Conversion Characteristics of the Conjugates of Mitomycin C with Estradiol and Estradiol Benzoate in Various pH Media

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Novel conjugates of mitomycin C (MMC) with 4-[3-benzoyloxy-1,3,5(10)-estratrien-17β-yloxycarbonyl]butyric acid (EB-glu) and 4-[3-hydroxy-1,3,5(10)-estratrien-17β-yloxycarbonyl]butyric acid (E-glu) at the 1a-N position, abbreviated as EB-glu–MMC and E-glu–MMC, respectively, were synthesized, and the kinetics on their decomposition and the MMC regeneration in various buffered solutions were investigated. EB-glu was synthesized by the reaction of estradiol benzoate (EB) and glutaric anhydride, and EB-glu–MMC was obtained by the condensation of MMC with EB-glu using *N,N'*-carbonyldiimidazole. E-glu was obtained by the alkaline hydrolysis of EB-glu, and E-glu–MMC was synthesized by the condensation of MMC with E-glu using *N,N'*-carbonyldiimidazole. The buffered solutions of pH 4, 6, 7, 7.4, 9 and 11 were used as buffered systems. In the stability study, EB-glu–MMC and E-glu–MMC were dissolved in a mixture of the buffer and propylene glycol (1:1, v/v) and incubated at 37 °C. E-glu–MMC and EB-glu–MMC were subjected to degradation at pH4, and further, their degradation became faster with the increase of pH at the basic condition. MMC was regenerated more easily with the increase of pH, and its regeneration was not detected at pH4. The analysis using the pseudo-first order kinetics elucidated the conversion rates. The direct conversion rate from EB-glu–MMC to MMC was found to be close to the regeneration rate from E-glu–MMC to MMC.

Key words conjugate; mitomycin C; estradiol; estradiol benzoate; stability study; conversion rate

Mitomycin C (MMC) is known as a strong and broadly effective antitumor agent. Many lipophilic[1–6] or macromolecular derivatives[7–13] of MMC have been developed in order to modify the pharmacodynamic or pharmacokinetic properties. They release MMC at their own regeneration rate. Some of them exhibit a passive targeting ability.[9,11,14] Some studies have been reported on active targeting systems,[15–17] which can be realized by using molecules showing specific binding to the target site. Drug delivery using hormone–drug conjugates is known as one of the ways for the active targeting.[18] Estradiol is an agent specific to the estrogen receptor. Several analogues of estradiol are effective as antiestrogenic agents against breast cancer.[19,20] Antiestrogenic action is known to be mediated through the estrogen receptor. In addition, the derivatives of an antitumor drug with estradiol or estradiol derivatives have been reported to improve the chemotherapeutic activity of the parent antitumor drug.[21,22] For example, Bestrabucil, which is a conjugate of chlorambucil with estradiol benzoate, showed a strong affinity to various tumors and exhibited a marked effect.[22] Since MMC is a very strong antitumor agent, it is suggested that the conjugate of MMC with estradiol or estradiol analogues should be of possible use in order to increase the chemotherapeutic activity or to permit MMC to move to the estrogen receptor-positive tumor cells specifically. In this study, the conjugates of MMC with estradiol benzoate and estradiol were synthesized, and their stability in the soluble form was investigated using mixtures of buffered solutions of various pH and propylene glycol (PG) as solvents.

Experimental

Materials MMC, supplied by Kyowa Hakko Kogyo Co. or its commercial product from the same company, Mitomycin Kyowa S, containing only NaCl with MMC was used. Estradiol benzoate (EB) and estradiol (E) were purchased from Wako Pure Chemical Industries, Ltd. All other chemicals used were of a reagent grade.

General Procedures UV spectra were recorded with a Beckman DU® 640 spectrophotometer. ^{1}H-NMR spectra were recorded with a JEOL JNM-GX270 spectrometer using tetramethylsilane (TMS) as a reference. The chemical shifts are reported in ppm on the δ scale from TMS. Electron impact (EI) mass spectra were obtained on a JEOL JMS-D300 spectrometer, and FAB mass spectra were obtained on a JEOL JMS-SX102 spectrometer. Silica Gel 60 F_{254} precoated plates (Art. 5715, Merck) were used in thin layer chromatography (TLC).

