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Development of lipophilic prodrugs of mitomycin C. I. Synthesis and antitumor activity of 1a-N-substituted derivatives with aromatic pro-moiety

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Summary

Five lipophilic 1a-N-substituted derivatives of mitomycin C possessing aromatic pro-moieties including benzyl, benzoyl, benzylcarbonyl, benzyloxycarbonyl, and benzoyloxymethyl groups were synthesized, and their physicochemical and biological characteristics were examined. All compounds showed increased octanol/water partition coefficients, lipophilic indexes (k') in HPLC system, and lipid solubilities. Their antimicrobial activities against *Escherichia coli B* were markedly less than that of mitomycin C except for benzoyloxymethyl mitomycin C. All of the compounds showed significant activity in vivo against P388 or L1210 leukemia system, and especially benzyloxycarbonyl and benzoyloxymethyl mitomycin C showed equal or superior activity to the parent drug at approximately the same low dose area. These results suggested that most of the compounds exhibit their cytotoxicity against tumor cells after being regenerated to their parent drug in the body.

Introduction

In cancer chemotherapy, it is necessary to control pharmacokinetic behavior of the cytotoxic drug for effective treatment and many attempts have been made to

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deliver the drug into the tumor site by means of drug delivery systems (Gregoriadis, 1977; Juliano, 1980).

For the past decade we have been engaged in studies on cancer drug delivery systems from viewpoints of utilization of physical devices such as various types of emulsion (Hashida et al., 1977a and b, 1979; Sezaki et al., 1982; Yoshioka et al., 1982) and gelatin spherical carriers (Yoshioka et al., 1981), or chemical transformation of drug molecules to prodrugs. In a chemical approach, an improvement of the drug effectiveness can result from alteration of its biopharmaceutical characteristics by introducing various kinds of carrier moieties using a covalent, but reversible bond (Stella and Higuchi, 1975; Sinkula and Yalkowsky, 1975; Roche, 1977). On the basis of this consideration, we have developed various types of derivatives of antitumor agents in which the parent drug was led to immobile depot forms by attaching it to agarose beads (Hashida et al., 1977c and 1978; Kojima et al., 1978) or to a high molecular weight compound by linking it to a polysaccharide or a polypeptide (Kojima et al., 1980; Hashida et al., 1981; Kato et al., 1982).

Among various physicochemical properties, lipophilicity or lipid solubility as well as molecular size and the extent of ionization can be considered as one of the most important factors controlling the biological behavior of a drug. Furthermore, an improvement of lipophilicity of the drug would expand its possibility to be combined into useful delivery systems such as liposomes and emulsions. In the present investigation therefore, antitumor antibiotic mitomycin C (I) was derivatized to form more lipophilic forms by substituting the 1a-N-position with aromatic carrier moieties such as benzyl(II), benzoyl(III), benzylcarbonyl(IV), benzyloxycarbonyl(V), and benzoyloxymethyl(VI) groups, and their fundamental physicochemical and biological characteristics are compared.

Materials and Methods

General procedures

Melting points were determined in capillary tubes using a Yanagimoto (serial no. 17) micro-melting point apparatus and uncorrected. UV absorption spectra were recorded on a Hitachi Model 220 UV-VIS spectrophotometer. NMR spectra were made on a JEOL FX-200 spectrometer and FD mass spectra data were obtained on a JEOL JMS-01SG-2 mass spectrometer. Elemental analyses were performed by the Center for Organic Elemental Microanalysis, Kyoto University. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within $\pm 0.4\%$ of the theoretical values. TLC was carried out on TLC aluminium sheets pre-coated with a 0.2 mm layer of silica gel 60 F_{254} (E. Merck), using the following solvent system: (A) ethyl acetate–isopropanol (1 : 2); and (B) *n*-propanol–petroleum ether–10% NH_4OH (5 : 4 : 1).

Chemicals

Mitomycin C (I) was supplied by Kyowa Hakko Kogyo and used without further purification; m.p. above 270°C; TLC R_f(A) 0.30, R_f(B) 0.33; NMR(pyridine-d_5) δ:

5.42 (1H, d-d, J = 4.0, 11.0 Hz, 10'-H), 5.11 (1H, d-d, J = 11.0, 11.0 Hz, 10-H), 4.54 (1H, d, J = 12.5 Hz, 3-H), 4.02 (1H, d-d, J = 4.0, 11.0 Hz, 9-H), 3.60 (1H, d, J = 12.5 Hz, 3'-H), 3.21 (3H, s, OMe), 3.13 (1H, m, 1-H), 2.73 (1H, m, 2-H), 2.01 (3H, s, CH_3). All other reagents were of reagent grade quality and obtained commercially (Nakarai Chemicals).

