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# Liposomal Sustained-Release Delivery Systems for Intravenous Injection. I. Physicochemical and Biological Properties of Newly Synthesized Lipophilic Derivatives of Mitomycin C

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1a-*N*-Stearoyl mitomycin C (MMC) and six 1a-*N*-substituted derivatives of MMC possessing the cholesteryl moiety with different spacers were synthesized, and their biopharmaceutical properties were studied to assess the feasibility of such derivatives as prodrugs for intravenously injectable liposomal sustained-release carrier systems. All compounds showed increased lipophilic indices ($\log k'_0$) in high performance liquid chromatography. It was found that all the derivatives could be almost completely entrapped in liposomes, although MMC itself was hardly encapsulated. The derivatives with the exception of cholesteryloxycarbonyl MMC (II) and *N*-(cholesteryloxycarbonyl)-4-aminophenylacetyl MMC (VI) were converted to the parent drug in rat serum. The suceptibility of the compounds to hydrolysis was strongly affected by the spacer structure between MMC and the cholesteryl moiety. Cholesteryloxyacetyl MMC (IV) was converted to MMC mainly by chemical hydrolysis. *N*-(Cholesteryloxycarbonyl)glycyl MMC (III) was also hydrolyzed to MMC chemically but in this case hydrolysis was accelerated in the presence of mouse, rat and human serum. No species differences were observed in these bioactivation phenomena. Entrapment of derivatives III and IV in liposomes resulted in enhancement of the stability against both chemical and enzymatic hydrolysis. The derivatives possessing the cholesteryl moiety were firmly associated with liposomes in the circulation, while stearoyl MMC (VIII) was rapidly removed. These results suggest that derivatives III and IV have the potential to be utilized as lipophilic prodrugs for liposomal sustained-release carrier systems to be delivered by intravenous injection.

**Keywords**——mitomycin C; prodrug; liposome; sustained release; intravenous injection; entrapment; affinity; conversion property; lipophilic moiety; spacer structure

Mitomycin C (MMC) is a widely used antitumor antibiotic in the treatment of not only chronic myelogeneous leukenia but also a variety of solid tumors including gastrointestinal and breast cancer.[1] Its clinical usefulness is, however, limited by its toxic side effects such as delayed cumulative myelosuppression and gastrointestinal damage.[2] As a result, it has proved necessary to control the pharmacokinetic behavior of MMC for effective treatment.

Barlogie and Drewinko indicated that the cytotoxicity of MMC is related to the given exposure dose regardless of the specific drug concentration and exposure time.[3] Furthermore, MMC has been found to be selectively toxic to hypoxic tumor cells at a relatively low concentration *in vitro*.[4] These findings indicate the possibility that maintaining blood MMC levels by the use of sustained-release dosage forms might improve the therapeutic efficacy of MMC.

One promising approach to sustained drug release is the use of liposomes as drug reservoirs.[5] However, encapsulation of the drug into liposomes has some disadvantages in that most antitumor drugs including MMC show low entrapment efficiency and relatively fast leakage out of lipid vesicles.[6] One of many possible ways of overcoming these disadvantages

may be chemical modification of MMC into lipophilic prodrugs.[7] Sasaki and his coworkers[8] have synthesized a variety of lipophilic derivatives of MMC in an attempt to improve its applicability to lipidic dosage forms such as liposomes and emulsions. They obtained specific lymphatic delivery of the parent drug following topical administration of lipidic carriers loaded with nonyloxycarbonyl MMC, the derivative which showed the best biopharmaceutical properties as a prodrug for these lipidic carrier systems.[9] However, there remained some problems such as the rapid removal of the prodrug from lipidic carriers in the circulation and low lability of the derivative in human plasma.[10] An integrated design of carrier moiety and spacer structure could lead to the solution of such problems: introduction of more lipophilic carrier moieties such as a long-chain aliphatic acyl group or cholesteryl group might lead to improvement in retention of the derivatives within the lipid vesicles in the circulation, and the lability characteristics of the compounds in human plasma could be controlled by manipulation of the spacer structure between MMC and the lipophilic moiety. Although a number of 1a-*N*-acyl derivatives have been reported,[8a,11] little is known about their biopharmaceutical characteristics with the exception of benzoylcarbonyl MMC. Among cholesteryl derivatives, only cholesteryloxycarbonyl MMC, which is stable to chemical and enzymatic hydrolysis, has been reported on.[8c]

In the present study, we synthesized 1a-*N*-stearoyl MMC as a typical compound with a long-chain aliphatic acyl group and six 1a-*N*-substituted derivatives of MMC possessing the cholesteryl moiety with different spacers, and examined their biopharmaceutical properties in order to assess the feasibility of employing them as prodrugs for liposomal sustained-release carrier systems to be delivered by intravenous injection.

## Experimental

**General Procedures**——Melting points were determined using a Thomas Hoover capillary melting point apparatus and are uncorrected. Ultraviolet (UV) absorption spectra were recorded on a Hitachi U-3200 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AM-200 spectrometer. Mass spectra (MS) were obtained using a VG Analytical ZAB-SE mass spectrometer.