Synthesis of Conjugates of MMC with Estradiol Benzoate and Estradiol (EB-glu–MMC, E-glu–MMC) The synthetic procedure of conjugates of MMC with estradiol benzoate and estradiol, called EB-glu–MMC and E-glu–MMC, respectively, is shown in Fig. 1. First, glutaric acid was introduced at the 17β position of estradiol benzoate. The obtained ester, EB-glu, was used for the synthesis of EB-glu–MMC. The benzoyl ester of EB-glu was hydrolyzed to give estradiol having 4-carboxybutyryl group attached at 17β position, termed E-glu. E-glu was converted to E-glu–MMC.

4-[3-Benzoyloxy-1,3,5(10)-estratrien-17β-yloxycarbonyl]butyric Acid (EB-glu) Glutaric anhydride (7.6 g, 66.6 mmol) and triethylamine (4.9 ml) were added to dried pyridine (20 ml) containing estradiol benzoate (5.0 g, 13.3 mmol), and the mixture was stirred at 80 °C for 24 h. After the solvent was evaporated, water was added and the mixture was extracted with chloroform. The chloroform layer was dried on anhydrous sodium sulfate, and then the solvent was evaporated. The residue was recrystallized from methanol to give 3.79 g (yield 58.1%) of EB-glu. ^{1}H-NMR ($CDCl_3$) δ: 8.19 (2H, d, J=7.9 Hz, 2-, 6-H of benzoyl), 7.61 (1H, t, J=7.9 Hz, 4-H of benzoyl), 7.50 (2H, dd, J=7.9, 7.9 Hz, 3-, 5-H of benzoyl), 7.33 (1H, d, J=8.5 Hz, 1-H of 1,3,5(10)-estratrien-17β-yl), 6.92—7.00 (2H, m, 2-, 4-H of 1,3,5(10)-estratrien-17β-yl), 4.72 (1H, dd, J=9.5, 9.5 Hz, 17-H of 1,3,5(10)-estratrien-17β-yl), 2.84—2.92 (2H, m, 6-H_2 of 1,3,5(10)-estratrien-17β-yl), 1.24—2.49 (19H, m, $-COCH_2CH_2CH_2CO-$, 8-, 9-, 14-H of 1,3,5(10)-estratrien-17β-yl, 7-, 11-, 12-, 15-, 16-H_2 of 1,3,5(10)-estratrien-17β-yl), 0.84 (3H, s, 18-CH_3 of 1,3,5(10)-estratrien-17β-yl). EI-MS m/z: 490 (M^+).

1a-{4-[3-Benzoyloxy-1,3,5(10)-estratrien-17β-yloxycarbonyl]butyryl}-mitomycin C (EB-glu–MMC) EB-glu (734 mg, 1.5 mmol) and *N,N'*-carbonyldiimidazole (CDI) (243 mg, 1.5 mmol) were added to dried tetrahydrofuran (THF) (10 ml), and the mixture was stirred at 0 °C for 4 h. MMC (100 mg, 0.3 mmol) and dimethylaminopyridine (DMAP) (5 mg) were added, and the mixture was further stirred at room temperature for 4 d. After the solvent was evaporated, water was added and the mixture was extracted with chloroform. The chloroform layer