Synthesis

Benzyl mitomycin C (II). To a solution of 50 mg of I in 2 ml of anhydrous DMSO, 150 mg of benzylchloride and 200 mg of K_2CO_3 were added and stirred at room temperature for 5 days shielding from the light. The reaction mixture was chromatographed on a silica gel column, eluting with a mixture of acetone–chloroform. The first purple fraction was recrystallized from chloroform–isopropyl ether to give II (23.5 mg, 37%) as a brown solid: m.p. 119–121°C; TLC R_f(A) 0.60, R_f(B) 0.61; NMR(pyridine-d_5) δ: 7.50–7.00 (5H, m, aromatic protons), 5.42 (1H, d-d, J = 4.0, 11.0 Hz, 10'-H), 4.87 (1H, d-d, J = 11.0, 11.0 Hz, 10-H), 4.49 (1H, d, J = 13.0 Hz, 3-H), 4.32 (1H, d, J = 13.0 Hz, N–CH_2–), 4.04 (1H, d-d, J = 4.0, 11.0 Hz, 9-H), 3.61 (1H, d-d, J = 2.0, 13.0 Hz, 3'-H), 3.22 (3H, s, OMe), 2.89 (1H, d, J = 13.0 Hz, N–CH_2–), 2.89 (1H, d, J = 5.0 Hz, 1-H), 2.44 (1H, d-d, J = 2.0, 5.0 Hz, 2-H), 1.98 (3H, s, CH_3).

Benzoyl mitomycin C (III) (Method A). To a solution of 50 mg of I in 14 ml of anhydrous THF, 21 mg of benzoyl chloride and 15 mg of triethylamine were added and stirred for 0.5 h. The reaction mixture was chromatographed and recrystallized from ethanol or chloroform–isopropyl ether to yield 53.8 mg (82%) of III, as purple crystals: m.p. above 270°C; TLC R_f(A) 0.58, R_f(B) 0.60; NMR(pyridine-d_5) δ: 8.20–7.20 (5H, m, aromatic protons), 5.70 (1H, d-d, J = 4.5, 11.0 Hz, 10'-H), 5.19 (1H, d, J = 13.5 Hz, 3-H), 4.75 (1H, d-d, J = 11.0, 11.0 Hz, 10-H), 4.20 (1H, d-d, J = 4.5, 11.0 Hz, 9-H), 4.16 (1H, d, J = 4.5 Hz, 1-H), 3.74 (1H, d, J = 13.5 Hz, 3'-H), 3.58 (1H, d, J = 4.5 Hz, 2-H), 3.27 (3H, s, OMe), 2.05 (3H, s, CH_3).

(Method B). To a solution of 50 mg of I in 50 ml water, 183 mg of benzoic acid and 860 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride were added and stirred overnight, maintaining at pH 5.5. The precipitate produced was recrystallized from ethanol to give 47 mg (71%) of III as purple crystals, which was identical in m.p., UV spectra, and TLC with the sample obtained by the method A.

Benzylcarbonyl mitomycin C (IV). IV was synthesized in accordance with the method A of III to give purple crystals in 83% yield; m.p. 154–156°C; TLC R_f(A) 0.61, R_f(B) 0.63; NMR(pyridine-d_5) δ: 7.40–7.20 (5H, m, aromatic protons), 5.69 (1H, d-d, J = 4.0, 11.0 Hz, 10'-H), 4.70 (1H, d-d, J = 11.0, 11.0 Hz, 10-H), 4.63 (1H, d, J = 13.0 Hz, 3-H), 4.12 (1H, d-d, J = 4.0, 11.0 Hz, 9-H), 3.93 (2H, s, –CO–CH_2–), 3.92 (1H, d, J = 4.5 Hz, 1-H), 3.54 (1H, d, J = 13.0 Hz, 3'-H), 3.50 (1H, d, J = 4.5 Hz, 2-H), 3.20 (3H, s, OMe), 2.09 (3H, s, CH_3).

