Preparation and bioactivity of pyrazole derivatives as potential cross-linking agent

H. Katayama and T. Oshiyama

Abstract: A series of pyrazole derivatives with two and three alkylating sites were prepared and their cytotoxicity against cancer cells as well as antitumor activity against L1210 was investigated. The best result of cytotoxicity was obtained with the trichloride **5** and the best antitumor activity (ILS 33%) with the dibromide **3**. These results can provide valuable information for further study of the more effective antitumor agent.

Key words: pyrazole, synthesis, cytotoxicity, antitumor activity, polyalkylator.

Résumé : On a préparé une série de dérivés du pyrazole avec deux et trois sites d'alkylation et on a étudié leur cytotoxicité contre les cellules cancéreuses aussi bien que leur activité antitumorale vis à vis du L1210. On a obtenu les meilleurs résultats de cytotoxicité avec le trichlorure **5** et la meilleure activité antitumorale (ILS 33%) avec le dibromure **3**. Ces résultats peuvent fournir une information valable pour une étude plus poussée de l'agent antitumoral le plus efficace.

Mots clés : pyrazole, synthèse, cytotoxicité, activité antitumorale, polyalkylateur.

[Traduit par la rédaction]

Introduction

Among alkylating agents with antitumor activities, the agents that are able to cross-link DNA are believed to be much more effective than singly alkylating agents (e.g., nitrogen mustard) (1). The degree of DNA cross-linking was correlated with the rate of cell death and it was suggested that the cross-linking of DNA represents the molecular basis for the inhibition of DNA replication and consequent cell death (e.g., mitomycin C) (2). The cross-linking distance is quite important for clinically effective anticancer agents and the distance of four carbons was reported to be quite important for effective clinical treatment as demonstrated in an activated form of mitomycin C (MMC), pyrrolizidine alkaloid, and busulphan (Chart 1) (3). The DNA cross-linking action of the mitomycins remains unique among the known naturally occurring antibiotics (2). This action was confirmed with simplified molecular models such as 2,3-bis(acetoxymethyl)-1-methylpyrrole (BAMP). Similar simplification of the pyrrolizidine alkaloid was also accomplished with the synthesis of dehydroretronecine diacetate (DHRA). These compounds were able to cross-link the DNA with some sequence selectivity (Chart 1, cross-linking sites are marked with full circle) (4). We had planned to introduce the cross-linking distance of these compounds into the skeleton of pyrazolo[1,5-*a*]indole, which has been attracting our interest from the chemical and bioactive standpoint (5), and elaborated the target compounds **A** and **B**. Compound **A** can be regarded as an 3-azamitosene. We are also interested in compound **C**, because it is formed by cleaving the C4—C4a bond of **B** and may provide useful information for evaluating the bioactivities of compound **B**. The nonplanarity of the 1-phenyl group in **C** is also important in this evaluation, because the twist angle between the 1-phenyl and pyrazole rings was estimated to be around 50° (6), but these two rings are planar in **B**. In this article we would like to report the preparation and bioactivity of compound **C**.

Results and discussion

Compounds **1–5** were synthesized according to the scheme summarized in Chart 2. Starting compound **6** was prepared by coupling benzaldehyde phenylhydrazone and ethyl acetoacetate at 120°C for 3.5 h in an open flask (7*a*). Introduction of bromide at the 5-methyl group was carried out by radical bromination with *N*-bromosuccimide (NBS) (8), and the bromide **7** was hydrolyzed with $NaHCO_3$ in aqueous tetrahydrofuran (THF) at refluxing temperature to give hydroxyester **8**. Addition of tetra-*n*-butylammonium salt was essential for this selective reaction. This reaction is analogous with the esterification of carboxylate with alkyl halide catalyzed with phase transfer catalyst (9). Reduction of hydroxyester **8** with lithium aluminum hydride (LAH) in ether yielded diol **9** in 51% yield. Transformation of the diol to the dicarbamate was carried out by the reported method (10). Thus diol **9** was esterified with phenyl chloroformate, and the bis-phenyl carbonate **10** so obtained was treated with ammonia to give the bis-carbamate **1** in good yield. When diol **9** was mesylated with methanesulfonyl chloride and triethylamine in dichloromethane, dimesylate was not obtained but the stable dichloride **2** was formed as a single product. This type of substitution readily takes place when the mesylate is treated with nucleophiles (e.g.,