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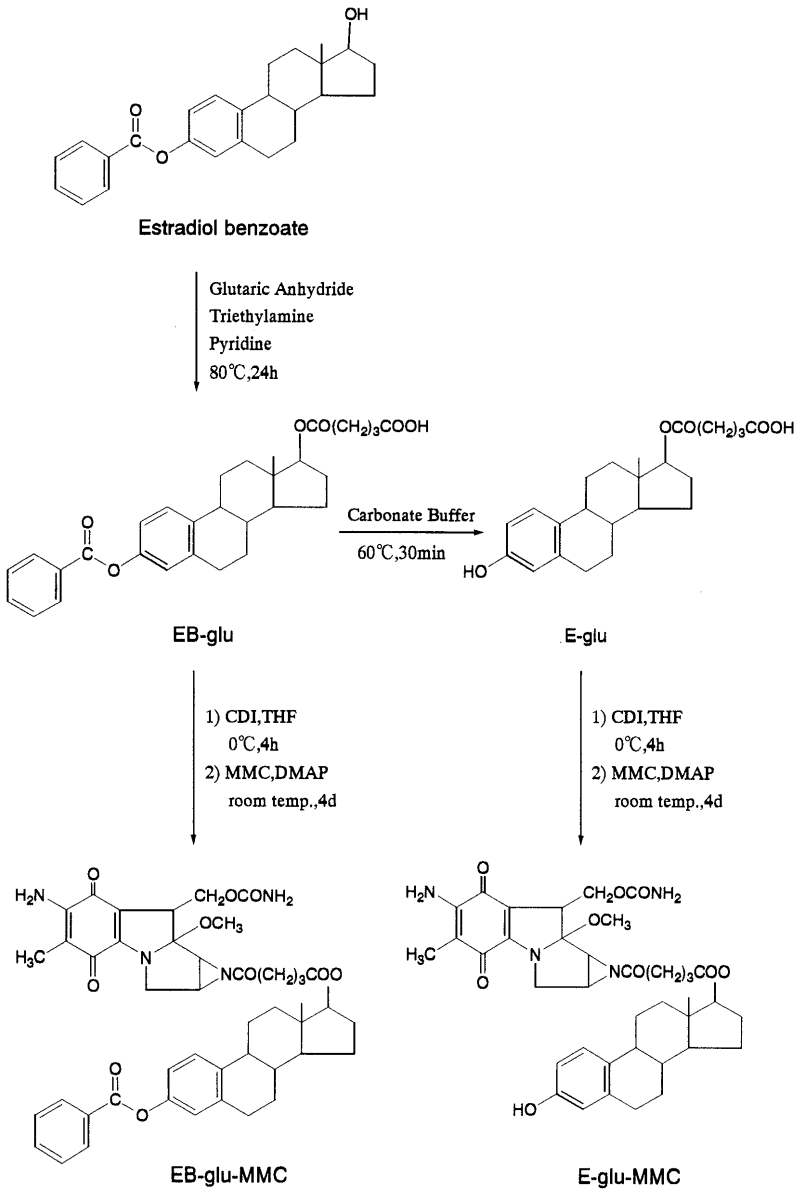

Fig. 1. Synthetic Procedures of EB-glu–MMC and E-glu–MMC

was washed with water, dried on anhydrous sodium sulfate and evaporated. After the resulting residue was dissolved in a small amount of chloroform, the solution was chromatographed on a silica gel column (2.5 × 25 cm; silica gel, 230—400 mesh from Merck) using the chloroform–methanol mixture as elution solvent. The fractions expected to be the product were collected, and the solvent was evaporated to give 84.1 mg (yield 34.8%) of EB-glu–MMC. UV λ_{max} (MeOH) nm (log ε): 356 (4.33). ^{1}H-NMR ($CDCl_3$) δ: 8.19 (2H, d, J=7.3 Hz, 2-, 6-H of benzoyl), 7.63 (1H, t, J=7.3 Hz, 4-H of benzoyl), 7.50 (2H, dd, J=7.3, 7.3 Hz, 3-, 5-H of benzoyl), 7.33 (1H, d, J=8.5 Hz, 1-H of 1,3,5(10)-estratrien-17β-yl), 6.92—6.99 (2H, m, 2-, 4-H of 1,3,5(10)-estratrien-17β-yl), 4.86—5.05 (3H, m, 10′-H of MMC, $-NH_2$), 4.68 (1H, dd, J=8.2, 8.2 Hz, 17-H of 1,3,5(10)-estratrien-17β-yl), 4.46 (1H, d, J=13.4 Hz, 3-H of MMC), 4.05 (1H, dd, J=11.0, 11.0 Hz, 10-H of MMC), 3.70 (1H, dd, J=4.9, 11.0 Hz, 9-H of MMC), 3.50—3.57 (2H, m, 1-, 3′-H of MMC), 3.28 (1H, dd, J=1.8, 4.5 Hz, 2-H of MMC), 3.20 (3H, s, $-OCH_3$), 2.80—2.94 (2H, m, 6-H_2 of 1,3,5(10)-estratrien-17β-yl), 1.26—2.63 (22H, m, $-COCH_2CH_2CH_2CO-$, 8-, 9-, 14-H of 1,3,5(10)-estratrien-17β-yl, 7-, 11-, 12-, 15-, 16-H_2 of 1,3,5(10)-estratrien-17β-yl, 6-CH_3 of MMC), 0.81 (3H, s, 18-CH_3 of 1,3,5(10)-estratrien-17β-yl). FAB-MS m/z: 808 (M+2).

