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Development of Lipophilic Prodrugs of Mitomycin C. III. Physicochemical and Biological Properties of Newly Synthesized Alkoxycarbonyl Derivatives

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Five alkoxycarbonyl derivatives of mitomycin C possessing various lipophilic pro-moieties including benzyloxycarbonyl, propyloxycarbonyl, pentyloxycarbonyl, nonyloxycarbonyl, and cholesteryloxycarbonyl groups were synthesized and their physicochemical and biological characteristics were examined. All compounds showed increases of octanol/water partition coefficients, lipophilic indexes (k'_0) in HPLC, and lipid solubilities to various degrees depending on their pro-moiety structure. They showed only slight antimicrobial activities against *Escherichia coli* B, but all the compounds except for cholesteryloxycarbonyl mitomycin C showed significant activity *in vivo* against a L1210 leukemia system at a relatively low dose range. These derivatives showed enzyme-mediated conversion to the parent compound in rat plasma and liver homogenate, while they were chemically stable in neutral aqueous media. Essentially no bioactivation was observed for cholesteryloxycarbonyl mitomycin C. Species differences were observed in these bioactivation phenomena. These results suggested the potential utility of the derivatives as lipophilic prodrugs.

Keywords——mitomycin C; alkoxycarbonyl derivative; lipophilic prodrug; partition coefficient; solubility; antimicrobial activity; antitumor activity; enzyme-mediated conversion; drug delivery system

In cancer chemotherapy, it is necessary to control the pharmacokinetic behavior of a cytotoxic drug for effective treatment, and many attempts have been made to deliver such drugs to the tumor site by means of drug delivery systems.[1] For the past decade we have been engaged in studies on cancer drug delivery systems either by utilizing physical devices[2] or by chemical transformation of drug molecules to prodrugs.[3] In the prodrug approach, an improvement of the drug effectiveness and ultimate therapeutic success can result from alteration of the biopharmaceutical characteristics through the introduction of pro-moieties with suitable physicochemical properties.[4] Among various physicochemical parameters, lipophilicity or lipid solubility plays a dominant role in determining biopharmaceutical properties.[5] Combined application of lipophilic derivatives to lipoidal delivery systems should enhance the utility of this approach.[6]

In a previous study,[7] the antitumor antibiotic mitomycin C(I) was derivatized to more lipophilic forms by introducing a model lipophilic functional group, a benzene ring, through various kinds of linkage structures. They showed a uniform increase of lipophilicity depending on the pro-moiety. Biological tests revealed that they exhibited characteristic antitumor activities after being regenerated to the parent drug, and a close relationship was observed between the activities and lability properties. Among the linkage structures of the prodrugs, the alkoxycarbonyl type linkage showed the best properties as regards chemical stability and biological lability.[8] In the present study, five lipophilic derivatives of I were synthesized with the above linkage structure and their biological and physicochemical characteristics were studied.

Experimental

General Procedures——Melting points were derermined in capillary tubes using a Yanagimoto NO17 micro-melting point apparatus and are uncorrected. Ultraviolet (UV) absorption spectra were recorded on a Hitachi model 220 UV-VIS spectrophotometer. Nuclear magnetic resonance (NMR) spectra were taken on a JEOL FX-200 spectrometer and elemental analyses were performed by the Center for Organic Elemental Microanalysis, Kyoto University. Where analyses are indicated only by the symbols of the elements, analytical results obtained for those elements were within $\pm 0.4\%$ of the theoretical values. Thin layer chromatography (TLC) was carried out on TLC aluminium sheets precoated with a 0.2 mm layer of Silica gel 60 F_{254} (E. Merck), using the solvent system of ethyl acetate–isopropanol (1 : 2, v/v).

Materials——Compound I was supplied by Kyowa Hakko Kogyo Co. and used without further purification. The synthesis and structure of benzyloxycarbonyl mitomycin C (II) were described in a previous paper.[7] All other reagents were of reagent grade quality and were obtained commercially (Nakarai Chemicals).