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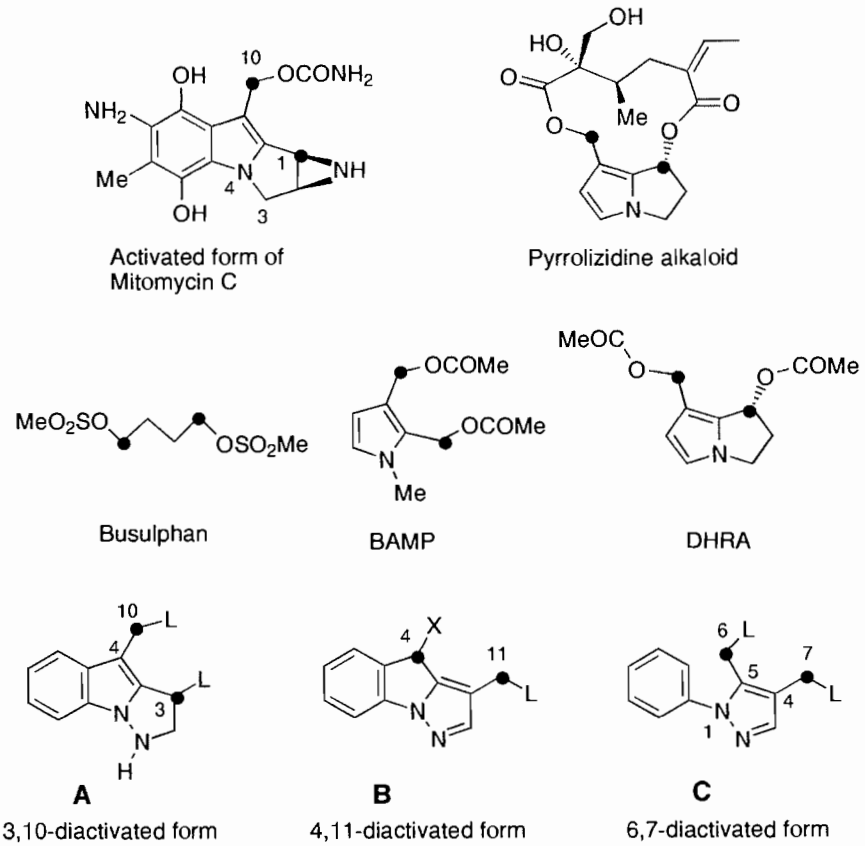
H. Katayama and T. Oshiyama. Niigata College of Pharmacy, 5-13-2 Kamishin'ei-cho, Niigata, 950-21, Japan.

[1] Author to whom correspondence may be addressed. Telephone: (025) 269-3170. Fax: (025) 268-1181. E-mail: Katayama@niigata-pharm.ac.jp

Chart 1.

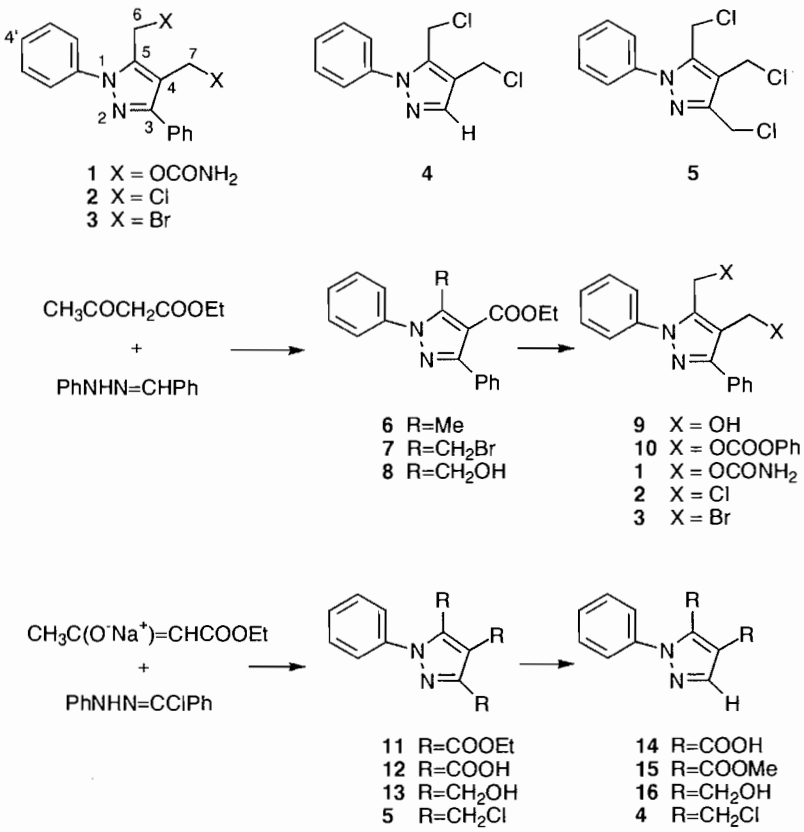


LiCl) in polar aprotic solvent (11), and this method is useful even at non-activated positions such as the C-11 of mitomycins (12). In the ^{13}C NMR of **2**, the difference in the chemical shifts of the C-7 (Δ 18.1 ppm) between diol **9** and dichloride **2** was about the same as that of the benzylic site (Δ 18.1 ppm) between benzyl alcohol and benzyl chloride (13). This similarity suggests that the chemical reactivity of **2** is comparable with that of benzyl chloride. As a compound that is more reactive than dichloride **2**, the dibromide **3** was prepared from the dialcohol **9** by refluxing in 48% HBr solution.