4-[3-Hydroxy-1,3,5(10)-estratrien-17β-yloxycarbonyl]butyric Acid (E-glu) EB-glu (1.0 g) was added to a solution of carbonate buffer of pH 10.0 and methanol, and the mixture was stirred at 60 °C for 30 min. After evaporation of the solvent, water was added and the mixture was extracted with ether. The ether layer was dried on anhydrous sodium sulfate. Then, after evaporation of the solvent, the residue was dissolved in a small amount of chloroform, and chromatographed on a silica gel column (2.5 × 25 cm; silica gel, 230—400 mesh from Merck) using the chloroform–methanol mixture as elution solvent. The fractions expected to be the product were collected, and the solvent was evaporated. Thus, E-glu (0.45 g) was obtained (yield 57.1%). ^{1}H-NMR ($CDCl_3$) δ: 7.12 (1H, d, J=8.2 Hz, 1-H of 1,3,5(10)-estratrien-17β-yl), 6.62 (1H, d, J=8.2 Hz, 2-H of 1,3,5(10)-estratrien-17β-yl), 6.56 (1H, s, 4-H of 1,3,5(10)-estratrien-17β-yl), 4.69 (1H, dd, J= 7.9, 7.9 Hz, 17-H of 1,3,5(10)-estratrien-17β-yl), 2.72—2.90 (m, 2H, 6-H_2 of 1,3,5(10)-estratrien-17β-yl), 1.27—2.44 (19H, m, $-COCH_2CH_2CH_2CO-$, 8-, 9-, 14-H of 1,3,5(10)-estratrien-17β-yl, 7-, 11-, 12-, 15-, 16-H_2 of 1,3,5(10)-estratrien-17β-yl), 0.84 (3H, s, 18-CH_3 of 1,3,5(10)-estratrien-17β-yl). EI-MS m/z: 386 (M^+).

1a-{4-[3-Hydroxy-1,3,5(10)-estratrien-17β-yloxycarbonyl]butyryl}-mitomycin C (E-glu–MMC) E-glu (578 mg, 1.5 mmol) and CDI (242.5 mg, 1.5 mmol) were added to anhydrous THF (10 ml), and stirred at 0 °C for 4 h. MMC (100 mg, 0.3 mmol) and DMAP (5 mg) were added, and the reaction mixture was stirred at room temperature for 4 d. Then, after the solvent was evaporated, the residue was dissolved in a small amount of chloroform and the solution was chromatographed on a silica gel column (2.5 × 25 cm; silica gel, 230—400 mesh from Merck) using the chloroform–methanol mixture as elution solvent. Thus, E-glu–MMC

(58.7 mg) was obtained (yield 27.9%). UV λ_{max} (MeOH) nm (log ε): 356 (4.28). ^{1}H-NMR ($CDCl_3$) δ: 7.33 (1H, d, J=8.2 Hz, 1-H of 1,3,5(10)-estratrien-17β-yl), 6.63 (1H, d, J=8.2 Hz, 2-H of 1,3,5(10)-estratrien-17β-yl), 6.57 (1H, s, 4-H of 1,3,5(10)-estratrien-17β-yl), 4.85—4.92 (3H, m, 10′-H of MMC, $-NH_2$), 4.66 (1H, dd, J=8.2, 8.2 Hz, 17-H of 1,3,5(10)-estratrien-17β-yl), 4.45 (1H, d, J=13.4 Hz, 3-H of MMC), 4.06 (1H, dd, J=11.0, 11.0 Hz, 10-H of MMC), 3.71 (1H, dd, J=5.2, 11.0 Hz, 9-H of MMC), 3.49—3.57 (2H, m, 1-, 3′-H of MMC), 3.27 (1H, dd, J=1.8, 4.3 Hz, 2-H of MMC), 3.20 (3H, s, $-OCH_3$), 2.76—2.84 (2H, m, 6-H_2 of 1,3,5(10)-estratrien-17β-yl), 1.25—2.60 (22H, m, $-COCH_2CH_2-CH_2CO-$, 8-, 9-, 14-H of 1,3,5(10)-estratrien-17β-yl, 7-, 11-, 12-, 15-, 16-H_2 of 1,3,5(10)-estratrien-17β-yl, 6-CH_3 of MMC), 0.79 (3H, s, 18-CH_3 of 1,3,5(10)-estratrien-17β-yl). FAB-MS m/z: 704 (M+2).

