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Synthesis, Cytotoxicity and Structure-Activity Relationship Study of Terpyridines

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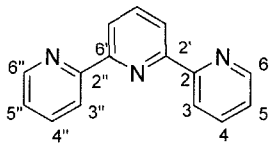
For the development of novel antitumor agents, we designed and synthesized terpyridines, and their biological activities were evaluated. Although most of the newly prepared terpyridines showed strong cytotoxicity against several human cancer cell lines, [2,2';6',2"]-terpyridine displayed the most significant cytotoxicity.

Key words: Terpyridines, Cytotoxicity, Topoisomerase I inhibition, Antitumor agent, Synthesis

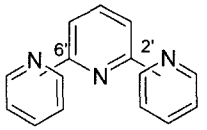
INTRODUCTION

The discovery of [2,2';6',2"]-terpyridine (α-terpyridine) in 1932 provided great interest to chemists due to its ability to form metal complexes, which has been extensively studied as a ligand in a wide range of metal complexes (Mukkala *et al.*, 1993; Lowe *et al.*, 1999) and DNA binding agents (Carter *et al.*, 1998; Liu *et al.*, 1995; McCoubrey *et al.*, 1996; Vliet *et al.*, 1995). Metal complexes of terpyridine can efficiently intercalate into nucleic acid (Jennette *et al.*, 1974) to be effectively performed as potential antitumor agents. Recently we reported that terpyridine and terthiophene derivatives and their bioisosteres showed a strong cytotoxicity against several human cancer cell lines as well as considerable topoisomerase I (Pommier *et al.*, 1998; Berger, 1998; Wang, 1996; Burden and Osheroff, 1998) inhibitory activities (Kim *et al.*, 1998; Zhao *et al.*, 2001, 2004). Although the cytotoxicity and topoisomerase I inhibitory activity of terpyridine derivatives and its bioisosteres have been reported, a systematic study on the effect of terpyridines has not yet been performed.

In the present study we prepared the six terpyridines, (Fig. 1) and evaluated their cytotoxicity against several human cancer cell lines and topoisomerase I inhibitory activity. Although most of the terpyridines showed strong cytotoxicity against several human cancer cell lines, [2,2';6',2"]-terpyridine displayed the most significant cytotoxicity and topoisomerase I inhibitory activity.



[2,2';6',2"]-terpyridine



X = 1, 2 or 3
X" = 1, 2, or 3

[X,2';6',X"]-terpyridines

Fig. 1. Structures of [2,2';6',2"]-terpyridine and other prepared terpyridines

MATERIALS AND METHODS

Chemistry

All reagents were obtained from Aldrich Chemical Co. (www.sigma-aldrich.com) and used without further purification. Unless otherwise indicated, anhydrous solvent were distilled over CaH_2 or sodium benzophenone ketyl prior to use. [2,2';6',2"]-Terpyridine was purchased from Aldrich Chemical Co. Thin-layer chromatography (TLC) and column chromatography were performed with Kieselgel

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60 F_{254} (Merck) and silica gel Kieselgel 60 (230~400 mesh, Merck) respectively. Compounds containing aromatic ring were visualized on TLC plates with UV light and compounds containing nitrogen were visualized on TLC plates with ninhydrin spray or Cl_2 exposure after spray with 1% KI starch solution, and all prepared compounds were detected on TLC plates with I_2. ^{1}H-NMR spectra were taken on a Bruker AMX 250 MHz, and tetramethylsilane was used as an internal standard. ^{13}C-NMR determined in DMSO-d_6 or $CDCl_3$ solutions with a Bruker AMX 250 spectrometer taken on 62.5 MHz. Chemical shifts (δ) were recorded in ppm, and coupling constants (*J*) in Hz. Melting points were determined in open capillary tubes on electrothermal 1A 9100 digital melting point apparatus and were uncorrected.

HPLC analyses were performed in Beckman HP controller and Beckmann UV detector using Beckmann ultraspere HPLC column (4.6 mm×150 mm, 5 mm Microsorb C-18 column) with a 20 μL injection volume with a gradient elution of 50% B in A to 100% B at a flow rate of 1.0 mL/min at 254 nm UV detection, where mobile phase A was doubly distilled water and mobile phase B was 90% acetonitrile in water. Retention time was displayed as min, and purity of compounds was described as percent.

