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Short Communication

Esters of chlorambucil with 2-substituted 1,4-dihydroxy-9,10-anthraquinones as multifunctional anticancer agents

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Abstract – Novel twelve esters of chlorambucil with 2-(1-hydroxyalkyl)-1,4-dihydroxy-9,10-anthraquinone were synthesized and tested for their antitumor activity in mice bearing S-180 ascitic cells as well as cytotoxic activity against L1210 cells. Eight of them were highly cytotoxic on L1210 cells (ED_{50}, <6 μg mL^{-1}) and derivatives **1** and **12** (T/C, 200 and 205%) appeared more active in vivo than chlorambucil (T/C, 168%). © 2001 Éditions scientifiques et médicales Elsevier SAS

anthraquinone / chlorambucil / ester / cytotoxicity / antitumor / structure–activity relationship

1. Introduction

1,4-Dihydroxy-9,10-anthraquinone (quinizarin) is a common structural moiety of adriamycin (ADR) and mitoxantrone. The main action mechanisms of adriamycin are inhibition of DNA topoisomerase-II [1] and DNA intercalation [2, 3]. Recently it was reported that 2-(1-hydroxyalkyl)- and 2-(1-acyloxyalkyl)-1,4-dihydroxy-9,10-anthraquinone derivatives [4] and 2-(1-hydroxyalkyl)-anthracene-1,4,9,10-tetraones [5] showed good antitumor activities, where bioreductive alkylation was suggested to be one of their action mechanisms. In the mean time, chlorambucil has shown good activities on a variety of human malignancies [6], but unfortunately it manifested bone marrow depression including neutropenia and thrombocytopenia in clinical studies, which was the dose-limiting toxicity. In this context, it will be interesting to synthesize chlorambucil esters with 1,4-dihydroxy-9,10-anthraquinones (DHAQ) to observe an enhanced antitumor effect. It is expected that such an ester could exert a multiple function such as easy transportation and DNA intercalation, which might not be possible from chlorambucil and from there, that the intercalated ester might exert a rapid intramolecular DNA-alkylation. Furthermore, the reductive bioactivation of these esters in target tissues leading to the release of two alkylating species of a quinone methide and chlorambucil also could be anticipated. Based on these expectations, we synthesized a series of chlorambucil esters of 2-(1-hydroxyalkyl)-DHAQ and evaluated their antitumor activity.

2. Chemistry

Chlorambucil was esterified with 2-(1-hydroxyalkyl)-1,4-dihydroxy-9,10-anthraquinone under the presence of 1,3-dicyclohexycarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) to produce esters (**1–12**). The structures were confirmed by NMR interpretation. 1′-Protons in the side chain of DHAQ derivatives, appeared from 5.85 to 6.10 ppm, were shifted to around 6.15 ppm by esterification. The ester carbonyl group was confirmed at 1725 cm^{-1} of IR spectrum. The peak at 3.66 ppm corresponding to eight protons of bis-(2-chloroethylamino) group confirmed that the alkylating moiety was not affected by the synthetic process.

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3. Results and discussion

3.1. Cytotoxicity

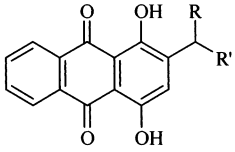
The cytotoxicity and antitumor activity of the esters (R′ = Cb, **1**–**12**) and their starting materials[3] (R′ = OH, **1a**–**12a**) were shown in *table I*. Compared to the cytotoxicity of the starting materials, introduction of Cb ((*p*-bis(2-chloroethyl)aminophenyl)-butanoyloxy group) enhanced the cytotoxic activity to a great extent. For example, 2-[(*p*-bis(2-chloroethyl)aminophenyl)-butanoyloxymethyl]-DHAQ **2** exhibited the cytotoxic activity seven times higher than 2-hydroxymethyl-DHAQ. The cytotoxic activity of the esters tends to be dependent upon the size of alkyl group, decreasing with larger R. The esters **1**–**6** with shorter chain than pentyl group showed either as strong or stronger activities than chlorambucil. The cytotoxic activities of the esters **8**–**11** having larger R than heptyl group decreased abruptly. This phenomenon might occur due to the remarkable decrease of their solubility in the aqueous system.