In preliminary cytotoxic tests a better level of activity was detected with chloride **2** than with carbamate **1**, so only chlorides were synthesized for the following pyrazole derivatives. Dichloride **4** was required to investigate the effect of the 2-substituent and trichloride **5** to explore the effect of an additional alkylating site on bioactivities. Compound **5** is unique in having three alkylating sites within the same molecule, all in a 1,4 relationship. Few alkylating agents with more than three alkylating sites in the molecule, e.g., polyaziridinyl compounds (14), have been investigated. Compounds **4** and **5** were prepared from triester **11**, which was obtained by the coupling reaction of hydrazonylchloride with the sodium salt of the β-ketoester as shown in Chart 2 (15). Since the formation of anhydride may takes place readily between two carboxyl groups and the removal of the 3-carboxyl group at 120°C was reported with 1-*p*-nitrophenylpyrazole-3,4,5-tricarboxylic acid (15), the triester **11** was converted to the triacid **12** (16) and **12** was reacted with acetic anhydride at the boiling point (138–140°C) for 7 h. During the reaction a stream of argon was introduced to the reaction vessel and CO_2 generation was monitored by bubbling the argon stream into an aqueous solution of barium hydroxide. The reaction was halted when no generation of CO_2 was detected (7 h). The crude reaction product of **14** obtained by the removal of acetic anhydride was dissolved in methanol and methylated by refluxing with sulfuric acid. The diester **15** (16) thus obtained was reduced with LAH to give the diol **16**. In the ^1H NMR spectrum of **16** a characteristic singlet signal for the 3-H (δ 7.62) was observed, which supports the removal of the 3-carboxyl group. The transformation of diol **16** to dichloride **4** was effected with MsCl, *s*-collidine, and LiCl in DMF (11). Triol **13** was prepared by the reduction of the triester **11** with LAH and was transformed to the trichloride **5** by a reaction similar to that used in the preparation of **4**. Special care was taken to isolate the diols **9** and **16** and the triol **13** after LAH reduction. Aqueous work-up of the LAH reduction product caused difficulties in the isolation of these alcohols and resulted in poor yields. Thus the work-up procedure using NaF and a controlled amount of water (17) was adopted and only moderate yields of these alcohols were obtained. An alternative procedure reported for isolating intractable aminodiol by using triethanolamine (18) was not effective in our case. Reduction of **11** with $NaBH_4$ in *t*-BuOH–MeOH (19) was used to avoid this work-up problem but was also not effective in reducing the ester group.

Cytotoxicity was measured by the microculture tetrazolium assay (20). Results for pyrazole derivatives **1–5** against cancer cells are summarized in Table 1. The activities of these compounds are relatively weak compared with the reference compound, MMC. The dichloride **2** was found to be more active than the dicarbamate **1**. The low activity of **1** may be a result of the poor reactivity of carbamate as a leaving group. But the

Chart 2.



dibromide **3**, which is chemically more reactive than the dichloride **2**, did not show a higher activity than **2**. The activity of **3** was found to be as low as that of the carbamate **1**. The small difference in activity between the two dichlorides, **2** and **4**, indicates that the contribution of the 2-phenyl group to the cytotoxic activity may be poor. Selectivity of activity against cancer cells was small. Since the trichloride **5** showed the highest activity against cancer cells, the number of alkylating sites may play an important role in the cytotoxicity. The activity of the trichloride **5** against uterus cervical cancer cells (HeLa) was noteworthy, since the increase of the alkylating sites from two to three increased the activity of the trichloride **5** 36 times more than that of the dichloride **2**. The activity of **5** against HeLa cell was found to be 2.8 times stronger than that of MMC.

Antitumor activities of the selected pyrazole derivatives **1–3** and **5** against murine leukemia L1210 are cited in Table 2 and the effect is expressed by ILS (increase of life span in %). The carbamate **1** did not show antitumor activity but it showed toxicity in mice. ILS of the dichloride **2** was proportional to the dose and 25.6% of ILS was obtained when a dose of 200 mg/kg was applied. At the same dose, the dibromide **3** showed a slightly better ILS value of 33.3%, which is thought to be effective. Although these ILS were relatively low compared with mitomycin C (74.4%), this dose dependence suggests that the intrinsic toxic property of the skeleton **C** is small. Contrary to the encouraging effects of **2** and **3**, the ILS of trichloride **5** was disappointingly small (4.8%) at maximum dose (12.5 mg/kg). In **5**, toxic effects became more significant than the desired activity at higher dose. The high chemical reactivity could be the reason for the toxic effect of **5**. Since the electron density at N-1 can control the reactivity of the alkylating sites, it may be possible to explore the desirable properties of the more effective antitumor agent by introducing a proper substituent at the N-1 position. Finally, the trichloride **5** attracted the attention of NCI in its in vitro screening test against 60 cancer cell lines but the potency in the test against prostate cell strains was disappointing. In conclusion, we have prepared a series of 1-phenylpyrazole derivatives with two and three alkylating sites and evaluated their cytotoxicities against cancer cells and antitumor activities against murine leukemia L1210. The best result of cytotoxicity was obtained with the trichloride **5** and the best in vivo result (ILS 33%) was obtained with the dibromide **3**. These results could provide valuable information for the further study of a series of **B** compounds.

Experimental

All melting points (mp) were determined with a Yanaco micro melting point apparatus without correction. Spectra were measured with the following spectrometers: IR (KBr pellet unless otherwise stated), Perkin–Elmer FT-IR 1720; ^{1}H and ^{13}C NMR, JEOL JNM-FT 200 and JNM-ALPHA 400 in $CDCl_3$ (unless otherwise mentioned) at ambient temperature (25–27°C) with tetramethylsilane as an internal standard; MS and high-resolution MS (HRMS), Hitachi RMU-7MG; UV and visible spectra (VIS), Shimadzu UV-200.

Ethyl 1,3-diphenyl-5-methylpyrazole-4-carboxylate 6 (ref. 7*a*)

Benzaldehyde phenylhydrazone (mp 156.0–158°C, 6.0 g, 0.031 mol), ethyl acetoacetate (6.0 g, 0.046 mol), and

Table 1. Cytotoxicities of pyrazole derivatives **1–5** against cancer cells.

	IC_{50} (μg/mL)[b]					
Compound[a]	L1210	Hela	PC-1	KATOIII	LoVo	MCF-7
MMC	0.085	0.148	0.117	0.333	0.279	0.065
1	>10	9.51	>10	7.09	9.15	>10
2	0.85	1.91	2.25	3.16	3.44	1.72
3	>2.5	>10	>5	>10	>10	>5
4	2.93	1.64	4.59	6.16	0.61	3.78
5	0.36	0.053	0.26	1.46	0.27	0.51

[a]MMC: Mitomycin C.