**Materials**——MMC (I) was purchased from Wako Pure Chemical Industries. Egg phosphatidylcholine (PC) and egg sphingomyelin (SM) were obtained from Sigma Chemical Co., *N*-4-Nitrobenzo-2-oxa-1,3-diazole-dipalmitoylphosphatidylethanolamine (NBD-PE) was synthesized by the method of Monti *et al.*[12] All other chemicals were of reagent grade or better.

**Synthesis**——Cholesteryloxycarbonyl MMC (II): II was prepared from cholesteryl chlorocarbonate (CC) and I as reported by Sasaki *et al.*[8]; yield 80%, mp 141—143 °C. $^1$H-NMR ($CDCl_3$) $\delta$ 5.33 (1H, br d, $J$=4.4 Hz, cholesteryl 6-H), 4.92 (1H, dd, $J$=4.6, 10.8 Hz, 10′-H), 4.45 (1H, d, $J$=13.4 Hz, 3-H), 4.30 (1H, dd, $J$=10.9, 10.9 Hz, 10-H), 3.67 (1H, dd, $J$=4.6, 10.9 Hz, 9-H), 3.49 (1H, dd, $J$=1.6, 13.4 Hz, 3′-H), 3.40 (1H, d, $J$=4.6 Hz, 1-H), 3.27 (1H, dd, $J$=1.6, 4.6 Hz, 2-H), 3.20 (3H, s, $OCH_3$), 1.77 (3H, s, –C=C–C$\underline{H}_3$), 0.67 (3H, s, cholesteryl 18-$CH_3$). FAB-MS $m/z$: 748 ($M^+$).

*N*-(Cholesteryloxycabonyl)glycyl MMC (III): Triethylamine (4.04 g) was added to a suspension of glycine (1.5 g) in water (30 ml). A solution of CC (8.98 g) in dioxane (200 ml) was added dropwise to the resulting clear solution at 0 °C and stirring was continued for 3 h. The mixture was concentrated *in vacuo*, and chloroform (100 ml), followed by 1 N hydrochloric acid (23 ml), was added to the residue. The organic layer was separated, washed repeatedly with water and dried over $MgSO_4$. After evaporation of the solvent, the product was suspended in ethanol and filtered to give *N*-(cholesteryloxycarbonyl)glycine (IIIa) as white crystals; yield 66% (6.44 g), mp 175—177 °C. (dec.). *N*-Hydroxysuccinimide (0.69 g) and dicyclohexylcarbodiimide (1.24 g) were added successively to a solution of IIIa (2.92 g) in a mixture of dioxane (40 ml) and chloroform (6 ml) at 0 °C. The mixture was left standing for 16 h in a refrigerator and the resulting precipitate was removed by filtration. The filtrate was concentrated *in vacuo* to give succinimidyl-*N*-(cholesteryloxycarbonyl)glycinate (IIIb); yield 92% (3.23 g), mp 159—164 °C. IIIb (1.75 g) and triethylamine (0.33 g) were added to a solution of I (1.0 g) in *N*,*N*-dimethylformamide (20 ml) at room temperature and the reaction mixture was stirred for 4 h. The solvent was evaporated off, and chloroform (50 ml) and water (50 ml) were added to the residue. The organic layer was separated and dried over $MgSO_4$. After evaporation of the solvent, the product was dissolved in chloroform and placed in a silica gel column. Elution with a mixture of chloroform and methanol gave III (1.74 g, 72%) as a dark purple solid; mp above 250 °C. $^1$H-NMR ($CDCl_3$) $\delta$: 5.36—5.31 (4H, m, cholesteryl 6H, –$COCH_2N\underline{H}COO$–, $NH_2$), 4.86 (1H, dd, $J$=4.6, 10.8 Hz, 10′-H), 4.43 (1H, d,

$J$=13.5 Hz, 3-H), 4.16-3.84 (2H, m, –COCH$_2$NH–), 3.97 (1H, dd, $J$=10.9, 10.9 Hz, 10-H), 3.71 (1H, dd, $J$=4.6, 10.8 Hz, 9-H), 3.59 (1H, d, $J$=4.7 Hz, 1-H), 3.57 (1H, dd, $J$=1.6, 13.4 Hz, 3′-H), 3.48 (1H, dd, $J$=1.6, 4.3 Hz, 2-H), 3.20 (3H, s, OCH$_3$), 1.78 (3H, s, –C=C–CH$_3$), 0.67 (3H, s, cholesteryl 18–CH$_3$). FAB-MS $m/z$: 805 (M+2).