Synthesis of Derivatives——The preparation of propyloxycarbonyl mitomycin C (III) was carried out as follows: 18 mg of propyl chlorocarbonate and 15 mg of triethylamine were added to a solution of 50 mg of I in 14 ml of anhydrous tetrahydrofuran (THF) and the mixure was stirred for 15 min, then 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 III (46.5 mg, 74% yield) as a brown solid; mp 203—207 °C; TLC *Rf* 0.59; ^{1}H-NMR (35 mM solution in pyridine-d_5) δ: 5.72 (1H, dd, J=4.5, 11.0 Hz, 10′-H), 4.87 (1H, dd, J=11.0, 11.0 Hz, 10-H), 4.78 (1H, d, J=13.0 Hz, 3-H), 4.09 (1H, dd, J=4.5, 11.0 Hz, 9-H), 4.08 (2H, t, J=4.5, –COOC$\underline{H}_2$–), 3.85 (1H, d, J=4.5 Hz, 1-H), 3.59 (1H, d, J=13.0 Hz, 3′-H), 3.55 (1H, d, J=4.5 Hz, 2-H), 3.23 (3H, s, OC$\underline{H}_3$), 2.03 (3H, s, –C=C–C$\underline{H}_3$), 1.62 (2H, 6, J=1.5 Hz, –COOCH$_2$C$\underline{H}_2$–), 0.77 (3H, t, J=7.5 Hz, –COOCH$_2$CH$_2$C$\underline{H}_3$).

The following alkoxycarbonyl mitomycins C were prepared in a similar manner.

Pentyloxycarbonyl mitomycin C (IV); 77% yield; mp 89—93 °C; TLC *Rf* 0.63; ^{1}H-NMR (35 mM solution in pyridine-d_5) δ: 5.72 (1H, dd, J=4.5, 11.0 Hz, 10′-H), 4.89 (1H, dd, J=11.0, 11.0 Hz, 10-H), 4.79 (1H, d, J=13.5 Hz, 3-H), 4.20—4.04 (3H, m, 9-H, –COOC$\underline{H}_2$–), 3.87 (1H, d, J=4.5 Hz, 1-H), 3.60 (1H, d, J=13.5 Hz, 3′-H), 3.56 (1H, d, J=4.5 Hz, 2-H), 3.22 (3H, s, OC$\underline{H}_3$), 2.03 (3H, s, –C=C–C$\underline{H}_3$), 1.73—1.53 (2H, m, –COOCH$_2$C$\underline{H}_2$–), 1.32—1.04 (4H, m, –COOCH$_2$CH$_2$(C$\underline{H}_2$)$_2$–), 0.82—0.68 (3H, m, –COO(CH$_2$)$_4$–C$\underline{H}_3$).

Nonyloxycarbonyl mitomycin C (V); 80% yield; mp 133—137 °C; TLC *Rf* 0.65; ^{1}H-NMR (35 mM solution in pyridine-d_5) δ: 5.73 (1H, dd, J=4.5, 11.0 Hz, 10′-H), 4.90 (1H, dd, J=11.0, 11.0 Hz, 10-H), 4.80 (1H, d, J=13.0 Hz, 3-H), 4.28—4.04 (3H, m, 9-H, –COOC$\underline{H}_2$–), 3.89 (1H, d, J=4.5 Hz, 1-H), 3.59 (1H, d, J=13.0 Hz, 3′-H), 3.57 (1H, d, J=4.5 Hz, 2-H), 3.22 (3H, s, OC$\underline{H}_3$), 2.03 (3H, s, –C=C–C$\underline{H}_3$), 1.76—1.56 (2H, m, –COOCH$_2$C$\underline{H}_2$–), 1.36—1.00 (12H, m, –COOCH$_2$CH$_2$(C$\underline{H}_2$)$_6$–), 0.84 (3H, t, J=7.0 Hz, –COO(CH$_2$)$_8$–C$\underline{H}_3$).

Cholesteryloxycarbonyl mitomycin C (VI); 83% yield; mp 140—142 °C; TLC *Rf* 0.70; ^{1}H-NMR (35 mM solution in pyridine-d_5) δ: 5.72 (1H, dd, J=4.5, 14.0 Hz, 10′-H), 5.33 (1H, b, cholesteryl 6-H), 4.95 (1H, dd, J=14.0, 14.0 Hz, 10-H), 4.84 (1H, d, J=13.0 Hz, 3-H), 4.11 (1H, dd, J=4.5, 14.0 Hz, 9-H), 3.92 (1H, d, J=4.5 Hz, 1-H), 3.70—3.56 (2H, m, 2-H, 3′-H), 3.24 (3H, s, OC$\underline{H}_3$), 2.04 (3H, s, –C=C–C$\underline{H}_3$), 2.80—2.60 (44H, m, cholesteryl group).

