Synthetic Inhibitors of DNA Topoisomerase I and II[1)]

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A new type of synthetic inhibitor of DNA topoisomerase I and II was examined and several of these derivatives exhibited strong dual activity against these enzymes. This series of compounds showed high cytotoxic activities against cancer cells, but only a limited number of compounds showed any noticeable activity in an *in vivo* test against murine P388. Non-specific toxicity was observed in the *in vivo* tests.

Key words dual inhibitor; DNA topoisomerase I; DNA topoisomerase II; pyrazolo[1,5-*a*]indole; cytotoxicity; antitumor activity

DNA topoisomerase has been a prime target for discovery of anticancer drugs[2)] and the efforts in this area have been fruitful in providing some useful anticancer agents.[3)] There are two types of DNA topoisomerase enzymes, type I (top I) and II (top II) which are classified according to whether they function by changing the topology of DNA helices by breaking one strand (top I) or two strands (top II) of the double helix. Most of the marketed topoisomerase inhibitors originated in natural products, and the number of totally synthetic agents is quite few compared with naturally-derived ones. Efforts at finding inhibitors has focused on the discovery of selective inhibitors of two types of topoisomerase, and the number of studies on dual inhibitors is quite limited.[4)] We have been involved in the chemistry of pyrazolo[1,5-*a*]indoles with the hope of discovering biologically active compounds.[5)] In this context, we have prepared a series of compounds and found that some derivatives have fairly strong inhibitory activity against DNA topoisomerase I and II, and have strong cytotoxic activity against cancer cells. We report these findings in this paper.

Chemistry We reported the preparation and characterization of 1-methyl-1*H*-pyrazolo[1,5-*a*]indole **1** as an isoelectronic analogue of azulene,[6)] *i.e.*, pseudoazulene,[7)] by treatment of the acid salt **6** with strong base (Chart 1). The organic salts of pseudoazulene were reported to condense with aldehyde *via* pseudoazulene to give condensation products.[8)] Three kinds of reaction conditions were employed for this condensation: a) refluxing in acetic acid, b) refluxing with piperidine in ethanol and c) refluxing with sodium methoxide in methanol. When we adopted the condition a) to the reaction of **6**[6)] with *p*-dimethylaminobenzaldehyde (**10**), the product **13** was obtained in 80% yield (Chart 2). The structure of **13** was supported from the ^{1}H-NMR spectrum, in particular the characteristic singlet signals for 10-H at δ 8.30 and 3-H at δ 7.45 together with two singlet N-Me signals at δ 3.11 (NMe_2) and 4.43 (NMe). Further support for this structure was obtained from the ^{13}C-NMR spectrum, *i.e.*, C-3 at δ 101.5 and C-10 at δ 140.0 ppm. The geometry of the side chain of **13** was determined to be *Z* from the following observations. When this condensation reaction was halted at shorter reaction time (1.5 h), the product was a mixture of two isomers (*ca.* 1 : 1). It was not possible to separate these isomers, but the detection of additional singlet signals for 10-H (δ 8.28 ppm) and 3-H (δ 7.49) for an isomer besides the signals for **13** in the ^{1}H-NMR spectrum supported the contamination by the isomer. Longer reaction times gave a single isomer **13** as the sole product, with total disappearance of the signals observed in incomplete reaction product. Since the *Z*-isomer is more stable than the *E*-isomer according to molecular modeling, the stereochemistry of **13** was determined to be *Z*-form. A similar stereochemical result was obtained with the product **12** and its stereochemistry was supported by the nuclear Overhauser effect spectroscopy spectrum (NOESY), *vide infra*. Compound **13** attracted our interest since molecular modeling revealed conformational similarity of **13** with 4-(9-acridinylamino)-*N*-methansulfonyl-*m*-anisidine (*m*-AMSA) (Chart 3) which is an anticancer agent clinically used nowadays. The preliminary cytotoxic activity for **13** showed promising results, thus we decided to prepare a series of this compound, as shown in Chart 2.

Compounds **12** and **14** were prepared in a similar manner as in **13**. The NOESY spectrum of **12** indicated nuclear Overhauser effects (NOE) between 3-H and aromatic protons on 10-phenyl group, and between 5-H and 10-H, thus supporting the *Z*-geometry.

For the preparation of 2-alkyl substituted derivatives, the quaternary salts **7** and **8** were prepared as shown in Chart 1. Dehydrogenation of tetrahydro derivative **2**[9a)] with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) gave **4** and methylation with methyl trifluoromethanesulfonate (methyl triflate) gave salt **7**. Dehydration of **3**[9b)] to **5** (methanesulfonyl chloride and triethylamine) and methylation of **5** gave **8** in good yield. The subsequent condensations of **7** and **8** with aldehyde **10** gave the products **15** and **16**, respectively. The yield in the condensation ranged from 51% to 99%. As the

