence of ammonium acetate in methanol to give **6**–**27** in a 89.4–97.6%

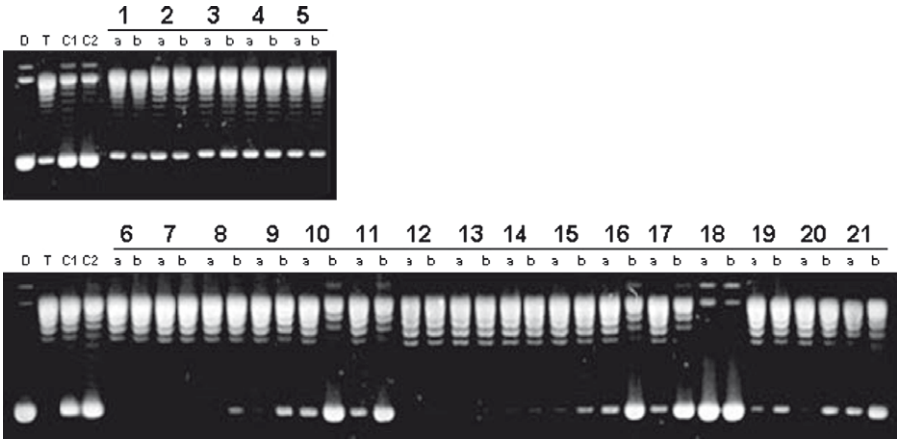


Scheme 1. Synthetic method.

yield. Pyridinium iodides **4a–c** were prepared in a quantitative yield by treatment of **1a–c** with iodine in pyridine.

Figure 2 shows the prepared 2,6-dithienyl-4-furyl pyridines (**6**–**27**).

Calf thymus DNA–topoisomerase I inhibitory activities[13] for the 21 prepared 2,6-dithienyl-4-furyl pyridines are shown in Figure 3, and summarized in Tables 1 and 2. Compounds **13**, **15**, **18**, and **21** exhibited moderate topoisomerase I inhibitory activities (42–52%



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 1- 38 Lane a: pBR322 DNA + Topo I + Compounds 20 μM

Lane 1- 38 Lane b: pBR322 DNA + Topo I + Compounds 100 μM

Lane	1	2	3	4	5
Compound	**8**	**10**	**7**	**9**	**11**

Lane	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Compound	**16**	**14**	**12**	**17**	**15**	**13**	**20**	**23**	**27**	**26**	**18**	**21**	**24**	**19**	**22**	**25**

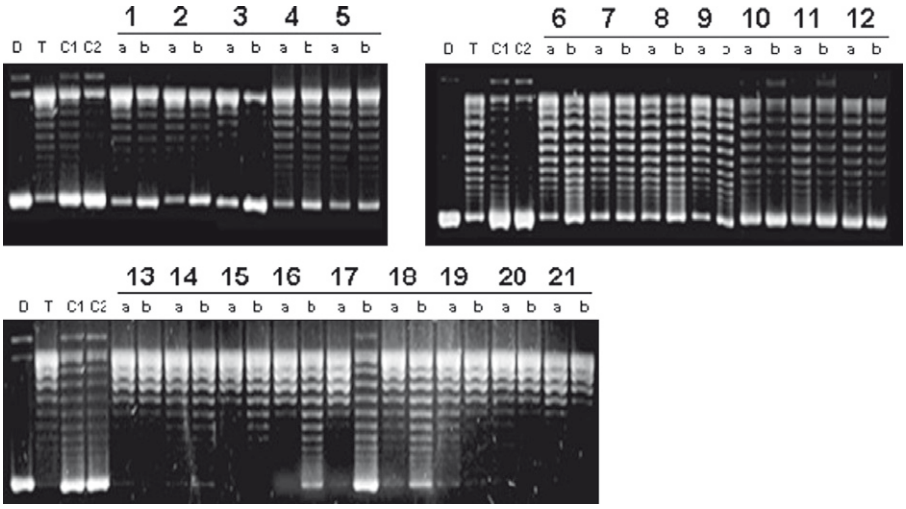
Figure 3. Topoisomerase I inhibitory activity of the prepared compounds.