Lipophilicity and Lipid Solubility Studies——Partition coefficients of alkoxycarbonyl derivatives were determined in an *n*-octanol/distilled water system at 25 °C according to Kakemi *et al.*[9] The relative lipophilic indexes (log k') were determined by high performance liquid chromatography (HPLC) employing the equation $\log k' = \log[(t_r - t_0)/t_0]$, where t_r is the retention time and t_0 is the elution time of solvent. The log k' values were extrapolated to 0% methanol concentration to obtain the lipophilic indexes (log k'_0).[10] The HPLC system and conditions used are described elsewhere. The solubilities of the compounds in *n*-hexane, isopropylmyristate, sesame oil, and distilled water determined by suspending excess amounts of them in a 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 were dissolved in methanol because of their low aqueous solubility for analysis. The antimicrobial activity was determined by measuring the diameter of the growth-inhibitory zone after a 24 h incubation at 37 °C.

Antitumor Activity Studies——L1210 leukemia was maintained by weekly transplantation of tumor cells into the peritoneal cavity of male DBA/2 mice. Animals used for tests were male hybrid BDF$_1$ mice (C57B1/6 × DBA/2). Six mice (weighing 20—25 g) per group were inoculated intraperitoneally with a suspension of 1×10^5 L1210 cells and the chemotherapy was given intraperitoneally at 24 h after inoculation. All drugs tested were administered in the form of sesame oil solution because of the low aqueous solubilities of V and VI. Activities were calculated as $T/C\%$, the ratio of the mean survival time of the treated group (T) to that of the control group (C). The observation period for determining survival time was 60 d.

Stability Measurements in Aqueous Solution and Biological Media——Stock solutions of all compounds were prepared in dimethylsulfoxide (DMSO) at a concentration of 5×10^{-3} M and an appropriate volume was mixed with an aqueous buffer or biological medium for kinetic studies.

Stability experiments were carried out in an isotonic pH 7.4 phosphate buffer at 37 ± 0.2 °C. The total buffer concentration was 0.1 M and the ionic strength was adjusted to 0.3. Degradation was initiated by the addition of the

stock solution to a preheated buffered solution to give a concentration of 1×10^{-5} M. Aliquots of the solution were withdrawn at suitable time intervals.

Male Wistar rats weighing 200—220 g were used to obtain plasma and liver homogenate. Human blood was obtained from healthy volunteers. The liver of rats was homogenized at 0—5 °C in a glass-Teflon homogenizer, then centrifuged at 600×9.8 kg/cm^2 for 10 min, and the supernatant was used for the experiments. An isotonic phosphate buffer (pH 7.4) containing 0.125 M sucrose was used to prepare tissue homogenate, diluted plasma, and tissue homogenate samples.

Bioactivation experiments were performed at 37 ± 0.2 °C and initiated by adding the stock solution to give a final concentration of 1×10^{-4} M. At appropriate time intervals, aliquots of the solution were withdrawn, acetonitrile was added to precipitate the protein, and the supernatant was subjected to analysis.

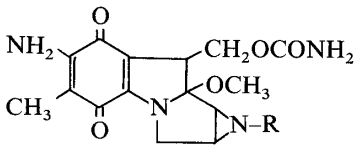
Analysis——In physicochemical and stability experiments, the prodrugs and I were determined by the use of an HPLC system (TRIROTAR, Jasco, Japan) equipped with a variable-wavelength UV detector (UVIDEC 100-II, Jasco) in a reverse phase mode. The stationary phase used was a Corasil C18 column (Nakarai Chemicals) and a short column packed with Lichrosorb RP-2 (E. Merck) was used to guard the main column. Mixtures of methanol–water were used as the mobile phase at a flow rate of 0.8 ml/min. The standard solutions were chromatographed and calibration lines were constructed on the basis of peak-area measurements.