Benzyloxycarbonyl mitomycin C (V). V was synthesized in accordance with the method A of III to give a brown solid in 62% yield; m.p. 102–104°C; TLC R_f(A) 0.62, R_f(B) 0.64; NMR(pyridine-d_5) δ: 7.60–7.20 (5H, m, aromatic protons), 5.69 (1H, d-d, J = 4.5, 11.0 Hz, 10'-H), 5.26 (2H, s, –COO–CH_2–), 4.89 (1H, d-d, J = 11.0, 11.0 Hz, 10-H), 4.72 (1H, d, J = 13.0 Hz, 3-H), 4.09 (1H, d-d, J = 4.5, 11.0

Hz, 9-H), 3.85 (1H, d, J = 4.5 Hz, 1-H), 3.56 (1H, d, J = 13.0 Hz, 3'-H), 3.53 (1H, d, J = 4.5 Hz, 2-H), 3.19 (3H, s, OMe), 2.03 (3H, s, CH_3).

Benzoyloxymethyl mitomycin C (VI). The synthesis of benzoyloxymethylchloride was carried out by the method of Ulich and Adams (1921) and the structure of the product was ascertained by NMR.

To a solution of 200 mg of I in 14 ml of anhydrous THF, 1020 mg of benzoyloxymethylchloride and 604 mg of triethylamine were added and stirred at room temperature for 3 months shielding from the light. The reaction mixture was chromatographed on a silica gel column using acetone–chloroform as an elution solvent. Two purple fractions were eluted and the first fraction was evaporated and recrystallized from chloroform–isopropyl ether to give 8.4 mg (3%) of VI as brown solid. The second fraction was identified as III by m.p. and TLC. The structure of VI was confirmed only by NMR and FD mass spectra because of its poor yield: m.p. 112–116°C; TLC R_f(A) 0.60; NMR(pyridine-d_5) δ: 8.10–7.30 (5H, m, aromatic protons), 5.49 (1H, d-d, J = 5.0, 12.0 Hz, 10'-H), 5.35 (1H, d, J = 10.0 Hz, N–CH$_2$–), 5.28 (1H, d-d, J = 12.0, 12.0 Hz, 10-H), 4.83 (1H, d, J = 10.0 Hz, N–CH$_2$–), 4.58 (1H, d, J = 13.5 Hz, 3-H), 4.08 (1H, d-d, J = 5.0, 12.0 Hz, 9-H), 3.65 (1H, d-d, J = 2.0, 13.5 Hz, 3'-H), 3.32 (1H, d, J = 5.0 Hz, 1-H), 3.20 (3H, s, OMe), 2.95 (1H, d-d, J = 2.0, 5.0 Hz, 2-H), 2.03 (3H, s, CH_3).

Lipophilicity and lipid solubility studies

Partition coefficients of compounds I–VI were determined in an *n*-octanol–distilled water system at 25°C according to the method of Kakemi et al. (1967). The lipophilic indexes (log k') were determined by high-performance liquid chromatography (HPLC) employing the following equation (Yamana et al., 1977):

$$\log k' = \log[(t_r - t_0)/t_0]$$

where t_r is the retention time and t_0 is the elution time of a solvent. An HPLC system (TRIROTAR, Jasco) equipped with a variable wavelength UV detector (UVIDEC 100-II, Jasco) was used in a reverse-phase mode with a stationary phase of silica gel bonded chemically with octadecyl chains prepacked into a 15 cm stainless steel column (Cosmosil 5C_{18} packed column; Nakarai Chemicals). A mixture of methanol and distilled water was used as the mobile phase at a flow rate of 1.0 ml/min. The solubilities in distilled water, sesame oil, isopropyl myristate and *n*-hexane were determined by suspending an excess amount of the compound in the solvent, followed by filtration and analysis.

Antimicrobial activity studies

Antimicrobial activities were determined by an ordinary paper disc method using *Escherichia coli B* as a test organism. The test compounds except for III were dissolved in saline and subjected to analysis. Saline containing DMSO (40%) was used as a medium of III because of its low aqueous solubility. The antimicrobial activity was determined by measuring a diameter of growth-inhibitory zone after 24 h incubation at 37°C.

Antitumor activity studies

P388 and L1210 leukemia were maintained by weekly transplantation of tumor cells into the peritoneal cavity of male DBA/2 mice. Animals used for test were male hybrid BDF_1 mice (C57Bl/6 × DBA/2). Six mice for each group weighing 20–25 g were inoculated intraperitoneally with a suspension of 1×10^6 P388 or 1×10^5 L1210 leukemia cells and the chemotherapy was given intraperitoneally at 24 h after inoculation. All drugs used were administered as a saline solution containing 40%(v/v) DMSO. Activities were calculated as T/C%, the ratio of the mean survival time of the treated group (T) divided by that of the control group (C). The observation period of survival time was 60 days.