Cholesteryloxyacetyl MMC (IV): Cholesteryloxyacetic acid was prepared according to the literature[13]; yield 73%, mp 164—166 °C. IV was synthesized in a similar manner to III to give a dark purple solid; yield 61%, mp 145—150 °C (dec.). $^1$H-NMR ($CDCl_3$) $\delta$: 5.33 (1H, br d, $J$=5.1 Hz, cholesteryl 6-H), 4.86 (1H, dd, $J$=4.6, 10.8 Hz, 10′-H), 4.50 (1H, d, $J$=13.4 Hz, 3-H), 4.12 (2H, s, –COCH$_2$–), 4.04 (1H, dd, $J$=10.8, 10.8 Hz, 10-H), 3.70 (1H, dd, $J$=4.6, 10.8 Hz, 9-H), 3.55 (1H, dd, $J$=1.6, 13.4 Hz, 3′-H), 3.52 (1H, d, $J$=4.4 Hz, 1-H), 3.36 (1H, dd, $J$=1.6, 4.4 Hz, 2-H), 3.21 (3H, s, OCH$_3$), 1.77 (3H, s, –C=C–CH$_3$), 0.67 (3H, s, cholesteryl 18–CH$_3$). FAB-MS $m/z$: 762 (M+2).

*N*-(Cholesteryloxycarbonyl)-4-aminomethylbenzoyl MMC (V): *N*-(Cholesteryloxycarbonyl)-4-aminomethylbenzoic acid (Va) was prepared from CC and *p*-aminomethylbenzoic acid in a similar manner to IIIa; yield 44%, mp 175—177 °C (dec.). Va (84 mg), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (57 mg) and triethylamine (30 mg) were added to a solution of I (50 mg) in a mixture of tetrahydrofuran (20 ml) and chloroform (3 ml) whereupon the mixture was stirred for 2 d at room temperature. After evaporation of the solvent, the residue was dissolved in chloroform and applied to a silica gel column. Elution of a mixture of chloroform and methanol gave V (62 mg, 47%) as a dark purple solid; mp 151—154 °C (dec.). $^1$H-NMR ($CDCl_3$) $\delta$: 7.83 (2H, d, $J$=8.1 Hz, 2″, 6″ aromatic protons), 7.34 (2H, d, $J$=8.1 Hz, 3″, 5″ aromatic protons), 5.39 (1H, br d, $J$=4.4 Hz, cholesteryl 6-H), 5.08—4.95 (1H, m, –COC$_6$H$_4$CH$_2$NH–), 4.91—4.64 (4H, m, 10′-H, 3-H, NH$_2$), 4.40 (2H, br d, $J$=5.6 Hz, –COC$_6$H$_4$CH$_2$–), 4.13 (1H, dd, $J$=10.9, 10.9 Hz, 10-H), 3.82—3.69 (2H, m, 1-H, 9-H), 3.62 (1H, dd, $J$=1.6, 13.4 Hz, 3′-H), 3.26 (3H, s, OCH$_3$), 3.25—3.20 (1H, m, 2-H), 1.81 (3H, s, –C=C–CH$_3$), 0.68 (3H, s, cholesteryl 18-CH$_3$). FAB-MS $m/z$: 822 (M+2).

*N*-(Cholesteryloxycarbonyl)-4-aminophenylacetyl MMC (VI): *N*-(Cholesteryloxycarbonyl)-4-aminophenylacetic acid (VIa) was prepared from CC and *p*-aminophenylacetic acid in a similar manner to IIIa; yield 80%, mp 170—172 °C (dec.). VI was synthesized from I and VIa in accordance with the method used to prepare V to give a dark purple solid; yield 18%, mp 136—139 °C (dec.). $^1$H-NMR ($CDCl_3$) $\delta$: 7.29 (2H, d, $J$=8.3 Hz, 3″, 5″ aromatic protons), 7.10 (2H, $J$=8.3 Hz, 2″, 6″ aromatic protons), 6.59 (1H, s, –COCH$_2$C$_6$H$_4$NH–), 5.41 (1H, br d, $J$=5.2 Hz, cholesteryl 6-H), 4.86 (1H, dd, $J$=4.7, 10.5 Hz, 10′-H), 4.15 (1H, d, $J$=13.6 Hz, 3-H), 4.06 (1H, dd, $J$=10.7, 10.7 Hz, 10-H), 3.68—3.59 (3H, m, 9-H, –COCH$_2$C$_6$H$_4$–), 3.50 (1H, d, $J$=4.4 Hz, 1-H), 3.34 (1H, dd, $J$=1.6, 13.2 Hz, 3′-H), 3.17 (3H, s, OCH$_3$), 3.02 (1H, d, $J$=4.5 Hz, 2-H), 1.80 (3H, s, –C=C–CH$_3$), 0.69 (3H, s, cholesteryl 18-CH$_3$). FAB-MS $m/z$: 882 (M+2).