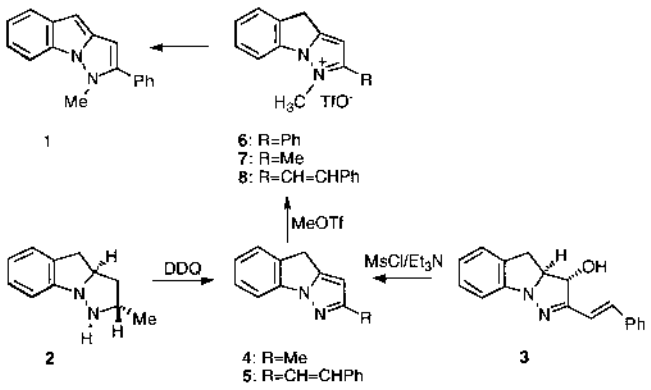

Chart 1

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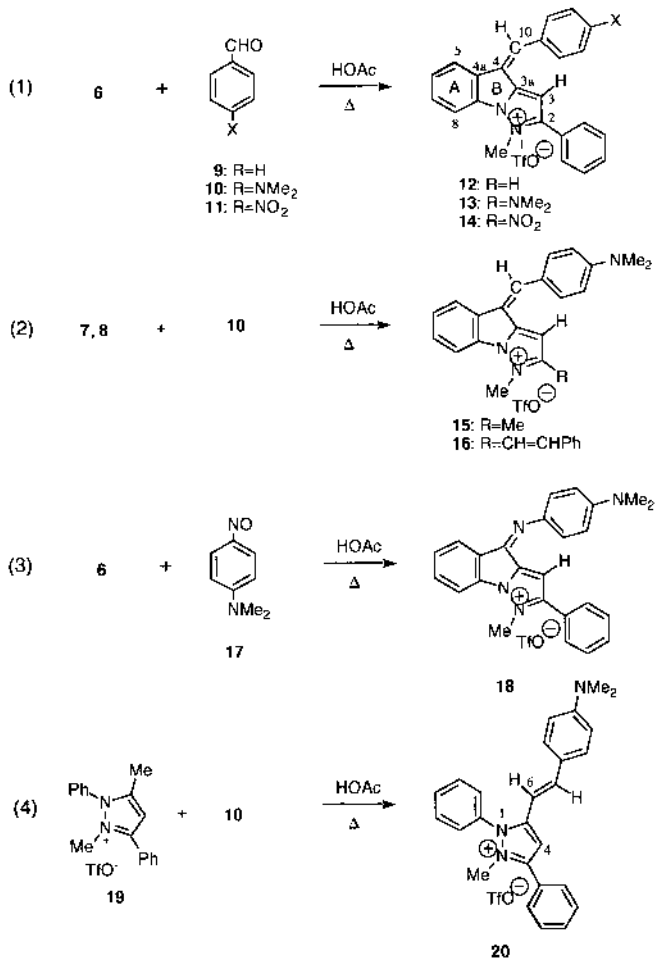

Chart 2

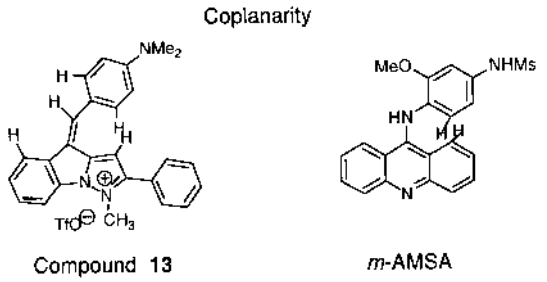

Chart 3

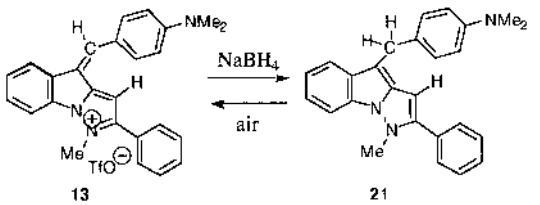

Chart 4

nitroso group can behave like an aldehyde in this type of coupling reaction,[10] 4-dimethylaminonitrosobenzene (**17**) was also employed in the above condensation reaction to introduce a nitrogen atom at position 10. Thus, the reaction of **6** with **17** under similar reaction conditions gave the product **18** in 90% yield. The imino group of **18** was reactive but an attempt at cleaving (acid hydrolysis) or reducing (sodium borohydride) the imino group of **18** led to intractable mixtures. In order to deduce the role of the five-membered ring of the indole nucleus of **13** in the biological activity the open-ring compound **20** was also prepared. The reaction of salt **19** with **10** in the presence of sodium ethoxide in ethanol, as reported,[8c] gave **20**. The UV spectrum of **20** revealed that there is more effective electron delocalization in open-ring compound **20** than in the closed-ring compound **13**. Thus the λ_{max} of **20** appeared at higher wavelength (408 nm, log ε 4.64) than that of **13** (323 nm, log ε 4.03) with stronger absorption. The flexible side chain of **20** can allow better electron delocalization than that for **13**. The presence of the conjugated system in **13** was confirmed by reduction, *i.e.*, the interruption of conjugation. When **13** was reduced with sodium borohydride in deuterated dimethyl sulfoxide (DMSO-d_6), the color of the solution changed from dark red to colorless (deconjugation) and the formation of the saturated product **21** was detected in the NMR spectra (Chart 4). In the ^{1}H-NMR spectrum of **21** the signal for 10-H of **13** (δ 8.30, s) was absent and a new signal for 10-CH_2 appeared at δ 4.01 (2H, s). By this transformation a singlet signal for 3-H shifted from δ 7.45 (for **13**) to δ 6.58 (for **21**). The proximity of the chemical shift for 3-H of **21** (δ 6.58, s) to that of 1*H*-pyrazolo[1,5-*a*]indole derivative **1** (δ 6.44, s)[6] supported this chemical transformation. The ^{13}C-NMR spectrum also agreed with this conclusion (δ 98.6 for C-3 of **21** and δ 99.3 for C-3 of **22**). No similar reduction was observed in the open-ring compound **20**. The UV-VIS spectrum of the reduction product **21** was obtained by adding sodium borohydride to a solution of **13** in ethanol. Bathchromic shifts of λ_{max} absorptions were observed, but they appeared at about 30 nm lower wavelength than those of **1**.[6] Attempts at both isolating and derivatizing **21** after reduction failed and led to recovery of the starting salt **13** (^{1}H-NMR identification). A similar reduction was reported to be successful with conjugated 3-phenylmethyleneoxyindoles.[11] Hydrolysis of **13** in acid or base resulted in the recovery of **13**.