Results

Chemistry

The prodrugs studied in the present paper were synthesized by ordinary acylation or alkylation methods (Montgomery and Temple, 1961; Matsui et al., 1968; Kinoshita et al., 1971). The structures, formulae, and physical data are summarized in Table 1. The benzene ring selected as a pro-moiety having high lipophilicity was linked to the 1a-N-position of the mitomycin C moiety through various linkage forms.

Each structure is supported by NMR, mass spectra, UV spectra and elemental analyses. The parent ion peak of each compound determined by FD mass spectrum agreed with its molecular weight. All compounds exhibited the UV maxima approximately at 360 nm in ethanol and pH 7.4 phosphate buffer due to the mitosane structure including aziridine ring.

Lipophilicity

Partition coefficients(P_{oct}), lipophilic indexes ($\log k'_0$), and solubilities in various solvents of synthesized compounds are summarized in Table 2.

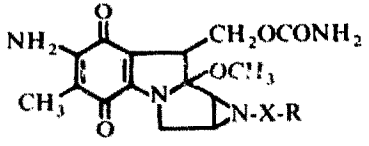
For all the tested compounds, plots of log k′ versus methanol concentration(v/v%) showed a reasonable linear relationship (Fig. 1). The $\log k'_0$ values extrapolated to 0% methanol concentration are listed in Table 2. The relationship between $\log k'_0$ and $\log P_{oct}$ is expressed by the following equation for the present and additional 3 alkoxycarbonyl derivatives of I (unpublished data).

$$\log P_{oct} = 1.074 \times \log k'_0 - 1.935 \quad n = 7,\ r = 0.9 \tag{1}$$

where n and r are number of experiment and correlation coefficient, respectively. The partition coefficients of III and VI were estimated from this equation which could not be determined experimentally because of low aqueous solubility (III) or instability in an aqueous medium (VI). As is obvious from these results, all derivatives showed relatively equally-increased lipophilicity comparing with I and $\log P_{oct}$ values, which varied between 1.14 and 1.35. They also exhibited increased lipid solubility.

TABLE 1

STRUCTURES AND PROPERTIES OF 1a-N-SUBSTITUTED MITOMYCIN C DERIVATIVES



Compound	X	R	Yield (%)	m.p. (°C)	Formula	[M⁺][a]	UV_{max} (nm) (ε × 10^{-3})	
							EtOH	pH7.4 buffer
I Mitomycin C	–	–H	–	> 270	$C_{15}H_{18}N_4O_5$	334	358(22.6)	364(22.9)
II Benzyl mitomycin C	$-CH_2-$		37	119–121	$C_{22}H_{24}N_4O_5$	424	360(21.3)	365(21.0)
III Benzoyl mitomycin C	–CO–		82	> 270	$C_{22}H_{22}N_4O_6$	438	356(23.6)	–
IV Benzylcarbonyl mitomycin C	$-COCH_2-$		83	154–156	$C_{23}H_{24}N_4O_6$	452	356(20.8)	363(23.8)
V Benzyloxycarbonyl mitomycin C	$-COOCH_2-$		60	102–104	$C_{23}H_{24}N_4O_7$	468	356(20.1)	360(20.0)
VI Benzoyloxymethyl mitomycin C	$-CH_2OCO-$		3	112–116	$C_{23}H_{24}N_4O_7$	468	359(21.0)	–

[a] Parent ion peaks determined by FD mass spectrum.

TABLE 2

LIPOPHILICITY AND SOLUBILITY OF 1a-N-SUBSTITUTED MITOMYCIN C DERIVATIVES

Compound	P_{oct}	log P_{oct}	log k'_0	Solubility (mM)			
				Water (25°C)	Sesame oil (25°C)	I.P.M. (37°C)	Hexane ($\times 10^3$) (25°C)
I	0.4	−0.398	1.93	2.730	0.004	0.013	0.005
II	21.0	1.322	2.83	0.972	4.165	3.740	1.390
III	(21.6)	(1.334)	3.04	0.007	0.0484	0.007	0.024
IV	14.0	1.146	2.52	1.590	1.351	0.406	0.125
V	22.0	1.342	3.53	0.489	3.291	2.610	0.415
VI	(18.4)	(1.264)	2.98	–	–	–	0.425

The value parenthesized was calculated from Eqn. 1.