An aromatic system seems to play a role in the cytotoxicity as shown by difference between DHAQ derivatives with R = phenyl and cyclohexyl groups. 2-{1-[4-(*p*-bis(2-chloroethyl)aminophenyl)butanoyloxy]-1-phenylmethyl}-DHAQ (R = phenyl) **12** showed an ED_{50} value of 1.5 μg mL^{-1}, while 2-{1-[4-(*p*-bis(2-chloroethyl)aminophenyl)butanoyloxy]-1-cyclohexymethyl}-DHAQ (R = cyclohexyl) **11** was inactive on the cells. Possibly the receptor affinity of the aromatic ring is important for the cytotoxic activity.

3.2. Antitumor activity

As demonstrated in *table I*, six of the 12 esters showed higher T/C values than 2-(1-hydroxyalkyl)-DHAQ derivatives. Moreover, two esters **1**, **12** were significantly more active than chlorambucil, suggesting that the chlorambucil and anthraquinone moieties of the ester functioned in a synergistic manner (*figure 1*). That is, in addition to the alkylating capability of chlorambucil component, the moiety of 2-(1-oxyalkyl)-DHAQ should contribute to the prolongation of the life span of the experimental mice through their own mechanisms. For enhancement of the antitumor acitivity other mechanisms such as oxidative stress, bioreductive alkylation

Table I. Antitumor effect of 2-{1-[4-(*p*-bis(2-chloroethyl)-aminophenyl)-butanoyloxy]alkyl}-1,4-dihydroxy-9,10-anthraquinones [a].



Number	R	R′ = Cb			Number	R′ = OH	
		ED_{50} (μg mL^{-1})	T/C (%)	SR		ED_{50} [b] (μg mL^{-1})	T/C [b] (%)
1	H	2.18	200	3/8	**1a**	15	125
2	Methyl	0.16	179	2/8	**2a**	1.9	139
3	Ethyl	1.22	174	2/8	**3a**	7.2	135
4	Propyl	1.05	191	3/8	**4a**	10.2	125
5	Butyl	2.85	172	2/8	**5a**	23.7	110
6	Pentyl	4.08	159	1/8	**6a**	58.0	108
7	Hexyl	5.93	158	0	**7a**	60	115
8	Heptyl	45	148	0	**8a**	>80	103
9	Octyl	60	134	0	**9a**	>80	99
10	Dodecyl	60	132	0	**10a**	>80	101
11	Cyclohexyl	>60	136	0	**11a**	>80	92
12	Phenyl	1.5	205	4/8	**12a**	5.6	138
VI	Chlorambucil	4.1	168	2/8			

[a] R; alkyl group, Cb; (*p*-bis(2-chloroethyl)aminophenyl)-butanoyloxy group of chlorambucil, SR; survival rate, the ratio of the number of the survived mice 50 days after sample treatment to eight mice tested.
[b] Data adopted from Ref. [3].

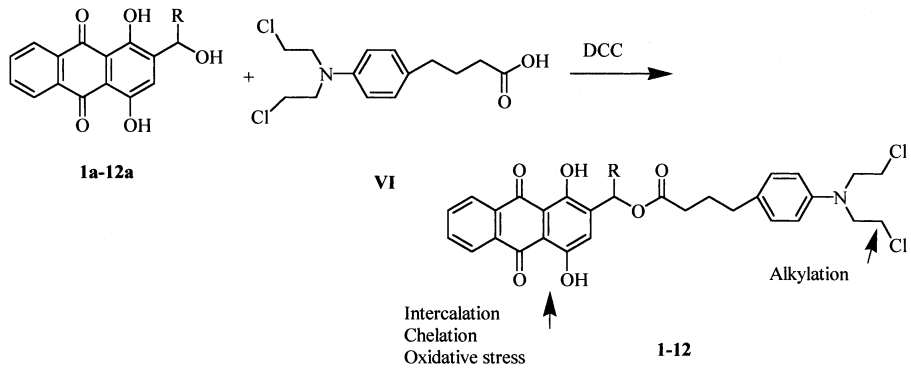


Figure 1. Synthesis of 2-{1-[4-(*p*-bis(2-chloroethyl)aminophenyl)butanoyloxy]alkyl}-1,4-dihydroxy-9,10-anthraquinones and their attacking points for bionucleophiles.