Biological Activity Inhibition tests for the compounds **12**—**16**, **18** and **20** against DNA topoisomerase I (top I) and II (top II) were carried out by measuring the relaxation of superhelical DNA.[12] The activity was evaluated as IC_{50} (μg/ml) and the results are summarized in Tables 1 and 2. The commercial antitumor agents SN-38 (11-ethyl-9-hydroxycamptothecin) and VP-16 (etoposide) were employed as references for the selective inhibition of top I (SN-38) and top II (VP-16). As shown in Table 1, all compounds, except **20**, displayed quite strong inhibitory activities against human DNA topoisomerases. The activity of compounds **13** and **18** were comparable, and about ten times stronger than the reference compounds. These compounds possessed activity against both types of DNA topoisomerases, and are unique in this sense among compounds having a quaternary nitrogen atom.[13] Lower activity against both topoisomerases was observed for **15** and **16**. When the source of topoisomerase was different (Table 2), the relative inhibitory activity varied but the activity of **13** remained strong. Compound **13** was more selective for top II obtained from murine P388 cancer cells. The poor inhibitory activity of **20** against top I and II (Table 1), indicates that the tricyclic system is important for high activity. However, introduction of a nitrogen at C-10, as in

Table 1. Inhibition of Human DNA Topoisomerase I and II (IC_{50}, μg/ml)

Compound[a]	Top I	(ratio[b])	Top II	(ratio[b])	Top I/Top II
SN-38	8.9	(17)	—		
VP-16	—		6.99	(18)	
12	>10	(>19)	3.98	(11)	>2.5
13	0.54	(1.0)	0.38	(1.0)	1.4
14	4.76	(8.8)	3.5	(9.2)	1.4
15	1.46	(2.7)	4.74	(13)	0.3
16	5.52	(10)	0.54	(1.4)	10.2
18	0.5	(0.9)	0.39	(1.0)	1.3
20	>10	(>19)	9.04	(24)	

a) SN-38, 11-ethyl-9-hydroxycamptothecin; VP-16, etoposide. *b*) Relative ratio was calculated from **13** as a standard.

Table 2. Inhibition of Murine P388 DNA Topoisomerase I and II (IC_{50}, μg/ml)

Compd.[a]	Top I	(ratio[b])	Top II	(ratio[b])	Top I/Top II
SN-38	2.67	(2.7)	—		
VP-16	—		6.11	(204)	
12	3.48	(3.6)	0.96	(32)	3.63
13	0.98	(1.0)	0.03	(1.0)	32.7
14	4.57	(4.7)	0.80	(27)	5.71

a) SN-38, 11-ethyl-9-hydroxycamptothecin; VP-16, etoposide. *b*) Relative ratio was calculated from **13** as a standard.

Table 3. Cytotoxic Activity against Cancer Cells[a] (GI_{50}, ng/ml)[b]

Compd.[c]	P388	PC-6	NUGC-3	SW620	MCF-7
5-FU	69.5	410	1791	2778	681
CDDP	24.9	232	73.1	1112	1819
12	197	211	962	967	506
13	8.6	37.6	153	141	62.9
14	668	828	1224	2060	720
5-FU	48.8	240	1290	1250	732
CDDP	13	105	76.5	403	1510
SN-38	1.79	0.4	0.8	1.11	4.27
VP-16	8.12	99	343	292	414
15	10	35.2	216	234	77.8
16	11.5	21	69.5	95.5	29.3
18	293	164	577	1280	329
20	0.85	28.7	292	399	98.5

a) Separate tests were carried out for two series of compounds and the results are summarized in one table. Cancer cells employed are P388, murine leukemia; PC-6, human lung; NUGC-3, gastric; SW-620, colon; MCF-7, breast cancer. *b*) GI_{50} is the concentration (ng/ml) for 50% growth inhibition of cells. *c*) Compounds used as reference are 5-FU, 5-fluorouracil; CDDP, cisplatin; SN-38, 11-ethyl-9-hydroxycamptothecin; VP-16 etoposide.

compound **18** did not alter the activity.

The cytotoxic activities of the above products were tested by measuring the proliferation of cancer cells in the presence of drug by the microculture tetrazolium method.[14] Separate experiments were conducted for two series of compounds and the results are summarized in Table 3.

The activity was expressed as GI_{50} (ng/ml). Anticancer drugs in clinical use, 5-fluorouracil (5-FU) and cisplatin (CDDP), were employed as reference compounds. Topoisomerase inhibition was principally reflected in the cytotoxic activity. The potencies of **13** and **16** were notable compared with the reference compounds and corresponded well with the inhibitory activities against topoisomerases (Table 1). The cytotoxicity of imine **18** was weak compared with that of **13**. The possible susceptibility of the imine **18** to hydrolysis under biological conditions may be the reason for reduced cytotoxicity. The cytotoxic activity of open ring compound **20** was comparable to the other compounds, even though poor inhibitory activity was observed against topoisomerases. However, the basic trend observed in these tricyclic compounds supports the hypothesis that topoisomerase inhi-

Table 4. Cytotoxic Activity against Cancer Cells[a] (GI_{50}, ng/ml)[b]

Compd.	P388	PC-6
CDDP	9.04	104
6	1330	1360
7	4370	24300
8	532	379

a) See the notation in Table 3.

Table 5. Antitumor Activity against P388

Compd.	*a*)	*b*)	*c*)	*d*)	*e*)
BTC	0	10—11	10.9	0	0/6
Cont.	0	11—12	11.4	5	0/6
12	12	6—11	8.3	−24	6/6
	8	11—13	12.3	13	0/6
	6	9—12	11.3	3	2/6
	4	11—13	12.1	12	0/6
	3	11—13	12.0	10	0/6
	2	11—12	11.7	7	0/6
13	12	8—13	12.3	13	1/6
	8	12—13	12.4	14	0/6
	6	12—15	12.8	17	0/6
	4	12—13	12.2	12	0/6
	3	11—14	12.3	13	0/6
	2	12—14	13.3	22	0/6
5-FU	100	20—21	20.9	84	0/6
	50	19—20	19.4	70	0/6
BTC	0	9—11	10.3	0	0/6
Cont.	0	10—11	10.7	3	2/6
15	6	11—12	11.4	10	0/6
	4	12—14	13.0	26	0/6
	3	11—13	12.3	19	0/6
	2	11—13	12.1	17	0/6
	1	10—12	11.0	6	1/6
16	4	6—13	12.0	16	0/6
	3	12—13	12.2	18	2/6
	2	12—14	13.0	26	0/6
	1.4	11—15	13.0	26	0/6
	1	11—15	14.3	38	0/6
18	35	5—10	10.0	−3	0/6
	21	10—11	10.7	3	0/6
	13	10—11	10.4	0	0/6
	8	10—14	10.2	−1	0/6
	5	10—11	10.2	−1	0/6
	3	10—11	10.9	5	0/6
20	3.0	7—12	11.3	3	4/6
	2.1	10—12	11.3	4	0/6
	1.5	10—12	11.1	2	0/6
	1.0	11—12	11.2	3	0/6
	0.7	10—11	10.9	0	0/6
	0.5	10—11	10.4	−5	0/6
5-FU	100	19	19.1	79	0/6
	50	17—19	17.4	63	