Antimicrobial activity

The antimicrobial activities of the prodrugs against *Escherichia coli B* are shown in Fig. 2. Each activity is expressed as a corresponding concentration of I (ordinate) which shows an equal growth inhibition activity to that of a test compound. VI showed almost the same activity as I. On the other hand, II–V exhibited remarkably less antimicrobial activities than I. The activity of IV, the most active derivative except for VI, was approximately one-tenth and those of II, III and V were only one-hundredth of I. These observations were in good agreement with the results obtained by the standard agar-dilution method.

Fig. 1.

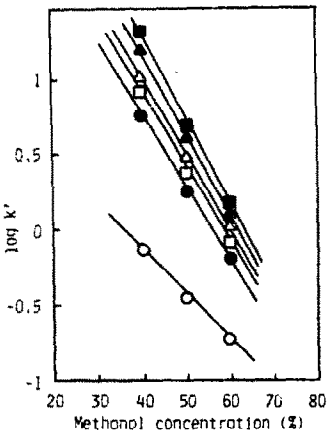


Fig. 2.

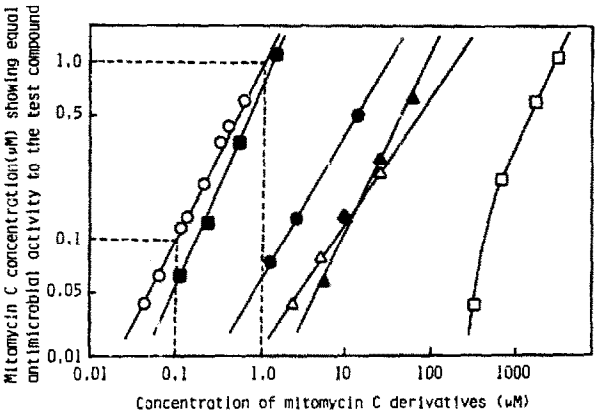


Fig. 1. Relationship between log k′ values of 1a-N-substituted mitomycin C derivatives and methanol concentration (v/v%) in the mobile phase. ○, I; △, II; □, III; ●, IV; ▲, V; ■, VI.

Fig. 2. Comparison of antimicrobial activities of 1a-N-substituted mitomycin C derivatives ○, I; △, II; □, III; ●, IV; ▲, V; ■, VI.

Fig. 3.

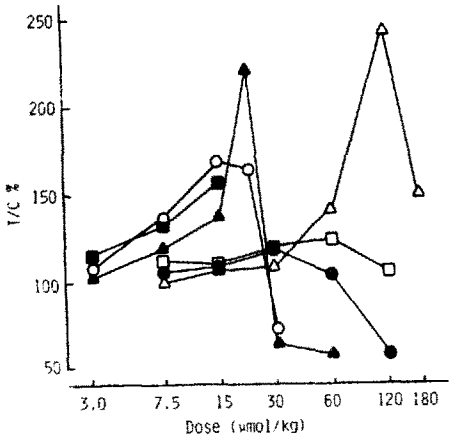


Fig. 4.

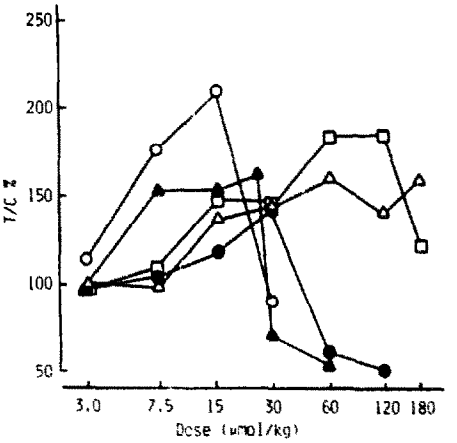


Fig. 3. Effect of 1a-N-substituted mitomycin C derivatives on survival time of mice bearing L1210 leukemia. ○, I; △, II; □, III; ●, IV; ▲, V; ■, VI.

Fig. 4. Effect of 1a-N-substituted mitomycin C derivatives on survival time of mice bearing P388 leukemia. ○, I; △, II; □, III; ●, IV; ▲, V.