Table 1
Topoisomerase I and II inhibitory activities of the prepared compounds (**7**–**11**)

Compounds	Topo I (% inhibition)		Topo II (% inhibition)	
	20 μM	100 μM	20 μM	100 μM
7	0	0	15	70
8	5	12	0	34
9	0	0	1	18
10	0	0	0	32
11	0	0	4	12
Camptothecin	64	76		
Etoposide			36	86

inhibition at 100 μM), whereas compound **24**[14] exhibited stronger topoisomerase I inhibitory activities (88% inhibition at 20 μM, 92% inhibition at 100 μM) compared to that of camptothecin (41% inhibition at 20 μM, 54% inhibition at 100 μM). It is generally recognized that such compounds have strong inhibitory activities as synthetic compounds, even though they have weaker inhibitory activities than that of camptothecin. Figure 4 shows human DNA–topoisomerase IIα inhibitory activities[13] for the 21 prepared 2,6-dithienyl-4-furyl pyridines, which are summarized in Tables 1 and 2. Compounds **7**, **15**, and **21** exhibited moderate topoisomerase II inhibitory activities (55–70% inhibition at 100 μM) compared to that of etoposide (73% inhibition at 100 μM). It is interesting to notice that compounds **15** and **21** exhibited inhibitory activities in both topoisomerase I and II.

In structure–activity relationship study of the prepared compounds for topoisomerase I and II inhibition, introduction of methyl or chloride functionality may increase the inhibitory activity, since compounds having strong or moderate topoisomerase I or II inhibitory activities possess chloride functionality on 4-furyl moiety (compounds **13** and **15**), or methyl functionality on 6-thienyl or both on 2- and 6-thienyl moiety (compounds **18**, **21** and **24**). However, we could not pull out any evident correlation regarding the introduction of methyl or chloride functionality. Evaluation of antitumor cytotoxicity[15] for the selected compounds (**7**, **13**, **15**, **18**, **21**, and **24**), which possess strong or moderate topoisomerase I or II inhibitory activities, were performed against five different human tumor cell lines with adriamycin, etoposide, and camptothecin as positive references: DU-145 (human prostate tumor), MCF-7 (human breast adenocarcinoma), HCT 116 (human colorectal carcinoma), MDA-MB231 (human breast tumor), and HeLa (human cervix tumor). The cytotoxic activities of the compounds were not very effective (Table 3). All the compounds exhibited moderate cytotoxicity in HCT 116, MDA-MB231, and HeLa cell lines, but they showed lower cytotoxicity than that of the references, which we could not pull out the structure correlation of cytotoxic activities among the prepared compounds. In addition,



Lane D: pBR322 DNA only

Lane T: pBR322 DNA +Topo II

Lane C1: pBR322 DNA + Topo II + Etoposide 20 μM

Lane C2: pBR322 DNA + Topo II + Etoposide 100 μM

Lane 1- 21 Lane a: pBR322 DNA + Topo II + Compounds 20 μM

Lane 1- 21 Lane b: pBR322 DNA + Topo II + Compounds 100 μM

Lane	1	2	3	4	5	6	7	8	9	10	11	12
Compound	**8**	**10**	**7**	**9**	**11**	**16**	**14**	**12**	**17**	**15**	**13**	**20**

Lane	13	14	15	16	17	18	19	20	21
Compound	**23**	**27**	**26**	**18**	**21**	**24**	**19**	**22**	**25**

Figure 4. Topoisomerase II inhibitory activity of the prepared compounds.

Table 2
Topoisomerase I and II inhibitory activities of the prepared compounds (**12**–**27**)

Compounds	Topo I (% inhibition)		Topo II (% inhibition)	
	20 μM	100 μM	20 μM	100 μM
12	0	3	0	8
13	12	42	0	35
14	0	0	0	2
15	12	50	7	55
16	0	0	0	5
17	0	14	0	8
18	12	51	9	28
19	3	9	7	3
20	0	0	15	25
21	9	52	11	58
22	0	13	0	0
23	0	0	0	0
24	88	92	14	23
25	15	30	0	0
26	0	5	0	0
27	0	0	0	7
Camptothecin	41	54		
Etoposide			65	73

there were no direct correlations between antitumor cytotoxicity and topoisomerase inhibitory activity.