3-(Cholesteryloxycarbonyl)propanoyl MMC (VII): Triethylamine (1.32 g) and succinic anhydride (1.29 g) were added to a solution of cholesterol (1.0 g) in chloroform (25 ml), and the mixture was refluxed with stirring for 3 h. After addition of water, the organic layer was separated, washed with water and dried over $MgSO_4$. Evaporation of the solvent gave crude crystals, which were recrystallized from isopropyl ether to afford 3-(cholesteryloxycarbonyl)propionic acid (VIIa); yield 55% (0.69 g), mp 178—180 °C. VII was prepared from I and VIIa in a similar manner to V to give a dark purple solid; yield 71%, mp 89—91 °C. $^1$H-NMR ($CDCl_3$) $\delta$: 5.36 (1H, br d, $J$=4.6 Hz, cholesteryl 6-H), 4.87 (1H, dd, $J$=4.6, 10.8 Hz, 10′-H), 4.45 (1H, d, $J$=13.7 Hz, 3-H), 4.06 (1H, dd, $J$=11.1, 11.1 Hz, 10-H), 3.69 (1H, dd, $J$=4.6, 11.2 Hz, 9-H), 3.62—3.50 (2H, m, 1-H, 3′-H), 3.21 (3H, s, OCH$_3$), 2.78—2.42 (4H, m, –COCH$_2$CH$_2$CO–), 1.78 (3H, s, –C=C–CH$_3$), 0.67 (3H, s, cholesteryl 18-CH$_3$). FAB-MS $m/z$: 805 (M+2).

Stearoyl MMC (VIII): VIII was synthesized from I and stearic acid in a similar manner to V to give a dark brown solid; yield 51%, mp 65—67 °C. $^1$H-NMR ($CDCl_3$) $\delta$: 4.86 (1H, dd, $J$=4.6, 10.9 Hz, 10′-H), 4.47 (1H, d, $J$=13.4 Hz, 3-H), 4.08 (1H, dd, $J$=10.9, 10.9 Hz, 10-H), 3.69 (1H, dd, $J$=4.7, 10.9 Hz, 9-H), 3.58—3.45 (2H, m, 3′-H, 1-H), 3.25—3.14 (4H, m, 2-H, OCH$_3$), 2.45—2.25 (2H, m, –COCH$_2$–), 1.78 (3H, s, –C=C–CH$_3$), 1.70—1.45 (2H, m, –COCH$_2$CH$_2$–), 0.93—0.79 (3H, m, –CO(CH$_2$)$_{16}$CH$_3$). FAB—MS $m/z$: 602 (M+2).

**Lipophilic Index**——The relative lipophilic indices ($\log k'$) were determined by high performance liquid chromatography (HPLC) employing Eq. 1.[14]

$$\log k' = \log[(t_R - t_0)/t_0] \quad (1)$$

where $t_R$ is the retention time of the solute and $t_0$ is the elution time of the solvent. The lipophilic indices ($\log k'_0$) were determined by extrapolation of the $\log k'$ values to 0% organic solvent concentration. The HPLC system and stationary phase used are described elsewhere. A mixture of isopropanol and distilled water was used as the mobile phase at a flow rate of 1.0 ml/min.

**Preparation of Liposomes**——Liposomes were prepared largely according to the controlled dialysis method described by Zumbuehl and Weder.[15] PC, SM and the derivative at a molar ratio of 7:3:0.5 were dissolved in chloroform–methanol (2:1, v/v), and in some cases a trace amount of NBD-PE was added as a liposomal membrane marker. The organic solvents were evaporated off under vacuum and the dried lipid film was suspended in 6 ml of pH 7.4 phosphate-buffered saline (PBS). Sodium cholate in a molar ratio of total phospholipids to detergent of 0.2 was added to the lipid suspension, and the mixture agitated by hand. The mixed micellar solution thus formed was dialyzed against PBS at 37 °C for 16 h using a Lipoprep dialyzer (Diachema). After completion of dialysis, the

liposome suspension was filtered through a Millex GV sterile filter (Millipore), and purged with nitrogen gas.

**Entrapment Measurement**——The extent of entrapment of the derivatives in liposomes was determined by gel filtration.[7] Liposome suspension (0.5 ml) was applied to a Sepharose CL-4B column (Pharmacia Fine Chemicals, 16 × 150 mm) and eluted with PBS. The eluent fractions were analyzed for derivative and NBD-PE by HPLC as described elsewhere. The entrapment efficiency was calculated from Eq. 2.

$$\text{entrapment } (\%) = D_e/D_t \times 100 \quad (2)$$

where $D_e$ is the amount of derivative recovered in the liposome (NBD-PE) fractions and $D_t$ is the initial amount of derivative applied to a column.

**Hydrolysis Test**——Hydrolysis experiments were carried out on the derivatives in PBS and various sera. Female ICR mice weighing 22—24 g and male Sprague-Dawley rats weighing 220—240 g were used to obtain sera. Human serum was obtained from healthy volunteers. Preheated rat serum was prepared by heating fresh serum at 60 °C for 1 h. All hydrolysis experiments were performed at 37 ± 0.2 °C and initiated by the addition of a stock ethanol solution or liposome suspension to prewarmed media to give a final drug concentration of $5 \times 10^{-5}$ M. At appropriate time intervals aliquots of the solution were withdrawn and added to acetonitrile (assay for MMC) or isopropanol (assay for derivative) to precipitate the protein. The supernatant after centrifugation was analyzed immediately for regenerated MMC and the derivative.