[b]Activities against cancer cells (L1210, murine leukemia; HeLa, uterus cervical; PC-1, lung; KATOIII, gastric; LoVo, colon; MCF-7, breast) were measured by MTT assay after 3 days of incubation and expressed as concentration for 50% growth inhibition (IC_{50}).

Table 2. Antitumor activities of pyrazole derivatives **1–3** and **5** against murine leukemia L1210

Compound	Dose[a] (mg/kg)	(i.p.)	MST[b] (Day)	ILS[b] (%)
Control			7.8 ± 0.2	
MMC	5	×1	13.6 ± 1.6	74.4
1	50	×1	7.4 ± 0.2	−5.1
	100	×1	7.4 ± 0.2	−5.1
	200	×1	7.2 ± 0.2	−7.7
2	50	×1	9.0 ± 0.6	15.4
	100	×1	9.0 ± 0.6	15.4
	200	×1	9.8 ± 0.8	25.6
Control			7.2 ± 0.2	
3	200	×1	9.6 ± 0.4	33.3
Control			8.4 ± 0.4	
5	12.5	×1	8.8 ± 0.2	4.8

[a]L1210 Cells (1×10^5) were inoculated i.p. into BDF1 mice (male, 6 weeks).

[b]Survival number was monitored daily and the increase in life span (ILS, %) was calculated from [(mean survival time of treated group) / (mean survival time of control group) − 1] × 100.

powdered anhydrous $ZnCl_2$ (0.6 g, 4.4 mmol) were placed in a beaker (100 mL) and the mixture was gradually heated to 120°C with stirring and kept at this temperature for 3.5 h. After cooling, ethanol was added, and the isolated crystals were collected to give **6** (4.15 g, yield: 44.3%), mp 102.5–105.0°C. IR: 3069, 1709, 1599, 1505, 1455, 1427, 1148, 1122, 1092, 772, 702 cm^{-1}. ^1H NMR δ: 1.21 (3H, t, J = 7.3, OCH_2CH_3), 2.58 (3H, s, 5-CH_3), 4.24 (2H, q, J = 7.3, OCH_2Me), 7.65–7.70 (2H, m, Ar-*H*), 7.44–7.52 (5H, m, Ar-*H*), 7.36–7.42 (3H, m, Ar-*H*); ^{13}C NMR δ: 12.7 (C-*9*), 14.1 (C-*6*), 60.0 (C-*8*), 110.6 (C-*4*), 125.8 (2 × C), 127.6 (2 × C), 128.2, 128.6, 129.2 (2 × C), 129.4 (2 × C), 133.1, 138.8 (C-*3*), 144.7 (C-*5*), 153.6, 164.1 (C-*7*). MS *m/e* (relative %): 307 (M^+ + 1, 19), 306 (M^+, 100), 262 (17), 261 (94), 260 (50), 259 (33), 234 (32), 233 (20), 118 (21), 77 (16).

Ethyl 5-bromomethyl-1,3-diphenylpyrazole-4-carboxylate 7

A mixture of **6** (15.0 g, 0.049 mol), *N*-bromosuccimide (13.35 g, 0.075 mol), and benzoylperoxide (4.1 g, 0.017 mol) in dry benzene (150 mL) was refluxed for 3 h. The solution was washed successively, with aqueous 1 M NaOH solution, water, and saturated brine and dried with anhydrous Na_2SO_4. Filtration and evaporation of the filtrate gave the crude product, which was recrystallized from EtOH to give the bromide **7** (17.4 g, 92.3%), mp 98.5–101.5°C. IR: 1717, 1595, 1532, 1496, 1458, 1259, 1147, 766, 694 cm^{-1}. ^1H NMR δ: 1.21 (3H, t, J = 7.1, OCH_2CH_3), 4.24 (2H, q, J = 7.1, OCH_2Me), 4.85 (2H, s, 6-*H*), 7.42–7.45 (3H, m, Ar-*H*), 7.62–7.70 (7H, m, Ar-*H*). ^{13}C NMR δ: 13.7 (C-*9*), 20.8 (C-*6*), 60.2 (C-*8*), 110.2 (C-*4*), 125.4 (2 × C), 127.7 (2 × C), 128.4, 129.0 (2 × C), 129.4, 129.5 (2 × C), 131.9, 137.7 (C-*3*), 143.2 (C-*5*), 152.5, 162.2 (C-*7*). MS *m/e* (relative %): 386 (M^+ + 2, 16), 384 (M^+, 19), 305 (68), 277 (21), 259 (91), 231 (12), 105 (79), 77 (100), 51 (18), 45 (13). Anal. calcd. for $C_{19}H_{17}BrN_2O_2$ (mw 385.25): C 59.24, H 4.45, N 7.27%; found: C 59.33, H 4.46, N 7.25.