To verify the binding mode of the potent inhibitor **24**, we carried out a docking study using Surflex-Dock in Sybyl version 8.05 by Tripos Associates, operating under Red Hat Linux 4.0 with an IBM computer (Intel Pentium 4, 2.8 GHz CPU, 1 GB memory) (Figs. 5 and 6). The structure of the inhibitor **24** was drawn into the Sybyl package and minimized with the Tripos force field and Gasteiger–Huckel charge on it. We chose the 1SC7 (PDB code) structure in Protein Data Bank and the structure was polished as follows: the phosphoester bond of G12 in 1SC7 was rebuilt, and the SH of G11 on the scissile strand was modified to OH. After running Surflex-Dock, 10 docked conformers were obtained. Among the conformations, the best total score (6.07) conformer was selected for speculating the detailed binding mode in the site. In our model, thienyl, pyridyl and furanyl rings intercalated between the −1 and +1 bases, parallel to the plane of the base pairs without having an H bond with topoisomerase I. In this model, the heterocyclic rings were considered to work as DNA intercalators to block the rewinding step of the phosphoester. The binding geometry of CPT

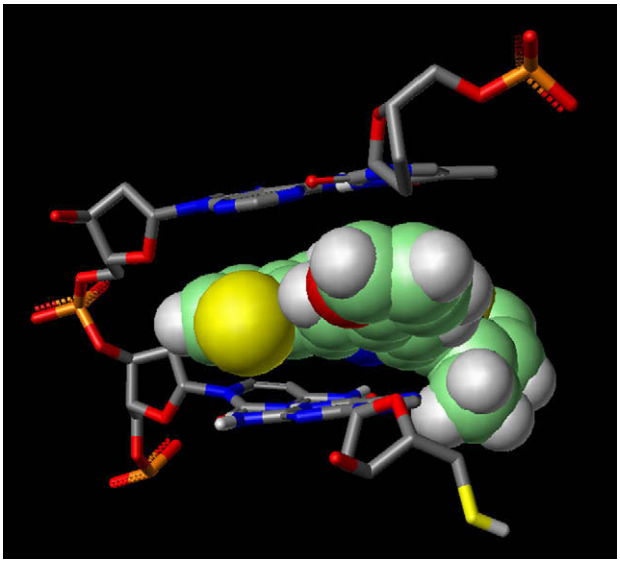


Figure 6. Space filling model of compound **24** docked in DNA sequence between C112, A113 and T10, TGP11.

Table 3
Cytotoxicities of selected compounds against various human cancer cell lines

	IC_{50}[a] (μM)				
Comp/cells (origin)	DU-145 (prostate)	MCF-7 (breast)	HCT 116 (colon)	MDA-MB231 (breast)	HeLa (cervix)
Adriamycin	1.30 ± 0.01	5.75 ± 0.29	1.10 ± 0.03	1.16 ± 0.12	1.60 ± 0.04
Etoposide	21.07 ± 2.58	18.01 ± 1.00	17.92 ± 0.38	16.85 ± 1.87	8.43 ± 0.65
Camptothecin	1.46 ± 0.08	7.42 ± 0.81	2.77 ± 0.29	1.10 ± 0.02	1.85 ± 0.28
7	>100	>100	50.57 ± 2.27	23.60 ± 2.46	8.62 ± 0.55
13	88.11 ± 4.82	>100	95.53 ± 5.84	54.83 ± 26	55.18 ± 1.32
15	>100	>100	>100	26.79 ± 0.981	43.68 ± 2.44
18	>100	>100	56.41 ± 1.33	59.61 ± 4.74	17.93 ± 1.30
21	>100	>100	51.06 ± 1.06	41.79 ± 2.35	19.19 ± 1.09
24	98.15 ± 5.09	>100	>100	38.34 ± 1.03	35.77 ± 2.38

[a] Each data point represents the mean ± S.D. from three different experiments performed in triplicate.