***In Vitro* Affinity Test**——Affinity of the derivatives to liposomes was estimated in the presence of PBS and fresh rat serum by the gel filtration method. Affinity experiments were performed at 37 ± 0.2 °C and initiated by adding the liposome suspension to give a drug concentration of $5 \times 10^{-5}$ M. The volume ratio of incubation medium to liposome suspension was 9 : 1. After a 10 min incubation, 0.5 ml of the incubation mixture was chromatographed on a Sepharose CL-4B column (16 × 150 mm). The eluent fractions were subjected to analysis for the derivatives and NBD-PE.

***In Vivo* Affinity Test**——Male Sprague-Dawley rats weighing 220—240 g were used. The animals were anesthetized with ether and injected with 1.0 ml of liposome suspension at a dose of 2.77 μmol drug/rat into the femoral vein. At various times after injection, blood samples (0.5 ml) were taken from the jugular vein and immediately frozen in liquid nitrogen to avoid postcollection degradation of the derivative. After lyophilization, the blood samples were homogenized in 3 ml of ethanol with a Polytron homogenizer (Kinematia, Gmbh), and the supernatant after centrifugation was taken for assay of the derivative and NBD-PE.

**HPLC Assay**——MMC and its derivatives were determined by using a Waters HPLC system equipped with a model 510 pump, a WISP 710B automatic sample injector and a Lamda-Max model 481 variable wavelength absorbance detector set at 355 nm. The chromatographic column was a stainless-steel tube (4.6 × 200 mm) filled with Develosil ODS-5 (Nomura Chemical Co.). Methanol–distilled water (35 : 65, v/v) and acetonitrile–isopropanol (50 : 50, v/v) were used as the mobile phases (flow rate, 1.5 ml/min) for MMC and its derivatives, respectively. NBD-PE was determined by using a Waters HPLC system equipped with a Hitachi model 650-10S fluorometer. Detection of NBD-PE was performed at excitation and emission wavelengths of 465 nm and 535 nm, respectively. The stationary phase used was a Develosil ODS-5 packed column (4.6 × 100 mm). Ethanol–distilled water–phosphoric acid (1000 : 15 : 10, v/v) was used as the mobile phase with a flow rate of 1.0 ml/min. Standard solutions were chromatographed and calibration lines were constructed on the basis of peak-area measurements.

## Results

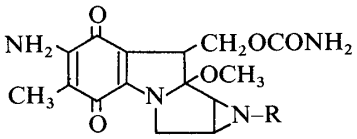
### Chemistry

The structures and physical data are listed in Table I. The structures of the compounds were supported by NMR, mass and UV spectra. Each derivative exhibited a UV maximum at approximately 360 nm due to the mitosane structure including the aziridine ring.[16]

### Lipophilicity and Entrapment Efficiency

The lipophilicity of the derivatives was evaluated by reversed-phase HPLC. In this method the log $k'$ value could be used as a parameter for characterizing the relative lipophilicity of the drug molecule.[17] For all the tested compounds, plots of log $k'$ *versus* isopropanol concentration showed a reasonable linear relationship ($r > 0.999$). The log $k'_0$ values (lipophilic indices) extrapolated to 0% isopropanol concentration are summarized in Table II. All derivatives showed increased lipophilicity compared with the parent drug, as expected, due to the introduction of the lipophilic moieties. Partition coefficients of the derivatives could not be determined experimentally because of their extremely low aqueous solubility ($< 1 \times 10^{-7}$ M). All derivatives were almost completely (>99.8%) entrapped in

TABLE I. Structures and Physical Data of 1a-*N*-Substituted MMC Derivatives



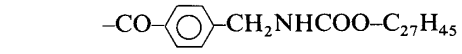
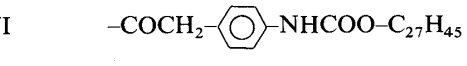
| Compound | R | mp (°C) | $M_r$ | $UV_{max}$ ($\varepsilon \times 10^{-3}$) (nm), EtOH |
|---|---|---|---|---|
| I | –H | >250 | 334.3 | 358 (22.4) |
| II | $-COO-C_{27}H_{45}$ | 141—143 | 747.0 | 356 (22.6) |
| III | $-COCH_2NHCOO-C_{27}H_{45}$ | >250 | 804.0 | 357 (23.3) |
| IV | $-COCH_2O-C_{27}H_{45}$ | 146—149 (dec.) | 761.0 | 357 (22.9) |
| V |  | 151—154 (dec.) | 880.1 | 357 (23.6) |
| VI |  | 136—139 (dec.) | 880.1 | 357 (23.4) |
| VII | $-COCH_2CH_2COO-C_{27}H_{45}$ | 89—91 (dec.) | 803.1 | 357 (23.2) |
| VIII | $-CO(CH_2)_{16}CH_3$ | 65—67 | 600.8 | 356 (23.8) |

TABLE II. Lipophilic Indices ($\log k_0'$) of 1a-*N*-Substituted MMC Derivatives

| Compound | $\log k_0'$[a)] |
|---|---|
| I | 0.913 |
| II | 3.461 |
| III | 3.463 |
| IV | 3.349 |
| V | 3.828 |
| VI | 3.964 |
| VII | 3.583 |
| VIII | 3.014 |

*a*) Each value represents the mean of three separate experiments. Each S.D. value was less than 2%.