Ethyl 5-hydroxymethyl-1,3-diphenylpyrazole-4-carboxylate 8

Bromide **7** (3.00 g, 7.8 mmol) and tetra-*n*-butylammonium bromide (0.75 g, 2.3 mmol; 29 mol%) were dissolved in tetrahydrofuran (THF, 30 mL). To this solution was added saturated aqueous $NaHCO_3$ solution (30 mL) and the mixture was refluxed until the total disappearance of **7** was observed on TLC (27 h). The solution was evaporated and the residue was diluted with water. Following extraction with CH_2Cl_2 (60 mL × 3), usual work-up of the extracts gave the crude product. Purification with flash column chromatography (SiO_2, petroleum ether – ethyl acetate 4:1) yielded the alcohol **8** (2.51 g, 41.0%), mp 80.5–81.5°C (petroleum ether – ethyl acetate 10:1). IR: 3451, 1707, 1598, 1538, 1501, 1216, 1185, 775, 769, 764, 759, 697 cm^{-1}. ^1H NMR δ: 1.20 (3H, t, J = 7.1, OCH_2CH_3), 4.14 (1H, t, J = 7.3, OH), 4.27 (2H, q, J = 7.1, OCH_2Me), 4.77 (2H, d, J = 7.1, 6-*H*; singlet by D_2O addition), 7.36–7.43 (3H, m, Ar-*H*), 7.45–7.61 (5H, m, Ar-*H*), 7.63–7.68 (2H, m, Ar-*H*). ^{13}C NMR δ: 13.9 (C-*9*), 55.3 (C-*6*), 60.9 (C-*8*), 111.7 (C-*4*), 125.6 (2 × C), 127.6 (2 × C), 128.5, 129.0, 129.4 (2 × C), 129.6 (2 × C), 132.7, 138.2 (C-*3*), 147.7 (C-*5*), 153.5,

165.1 (C-7). MS *m/e* (relative %): 323 (M^+ + 1, 7), 322 (M^+, 45), 276 (36), 275 (100), 247 (4), 104 (7), 77 (55), 43 (78). Anal. calcd. for $C_{19}H_{18}N_2O_3$ (mw 322.36): C 70.79, H 5.63, N 8.69%; found: C 70.84, H 5.63, N 8.66.

4,5-Dihydroxymethyl-1,3-diphenylpyrazole 9

A solution of hydroxyester **8** (0.285 g, 0.88 mmol) in anhydrous ether (60 mL) was added gradually to a solution of lithium aluminum hydride (0.8 g, 21.1 mmol) in dry ether (10 mL) for 1 h. The reaction mixture was stirred overnight. After cooling with an ice-bath, the reaction was successively quenched with NaF (2.5 g, 59.5 mmol) and water (5 mL). The resulting solution was stirred vigorously at room temperature until the inorganic precipitates became totally white. The inorganics were removed by filtration and washed well with ether (50 mL). The filtrate and washings were combined, dried with anhydrous $MgSO_4$ overnight, and evaporated. Chromatographic purification of the crude product gave the diol **9** (0.127 g, 51.3%), mp 167.0–168.0°C (from EtOH). IR: 3379, 3338, 3181, 3060, 1596, 1559, 1503, 1117, 1008, 993, 775, 715 cm^{-1}; ^1H NMR δ: 4.53 (2H, d, J = 5.0, 7-H; singlet by irradiation at δ 5.00), 4.54 (2H, d, J = 5.2, 6-H; singlet when irradiated at δ 5.44), 5.00 (1H, t, J = 5.0, O*H*), 5.44 (1H, t, J = 5.2, O*H*), 7.86–7.93 (2H, m, Ar-*H*), 7.74–7.80 (2H, m, Ar-*H*), 7.34–7.60 (6H, m, Ar-*H*). ^{13}C NMR δ: 51.7 (C-*7*), 52.7 (C-*6*), 119.1 (C-*4*), 123.8 (2 × C), 127.4, 127.5 (2 × C), 127.7, 128.4 (2 × C), 129.1 (2 × C), 133.1, 139.4 (C-*3*), 142.0 (C-*5*), 150.3. MS *m/e* (relative %): 281 (M^+ + 1, 14), 280 (M^+, 100), 262 (35), 261 (57), 233 (49), 130 (31), 104 (26), 77 (86). Anal. calcd. for $C_{17}H_{16}N_2O_2$ (mw 280.33): C 72.84, H 5.75, N 9.99%; found: C 72.90, H 5.89, N 9,99.

4,5-Diphenoxycarbonyloxymethyl-1,3-diphenylpyrazole 10

Phenyl chloroformate (2.98 mL, 23.8 mmol) was introduced into a solution of the dialcohol **9** (2.20 g, 7.8 mmol) in dry pyridine (15 mL) that was precooled in an ice-bath. The resulting solution was stirred at room temperature for 46 h. The solution was diluted with water (100 mL), and precipitates were collected. Recrystallization of the crude product from acetone–hexane (1:5) gave **10** (3.797 g, 93.0%), mp 138.5–139.0°C. IR: 1765, 1598, 1501, 1471, 1376, 1257, 1204, 779, 706 cm^{-1}. ^1H NMR δ: 5.45 (4H, s, 6-H_2 + 7-*H*), 7.07–7.58 (16H, m, Ar-*H*), 7.64 (2H, m, Ar-*H*), 7.83 (2H, m, Ar-*H*). ^{13}C NMR δ: 58.5 (C-*7*), 60.5 (C-*6*), 114.6 (C-*4*), 121.0 (2 × C), 121.1 (2 × C), 125.5 (2 × C), 126.0, 126.1, 128.3 (2 × C), 128.6, 128.8 (2 × C), 128.9, 129.4 (4 × C), 132.1, 137.7 (C-*3*), 138.9 (C-*5*), 151.1, 151.2, 152.7, 153.2 (C-*8*), 153.6 (C-*9*). MS *m/e* (relative %): 520 (M^+, <1), 384 (28), 383 (100), 339 (12), 246 (26), 245 (30), 94 (21), 43 (55). Anal. calcd. for $C_{31}H_{24}N_2O_6$: C 71.53, H 4.65, N 5.38%; found: C 71.74, H 4.65, N 5.49.