Results

Chemistry

The alkoxycarbonyl derivatives of I were prepared by an ordinary method.[11] Mitomycin C was condensed with reactive alkyl chlorocarbonates in the presence of triethylamine as a

Table I. Structures and Physical Data of Alkoxycarbonyl Mitomycin C Derivatives



	Compound	R	Yield (%)	mp (°C)	mw	UV_{max} ($\varepsilon \times 10^{-3}$) (nm), EtOH
I	Mitomycin C (MMC)	–H	—	>270	334	358 (22.6)
II	Benzyloxycarbonyl MMC	–COO–$CH_2C_6H_5$	60	102—104	468	356 (20.1)
III	Propyloxycarbonyl MMC	–COO–C_3H_7	74	203—207	420	356 (20.1)
IV	Pentyloxycarbonyl MMC	–COO–C_5H_{11}	77	89—93	448	356 (22.5)
V	Nonyloxycarbonyl MMC	–COO–C_9H_{19}	80	139—141	504	356 (23.7)
VI	Cholesteryloxy-carbonyl MMC	–COO–$C_{27}H_{45}$	83	140—142	747	356 (23.1)

Table II. Lipophilicity and Solubility of Alkoxycarbonyl Mitomycin C Derivatives

Compound	P_{oct} [a]	log P_{oct}	log k_0' [b]	Solubility (mM)			
				Water (25 °C)	Sesame oil (25 °C)	I.P.M. (37 °C)	Hexane ($\times 10^3$) (25 °C)
I	0.414	−0.386	1.93	2.730	0.0180	0.0193	0.0234
II	112.95	2.053	3.53	0.528	3.291	3.283	0.415
III	32.73	1.515	2.91	0.333	0.104	0.106	<0.0200
IV	279.19	2.446	3.59	0.590	3.480	8.220	2.020
V	3673	3.561	4.79	0.00025	15.170	12.620	0.304
VI	7606	3.881	10.86	0.00025	12.100	18.020	6.610

a) Partition coefficient between *n*-octanol and water (see the text).
b) Lipophilic index in HPLC (see the text).

base. The reaction progressed rapidly and was completed within 30 min at room temperature. The acylated products were obtained in high yields and recrystallized. The structures of the purified compounds were derermined by elemental and spectral (NMR) analyses, as summarized in Table I. All compounds exhibited a UV maximum at approximately 360 nm due to the mitosane structure including the aziridine ring.[12]

Physicochemical constants such as partition coefficients (P_{oct}), lipophilic indexes ($\log k'_0$) in HPLC, and solubilities in various solvents are summarized in Table II. All derivatives showed increased lipophilicity, as expected, due to the introduction of the carrier moiety, and the $\log P_{oct}$ values varied between 1.515 and 3.881. They also exhibited increased lipid solubilities to various degrees.

Biological Activity

The antimicrobial activities of the compounds against *Escherichia coli* B are shown in Fig. 1. Each activity is expressed as the corresponding concentration of I (ordinate) which shows growth inhibition activity equal to that of the test compound. All the compounds exhibited remarkably lower antimicrobial activities than I. These observations were in good agreement with the results obtained by the standard agar-dilution method.

The effects of the prodrugs on the survival time of mice given intraperitoneal inoculation of L1210 leukemia are summarized in Table III. The average survival time of the untreated control groups were 8.0 d. Compound I exhibited maximum activity at a dose of 15 μmol/kg and afforded a $T/C\%$ value of 141. Above this dose, I exhibited a marked toxicity. Treatment with derivatives II—VI significantly increased the survival time of mice bearing L1210, but VI had no effect. Compounds II—IV showed maximum activities of approximately 140% at a dose of 22.5 μmol/kg, which surpassed the usual criterion of $T/C\% > 130$ and are comparable to the effect of I. The dose-response curves of II—IV were relatively similar to that of I. Compound V gave a $T/C\%$ value of 118 at a dose of 60 μmol/kg against L1210 leukemia, but VI scarcely showed biological activity under these experimental conditions.