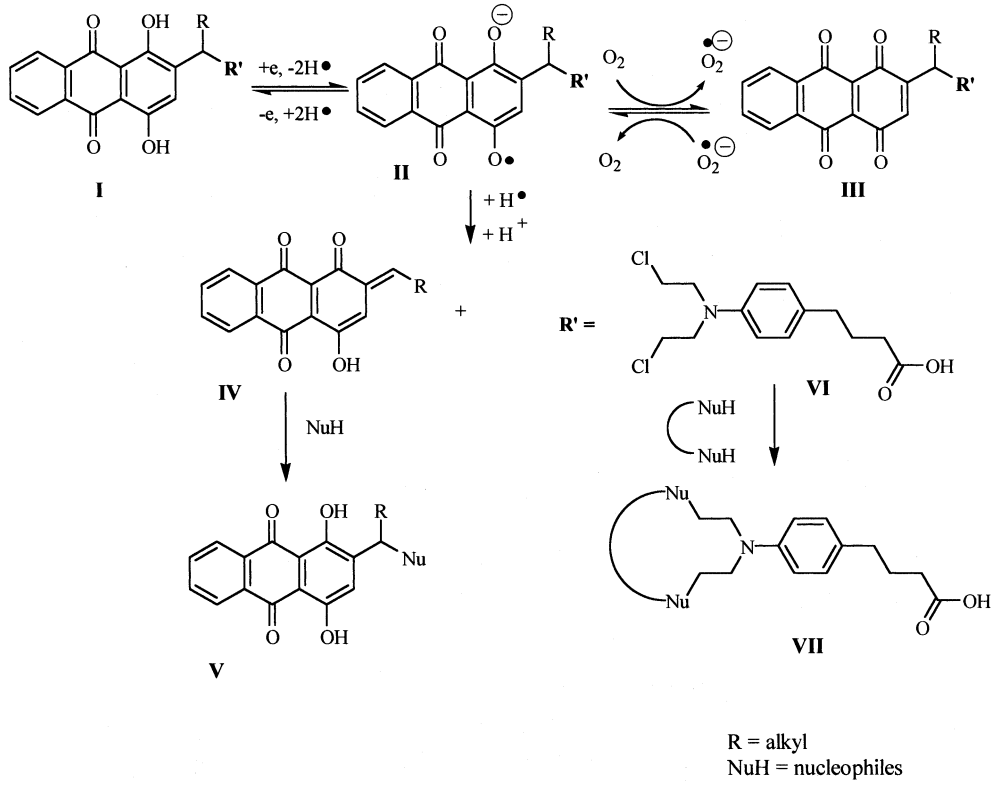


Figure 2. Proposed mechanism of bioreductive alkylation of 2-{1-[4-(*p*-bis(2-chloroethyl)amino-phenyl)butanoyloxy] alkyl}-1,4-dihydroxy-9,10-anthraquiones.

(*figure 2*) and DNA intercalation could also be expected from 1,4-dihydroxy-9,10-anthraquinone moiety.

T/C values were dependent upon the size and electronic state of the side chain. The esters with a shorter side chain showed higher T/C values than those with a longer side chain; T/C>150% for DHAQ having R from H to hexyl versus T/C<150% for DHAQ having R from heptyl to dodecyl. As mentioned in the cytotoxic activity, presence of phenyl group at R (T/C, 205%) also enhanced the antitumor activity to a great extent compared to cyclohexyl group (136%). Aromatic ring system in the side chain seemed to be important for the antitumor activity. DHAQ with R = phenyl, 2-{1-[4-(*p*-bis-(2-chloroethyl) aminophenyl)butanoyloxy]-1-phenyl-

methyl}-DHAQ **12**, showed the highest antitumor activity (T/C, 205%; SR, 4/8).