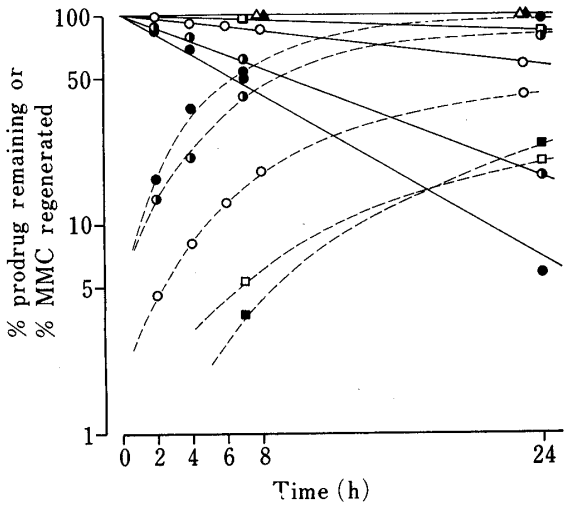



Fig. 1. Stability of 1a-*N*-Substituted MMC Derivatives in Rat Serum

The solid lines represent time-courses of degradation of derivatives and the dashed lines are for regeneration of MMC (I) from derivatives. ▲, II; ●, III; ◑, IV; ■, V; △, VI; □, VII; ○, VIII.

liposomes. Liposomes incorporating MMC proved impossible to prepare by the controlled dialysis method because MMC rapidly diffused out through the dialysis membranes during detergent removal.

**Hydrolysis in Rat Serum**

Hydrolysis of the derivatives was examined in fresh rat serum at 37 °C. The time-courses of removal of the compounds and appearance of MMC are shown in Fig. 1. The derivatives except for II and VI were removed at various rates and quantitative formation of the parent drug was observed simultaneously. As confirmed by HPLC, no peaks in the chromatograms

TABLE III. Degradation Rate Constants ($k_{obs}$) and Half-Lives ($t_{1/2}$) of 1a-*N*-Substituted MMC Derivatives in Rat Serum

| Compound | $k_{obs}$ ($h^{-1}$) | $t_{1/2}$ (h) | Compound | $k_{obs}$ ($h^{-1}$) | $t_{1/2}$ (h) |
|---|---|---|---|---|---|
| I | <0.0014 | >500 | V | 0.0091 | 76.2 |
| II | <0.0014 | >500 | VI | <0.0014 | >500 |
| III | 0.1216 | 5.7 | VII | 0.0082 | 83.6 |
| IV | 0.0745 | 9.3 | VIII | 0.0229 | 30.2 |

TABLE IV. Degradation Half-Lives of Derivatives III and IV in PBS and Various Sera

| Compound | Degradation half-life,[a] $t_{1/2}$ (h) | | | | |
|---|---|---|---|---|---|
| | PBS | Mouse serum | Rat serum | Human serum | Preheated rat serum[b] |
| III | 18.9 | 7.1 | 5.7 | 4.6 | 19.1 |
| IV | 10.9 | 8.3 | 9.3 | 4.9 | 9.9 |

*a*) Each value represents the mean of three separate experiments. Each S.D. value was less than 6%. *b*) Rat serum preheated at 60 °C for 1 h.

TABLE V. Effect of Liposome Entrapment on Stability of Derivatives III and IV in PBS, Rat Serum and Human Serum

| Compound | Form | Degradation half-life,[a] $t_{1/2}$ (h) | | |
|---|---|---|---|---|
| | | PBS | Rat serum | Human serum |
| III | Free | 18.9[b] | 5.7[b] | 4.6[b] |
| | Entrapped | 52.3 | 10.0 | 7.1 |
| IV | Free | 10.9[b] | 9.3[b] | 4.9[b] |
| | Entrapped | 35.9 | 17.3 | 10.1 |

*a*) Each value represents the mean of three separate experiments. Each S.D. value was less than 7%. *b*) Data from Table IV.

other than that corresponding to MMC appeared during the degradation of the derivatives. Regeneration of MMC may be due to the direct cleavage of the 1a-*N*-acyl linkage. The degradation of the derivatives followed apparent pseudo-first order kinetics with respect to concentration. Table III summarizes the observed pseudo-first order rate constants ($k_{obs}$) and half-lives ($t_{1/2}$) for the overall degradation of the compounds. The $k_{obs}$ values were calculated by linear regression analysis of semilogarithmic plots of drug concentration against time. Although compounds V, VII and VIII were hydrolyzed to the parent drug, their degradation rates were rather slow. Derivatives III and IV exhibited relatively rapid conversion to MMC compared with V, VII and VIII. Therefore, further study on the hydrolysis of III and IV was performed.