Stability and Bioactivation

The stability of prodrugs were investigated in an isotonic pH 7.4 phosphate buffer at

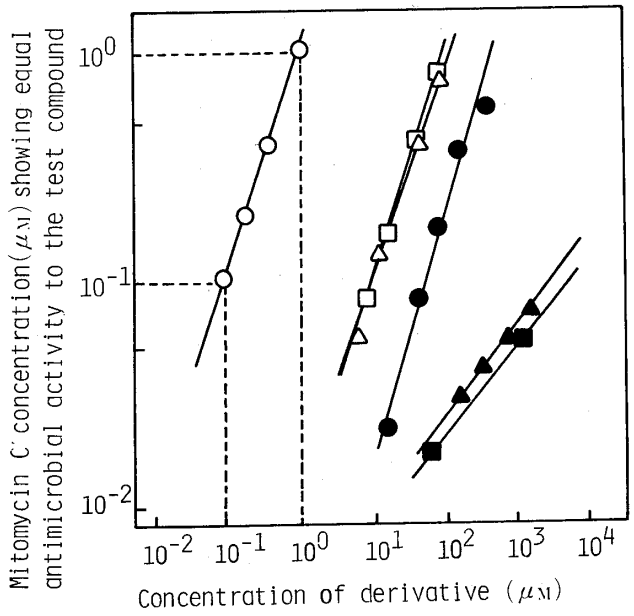


Fig. 1. Comparison of Antimicrobial Activities of Alkoxycarbonyl Derivatives
○, I; △, II; □, III; ●, IV; ▲, V; ■, VI.

TABLE III. Effects of Alkoxycarbonyl Derivatives on the Survival Time of Mice with Intraperitoneally Implanted L1210 Leukemia[a)]

Compound	Dose (μmol/kg)	Mean survival time (d)	T/C %	60-d survivors/total
Control		8.0	100	0/16
Sesame oil		7.9	99	0/16
I	3.0	8.6	108	0/6
	7.5	10.9	136	0/6
	15.0	10.9	141	0/6
	30.0	5.7	71	0/6
II	7.5	8.0	100	0/6
	15.0	8.7	109	0/6
	22.5	10.1	126	0/6
	30.0	10.3	129	0/6
	60.0	6.0	75	0/6
III	7.5	7.8	98	0/6
	15.0	8.5	106	0/6
	22.5	10.8	135	0/6
	30.0	10.5	131	0/6
	60.0	6.2	78	0/6
IV	7.5	8.7	109	0/6
	15.0	9.2	115	0/6
	22.5	10.3	129	0/6
	30.0	10.8	135	0/6
	60.0	6.3	79	0/6
	120.0	4.9	61	0/6
V	7.5	7.5	94	0/6
	15.0	7.7	96	0/6
	22.5	8.3	104	0/6
	30.0	8.8	110	0/6
	60.0	9.4	118	0/6
	120.0	5.0	63	0/6
VI	7.5	8.0	100	0/6
	15.0	8.2	103	0/6
	22.5	8.7	109	0/6
	30.0	8.2	103	0/6
	60.0	8.3	104	0/6
	120.0	8.2	103	0/6

a) BDF_1 mice were inoculated intraperitoneally with 1×10^5 cells of L1210 leukemia. The drug used in chemotherapy was dissolved in sesame oil and doses were started 24 h after tumor implantation.

37 °C. The relative susceptibility of the derivatives to enzymatic hydrolysis was also studied *in vitro* in rat and human plasma, and in the supernatant of rat liver homogenate at 37 °C, and the results are summarized in Table IV. The observed pseudo-first order rate constants for the overall degradation of these compounds were calculated by linear regression analysis of a logarithmic plot of the concentration against time.

Compound II degraded with a half-life of 190 h in buffer solution and the other compounds were stabler than II. In contrast with the chemical stability, these prodrugs (except for VI) disappeared at various rates in both plasma and liver homogenate and formation of I was observed simultaneously. Mouse plasma and liver homogenate gave

Table IV. Conversion Rates of Alkoxycarbonyl Derivatives of Mitomycin C in Various Media

Compound	Conversion rate constant, k_{obs} (h^{-1})			
	pH 7.4 phosphate buffer	Rat		Human
		2% plasma	2% liver	50% plasma
I	0.00148	0.0108	0.0461	0
II	0.00362	5.15	12.7	0.0136
III	0.00139	1.15	1.55	0.00603
IV	0.00202	2.92	4.35	—
V	0.0000801	1.03	2.62	0.0174
VI	0.0000151	0.0501	0.0762	0.0148

almost the same results as those obtained with rats (not shown). However, these compounds proved to be stable in human plasma, as shown in Table IV.