4,5-Diaminocarbonyloxymethyl-1,3-diphenylpyrazole 1

A solution of **10** (2.00 g, 3.84 mmol) in dry CH_2Cl_2 (100 mL) was cooled to −78°C by Dry Ice – acetone, and dry ammonia was introduced under a stream of nitrogen to this solution to collect about 200 mL of ammonia. The cooling bath was removed and the flask equipped with the Dry Ice condenser was left at room temperature for 5 h. Ammonia was evaporated during this period and the crystals isolated in the residual solution were collected. Usual work-up of the filtrate yielded the second crop of the crystals. The combined crystals were recrystallized from ethanol to give **1** (1.34 g, 95.0%), mp 226.0–227.0°C. IR: 3437, 3339, 3279, 3215, 1703, 1692, 1613, 1504, 1422, 1344, 1339, 1063, 1009, 779, 697, 571 cm^{-1}. ^1H NMR δ: 5.07 (2H, s, 6-*H*), 5.10 (2H, s, 7-*H*), 6.60–6.66 (4H, br s, 2 × NH_2), 7.42–7.65 (8H, m, Ar-*H*), 7.74–7.79 (2H, m, Ar-*H*). ^{13}C NMR δ: 53.8 (C-*6*), 55.1 (C-*7*), 115.2 (C-*4*), 124.8 (2 × C), 127.5 (2 × C), 128.1, 128.3, 128.5 (2 × C), 129.2 (2 × C), 132.3, 138.8 (C-*3*), 138.9 (C-*5*), 150.9, 155.7 (*C*O), 156.4 (*C*O). MS *m/e* (relative %): 366 (M^+, 6), 305 (13), 262 (100), 233 (19), 142 (7), 130 (16), 77 (46), 57 (36), 43 (80). Anal. calcd. for $C_{19}H_{18}N_4O_4$: C 62.29, H 4.95, N 15.29%; found: C 62.09, H 4.89, N 15.32.

4,5-Dichloromethyl-1,3-diphenylpyrazole 2

Methanesulfonyl chloride (0.63 mL, 8.0 mmol) was dropped for 5 min into a solution of the dialcohol **9** (0.84 g, 3.0 mmol) and triethylamine (1.26 mL, 9.1 mmol) in dry CH_2Cl_2 (30 mL) that was cooled in an ice-bath, and the reaction mixture was stirred at room temperature until the solution showed a single spot on TLC (22 h). The solution was washed successively with water, aqueous 10% HCl solution, aqueous $NaHCO_3$-saturated solution, and a saturated brine. The crude product obtained was recrystallized from ether–pentane to give the dichloride **2** (0.51 g, 39%), mp 119.0–120.0°C. IR: 3068, 2968, 1596, 1559, 1503, 1471, 1373, 1284, 1193, 1110, 1075, 1028, 917, 876, 766, 756, 695, 664, 514 cm^{-1}. ^1H NMR δ: 4.69 (2H, s, CH_2Cl), 4.77 (2H, s, CH_2Cl), 7.41–7.58 (6H, m, Ar-*H*), 7.61–7.67 (2H, m, Ar-*H*), 7.75–7.82 (2H, m, Ar-*H*); ^{13}C NMR δ: 33.6 (C-*7*), 36.3 (C-*6*), 116.2 (C-*4*), 125.2 (2 × C), 128.1 (2 × C), 128.5, 128.8 (2 × C), 128.9, 129.5 (2 × C), 131.9, 138.6 (C-*3*), 138.7 (C-*5*), 151.6. MS *m/e* (relative %): 320 (M^+ + 4, 4), 318 (M^+ + 2, 26), 316 (M^+, 39), 283 (36), 282 (20), 281 (100), 245 (18), 142 (11), 115 (6), 77 (16). Anal. calcd. for $C_{17}H_{14}N_2Cl_2$: C 64.67, H 4.45, N 8.63%; found: C 64.37, H 4.74, N 8.83.

4,5-Dibromomethyl-1,3-diphenylpyrazole 3

A solution of **9** (500 mg, 1.78 mmol) in aqueous 48% HBr solution (5 mL) was refluxed for 0.5 h. The solution was diluted with water, basified to pH 7–8 with Na_2CO_3, and extracted with ether. The extract was washed with water and aqueous 50% NaBr solution and, following the usual work-up, gave the crude product (0.73 g). Chromatographic purification (silica gel, CH_2Cl_2–hexane 1:1) yielded pure **3** (0.473 g, 65.3%), mp 94.5–95.5°C (hexane–ether 5:2). IR: 1597, 1550, 1503, 1457, 1375, 1212, 1198, 916, 779, 761, 699, 685, 590 cm^{-1}. ^1H NMR δ: 4.58 (2H, s, CH_2Br), 4.69 (2H, s, CH_2Br), 7.42–7.60 (6H, m, Ar-*H*), 7.65–7.69 (2H, m, Ar-*H*), 7.79–7.83 (2H, m, Ar-*H*). ^{13}C NMR δ: 19.3 (C-*7*), 23.5 (C-*6*), 116.3 (C-*4*), 125.1 (2 × C), 128.1 (2 × C), 128.6, 128.8 (2 × C), 128.9, 129.5 (2 × C), 131.9, 138.5 (C-*3*), 138.8 (C-*5*), 151.5. MS *m/e* (relative %): 327 (91), 325 (90), 246 (100), 245 (95), 142 (27), 123 (20), 115 (19), 77 (54), 51 (20). Anal. calcd. for $C_{17}H_{14}Br_2N_2$: C 50.28, H 3.47, N 6.90%; found: C 50.39, H 3.54, N 6.83.