a) Dose (mg/kg×2). Ascitic fluid containing 1×10^6 cells was inoculated i.p. into CSDF1 mice (day 0), and the compound in BTC suspension was administered i.p. at day 1 and 5. *b*) Survival days range. *c*) MST (mean survival terms in days). *d*) ILS (increase of life span in %). *e*) Number of mice that died by toxicity after the operation, over the number of mice tested. The rest of the mice all died after the days indicated.

bition may play an important role in cytotoxic activity. The principal role of the substituent at C-4 of this series of compounds on cytotoxic activity was investigated with the skeletal compound, 1-methyl-4*H*-pyrazolo[1,5-*a*]indolenium salt. The cytotoxicity of the salt with different substituents at C-2, **6** (2-phenyl), **7** (2-methyl) and **8** (2-styryl) against cancer cells is cited in Table 4. It is apparent from this table that the presence of a C-4 substituent is important for expressing cytotoxic acitvities. The weak activity of **8** suggests the possibility of improving cytotoxic activities by introducing a suitable substituent at the C-2 position.

The *in vivo* antitumor activity against P388 was also investigated. P388 cells were inoculated interperitoneally (i.p.) into CDF1 male mice and the drugs were given i.p. on days 1 and 5. Survival was monitored daily and the increase in life span (ILS) was calculated using the control group. The results are listed in Table 5. A favorable ILS value (38%) was obtained for **16** at a dose of 1 mg/kg. Small ILS values, 22% and 26%, were observed for **13** and **15** respectively. Dose escalation did not improve the ILS values probably due to the non-specific toxicity of the compounds. A good result both in *in vitro* and *in vivo* tests was obtained with **16**. The mechanism of DNA topoisomerase inhibition has not been clarified yet, but in a preliminary experiment, the binding of compound **13** to calf thymus DNA was detected by UV-VIS spectra, suggesting a possible cause of inhibition. It is essential to resolve the mechanism of inhibition of topoisomerase I and II for the exploration of new types of antitumor agents. Also, analysis of structure-activity relationships (SAR) and subsequent removal of non-specific toxicity are an essential requirement for discovering a potential antitumor agent.

Experimental

Melting points (mp) were determined on a Yanaco micro-melting point apparatus without correction. Infrared spectra (IR) (KBr pellet unless stated otherwise) were measured with a Perkin–Elmer FT-IR 1720. Ultraviolet-Visible spectra were recorded on a Shimadzu UV-200S double beam spectrophotometer. ^{1}H- and ^{13}C-nuclear magnetic resonance spectra (NMR) were obtained at ambient temperature (25—27 °C) with JEOL JNM-FT 200 and JNM-ALPHA 400 instrument in $CDCl_3$, unless otherwise specified, with tetramethylsilane as an internal standard. Mass spectra (MS) and high resolution MS (HR-MS) were recorded on a Hitachi RMU-7MG and the figures in parentheses indicate the relative intensities. Anhydrous tetrahydrofuran (THF) was prepared by distilling in the presence of ketyl radical and dry acetonitrile by distilling from calcium hydride.

1,2-Dimethyl-4*H*-pyrazolo[1,5-*a*]indolium Trifluoromethanesulfonate (7) 2-Methyl-1,2,3,3a-tetrahydro-4*H*-pyrazolo[1,5-*a*]indole (**2**)[9a] (2.23 g, 12.8 mmol) and DDQ (6.39 g, 28.2 mmol) in dry dichloromethane (80 ml) was stirred at room temperature for 2 h. Dilution with ether (30 ml), filtration to remove insoluble material, washing of the filtrate (aqueous 10% NaOH solution and saturated brine), drying (anhydrous sodium sulfate) and evaporation *in vacuo* gave 2-methyl-4*H*-pyrazolo[1,5-*a*]indole (**4**) (1.00 g, 45.9%), mp 66.0—67.5 °C (ether–hexane). MS *m/z*: 170(M^+, 96), 155 (60), 143 (25), 129 (100), 115 (33), 102 (76). IR (KBr) cm^{-1}: 1619, 1592, 1565, 1545, 1472, 1402, 1332, 1301, 1199, 1129, 1097, 1003, 965, 777, 759, 697, 642. ^{1}H-NMR δ: 2.42 (3H, s, Me), 3.81 (2H, s, 4-H_2), 6.03 (1H, s, 3-H), 7.13 (1H, dt, *J*=1.0, 7.4 Hz), 7.36 (1H, t, *J*=7.6 Hz), 7.41 (1H, d, *J*=7.3 Hz), 7.56 (1H, d, *J*=7.6 Hz). ^{13}C-NMR δ: 14.3, 28.2 (C-4), 100.5 (C-3), 109.9, 123.7, 125.7, 127.9, 133.1, 140.6, 145.4, 153.7.