Discussion

Mitomycin C is an antitumor antibiotic that has been demonstrated to have activity against a number of human neoplasma, but its toxic effects, such as delayed cumulative myelosuppression and gastrointestinal damage, have impeded its utilization.[13] Although numerous analogues have been prepared in attempts to improve the therapeutic properties of mitomycin C, few of them have emerged as clinical agents.[14]

Among chemical approaches, prodrug preparation seems to be a promising one, but it is necessary to satisfy the following two criteria to obtain a useful prodrug: a linkage structure with adequate stability and lability and a pro-moiety with suitable physicochemical properties must be used.[15] In a previous investigation on linkage structures,[7,8] it was demonstrated that the alkoxycarbonyl group, *i.e.* a carbamate linkage, is the most suitable one for derivatizing I to a lipophilic form among various linkage groups tested including alkyl, acyl, and acyloxymethyl groups. Among alkoxycarbonyl derivatives, however, only ethoxycarbonyl I had been reported,[16] and no detailed information about its biopharmaceutical characteristics was available. In the present investigation, we newly synthesized four alkoxycarbonyl type derivatives of I (compounds III—VI) with various pro-moieties, and examined their utility in relation to the properties of their leaving groups. As shown in Table II, prodrugs (III, IV, and V) showed increased lipophilicity with increase of the alkyl chain length from three to nine, and these results confirmed the feasibility of selecting physicochemical properties such as lipid solubility and lipophilicity by the use of an appropriate pro-moiety.

Compound I has been shown to cross-link double-helical DNA after enzymatic reduction to the corresponding hydroquinone.[17] Positions 1a and 10 appear to be the alkylating sites of I, and their alkylating ability is enhanced when methanol is eliminated from the produced hydroquinone to give the indolohydroquinone.[18] Consequently it is considered that substitution at the 1a position leads to diminution of the biological activity, and this was confirmed by the results shown in Fig. 1. All of the compounds showed markedly reduced antimicrobial activities, which can be considered to be parallel to *in vitro* antitumor activity,[19] suggesting that they are latent forms of I.

In contrast with the antimicrobial activities, II, III, IV, and V showed significant antitumor activities at a relatively low dose range (Table III). Compound VI showed no biological activity *in vitro* or *in vivo* under these experimental conditions. These results suggest

the existence of a rapid conversion process after their administration except in the case of VI.

In fact, the present results revealed peculiar bioactivation profiles of these prodrugs under physiological conditions. The rat plasma and liver homogenate successfully catalyzed the hydrolysis of the present prodrugs having the carbamate linkage. Non-specific esterase or carbamidase might be responsible for this reaction.[4] On the other hand, the rates of hydrolysis to the prodrugs varied with the structure of the pro-moiety. These results suggested that the leaving group could influence the hydrolysis rate of the linkage. In the case of VI, I was not produced in any tissue medium, suggesting that substitution by the cholesteryl group hinders the enzymatic attack. Thus, some relationships between biological activities and lability characteristics were suggested in the present investigation, but it is necessary to consider the complicated effect of differences in lipophilicity, which was dependent on the pro-moiety.

Although further work is necessary, the present report raises the possibility of controlling biopharmaceutical and chemotherapeutic properties of mitomycins by introducing a suitable lipophilic pro-moiety. An integrated design of linkage bond and leaving group could lead to a promising prodrug with excellent activity by employing various routes of administration and/or combining various dosage forms with lipoidal ones. In a previous investigation of this series, we compared the percutaneous permeabilities of these compounds and found that those with adequate lipophilicity and low melting point showed excellent absorbability.[20] Liposomes and emulsions loaded with lipophilic prodrugs of I successfully showed specific lymphatic delivery of the parent drug following topical administration. The results will be presented in detail in subsequent papers.

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