SR, the survival rate, means the ratio of the number of the survived mice for 50 days after sample treatment to eight experimental mice. For example, among eight mice treated with 2-{1-[4-(*p*-bis(2-chloroethyl)aminophenyl)butanoyloxy]-1-phenylmethyl}-DHAQ **12** at a dose of 9.5 μmol kg^{-1} day^{-1} for consecutive seven days, four mice, 50% of the experimental mice, lived longer than 50 days yielding SR of 4/8.

The bioreductive alkylation might contribute to the enhanced antitumor activity of the esters. According to the proposed mechanism of the bioreductive activation [7, 8], ester accepts an electron, then produces a anion radical (**II**) which transforms to a quinone methide, as an electrophilic intermediate (**IV**), and chlorambucil (**VI**). Both of them can alkylate bionucleophiles in DNA, enzymes, and other molecules (**V**, **VII**). Furthermore, the radical anion transforms molecular oxygen to superoxide, which triggers a oxidative stress in cells.

4. Experimental

4.1. Chemistry

Melting points were determined on an electrothermal melting point apparatus and were uncorrected. The IR spectra were recorded on a Jasco Report-100 FT IR spectrometer, and only the principal bands were described. The ^{1}H-NMR spectra were recorded on either JEOL EX-90 (90 MHz) or Bruker ARX (300 MHz) NMR spectrometer, and proton chemical shifts are relative to tetramethylsilane as an internal standard in $CDCl_3$ or in DMSO-d_6. All fractions from column chromatography (silica gel 60, 230–400 mesh, E. Merck) were monitored by thin layer chromatography (silica gel 60 GF-254, E. Merck). All reagents, commercially available, were used without further purification unless otherwise stated.

4.1.1. General procedure for the synthesis of 2-{1-[4-(p-bis(2-chloroethyl)aminophenyl)-butanoyloxy]-alkyl}-1,4-dihydroxy-9,10-anthraquinones

To a stirred solution of 2-hydroxyalkyl-1,4-dihydroxy-9,10-anthraquinine (0.55 mmol) in dichloromethane was added DCC (0.605 mmol) and DMAP (0.275 mmol) at 0–5°C, followed by the addition of chlorambucil (0.55 mmol). The solution was stirred for 4 h at 0–5°C and for 4 h at room temperature (r.t.). After this 20 mL hexane was added and stirred for 5 min, the solution was left aside for 30 min and then filtered. The filterate was dried with Na_2SO_4, concentrated, and eluted through silica gel column using hexane/ethyl acetate to give the products. Spectral data of esters **1**–**12** are given below.

*4.1.1.1. 2-{1-[4-[p-Bis(2-chloroethyl)aminophenyl]-butanoyloxy]methyl}-1,4-dihydroxy-9,10-anthraquinone **(1)***

Yield: 63.6%, m.p. 113.8–114.1°C, ^{1}H-NMR ($CDCl_3$): $\delta = 13.14$ (s, 1H), 12.79 (s, 1H), 8.33–8.23 (m, 2H), 7.85–7.75 (m, 2H), 7.27 (s, 1H), 7.09 (d, 2H, $J = 8.64$ Hz), 6.64 (d, 2H, $J = 8.64$ Hz), 5.24 (s, 2H), 3.66 (s, bro, 8H), 2.62–2.39 (m, 4H), 2.05–1.99 (m, 2H), IR (KBr, cm^{-1}) ν_{max}: 3440, 1738, 1622, 1582, 1510.