A solution of the above product **4** (0.500 g, 2.9 mmol) in dry dichloromethane (20 ml) was reacted with methyl trifluoromethanesulfonate (2.66 ml, 23.5 mmol) in two portions (interval 42 h). The precipitated crystals (0.900 g, 91.6%) were recrystallized to give **7** (0.45 g, 45.8%), mp 210.0—212.0 °C (MeOH). MS *m/z*: 185 ($M^+-CF_3SO_3$, 15), 184 (100), 169 (25), 154 (10), 143 (25), 129 (7), 115 (12), 101 (5), 92 (6). IR cm^{-1}:1545, 1485, 1398, 1267, 1227, 1157, 1029, 768, 637, 575, 519. ^{1}H-NMR δ: 2.57 (3H, s, 2-Me), 4.31 (2H, s, 4-H_2), 4.32 (3H, s, 1-Me), 6.91 (1H, s, 3-H), 7.48 (1H, t, *J*=7.6 Hz), 7.59 (1H, t, *J*=7.6 Hz), 7.95 (1H, d, *J*=7.3 Hz), 8.06 (1H, d, *J*=8.1 Hz). ^{13}C-NMR δ: 11.6 (2-Me), 29.1 (C-4), 25.5 (1-Me), 103.3 (C-3), 112.5, 127.0 (C×2), 128.1, 133.5, 136.8, 149.5, 150.6. *Anal*. Calcd for $C_{13}H_{13}F_3N_2O_3S$: C, 46.71; H, 3.92; N, 8.38. Found: C, 46.41; H, 3.91; N, 8.21.

1-Methyl-2-*E*-styryl-4*H*-pyrazolo[1,5-*a*]indolium Trifluoromethane-sulfonate (8) 2-*E*-Styryl-3-hydroxy-3a,4-dihydro-4*H*-pyrazolo[1,5-*a*]indole (**3**)[9b] (0.63 g, 2.1 mmol) was reacted with methanesulfonyl chloride (0.25 ml, 3.1 mmol) and triethylamine (0.60 ml, 4.3 mmol) in dry dichloromethane (38 ml) at ice-cooling temperature for 4 h. The solution was diluted with water, basified with sodium carbonate and extracted with dichloromethane. The organic layer was washed (saturated brine), dried (anhydrous sodium sulfate), and evaporated in vacuo. The crude product was purified by flash column chromatography (silica gel 20 g, hexane–ethyl acetate 5 : 1) to give 2-*E*-styryl-4*H*-pyrazolo[1,5-*a*]indole (**5**) (0.48 g, 91%) as yellow crystals, mp 148.0—149.0 °C (CH_2Cl_2–iso-Pr_2O). MS *m/z*: 258 (M^+, 100), 257 (84.8), 256 (16.6), 230 (5.6), 155 (3.7), 129 (10.5), 128 (8.7). IR cm^{-1}: 2908, 1619, 1591, 1480, 1401, 1306, 982, 968, 796, 756, 694. ^{1}H-NMR δ: 3.86 (2H, s, 4-H), 6.50 (1H, s, 3-H), 7.11—7.55 (10H, m), 7.62 (1H, br d, *J*=8.0 Hz). ^{13}C-NMR δ: 28.2, 98.1, 110.3, 121.1, 124.3, 125.8, 126.4 (2×C), 127.7, 128.1, 128.7 (2×C), 130.2, 133.3, 137.1, 140.3, 145.5, 155.3. *Anal*. Calcd for $C_{18}H_{14}N_2$: C, 83.69; H, 5.46; N, 10.84. Found: C, 83.56; H, 5.43; N, 10.94.

The above product **5** (1.00 g, 3.87 mmol) was reacted with methyl trifluoromethanesulfonate (0.88 ml, 7.74 mmol) as described above and crystallization of the product from MeOH–EtOAc gave the salt **8** (1.345 g, 82.2%), mp 223.5—225.0 °C (MeOH–EtOAc). IR cm^{-1}: 1539, 1468, 1291, 1247, 1224, 1154, 1027, 756, 747, 635. ^{1}H-NMR (DMSO-d_6) δ: 4.38 (2H, s, 4-H), 4.49 (3H, s, NMe), 7.43—7.86 (10H, m), 8.10 (1H, br d, *J*=7.8 Hz). ^{13}C-NMR (DMSO-d_6) δ: 24.6 (C-4), 31.4 (NMe), 95.0 (C-9), 107.4 (C-3), 108.3, 122.5, 122.6, 123.4 (C×2), 124.4, 125.5 (C×2), 129.3, 130.5, 132.4, 135.6, 145.1, 145.6. *Anal*. Calcd for $C_{20}H_{17}F_3N_2O_3S$: C, 56.87; H, 4.06; N, 6.63%. Found: C, 56.98; H, 3.97; N, 6.63.

Typical Procedure for Condensation Reactions. 4-(*Z*)-(*p*-Dimethylaminophenylmethylene)-1-methyl-2-phenyl-4*H*-pyrazolo[1,5-*a*]indolium Trifluoromethanesulfonate (13) A suspension of 1-methyl-2-phenyl-4*H*-pyrazolo[1,5-*a*]indolinium trifluoromethane sulfonate (**6**)[6] (0.30 g, 0.82 mmol) and *p*-dimethylaminobenzaldehyde (**10**) (0.14 g, 0.94 mmol) in acetic acid (30 ml) was refluxed for 20 h under an atmosphere of nitrogen. The solution was concentrated under reduced pressure and the residue was crystallized from ethanol to give **13** as dark red-purple crystals (0.32 g, 80%), mp 237.0—237.5 °C. UV-VIS λ_{max}^{MeOH} nm (log ε): 246 (4.42), 259 (4.43), 323 (4.03), 500 (4.65). IR cm^{-1}: 1583, 1529, 1464, 1360, 1325, 1188, 1161, 1095 (s), 765. ^{1}H-NMR (DMSO-d_6) δ: 3.11 (6H, s, NMe_2), 4.43 (3H, s, NMe), 6.93 (2H, d, *J*=9.0 Hz), 7.45 (1H, s, 3-H), 7.56 (2H, m), 7.70 (3H, m), 7.82 (2H, m), 7.84 (2H, d, *J*=9.0 Hz), 8.14 (1H, m), 8.22 (1H, m), 8.30 (1H, s, 10-H). ^{13}C-NMR (DMSO-d_6) δ: 37.5, 39.5 (2×C), 101.5 (C-3), 112.1, 112.2, 120.8, 121.2, 122.6, 125.9, 126.7, 127.4, 129.3, 129.6, 131.2, 131.6, 132.4, 133.4, 140.0 (C-10), 141.9, 151.6, 152.5. *Anal*. Calcd for $C_{27}H_{24}F_3N_3O_3S$: C, 61.47; H, 4.59; N, 7.97. Found: C, 61.44; H, 4.66; N, 7.98. *E*-Isomer of **13**, ^{1}H-NMR (DMSO-d_6) δ: 3.10 (s), 4.43 (s), 6.52 (d, *J*=9 Hz), 7.49 (s), 7.54—7.58 (m), 7.68—7.19 (m), 7.81 (d, *J*=9 Hz), 7.81—7.82 (m), and 8.28 (s). ^{13}C-NMR (DMSO-d_6) δ: 37.5*, 39.5*, 101.5*, 112.1, 112.3, 112.6, 120.8, 121.2, 126.0, 126.7, 127.4, 129.3*, 129.6*, 131.2, 131.6, 132.5, 133.4, 140.0, 142.0, 151.6, 152.6. The signals marked with an asterisk overlapped with those of the *Z* isomer.