β-(Dimethylamino)vinyl 3-pyridyl ketone (3)

General procedure: A solution of **1** (165 mmol) and *N,N*-dimethylformamide dimethyl acetal (201 mmol) (Donald and Guise, 1991) in toluene (100 mL) was heated to reflux. Methanol was gradually removed by fractional distillation. The toluene was removed on a rotary evaporator and the product was crystallized by addition of cyclohexane. Filtration of crystal formed afforded a yellow crystalline enaminone.

Yield; 96.0%; mp >300°C; ^{1}H-NMR (250 MHz, $CDCl_3$) δ 9.08 (d, *J* = 2.1 Hz, 1H, pyridine H-2), 8.67 (dd, *J* = 4.8, 1.7 Hz, 1H, pyridine H-6), 8.19 (dt, *J* = 7.9, 1.9 Hz, 1H, pyridine H-4), 7.85 (d, *J* = 12.3 Hz, 1H, =CH-N=), 7.36 (ddd, *J* = 7.9, 4.8, 0.7 Hz, 1H, pyridine H-5), 5.69 (d, *J* = 12.3 Hz, 1H, -CO-CH=), 3.07 (d, 6H, -N(CH_3)$_2$).

β-(Dimethylamino)vinyl 4-pyridyl ketone (4)

Yield; 93.2%; mp 115.0~117.0°C; ^{1}H-NMR (250 MHz, $CDCl_3$) δ 9.08 (d, *J* = 2.1 Hz, 1H, pyridine H-2), 8.67 (dd, *J* = 4.8, 1.7 Hz, 1H, pyridine H-6), 8.19 (dt, *J* = 7.9, 1.9 Hz, 1H, pyridine H-4), 7.85 (d, *J* = 12.3 Hz, 1H, =CH-N=), 7.36 (ddd, *J* = 7.9, 4.8, 0.7 Hz, 1H, pyridine H-5), 5.69 (d, *J* = 12.3 Hz, 1H, -CO-CH=), 3.07 (d, 6H, -N(CH_3)$_2$).

[2,2';6',3"]-Terpyridine (8)

General procedure: To a solution of 1-pyridin-2-yl-ethanone (**5**, 4.03 g, 33.27 mmol) in tetrahydrofuran (150 mL) was added potassium *t*-butoxide (7.47 g, 66.57 mmol), then 3-dimethylamino-1-pyridin-3-yl-propenone (**3**, 5.87 g, 33.31 mmol) was added, after stirring for 14 h at 20°C, the solution of ammonium acetate (25.6 g, 332.1 mmol) in glacial acetic acid (83.3 mL) was added dropwise to the mixture. The tetrahydrofuran and acetic acid remained was removed by rotary evaporator to give black residue. To the residue was added water (180 mL) and neutralized by sodium carbonate, and the solution was extracted with dichloromethane (150 mL×2) and the organic layer was washed with water (100 mL×2) and brine (30 mL), and dried over sodium sulfate and filtered. The filtrate was concentrated *in vacuo* and the residue was purified by alumina column chromatography with a gradient elution of EtOAc/*n*-hexane (1:5 to 1:2) to afford a colorless solid (2.1 g, 27.1%).

TLC (Al_2O_3): EtOAc:*n*-hexane (1:5), R_f = 0.15, mp: 103.8~105.8°C

HPLC rt: 8.98min. purity: 99.9%

^{1}H-NMR (250 MHz, $CDCl_3$): δ 9.35 (dd, *J* = 2.3, 0.7 Hz, 1H, H-2"), 8.70 (ddd, *J* = 4.8, 1.7, 0.9 Hz, 1H, H-6), 8.68 (dd, *J* = 4.8, 1.7 Hz, 1H, H-6"), 8.60 (dt, *J* = 8.0, 1.0 Hz, 1H, H-5'), 8.44 (ddd, *J* = 8.0, 2,3, 1.8 Hz, 1H, H-4"), 8.43 (dd, *J* = 7.8, 0.8 Hz, 1H, H-3), 7.92 (t, *J* = 7.8 Hz, 1H, H-4'), 7.85 (dt, *J* = 7.8, 1.8 Hz, 1H, H-4), 7.78 (dd, *J* =7.8, 1.0 Hz, 1H, H-3'), 7.43 (ddd, *J* = 8.0, 4.8, 0.8 Hz, 1H, H-5"), 7.33 (ddd, *J* = 7.5, 4.8, 1.2 Hz, 1H, H-5).