4,5-Bis (hydroxymethyl)-1-phenylpyrazole 16

The triester **11** (2.00 g, 5.55 mmol) was dissolved in the KOH solution prepared from KOH (12.46 g) and aqueous ethanol

(water-EtOH 1:1, 200 mL), and the resulting solution was refluxed for 4 h. After evaporation the residual solution was acidified with 35% HCl solution and the isolated crystals were collected to give the triacid **12** (1.32 g, 86.1%), mp 228.0–229.0°C (Lit. (14) mp 213.0–214.0°C). IR: 3442 (br), 1718, 1598, 1498, 1429, 1384, 1320, 1132, 1017, 770 cm^{-1}. ^1H NMR δ: 7.46–7.53 (5H, m, Ar-*H*). ^{13}C NMR δ: 118.1, 126.3 (2 × C), 128.6 (2 × C), 129.0, 137.5 (C-*4*), 141.0 (C-*5*), 144.0 (C-*3*), 158.4 (C-*7*), 160.9 (C-*5*), 167.6 (C-*6*).

The triacid **12** (2.00 g, 7.24 mmol) was added to acetic anhydride (50 mL), and the solution was refluxed for 7 h under a stream of argon. Reaction was halted when the stream of argon was introduced to the barium hydroxide solution and no generation of CO_2 was detected. Solvent was removed and the residue containing diacid **14** was dissolved in methanol (50 mL) containing sulfuric acid (3 mL). The solution was refluxed for 5 h, and evaporated. Basification with aqueous 1 M sodium carbonate solution and extraction with ethyl acetate gave, after the usual work-up, 1.41 g of **15** (14).

A solution of the diester **15** (0.30 g, 1.2 mmol) in dry THF (100 mL) was slowly added to a solution of LAH (0.175 g, 4.6 mmol) in dry THF (200 mL) for 30 min. The solution was refluxed for 2.5 h, and the reaction was quenched by the addition of NaF (0.773 g, 18.4 mmol) and water (1 mL). After stirring the solution for 2 h, the inorganic precipitates were removed and the filtrate was evaporated to give the oily dialcohol **16** (0.157 g, 66.8%). IR: 3338, 1599, 1505, 1392, 1017, 757, 692 cm^{-1}. ^1H NMR δ: 4.47 (2H, d, *J* = 5.4, 7-*H*), 4.48 (2H, d, *J* = 5.4, 6-*H*), 4.84 (1H, t, *J* = 5.4, 7-O*H*), 5.35 (1H, t, *J* = 5.4, 6-O*H*), 7.36–7.44 (1H, m, Ar-*H*), 7.48–7.55 (2H, m, Ar-*H*), 7.62 (1H, s, 3-*H*), 7.65–7.69 (2H, m, Ar-*H*). ^{13}C NMR δ: 51.7 (C-*7*), 53.6 (C-*6*), 122.4 (s, C-*4*), 123.7 (d, 2 × C), 127.2 (d), 129.0 (d, 2 × C), 139.2 (s, C-*5*), 139.5 (s + d, *ipso*-C + C-*3*). MS *m/e* (relative %): 204 (M^+, 44), 188 (50), 187 (39), 186 (45), 185 (53), 171 (21), 159 (40).

4,5-Bis (dichloromethyl)-1-phenylpyrazole 4

Under an argon atmosphere, methanesulfonyl chloride (1.13 mL, 8.1 mmol) was gradually dropped into a solution of the dialcohol **16** (0.75 g, 3.7 mmol), *s*-collidine (1.07 mL, 8.8 mmol), and LiCl (0.31 g, 7.6 mmol) in dry dimethylformamide (DMF, 35 mL) at 0°C. The solution was stirred at the same temperature for 1 h, then left at room temperature overnight. Dilution with ice-water and extraction with ether–pentane yielded the crude product (1.17 g), which was crystallized from isopropyl ether – ethyl acetate to give **4** (0.20 g, 22.6%), mp 90.0–91.5°C. IR: 1598, 1570, 1407, 1288, 1260, 1206, 1131, 1070, 987, 911, 868, 772 cm^{-1}. ^1H NMR δ: 4.62 (2H, s, 7-*H*), 4.67 (2H, s, 6-*H*), 7.45–7.60 (5H, m, Ar-*H*), 7.71 (1H, s, 3-*H*). ^{13}C NMR δ: 33.3 (C-*7*), 35.7 (C-*6*), 119.0, 125.1 (2 × C), 128.9, 129.5 (Ar-*H*), 137.0 (C-*4*), 138.7 (C-*5*), 140.4 (C-*3*). MS *m/e* (relative %): 242 (M^+ + 2, 12), 240 (M^+, 19), 207 (35), 206 (14), 205 (100), 170 (11), 169 (20), 142 (11), 115 (7), 77 (23). Anal. calcd. for $C_{11}H_{10}N_2Cl_2$: C 54.79, H 4.18, N 11.62, Cl 29.41%; found: C 54.96, H 4.19, N 11.56.