Preparation of Media for Stability Studies One fifteenth M acetate buffer of pH 4.0, 1/15 M phosphate buffer of pH 6.0, 7.0 and 7.4 and 1/15 M carbonate buffer of pH 9.0 and 11.0 were prepared and the ionic stength (μ) of all the buffers was adjusted to 0.3 with NaCl. The resulting buffered solutions (μ=0.3) were used to prepare samples in the incubation study for the stability test.

Stability Tests EB-glu–MMC (2 mg) and E-glu–MMC (2 mg) were dissolved in 10 ml of PG, and each solution was diluted to 40 μg/ml with PG. To 5 ml of these solutions was added 5 ml of the buffered solution (μ=0.3) described above. The obtained solution was filtered out using a membrane filter (0.45 μm pore diameter). The filtrate (5 ml) was mixed with 2 ml of the mixture of the buffer and PG (1 : 1, v/v). The resulting solution was incubated at 37 °C. MMC was dissolved in the mixture of the buffer and PG (1 : 1, v/v) to obtain a solution with the concentration of 20 μg MMC/ml. This solution was incubated at 37 °C, during which time aliquot samples were withdrawn at appropriate times. The aliquot samples obtained in the incubation of MMC, E-glu–MMC and EB-glu–MMC were analyzed by high performance liquid chromatography (HPLC) on MMC, on MMC and E-glu–MMC, and on MMC, E-glu– MMC and EB-glu–MMC, respectively.

HPLC Analysis MMC, E-glu–MMC and EB-glu–MMC in each sample were determined by directly injecting the sample on HPLC. Briefly, HPLC analysis was done at room temperature using a Shimadzu LC-6A with a Shimadzu SPD-6A detector set at 364 nm. The reversed phase column, SUMIPAX Nucleosil 5C_{18}, (4 mm ϕ × 250 mm) connected with a guard column, Applied Biosystems RP-18 NEW GUARD 7 micron, (3.2 mm ϕ × 15 mm) was used as an analytical column. The mixtures of 10 mM phosphate buffer of pH 6.0 and methanol at ratios of 13 : 7, 1 : 9 and 1 : 4 (v/v) were used as mobile phases for the determination of MMC, EB-glu–MMC and E-glu–MMC, respectively. The flow rate was set at 0.6, 0.9 and 0.7 ml/min for the respective determinations. Each compound was determined using an absolute calibration curve method.

Results and Discussion

Conversion Profiles EB-glu–MMC and E-glu–MMC are quite water-insoluble but are soluble in 50% PG. Therefore, their stability and MMC regeneration from them were investigated in their soluble form in 50% PG. Figure 2 shows the degradation of MMC during its incubation. The degradation profiles and the MMC regeneration profiles for E-glu–MMC and EB-glu–MMC are shown in Figs. 3 and 4, respectively. E-glu–MMC and EB-glu–MMC decomposed at similar profiles following the pseudo-first order kinetics; in each degradation, the main regenerating compound was MMC. However, during the incubation of EB-glu–MMC, E-glu–MMC was found to be regenerated to a certain amount under some pH conditions. This indicated that the ester bond between benzoate and estradiol moieties was subjected to hydrolysis in the incubation. Considering the structure of each compound, the ester bond at the 17β position of the estradiol moiety might be cleft. This was checked by HPLC which enabled the detection of 1a-(4-carboxybutyryl) MMC (glu–MMC). Since glu–MMC was not observed during the incubation, MMC was considered to be re-

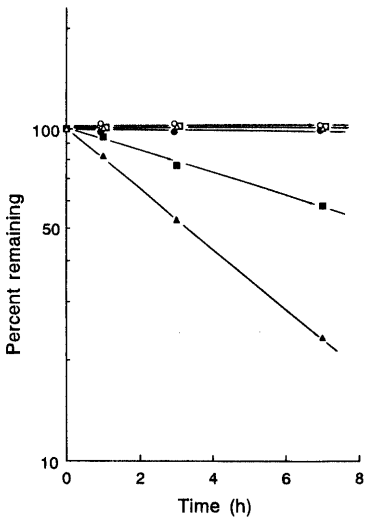

Fig. 2. Semi-logarithmic Plots for the Conversion of MMC during the Incubation of MMC in a Mixture of 1/15 M Phosphate Buffer of Various pH (μ=0.3)–PG (1 : 1, v/v) at 37 °C

▲, pH 4; △, pH 6; ○, pH 7; □, pH 7.4; ●, pH 9; ■, pH 11.