^{13}C-NMR (62.5 MHz, $CDCl_3$): δ 156.18, 155.91, 153.85, 149.94, 149.14, 148.41, 137.97, 136.94, 134.76, 134.28, 123.95, 123.54, 121.25, 120.30, 120.03.

[2,2';6',4"]-Terpyridine (9)

Yield; 28.4%, TLC: EtOAc:*n*-hexane (1:2), R_f = 0.3, mp: 93~94.2°C. HPLC rt: 10.72 min. purity: 99.7%. ^{1}H-NMR (250 MHz, $CDCl_3$): δ 8.77 (dd, *J* = 4.5, 1.6 Hz, 2H, H-2" and H-6"), 8.71 (ddd, *J* = 4.8, 1.7, 0.9 Hz, 1H, H-6), 8.62 (dt, *J* = 8.0, 0.9 Hz, 1H, H-4), 8.49 (dd, *J* = 7.8, 1.0 Hz, 1H, H-3'), 8.04 (dd, *J* = 4.5, 1.7 Hz, 2H, H-3" and H-5"), 7.95 (t, *J* = 7.8 Hz, 1H, H-4'), 7.87 (dt, *J* = 7.8, 1.8 Hz, 1H, H-2"), 7.84 (dd, *J* = 7.8, 1.0 Hz, 1H, H-5'), 7.35 (ddd, *J* = 7.5, 4.8, 1.2 Hz, 1H, H-5). ^{13}C-NMR (62.5 MHz, $CDCl_3$): δ 156.26, 155.78, 153.61, 150.43, 149.16, 146.30, 138.01, 136.93, 124.01, 121.25, 121.03, 120.57.

[3,2';6',3"]-Terpyridine (10)

Yield; 27.1%, TLC (Al_2O_3): EtOAc:*n*-hexane (1:1), R_f = 0.3, mp: 82.7~84.2°C. HPLC rt: 6.78 min. purity: 99.6%. ^{1}H-NMR (250 MHz, $CDCl_3$): d 9.33 (dd, *J* = 2.3, 0.7 Hz, 2H, H-2 & H-2'), 8.69 (dd, *J* = 4.8, 1.6 Hz, 2H, H-6 & H-6'), 8.47 (ddd, *J* = 8.0, 2.2, 1.6 Hz, 2H, H-4 & H-4'), 7.92 (dd, *J* = 8.6, 6.9 Hz, 1H, H-4'), 7.78 (dd, *J* = 8.2, 7.1 Hz, 2H, H-3' & H-5'), 7.45 (ddd, *J* = 8.0, 4.8, 0.8 Hz, 2H, H-5 & H-5'). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 154.72, 150.14, 148.31, 138.04, 134.49, 134.35, 123.59, 119.36.

[3,2';6',4"]-Terpyridine (11)

Yield; 25.8%, TLC: EtOAc:*n*-hexane (1:5), R_f = 0.3, mp: 111~113°C. HPLC rt: 6.70 min. purity: 99.7%. ^{1}H-NMR (250 MHz, $CDCl_3$): δ 9.33 (dd, *J* = 2.2, 0.6 Hz, 1H, H-2), 8.77 (dd, *J* = 4.5, 1.7 Hz, 2H, H-2" and H-6"), 8.70 (dd, *J* = 4.8, 1.6 Hz, 1H, H-6), 8.47 (ddd, *J* = 8.0, 2.3, 1.7 Hz, 1H, H-4), 8.02 (dd, *J* = 4.5, 1.6 Hz, 2H, H-3" and H-5"), 7.93 (dd, *J* = 8.9, 6.6 Hz, 1H, H-4'), 7.81-7.83 (m, 2H, H-3' and H-5'), 7.46 (ddd, *J* = 8.0, 4.8, 0.7 Hz, 1H, H-5). ^{13}C-NMR (62.5 MHz, $CDCl_3$): δ 154.82, 154.52, 150.49, 150.24, 148.34, 145.99, 138.12, 134.34, 123.60, 121.00, 120.62, 120.32, 119.66.