*4.1.1.2. 2-{1-[4-(p-Bis(2-chloroethyl)aminophenyl)-butanoyloxy]ethyl}-1,4-dihydroxy-9,10-anthraquinone **(2)***

Yield: 61.5%, amorphous powder, ^{1}H-NMR ($CDCl_3$): $\delta = 13.29$ (s, 1H), 12.86 (s, 1H), 8.31–8.21 (m, 2H), 7.85–7.75 (m, 2H), 7.35 (s, 1H), 7.10 (d, 2H, $J = 8.19$ Hz), 6.64 (d, 2H, $J = 8.64$ Hz), 6.29 (m, 1H), 3.68 (s, bro, 8H), 2.61–2.38 (m, 4H), 2.05–1.97 (m, 2H), 1.58 (d, 3H, $J = 6.39$ Hz), IR (KBr, cm^{-1}) ν_{max}: 3350, 2920, 2850, 1732, 1622, 1582, 1515.

*4.1.1.3. 2-{1-[4-(p-Bis(2-chloroethyl)aminophenyl)-butanoyloxy]propyl}-1,4-dihydroxy-9,10-anthraquinone **(3)***

Yield: 58.2%, amorphous powder, ^{1}H-NMR ($CDCl_3$): $\delta = 13.31$ (s, 1H), 12.86 (s, 1H), 8.34–8.24 (m, 2H), 7.85–7.75 (m, 2H), 7.30 (s, 1H), 7.08 (d, 2H, $J = 8.64$ Hz), 6.64 (d, 2H, $J = 8.64$ Hz), 6.16 (m, 1H), 3.68(s, bro, 8H), 2.70–2.39 (m, 4H), 2.13–1.89 (m, 4H), 1.00 (m, 3H), IR (KBr, cm^{-1}) ν_{max}: 3310, 1734, 1618, 1582, 1516.

*4.1.1.4. 2-{1-[4-(p-Bis(2-chloroethyl)aminophenyl)-butanoyloxy]butyl}-1,4-dihydroxy-9,10-anthraquinone **(4)***

Yield: 56.4%, amorphous powder, ^{1}H-NMR ($CDCl_3$): $\delta = 13.34$ (s, 1H), 12.89 (s, 1H), 8.39–8.29 (m, 2H), 7.87–7.77 (m, 2H), 7.29 (s, 1H), 7.07 (d, 2H, $J = 8.64$ Hz), 6.63 (d, 2H, $J = 8.64$ Hz), 6.21 (m, 1H), 3.66 (s, bro, 8H), 2.59–2.35 (m, 4H), 1.99–1.73 (m, 4H), 1.26 (m, 2H), 1.03 (m, 3H), IR (KBr, cm^{-1}) ν_{max}: 3400, 2922, 1722, 1618, 1582, 1512.

4.1.1.5. 2-{1-[4-(p-Bis(2-chloroethyl)aminophenyl)-butanoyloxy]pentyl}-1,4-dihydroxy-9,10-anthraquinone ***(5)***

Yield: 56.1%, m.p. 110.8–111.7°C, ^{1}H-NMR ($CDCl_3$): $\delta = 13.34$ (s, 1H), 12.90 (s, 1H), 8.41–8.31 (m, 2H), 7.88–7.78 (m, 2H), 7.29 (s, 1H), 7.07 (d, 2H, $J = 8.10$ Hz), 6.63 (d, 2H, $J = 9.1$ Hz), 6.21 (m, 1H), 3.66 (s, bro, 8H), 2.59–2.35 (m, 4H) 2.16–1.87 (m, 4H), 1.23 (m, 4H), 0.97 (m, 3H), IR (KBr, cm^{-1}) ν_{max}: 3420, 2952, 2925, 1725, 1618, 1582, 1516.

4.1.1.6. 2-{1-[4-(p-Bis(2-chloroethyl)aminophenyl)-butanoyloxy]hexyl}-1,4-dihydroxy-9,10-anthraquinone ***(6)***

Yield: 51.0%, m.p. 197.9–198.9°C, ^{1}H-NMR ($CDCl_3$): $\delta = 13.32$ (s, 1H), 12.88 (s, 1H), 8.36–8.26 (m, 2H), 7.85–7.75 (m, 2H), 7.29 (s, 1H), 7.06 (d, 2H, $J = 8.82$ Hz), 6.63 (d, 2H, $J = 8.82$ Hz), 6.19 (m, 1H), 3.66 (s, bro, 8H), 2.66–2.35 (m, 4H), 1.90–1.75 (m, 4H), 1.26 (m, 6H),1.03 (m, 3H), IR (KBr, cm^{-1}) ν_{max}: 3400, 2920, 1732, 1618, 1582, 1515.