1-Methyl-2-phenyl-4-phenylmethylene-4*H*-pyrazolo[1,5-*a*]indolium Trifluoromethanesulfonate (12) The salt **6** (0.50 g, 1.26 mmol) and benzaldehyde (**9**) (0.17 ml, 1.67 mmol) were reacted in acetic acid (50 ml) for 20 h and purified as described above to give **12** (0.61 g, 99.8%), mp 233.0—235.0 °C (CH_2Cl_2–iso-Pr_2O). UV-VIS λ_{max}^{MeOH} nm (log ε): 270 (4.38), 356 (4.34). IR cm^{-1}: 1627, 1544, 1467, 1383, 1263, 1226, 1157, 1034, 766, 700. ^{1}H-NMR (DMSO-d_6) δ: 4.46 (3H, s, NMe), 7.38 (1H, s, 3-H), 7.60—7.71 (4H, m), 7.78 (2H, d, *J*=7.2 Hz with small splittings, 2,6-H of 2-phenyl group), 7.90 (2H, d, *J*=7.0 Hz, 2,6-H of 10-phenyl ring), 8.17 (1H, d, *J*=7.8 Hz, 8-H), 8.34 (1H, d, *J*=7.3 Hz, 5-H), 8.54 (1H, s, 10-H). Assignments are based upon COSY and NOESY spectra: ^{13}C-NMR (DMSO-d_6) δ: 37.7 (NMe), 103.0 (C-3), 113.0 (C-8), 120.2 (C-4), 122.5 (C-5), 125.5 (C-6), 127.5 (C-7), 129.3 (2×C, 3,5-C of 2-phenyl ring), 129.4 (2×C, 3,5-C of 10-phenyl ring), 129.5 (2×C, 2,6-C of 2-phenyl ring), 129.6 (C-8a), 129.9 (2×C, 2,6-C of 10-phenyl ring), 130.4 (C-4a), 131.1 (C-4 of 2-phenyl ring), 131.4 (C-4 of 2-phenyl ring), 133.8 (C-1 of 2-phenyl ring), 134.1 (s, C-4), 138.7 (C-10), 141.8 (C-3a), 152.0 (C-2). Assignments were obtained by detailed analyses of CHCOSY and long range CHCOSY spectra. *Anal*. Calcd

for $C_{25}H_{19}F_3N_2O_3S$: C, 62.07; H, 3.95; N, 5.78%. Found: C, 62.07; H, 4.01; N, 5.82%.

1-Methyl-4-(*p*-nitrophenylmethylene)-2-phenyl-4*H*-pyrazolo[1,5-*a*]indolium Trifluoromethanesulfonate (14) The salt **6** (0.50 g, 1.37 mmol) and *p*-nitrobenzaldehyde (**2c**) (0.25 g, 1.26 mmol) in AcOH (50 ml) were reacted for 10 h to give yellowish brown crystal (0.53 g, 79.4%) of **14**, mp 188.0—190.0 °C (MeOH–iso-Pr_2O). UV-VIS λ_{max}^{MeOH} nm (log ε): 270 (4.47), 360 (4.26). IR cm^{-1}: 1518, 1338, 1262, 1153, 1028, 763. ^{1}H-NMR (DMSO-d_6) δ: 4.48 (1H, s, NMe), 7.45 (1H, s, 3-H), 7.61—7.81 (7H, m), 8.09 (2H, d, J=8.8 Hz), 8.19 (1H, d, J=7.8 Hz), 8.33 (1H, d, J=7.1 Hz), 8.45 (2H, d, J=8.8 Hz), 8.61 (1H, s, 10-H). ^{13}C-NMR (DMSO-d_6) δ: 37.9, 103.9 (C-3), 113.3, 123.1, 123.2, 124.6, 125.4, 127.7, 129.3 (C×2), 129.6 (C×2), 130.0, 130.5, 130.8 (C×2), 131.5, 134.7, 135.5 (C-10), 140.1, 141.4, 147.9, 152.2. *Anal*. Calcd for $C_{25}H_{18}F_3N_3O_5S$: C, 56.71; H, 3.43; N, 7.94%. Found: C, 56.57; H, 3.47; N, 7.88.