Antitumor activity

The effects of prodrugs on the survival time of mice having intraperitoneal inoculation of L1210 and P388 leukemia are summarized in Figs. 3 and 4. The average survival time of the untreated control groups were 8.7 days for L1210 leukemia and 10.7 days for P388 leukemia, respectively. I exhibited maximum activity on both tumors at a dose of 15 μmol (5 mg)/kg and afforded T/C% values of 169% (L1210) and 209% (P388). Over this dose, I exhibited a marked toxicity. Treatment with derivatives II–VI demonstrated significant increase of the survival time of mice bearing L1210 and P388 and the dose–response relationship of each compound was relatively similar between both tumor types. II gave a T/C% value of 180% at a dose of 120 μmol/kg against L1210 leukemia and 1 of 6 mice was still surviving at 60 days after inoculation. The dose–response curve of V was relatively similar to that of I and maximum T/C% values are recorded at the dose of 22.5 μmol/kg.

Discussion

Mitomycin C (I) is an antitumor antibiotic that has demonstrated activity against a number of human neoplasms including chronic myelogenous leukemia and solid tumors of various organs (Carter and Crooke, 1979). In clinical practice, however, the toxicity problems such as delayed cumulative myelosuppression and gastrointestinal damage have impeded its utilization. Numerous analogues of I have been prepared in the hope of obtaining compounds with improved therapeutic properties (Matsui et al., 1968; Kinoshita et al., 1971; Kojima et al., 1972) and their structure–activity relationships have been studied (Driscoll et al., 1974; Remers et

al., 1974). But none of these analogues has emerged as a clinical agent, although the 7-N-phenyl analogue of I has received intensive study recently in Japan (Imai et al., 1980).

One promising aspect for improving a distribution of I to tumor sites appears to be prodrug approach by which pharmacokinetic patterns and, ultimately, therapeutic success can be altered through the introduction of the pro-moieties with ideal physicochemical properties. In the present investigation, I was derivatized to the lipophilic form by introducing a model lipophilic functional group, benzene ring, through various kinds of linkage structures.

As shown in Table 1, 5 kinds of derivatives were synthesized; two (II and VI) by alkylation and 3 (III, IV and V) by acylation of 1a-position of I. The acylation reaction proceeded easily but the alkylation reaction needed a fairly long period. V and VI possess an ester bond in their linkage structures. Although numerous acylation products have been reported (Kinoshita et al., 1971), no detailed examination about biopharmaceutical characteristics of the present compounds have been carried out.

All of the compounds showed fairly higher lipophilicity than I and their partition coefficients were 12–50 times as much as that of I. Their melting points were considerably lower than I, except for III, and their aqueous solubilities did not decrease as much as the lipid solubility increased. III showed a high melting point and slight solubilities in both aqueous and organic media. It is noteworthy that an addition of one methylene group between the amide bond and the aromatic ring markedly alters its physicochemical properties as shown in III and IV.

I has been shown to cross-link double helical DNA after reduction to the corresponding hydroquinones (Iyer and Szybalsky, 1963). This process, known as bioreductive alkylation(Lin et al., 1973), is thought to be the main lethal event to tumor cells, although the generation of hydrogen peroxide by successive redox cycles of the DNA-bound mitomycin is also considered important. Position 1a and 10 appear to be the alkylating sites of I, with their alkylating ability enhanced when methanol is eliminated from produced hydroquinone to give the indolohydroquinone. Consequently it is considered that the substitution of 1a position leads to the diminution of biological activity and this was confirmed from the present results shown in Fig. 2. All of the compounds except for VI showed markedly reduced antimicrobial activities which can be considered to be parallel to an in vitro antitumor activity (Kosaki, 1962), suggesting that they are the latent forms of I. The high antimicrobial activity of VI is explained by a rapid conversion to I in an aqueous medium (unpublished data).

In contrast with the antimicrobial activities, the present compounds showed significant antitumor activities at a relatively low dose area (Figs. 3 and 4). Especially those with an ester bond (V and VI) showed good activities at almost the same dose area as I. These results suggest the existence of a rapid conversion process after their administration. The difference of activities among these derivatives can be explained in part by their conversion characteristics. On the basis of these considerations, the present compounds, except for II, can be concluded to be actual prodrugs of I. In the case of II, the protonation of the aziridine ring could have occurred

without dealkylation process (Kinoshita et al., 1971).

Although the present compounds failed to exhibit superior effectiveness to the parent drug (I) in a fundamental screening system, an improvement of their chemotherapeutic activities would be expected by certain routes of administration, such as percutaneous, oral, and topical injections. A combining modification with other delivery systems such as liposomes and emulsions will expand the utility of these compounds. Detailed examination of bioactivation properties would offer beneficial information for understanding the modes of action of these prodrugs.

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