4.1.1.7. 2-{1-[4-(p-Bis(2-chloroethyl)aminophenyl)-butanoyloxy]heptyl}-1,4-dihydroxy-9,10-anthraquinone ***(7)***

Yield: 49.1%, m.p. 176.1–176.9°C, ^{1}H-NMR ($CDCl_3$): $\delta = 13.30$ (s, 1H), 12.86 (s, 1H), 8.33–8.23 (m, 2H), 7.84–7.74 (m, 2H), 7.29 (s, 1H), 7.08 (d, 2H, $J = 8.91$ Hz), 6.63 (d, 2H, $J = 8.82$ Hz), 6.20 (m, 1H), 3.67 (s, bro, 8H), 2.61–2.38 (m, 4H), 2.03–1.85 (m, 4H), 1.32 (s, bro, 8H), 0.88 (m, 3H), IR (KBr, cm^{-1}) ν_{max}: 3420, 2920, 1732, 1618, 1582, 1515.

4.1.1.8. 2-{1-[4-(p-Bis(2-chloroethyl)aminophenyl)-butanoyloxy]octyl}-1,4-dihydroxy-9,10-anthraquinone ***(8)***

Yield: 48.2%, m.p. 118.5–120.1°C, ^{1}H-NMR ($CDCl_3$): $\delta = 13.31$ (s, 1H), 12.86 (s, 1H), 8.33–8.23 (m, 2H), 7.89–7.79 (m, 2H), 7.29 (s, 1H), 7.08 (d, 2H, $J = 8.37$ Hz), 6.63 (d, 2H, $J = 8.37$ Hz), 6.20 (m, 1H), 3.42 (s, bro, 8H), 2.69–2.32 (m, 4H), 2.11–1.82 (m, 4H), 1.34–1.18 (m, 10H), 0.91 (m, 3H), IR (KBr, cm^{-1}) ν_{max}: 3400, 2950, 2920, 1720, 1618, 1580, 1515.

4.1.1.9. 2-{1-[4-(p-Bis(2-chloroethyl)aminophenyl)-butanoyloxy]nonyl}-1,4-dihydroxy-9,10-anthraquinone ***(9)***

Yield: 47.3%, amorphous powder, ^{1}H-NMR ($CDCl_3$): $\delta = 13.32$ (s, 1H), 12.88 (s, 1H), 8.37–8.27 (m, 2H), 8.86–8.76 (m, 2H), 7.28 (s, 1H), 7.07 (d, 2H, $J = 8.55$ Hz), 6.62 (d, 2H, $J = 8.82$ Hz), 6.12 (m, 1H), 3.63 (s, bro, 8H), 2.67–2.35 (m, 4H), 2.01–1.86 (m, 4H), 1.24 (s, bro, 12H), 0.87 (m, 3H), IR (KBr, cm^{-1}) ν_{max}: 3425, 2950, 2908, 1732, 1620, 1580, 1514.

4.1.1.10. 2-{1-[4-(p-Bis(2-chloroethyl)aminophenyl)-butanoyloxy]tridecyl}-1,4-dihydroxy-9,10-anthraquinone ***(10)***

Yield: 34.5%, m.p. 77.6–78.5°C, ^{1}H-NMR ($CDCl_3$): $\delta = 13.32$ (s, 1H), 12.88 (s, 1H), 8.37–8.27 (m, 2H), 8.86–8.76 (m, 2H), 7.28 (s, 1H), 7.18 (d, 2H, $J = 8.55$ Hz), 6.62 (d, 2H, $J = 8.82$ Hz), 6.18 (m, 1H), 3.64 (s, bro, 8H), 2.67–2.35 (m, 4H), 2.01–1.86 (m, 4H), 1.24 (s, bro, 18H), 0.87 (m, 3H), IR (KBr, cm^{-1}) ν_{max}: 3420, 2908, 1732, 1618, 1580, 1515.