**Conversion Characteristics of Derivatives III and IV**

In order to investigate the susceptibility of compounds III and IV to enzymatic hydrolysis and species differences in the activity of serum samples for catalyzing the conversion of both derivatives, further stability tests were performed in PBS, mouse serum, human serum and preheated rat serum. Table IV summarizes the half-lives for hydrolysis of III and IV in various media. Differences in susceptibility to enzymatic hydrolysis were

observed between the two compounds. Derivative III was about 3—4 times more susceptible to hydrolysis in all fresh sera than in PBS. However, acceleration of hydrolysis was not observed in preheated (60 °C) rat serum. On the other hand, the conversion of derivative IV to MMC was only slightly accelerated in the presence of mouse or rat serum. Relatively rapid cleavage ($t_{1/2}=4.9$ h) was observed in human serum. No remarkable species differences were observed in the bioactivation of III.

**Effect of Liposome Encapsulation on Stability**

To investigate the effect of liposome incorporation on the hydrolysis of III and IV, the derivatives entrapped in liposomes were incubated at 37 °C with PBS and fresh sera. Apparent pseudo-first-order kinetics was observed for the hydrolysis of liposome-encapsulated compounds. Table V shows the half-lives for the hydrolysis of both derivatives in the liposome-entrapped form. The results for hydrolysis of the compounds in their free form are included for comparison. A stabilizing effect of liposomes was observed for the hydrolysis of both III and IV, and liposome entrapment increased the degradation half-lives of the compounds by a factor of about 2 to 3.

***In Vitro* Affinity Test**

The effect of lipophilic moieties on the affinity of the compounds for liposomes was studied *in vitro* by a gel filtration method. The elution of liposomes was monitored by determining NBD-PE (a liposomal membrane marker). Figure 2 illustrates typical elution patterns of liposome-entrapped cholesteryl (III) and acyl (VIII) derivatives on a Sepharose CL-4B column after incubation with PBS and fresh rat serum. After incubation with PBS, derivatives and lipid vesicles were eluted simultaneously in fractions near the void volume (Fig. 2 A, C). Almost all of the compounds were recovered in the liposome fractions. On the

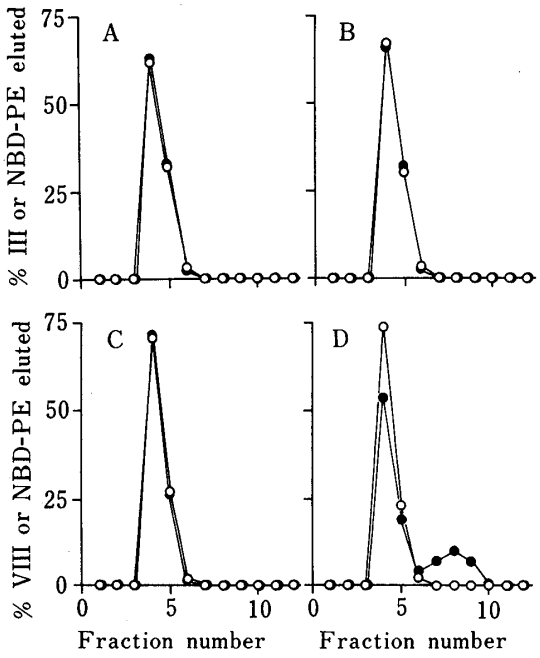



Fig. 2. Sepharose CL-4B Gel Filtration Patterns of Liposome-Entrapped Derivatives III and VIII after Incubation with PBS and Rat Serum

Liposomes entrapping III were incubated with PBS (A) and rat serum (B). Liposomes containing VIII were incubated with PBS (C) and rat serum (D). ●, derivative; ○, NBD-PE.

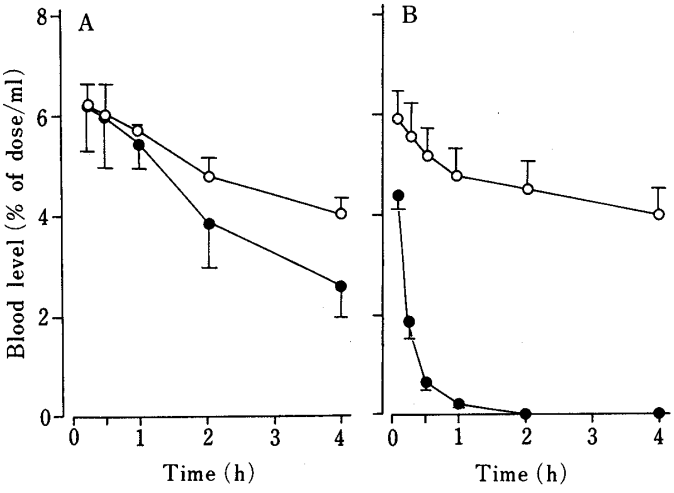



Fig. 3. Blood Levels of Derivatives III (A) and VIII (B) in Rats after Intravenous Injection in Liposome-Entrapped Form

●, derivative III or VIII; ○, NBD-PE (a liposomal membrane marker). Each point represents the mean ± S.D. of three rats.

other hand, after incubation with rat serum, derivative VIII was observed in not only the liposome fractions but also the lower-molecular fractions (Fig. 2D), while almost all of compound III was recovered in the liposome fractions (Fig. 2B). Similar elution profiles were observed for all other cholesteryl derivatives synthesized (data not shown).