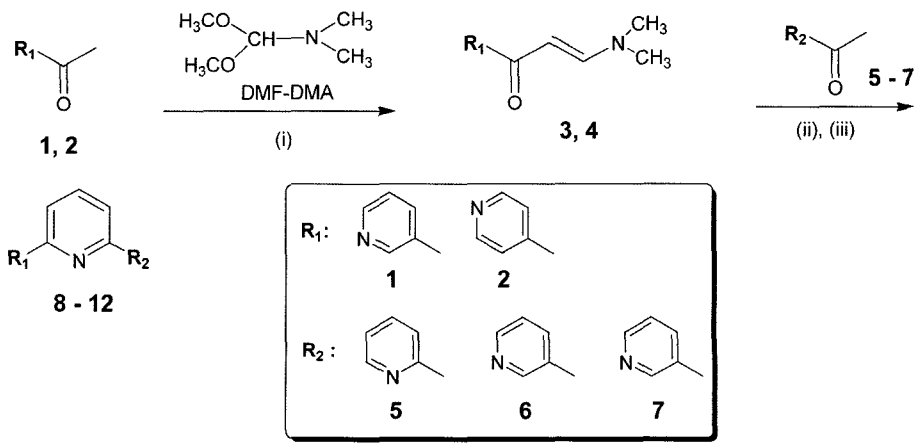
[4,2';6',4"]-Terpyridine (12)

Yield; 29.7%, TLC: EtOAc:*n*-hexane (1:1), R_f = 0.4, Mp: 146~148°C. HPLC rt: 4.22 min. purity: 98.8%. ^{1}H-NMR (250 MHz, $CDCl_3$): δ 8.77 (dd, *J* = 4.5, 1.7 Hz, 4H, H-2, H-6, H-2" and H-6"), 8.03 (dd, *J* = 4.5, 1.7 Hz, 4H, H-3, H-5, H-3" and H-5"), 7.97 (dd, *J* = 9.0, 6.3 Hz, 1H, H-4'), 7.87 (dd, *J* = 8.9, 6.5 Hz, 2H, H-3' and H-5'). ^{13}C-NMR (62.5 MHz, $CDCl_3$): δ 154.62, 150.53, 145.85, 138.23, 121.02, 120.63.

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 HCT-15 (human colon adenocarcinoma). All experimental procedures were followed up the NCI's protocol (Skehan *et al.*, 1990; Mosmann, 1983) based on the Sulforhodamine B (SRB) method. Briefly, 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 the concentrations of the compounds that inhibit 50% of cell growth. All data represent the average values for a minimum of three wells.

The topoisomerase I inhibitory activity was carried out as following (Fukuda *et al.*, 1996): 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.



Scheme 1. Synthetic pathway of terpyridines. (i) toluene, reflux, 8 h (ii) *t*-BuOK / THF, 25°C, 2 h (iii). NH_4OAc / AcOH, reflux, 14 h

RESULTS AND DISCUSSION

Chemistry

Enaminone intermediates **3** or **4** were prepared by an addition of 3- or 4-acetyl pyridine **1** or **2** in a toluene in the presence of DMF-DMA (Donald and Guise, 1991) and heating to reflux for 18 h to afford **3** or **4** in 96.0% and 93.2% yield, respectively (Scheme 1). An each solution of **5-7** in anhydrous THF in the presence of potassium *t*-butoxide was stirred for 2 h, and enaminone intermediates **3** or **4** were added to each solution and allowed to stir for 14 h. To the mixture were added NH_4OAc and AcOH to afford terpyridines **8-12** in 25.8~29.7% yields. The HPLC results of the prepared compounds displayed that they have high purity in a range of 98.8~99.9%. The prepared terpyridines are shown in Fig. 2.