4.1.1.11. 2-{1-[4-(p-Bis(2-chloroethyl)aminophenyl)-butanoyloxy]-1-cyclohexymethyl}

×-1,4-dihydroxy-9,10-anthraquinone ***(11)***

Yield: 58.2%, amorphous powder, ^{1}H-NMR ($CDCl_3$): $\delta = 13.39$ (s, 1H), 12.89 (s, 1H), 8.39–8.29 (m, 2H), 7.86–7.76 (m, 2H), 7.25 (s, 1H), 7.06 (d, 2H, $J = 8.64$ Hz), 6.62 (d, 2H, $J = 8.64$ Hz), 6.04 (d, 1H, $J = 5.76$ Hz), 3.66 (s, bro, 8H), 2.66–2.33 (m, 4H), 1.99–1.92 (m, 2H), 1.82–1.64 (m, 6H), 1.22 (m, 5H), IR (KBr, cm^{-1}) ν_{max}: 3410, 2950, 2920, 1720, 1616, 1580, 1515.

4.1.1.12. 2-{1-[4-(p-Bis(2-chloroethyl)aminophenyl)-butanoyloxy]-1-phenymethyl}-1,4-dihydroxy-9,10-anthraquinone ***(12)***

Yield: 60%, amorphous powder, ^{1}H-NMR ($CDCl_3$): $\delta = 13.30$ (s, 1H), 12.91 (s, 1H), 8.39–8.29 (m, 2H), 7.87–7.77 (m, 2H), 7.50–7.24 (m, 6H), 7.04 (d, 2H, $J = 9.1$ Hz), 7.00 (s, 1H), 6.61 (d, 2H, $J = 8.82$ Hz), 3.66 (s, bro, 8H), 2.52 (m, 4H), 1.98 (m, 2H), IR (KBr, cm^{-1}) ν_{max}: 3410, 3050, 2920, 1726, 1620, 1580, 1515.

4.2. Pharmacology

4.2.1. Measurement of the cytotoxicity against L1210 tumor cells

Cytotoxicity of compounds against L1210 cell lines was measured as described previously [9]. Fisher's medium supplemented with 10% horse serum was used for the proliferation of L1210 cells. One day before the test, a cell suspension ($2–3 \times 10^5$ cells mL^{-1}) in a logarithmic phase (viability, >95%) was prepared and incubated at 37°C in an atmosphere of 5% CO_2. For the test, the cell suspension was adjusted to 5×10^4 cells mL^{-1}. A sample (0.1 mg mL^{-1} in dimethylsulfoxide) was diluted by ten folds with fresh medium. About 15, 30, 60 μL of

the diluted sample was put in cell suspension (3 mL) and five wells were used for each concentration of the test sample. After 48 h incubation, viability was determined using a hemocytometer. ED_{50} value ($\mu g\,mL^{-1}$), calculated by an available computerized program, was defined as the concentration of drug to produce a 50% reduction in the viability relative to the control.

4.2.2. Antitumor activity in ICR mice bearing sarcoma 180 cells

Sarcoma 180 cells suspended in saline (1×10^7 cells mL^{-1}) were inoculated intraperitoneally to male ICR mice (injection volume; 0.2 mL per mouse) [10] 24 h after the transplantation, mice were divided so that each group contains eight mice. The sample, dissolved in a predetermined amount of 50% PEG200 and stored at 4°C, was administered into the intraperitoneal cavity of the mouse consecutively for seven days. The mice were kept for 51 days. The survival rate (T/C,%) was calculated by following equation:

$$T/C(\%) = \frac{\text{Average Survival Period in the Test Group}}{\text{Average Survival Period in the Control Group}} \times 100$$

The survival ratio (SR), the ratio of the number of the survived mice 50 days after sample treatment to eight mice tested, is expressed as an important parameter for antitumor activity.

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