***In Vivo* Affinity Test**

The affinity of the derivatives for lipid vesicles was investigated *in vivo*. The fate of liposomes was determined by monitoring NBD-PE. Figure 3 shows the blood levels of the derivatives (III and VIII) and NBD-PE after intravenous injection in the liposome-entrapped form into rats. Each drug concentration is expressed as a percent of dose/ml for comparison with liposome carriers. Compound III was cleared slowly from the circulation in a similar fashion to NBD-PE (Fig. 3A). All other derivatives possessing the cholesteryl moiety showed similar *in vivo* behavior to III (data not shown). In contrast to the cholesteryl derivatives, stearoyl MMC (VIII) disappeared rapidly from the circulation immediately after intravenous injection, becoming undetectable after 2 h, while liposomes remained in the circulation even 4 h after injection (Fig. 3B).

## Discussion

In the present study, lipophilic derivatives of MMC were synthesized by either acylation with stearic acid or introduction of the cholesteryl moiety through various spacers in an attempt to improve the applicability of MMC to intravenously injectable liposomal sustained-release carrier systems. Prodrugs suitable for these delivery systems had to satisfy at least the following three criteria: (1) high liposome entrapment efficiency, (2) adequate lability in various kinds of animal species and (3) firm association with liposome carriers in the systemic circulation.

Judging from the log $k'_0$ values (Table II) determined by HPLC, all the derivatives achieved a lipophilicity much higher than that of the original MMC. The derivatives having the cholesteryl moiety (compounds II—VII) showed relatively high log $k'_0$ values compared with stearoyl MMC (VIII). Almost complete entrapment of the derivatives tested in liposomes indicates that all the derivatives satisfy the first criterion.

In prodrug design, the structure of the spacer used for the linkage between the parent drug and carrier moiety is one of the most important factors that affect the physicochemical characteristics of a prodrug.[18] In fact, as shown in Table III and Fig. 1, the rates of hydrolysis of the cholesteryl derivatives varied widely with the spacer structures regardless of their relatively similar lipophilicity. In agreement with previous observations,[8c] compound II was stable in rat serum. These results demonstrated the feasibility of selecting lability properties by use of an appropriate spacer. Stearoyl MMC (VIII) was also converted to the parent drug in rat serum, but the rate of hydrolysis was relatively slow. 1a-*N*-Acyl derivatives showed decreased hydrolysis rates with increase of the substituent chain length from $C_{10}$ to $C_{18}$ (unpublished data).

Derivative III was hydrolyzed to MMC to some extent in PBS and its hydrolysis was accelerated in the presence of different sera. No acceleration was observed in the preheated rat serum (Table IV). This finding indicates that the cleavage of III occurs by not only chemical but also enzyme-mediated hydrolysis and that the enzymes which participate in the bioactivation are inactivated at 60 °C. On the other hand, in the case of compound IV, only a slight increase in hydrolysis was observed in the presence of mouse and rat sera (Table IV), suggesting that a significant fraction of its cleavage is due to chemical hydrolysis. Relatively rapid hydrolysis of IV was observed in human serum, implying the existence of specific enzyme(s) in the serum. Regardless of the hydrolysis mechanisms, derivatives III and IV were

regenerated to MMC at adequate rates in various kinds of sera, and these results reveal that these two compounds satisfy the second criterion. As shown in Table V, liposome-incorporated derivatives (III and IV) exhibited increased stability, suggesting that the liposomal membranes hinder both chemical and enzymatic attack against these prodrugs.

In systemic use of liposomal slow-release delivery systems, prodrugs might have to be firmly associated with liposome carriers in the circulation for the optimal sustained release of the active drug.[9] Retention of the derivatives within lipid vesicles is considered to be affected by the structures of the carrier moieties. As shown in Fig. 2D, some of VIII was eluted in non-liposome fractions after a 10 min incubation with rat serum. This finding suggests the possibility that derivative VIII might be removed from liposomes in the presence of serum. This possibility was confirmed by an *in vivo* affinity test. The observed difference in clearance behavior between VIII and liposome carriers (Fig. 3B) is considered to be due to rapid removal of the prodrug from lipid vesicles in the circulation. Similar removal phenomena have been observed for nonyloxycarbonyl MMC.[9] Serum components including albumin and high density lipoprotein might be responsible for the removal of VIII.[19] In contrast to VIII, compound III showed a similar elimination profile to liposomes under the same experimental conditions (Fig. 3A). *In vivo* behavior similar to that of III was observed for all other cholesteryl derivatives studied. These results reveal that cholesteryl derivatives seem to be firmly associated with lipid vesicles in the blood circulation. Therefore, the introduction of the cholesteryl moiety at the 1a-*N*-position of MMC proved useful for the firm retention of the derivatives within the liposome carriers, and all cholesteryl derivatives satisfy the third criterion.

On the basis of these results, compounds III and IV showed the best biopharmaceutical properties among the derivatives tested in meeting the requirements for prodrugs to be employed in intravenously injectable liposomal sustained-release delivery systems. Liposomes loaded with these prodrugs successfully maintained blood levels of MMC following intravenous injection. Detailed results will be published in subsequent papers.

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