Antitumor activities

The IC_{50} values of terpyridines against A-549, SK-OV-3, SK-MEL-2 and HCT-15 are shown in Table I. Most of the prepared compounds showed moderate cytotoxicity, generally 10~90 μM, against human cancer cell lines, which are less potent than doxorubicin or camptothecin used as control references. But cytotoxicity of [2,2';6',2"]-terpyridine was much stronger than that of doxorubicin, which indicated that the position of substituted pyridines at central pyridine ring would yield strong effect on cytotoxicity. However, the prepared compounds did not exhibit considerable topoisomerase I inhibitory activity, which were shown in Fig. 3.

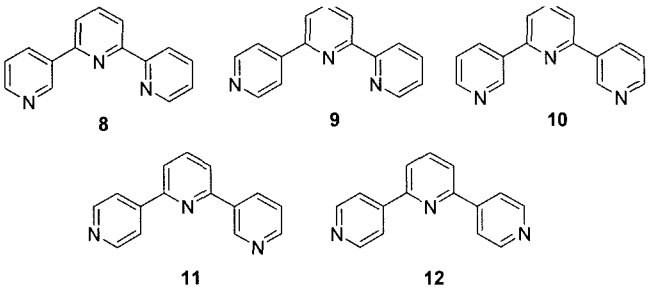
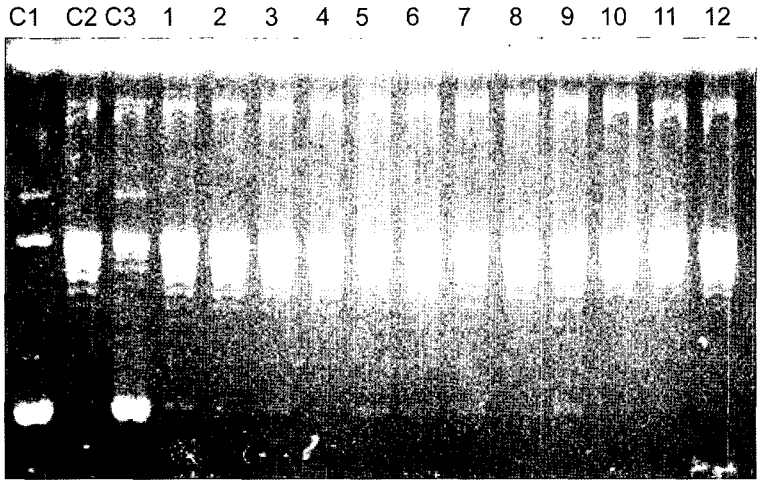


Fig. 2. Structure of prepared terpyridines

In conclusion, we have designed and prepared the terpyridines and evaluated them for their antitumor cytotoxicity and topoisomerase I inhibitory activity. Among them [2,2';6',2"]-terpyridine showed the strong cytotoxicity against several human cancer cell lines. Structure-activity

Table I. Cytotoxicity of the terpyridines against several human cancer cell lines

Compounds	EC_{50} (μM)			
	A549	SK-OV-3	SK-MEL-2	HCT15
[2,2';6',2"]-terpyridine	0.02	0.02	0.03	0.03
8	76.57	35.06	36.19	17.96
9	>100.0	72.26	102.83	>100.0
10	>100.0	66.41	92.16	104.62
11	109.71	62.24	70.81	45.50
12	37.08	29.23	92.91	41.26
doxorubicin	0.07	0.41	0.07	0.16
camptothecin	0.008	0.024	0.094	0.033



Lane C1. pBR322 DNA (supercoiled form)
Lane C2. pBR322 DNA + Topo I (relaxed form)
Lane C3. pBR322 DNA + Topo I + CPT (10 μM)
Lane1, 3, 5, 7, 9, 11. pBR322 DNA + Topo I + terpyridines (100 μM)
Lane2, 4, 6, 8, 10, 12. pBR322 DNA + Topo I + terpyridines (10 μM)
lane1, 2: [2,2';6',2"]-terpyridine, lane3, 4: **8**, lane5, 6: **9**, lane7, 8: **10**, lane9, 10: **11**, lane11,12: **12**

Fig. 3. Topoisomerase I inhibitory activity of terpyridines

relationship study of the prepared terpyridines indicated that substituted position of pyridines at central pyridine ring would contribute great effect on cytotoxicity. In addition, this study may provide valuable information to the researchers who are working on the development of antitumor agents.

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