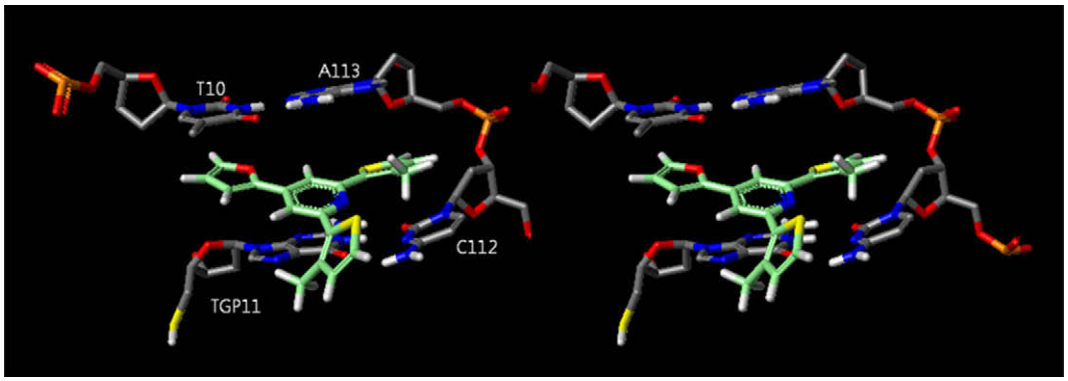


Figure 5. Model of the binding of compound **24** in the ternary complex consisting of DNA, topo I, and the inhibitor. The diagram is captured for stereoview.

in the DNA–topoisomerase I complex was studied by an ab initio quantum mechanics calculation to afford the result that the π−π stacking interactions, DNA intercalating forces were much more significant than the H bond with the amino acid residues of the protein.[16] In the compound **24**, location of oxygen in furan at C-4 and methyl group in thiophene at C-2 carbon of pyridine look enter into the base stacked space and these tricyclic system stabilize the DNA-Protein-compound ternary complex. And another 3-methylthiophen part at C-6 of pyridine controls the degree of intercalation and optimize the electrostatic π-π stacking inter action between tricylcic core of **24** and DNA base pairs. Our molecular docking study proved the importance of DNA intercalation of **24**.

In conclusion, we have designed and prepared 21 2,6-dithienyl-4-furyl pyridine derivatives by efficient synthetic routes, and evaluated their topoisomerase I and II inhibitory activity and antitumor cytotoxicity. Among the prepared compounds, **24** exhibited much stronger topoisomerase I inhibitory activity than camptothecin. Compounds **7**, **13**, **15**, **18**, and **21** exhibited moderate topoisomerase I or II inhibitory activity. The results suggest that introduction of methyl or chloride functionality on furyl or thienyl moiety may increase topoisomerase inhibitory activity. A docking study of compound **24** with topoisomerase I–DNA complex was also performed, which indicated that the π−π stacking interactions between thienyl and furyl rings with DNA were quite important for exhibiting topoisomerase I inhibition activities, and these results proved the importance of DNA intercalation ability of **24** in the binding site of topoisomerase I–DNA complex. There were no direct correlations between antitumor cytotoxicity and topoisomerase inhibitory activity. This study may provide valuable information to researchers working on the development of antitumor agents.