1-Phenyl-3,4,5-tris (hydroxymethyl)pyrazole 13

A solution of the triester **11** (5.40 g, 15 mmol) in dry THF (400 mL) was slowly dropped into a solution of LAH (2.28 g, 60 mmol) in dry THF (600 mL) at room temperature for 6 h, and the resulting solution was stirred overnight. Under vigorous stirring, NaF (10.08 g, 240 mmol) and water (15 mL) were added and the stirring was resumed until the inorganic precipitates became totally white. The inorganics were removed by filtration and washed with THF. The filtrate and washings were combined and evaporated. The oily product (3.02 g) was purified by column chromatography (silica gel 90 g, CH_2Cl_2 containing 10% methanol) to give 1.02 g (29.1%) of the trialcohol **13**, mp 102.0–103.0°C (ethyl acetate – isopropyl ether). IR: 3310, 3274, 1506, 1402, 1082, 1031, 1003, 830, 769, 701 cm^{-1}. ^1H NMR δ: 4.46 (2H, d, *J* = 5.1, 7-*H*; singlet by D_2O addition), 4.53 (4H, d, *J* = 5.3, 6-*H* and 8-*H*; singlet after addition of D_2O), 4.76 (1H, t, *J* = 5.4, O*H*), 5.05 (1H, t, *J* = 5.6, O*H*), 5.40 (1H, t, *J* = 5.4, O*H*), 7.41 (1H, d, *J* = 7.1, Ar-*H*), 7.52 (2H, t, *J* = 7.4, Ar-*H*), 7.67 (2H, d, *J* = 7.1, Ar-*H*). ^{13}C NMR δ: 51.8 (C-*7*), 52.8 (C-*6*), 56.1 (C-*8*), 120.0, 123.9 (2 × C), 127.3, 129.2 (2 × C), 139.5 (C-*4*), 140.7 (C-*5*), 151.4 (C-*3*). MS *m/e* (relative %): 234 (M^+, 59%), 216 (61), 198 (100), 187 (36), 169 (22), 157 (20), 142 (19), 130 (23), 117 (13), 104 (16), 77 (78), 51 (22). Anal. calcd. for $C_{12}H_{14}H_2O_3$ (mw 234.25): C 61.53, H 6.02, N 11.96%; found: C 61.34, H 6.01, N 12.00.

1-Phenyl-3,4,5-tris (chloromethyl)pyrazole 5

Under an argon atmosphere at ice-cooling temperature, methanesulfonyl chloride (0.924 mL, 6.6 mmol) was gradually dropped into a solution of trialcohol **13** (468 mg, 2.0 mmol), *s*-collidine (0.876 mL, 6.6 mmol), and LiCl (254 mg, 6.0 mmol) in dry DMF (30 mL) for 1 h, and the resulting solution was stirred overnight. The reaction was quenched with addition of ice-water (5 mL), and the solution was extracted with ether–pentane (1:1). The combined extracts were washed once with aqueous Cu $(NO_3)_2$ solution, and the usual work-up was followed. Crystallization of the crude product (436 mg, 75%) from ether–petroleum ether yielded the trichloride **5**, mp 53.0–54.0°C. IR: 3066, 1603, 1578, 1509, 1463, 1402, 1280, 1265, 1230, 1053, 1024, 813, 780, 770, 752 cm^{-1}. ^1H NMR δ: 4.60 (2H, s, 7-*H*), 4.74 (2H, s, 8-*H*), 4.76 (2H, s, 6-*H*), 7.47–7.58 (5H, m, Ar-*H*). ^{13}C NMR δ: 33.1 (C-*7*), 34.5 (C-*8*), 36.9 (C-*6*), 117.3, 125.1 (2 × C), 129.1 (2 × C), 129.5 (2 × C), 138.1 (C-*4*), 138.5 (C-*5*), 148.1 (C-*3*). MS *m/e* (relative %): 292 (M^+ + 4, 6), 290 (M^+ + 2, 18), 288 (M^+, 20), 255 (66), 253 (100), 217 (19), 183 (12), 77 (14), 51 (5). Anal. calcd. for $C_{12}H_{11}C_{l3}N_2$ (mw 289.59): C 49.77, H 3.83, N 9.67; found: C 50.07, H 3.78, N 9.74.

Cytotoxic activities

Cytotoxicity was measured by the microculture tetrazolium assay as described (20). Cancer cell lines of uterus cervical (HeLa), human lung (PC-1), gastric (KATOIII), colon (LoVo), breast (MCF-7), and murine leukemia cell (L1210) were maintained in RPM1 1640 (Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Utah, U.S.A.). Growth inhibition experiments were carried out in 96-well (flat bottom) microplates, and the amount of viable cells at the end of the incubation was determined using the MTT assay. Thus, tumor cells (L1210, 5×10^3 cells/well; HeLa, 2×10^3 cells/well, and other cells, 3×10^3 cells/well) were incubated in RPM1-1640 supplemented with 10% fetal calf serum. Drugs were dissolved in DMSO (2 mg/mL), diluted with cultural media (20 μg/mL), and introduced to the cell culture with gradual dilution. The

cells were exposed to drugs for 3 days at 37°C in air containing 5% carbon dioxide.

MTT (10 μL, 5 mg/mL in phosphate-buffered saline) was added to each culture well. The plates were incubated for an additional 4 h, and the medium was removed. The formazan product was dissolved in DMSO (150 μL/well) and the absorbance was measured at 540 nm using a Microplate Reader (Molecular Devices Corporation, California, U.S.A.). Each data point on the growth curves was an average of three wells. The 50% growth inhibitory concentration (IC_{50}) was calculated by the probit method.

Antitumor activity

The assays for L1210 leukemia in mice were performed as specified in the standard NCI protocols (21). L1210 Cells (1×10^5) were inoculated interperitoneally (i.p.) into BDF1 mice (male, 6 weeks old) on day 0 and mice divided into several groups (5 mice per group) on day 1. Test compounds were suspended in 0.5% CMC and given i.p. on days 1. Survival was monitored daily and the increase in life span (ILS) was calculated using the following formula: ILS (%) = [(mean survival time of treated group)/(mean survival time of control group) − 1] × 100.

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