4-(4-Dimethylaminobenzylidene)-1,2-dimethyl-4*H*-pyrazolo [1,5-*a*]indolium Trifluoromethanesulfonate (15) The salt **7** (500 mg, 1.50 mmol) was reacted with **10** (291 mg, 1.95 mmol) in AcHO (50 ml) for 31 h to give **15** as a dark red amorphous solid (576 mg, 84.6%), mp 249.0—251.0 °C (MeOH). IR cm^{-1}: 1585, 1532, 1378, 1362, 1266, 1192, 1164, 1142, 1031, 639. ^{1}H-NMR (DMSO-d_6) δ: 2.58 (3H, s, 2-Me), 3.02 (6H, s, NMe_2), 4.36 (3H, s, NMe), 6.88 (2H, d, J=8.8 Hz), 7.20 (1H, s, 3-H), 7.44—7.54 (2H, m), 7.72 (2H, d, J=8.8 Hz), 8.00—8.04 (1H, m), 8.01—8.15 (1H, m), 8.17 (1H, s, 10-H). ^{13}C-NMR (DMSO-d_6) δ: 11.6, 35.7, 101.5, 111.8 (C×2), 112.1, 112.8, 120.8, 120.1, 126.3, 127.3, 131.2, 132.6, 132.9 (C×2), 139.1 (C-10), 141.9, 150.3, 152.3. *Anal*. Calcd for $C_{22}H_{22}F_3N_3O_3S$: C, 56.76; H, 4.77; N, 9.03. Found: C, 56.57; H, 4.64; N, 9.03.

4-(4-Dimethylaminobenzylidene)-1-methyl-2-styryl-4*H*-pyrazolo [1,5-*a*]indolium Trifluoromethanesulfonate (16) The salt **8** (500 mg, 1.18 mmol) and **10** (229 mg, 1.53 mmol) were reacted in AcOH (50 ml) for 11.5 h to give dark red fine crystals (336 mg, 51.3%) of **16**, mp 144.5—146.5 °C (dec.) (EtOH). UV-VIS λ_{max}^{MeOH} nm (log ε): 279 (4.30), 313 (4.50), 501 (4.58). IR cm^{-1}: 1579, 1529, 1369, 1261, 1159, 1031, 638. ^{1}H-NMR (DMSO-d_6) δ: 3.13 (6H, s, NMe_2), 4.59 (3H, s, NMe), 6.96 (2H, d, J=8.8 Hz), 7.45—7.99 (12H, m), 8.07—8.12 (1H, m), 8.15—8.20 (1H, m), 8.24 (1H, s, 10-H). ^{13}C-NMR (DMSO-d_6) δ: 36.0 (NMe), 39.6 (NMe_2), 97.8, 111.9 (C-3), 112.2 (C×2), 112.5, 120.9, 121.1, 126.4, 127.4, 127.9 (2×C), 128.88 (C×2), 128.90, 131.8 (2×C), 132.6, 133.3 (C×2), 135.2, 139.2, 140.0 (C-10), 142.1, 149.7, 152.4. *Anal*. Calcd for $C_{29}H_{26}F_3N_3O_3S$: C, 62.92; H, 4.73; N, 7.59. Found: C, 62.46; H, 4.65; N, 7.62.

4-(*p*-Dimethylaminophenylimino)-1-methyl-2-phenyl-4*H*-pyrazolo [1,5-*a*]indolium Trifluoromethanesulfonate (18) The salt **6** (0.1 g, 0.27 mmol) and *N*,*N*-dimethyl-*p*-nitrosoaniline (**17**) (0.05 g, 0.25 mmol) in AcOH (10 ml) were reacted for 1.5 h. Column chromatography (silica gel, dichloromethane–methanol 95 : 5) of the crude blue-purple solid (0.18 g) gave 0.12 g (90.0%) of **18**, mp 204.0—206.0 °C (CH_2Cl_2–*n*-hexane). IR cm^{-1}: 1618, 1551, 1364, 1261, 1151, 1030, 773, 638. ^{1}H-NMR δ: 3.08 (6H, s, NMe_2), 4.45 (3H, s, NMe), 6.90 (2H, d, J=9 Hz), 7.23 (1H, s, 3-H), 7.45 (2H, d, J=9.0 Hz), 7.61 (1H, t, J=7.6 Hz), 7.65—7.77 (8H, m), 8.08 (1H, d, J=7.4 Hz), 8.15 (1H, d, J=8.0 Hz). ^{13}C-NMR δ: 38.0 (NMe_2), 39.7 (NMe), 104.2, 112.2 (2×C), 113.3, 123.4, 125.2, 125.7 (2×C), 128.2, 129.3 (2×C), 129.5 (2×C), 129.9, 131.52, 131.58, 135.7, 136.32, 136.39, 138.8, 151.0, 152.4. *Anal*. Calcd for $C_{26}H_{23}F_3N_4O_3S$: C, 59.08; H, 4.39; N, 10.60. Found: C, 60.02; H, 4.27; N, 10.10.

5-[2-(*p*-Dimethylaminophenyl)vinyl]-1,3-diphenyl-3-methyl-pyrazolium Trifluoromethanesulfonate (20) A solution of 2,5-dimethyl-1,3-diphenylpyrazolium trifluoromethanesulfonate (**19**) (0.30 g, 0.75 mmol)[8c] and **10** (0.45 g, 3.00 mmol) in ethanol containing sodium ethoxide, prepared by dissolving sodium metal (45.0 mg, 1.96 mmol) in dry ethanol (30 ml) was heated to reflux for 7 h. After removal of solvent, the residue was dissolved in dichloromethane and worked up to give the crude product (0.77 g). Column chromatography (silica gel, dichloromethane–methanol 95 : 5) gave the reddish purple crystals (0.31 g, 79.5%) of **20**, mp 213.0—214.0 °C (CH_2Cl_2–iso-Pr_2O). UV-VIS λ_{max}^{MeOH} nm (log ε): 257 (4.33), 408 (4.64). ^{1}H-NMR (DMSO-d_6) δ: 2.96 (6H, s, NMe_2), 3.66 (3H, s, NMe), 6.25 (1H, d, J=16.1 Hz), 6.69 (2H, d, J=8.8 Hz), 7.35 (2H, d, J=8.8 Hz), 7.67 (1H, s, 4-H), 7.72 (1H, d, J=16.2 Hz), 7.70—7.72 (3H, m), 7.79—7.82 (3H, m), 7.84 (5H, s). ^{13}C-NMR δ: 36.1 (NMe), 39.5 (NMe_2), 103.0 (C-4), 105.0, 111.8 (2×C), 121.8, 126.3, 129.1 (2×C), 129.2 (C×2), 129.3 (2×C), 130.6 (2×C), 131.0, 131.2, 132.5, 141.2, 147.9, 148.9, 152.5.