Acknowledgment

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13. Fukuda, M.; Nishio, K.; Kanzawa, F.; Ogasawara, H.; Ishida, T.; Arioka, H.; Bojanowski, K.; Oka, M.; Saijo, N. *Cancer Res.* **1996**, *56*, 789. The topoisomerase I inhibitory activity was carried out as following: 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 MgCl2, 5 mM dithiothreitol, 2 mM spermidine, 0.01% bovine serum albumin, 200 ng pBR322, 0.3 U calf thymus DNA–topoisomerase I (Amersham), and topoisomerase I inhibitors (prepared compounds) in a final volume of 10 μL. The reaction mixture was incubated at 37 °C for 30 min. The reactions were terminated by adding 2.5 μL of solution comprising 10% SDS, 0.2% bromophenol blue, 0.2% xylene cyanol, and 30% glycerol. The mixture was applied to 1% agarose gel and electrophoresed for 10 h with a running buffer of Tris–borate–EDTA. 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 by an image analyzer and LabWork 4.5 software (UVP).*The topoisomerase II inhibitory activity was carried out as following*: DNA–topoisomerase II inhibition was measured by assessing relaxation of supercoiled pBR322 plasmid DNA. The reaction mixture contained 50 mM Tris–HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 30 μg/mL bovine serum albumin, 0.2 μg pBR322 plasmid DNA, 0.3 U human DNA–topoisomerase IIα (TopoGEN), and topoisomerase II inhibitors (prepared compounds) in a final volume of 20 μL. The reactions were incubated for 30 min at 37 °C and terminated by the addition of 3 μL of solution containing 0.77% sodium dodecyl sulfate, 77 mM EDTA. Samples were mixed with 2 μL of solution containing 30% sucrose, 0.5% bromophenol blue, and 0.5% xylene cyanol, and subjected to electrophoresis on a 1% agarose gel at 1.5 V/cm for 10 h with a running buffer of Tris–borate–EDTA. Gels were stained for 30 min in a aqueous solution of ethidium bromide (0.5 μg/mL). DNA bands were visualized by transillumination with UV light and supercoiled DNA was quantitated by an image analyzer and LabWork 4.5 software (UVP).
14. *The spectral data of* **24**: TLC (EtOAC/*n*-hexane = 1:5, v:v), R_f = 0.43, mp 101.5–103.5 °C.^{1}H NMR (250 MHz, $CDCl_3$) δ 7.65 (s, 2H, pyridine H-3, H-5), 7.56 (d, J = 1.39 Hz, 1H, 4-furan H-5), 7.26(d, J = 5.05 Hz, 2H, 2-thiophene H-5, 6′-thiophene H-5), 6.93(d, J = 5.18 Hz, 2H, 2-thiophene H-4, 6′-thiophene H-4), 6.94–6.90 (m, 1H, 4-furan H-4), 6.54 (dd, J = 3.08, 1.72 Hz, 1H, 4-furan H-3), 2.61 (s, 6H, 6′-thiophene 3-CH_3).^{13}C NMR (62.5 MHz, $CDCl_3$) δ 153.54, 151.68, 143.72, 138.55, 137.91, 136.06, 132.26, 125.52, 112.78, 112.07, 108.58, 16.56, ESI LC/MS/MS: Retention time: 20.95 min, $[MH]^+$: 338.HPLC condition: column: C18 reverse phased, 1.5 × 150 mm, 5 μm, GL science, flow rate: 180 μl/min, injection volume: 5 μl of 100 μM solution, mobile phase: 0.1% formic acid in water (A), 0.1% formic acid in acetonitrile (B), 10% B in A to 90% B in A for 10 min and retaining for 10 min at 90% B in A.*MS ionization condition*: Sheath gas flow rate: 70 arb, Aux gas flow rate: 20 arb, I spray voltage: 4.5 KV, capillary temperature: 215 °C, capillary voltage: 21 V, tube lens offset: 10 V.
15. (a) Skehan, P.; Streng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warrenm, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107; (b) Woo, S.; Jung, J.; Lee, C.; Kwon, Y.; Na, Y. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1163. Cancer cells were cultured according to the supplier's instructions. Cells were seeded in 96-well plates at a density of 2–4 × 10^4 cells per well and incubated 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 1, culture medium in each well was exchanged with 0.1 mL aliquots of medium containing graded concentrations of compounds. On day 3, 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. Adriamycin, etoposide, and camptothecin were purchased from Sigma and used as positive controls.
16. Xiao, X. S.; Cushman, M. *J. Am. Chem. Soc.* **2005**, *127*, 9960.