4-(*p*-Dimethylaminophenylmethyl)-2-phenyl-1-methyl-1*H*-pyrazolo[1,5-*a*]indole (21) A solution of **13** (30 mg, 0.07 mmol) and sodium borohydride (27 mg, 0.71 mmol) in DMSO-d_6 (1.0 ml) was stirred at room temperature for 30 min. During this period, the dark red solution became colorless. The solution was transferred into an NMR tube under a stream of dry nitrogen and the NMR spectra of **21** measured. ^{1}H-NMR (DMSO-d_6) δ: 2.81 (6H, s, NMe_2), 3.45 (3H, s, NMe), 4.01 (2H, s, 10-H), 6.58 (1H, s, 3-H), 6.60 (2H, AB type, J=8.8 Hz), 7.01 (2H, m, 6,7-H), 7.10 (2H, AB type, J=8.8 Hz), 7.43 (1H, m), 7.51 (3H, m), 7.60 (2H, m), 7.70 (1H, m, 8-H). ^{13}C-NMR (DMSO-d_6) δ: 29.3 (C-10), 40.4 (NMe_2), 41.6 (NMe), 95.8 (C-4), 98.6 (C-3), 108.7 (C-8), 112.6 (2×C), 118.1, 118.8, 119.0, 127.1, 127.4 (2×C), 128.7 (2×C), 129.0, 129.1 (2×C), 129.8, 129.9, 137.6, 148.8, 153.6. UV-VIS $\lambda_{max}^{EtOH-NaBH_4}$ nm (log ε): 210 (end absorption, 4.51), 229 (4.41), 264 (4.46), 322 (4.00), 397 (3.95); $\lambda_{min}^{EtOH-NaBH_4}$ nm (log ε): 221 (4.40), 243 (4.36), 296 (3.90), 350 (3.70).

Topoisomerase Inhibition Test Commercial DNA topoisomerase I and II (TopoGen, U.S.A.) were used for the assays and activity was measured by the relaxation of super helical DNA.[12] DNA topoisomerase I and II from murine leukemia P388 were prepared as described by Ishii *et al*.[15] The assay mixture (20 μl) containing 25 mM Tris–HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.25 mM EDTA disodium salt, 0.25 mM dithiothreitol (DTT), 15 μg/ml bovine serum albumin (plus 10 mM ATP for top II test), and enzyme (sufficient units for relaxing 0.5 μg of SV40 DNA) was used as control. The assay mixture was treated with test sample in DMSO (0.5 μl) and left at 37 °C for 10 min, then treated with 0.5 μg of SV40 DNA, and incubated for 10 min. The reaction was terminated by addition of 5 μl of a solution containing 1% sodium dodecylsulfate (SDS), 20 mM EDTA disodium salt and 0.5 mg/ml proteinase K and by incubation for 30 min. Then the solution was mixed with a buffer solution (5 μl) containing 10 mM Na_2HPO_4, 31.3% sucrose and 0.3% bromophenolblue, and subjected to electrophoresis (50 mA, 20 V, overnight) on 0.8% agarose gel plates equilibrated with 2 mg/ml chloroquine, 10 mM EDTA, 30 mM Na_2HPO_4 and 36 mM Tris–HCl (pH 7.8). The plates were stained with 0.05% ethidium bromide solution and photographed under UV light (302 nm). The IC_{50} value is expressed as the concentration which caused 50% inhibition of enzyme activity, corresponding to 50% inhibition of relaxation of supercoiled SV40 DNA under the conditions used.

Cytotoxic Activity Test Cytotoxicity was measured by the microculture tetrazolium assay as described.[14] Growth inhibition experiments were carried out in 96-well (flat bottom) microplates (Falcon California, U.S.A.), and the amount of viable cells at the end of the incubation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Thus, tumor cells ($5.0\times10^2-5\times10^3$ cells/150 μl/well) were cultivated in supplement with 10% fetal calf serum, 2 mM L-glutamine, and 100 mg/ml kanamycin sulfate for 24 h, except P388 (2 h) prior to exposure to drugs. Graded concentrations of drugs (50 μl/well) were introduced to the cell culture and the wells were incubated for 3 d. After MTT (50 μl, 20 mg/ml in phosphate-buffered saline) was added to each culture well and the plates were incubated for an additional 4 h, the culture was centrifuged (800×***g***, 5 min) and medium were removed. The formazan product was dissolved in DMSO (150 μl/well) and the absorbance was measured at 540 nm using a Microplate Reader (model 3550 Bio-Rad, California, U.S.A.). Each data point on growth curves was an average of four wells. A measure was also made of the cell population density at time 0 (the time at which drugs were added). Concentration for 50% growth inhibition (GI_{50}) was calculated from the reduction of optical density of treated cells with respect to the density of untreated controls.

Antitumor Activity Test The assay for P388 leukemia in mice was conducted as specified in the standard NCI protocols.[16] P388 Cells (1×10^6) were inoculated interperitoneally (i.p.) into CDF1 male mice on day 0 and mice were divided into several groups (6 mice per group) on day 1. Test compounds were suspended in BTC solution (0.9% benzylalcohol, 0.4% Tween 80, 0.5% CMC, 0.97% NaCl) by sonification and given i.p. on days 1 and 5. The control group was given 10^6 tumor cells i.p. and injected with BTC solution (10 ml/kg) on the scheduled days. Survival was monitored daily and the ILS was calculated using the following formula: ILS (%)= [(median survival time of treated group)/(median survival time of control group)+1]×100. Statistical analysis was carried out by the Williams–Wilcoxon test.

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