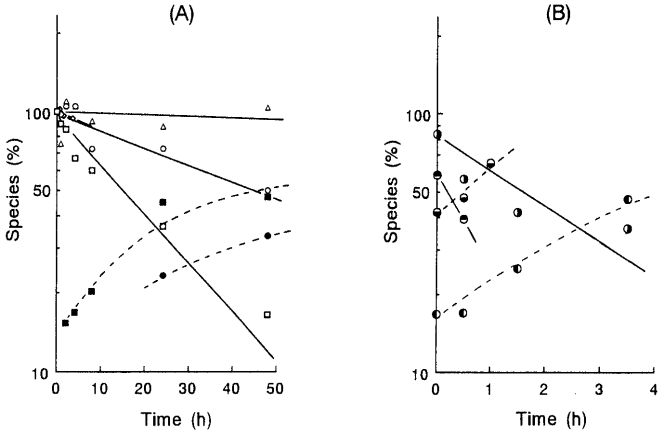

Fig. 3. Semi-logarithmic Plots for the Conversion of E-glu–MMC (——) and MMC (------) during the Incubation of E-glu–MMC in a Mixture of 1/15 M Phosphate Buffer of Various pH (μ=0.3)–PG (1 : 1, v/v) at 37 °C

(A) E-glu–MMC, pH 4 (◇), 6 (△), 7 (○), 7.4 (□); MMC, pH 7 (●), 7.4 (■). (B) E-glu–MMC, pH 9 (◑), 11 (◓); MMC, pH 9 (◐), 11 (◒).

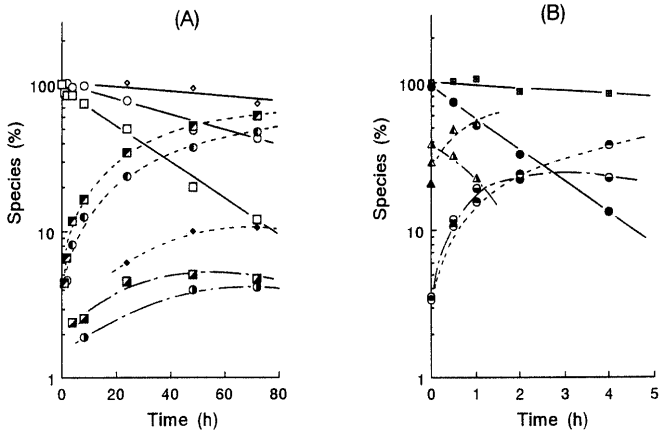

Fig. 4. Semi-logarithmic Plots for the Conversion of EB-glu–MMC (——), E-glu–MMC (—·—) and MMC (------) during the Incubation of EB-glu–MMC in a Mixture of 1/15 M Phosphate Buffer of Various pH (μ=0.3)–PG (1 : 1, v/v) at 37 °C

(A) EB-glu–MMC, pH 6 (◇), 7 (○), 7.4 (□); E-glu–MMC, pH 7 (◑), 7.4 (◪); MMC, pH 6 (◆), 7 (◐), 7.4 (◩). (B) EB-glu–MMC, pH 4 (⊞), 9 (●), 11 (▲); E-glu–MMC, pH 9 (◒), 11 (◭); MMC, pH 9 (◓), 11 (◮).

generated directly from EB-glu–MMC and E-glu–MMC. Under the conditions used, except at pH 4.0 and pH 11.0, MMC was very stable.

Kinetic Analysis The above degradation profiles of MMC, E-glu–MMC and EB-glu–MMC followed almost the pseudo-first order kinetics. In view of the detected compounds, the degradation or conversion schemes for MMC, E-glu–MMC and EB-glu–MMC may be proposed as shown in Fig. 5. The kinetic equations may be described using the pseudo-first order kinetic rate constants, k_1-k_6, in Fig. 5 as follows:

$$dM_a/dt = -k_1 M_a \quad (1)$$

This shows the kinetic equation based on scheme (A), and M_a is the concentration of MMC at time, t, in the incubation of MMC.

$$d(EGM_b)/dt = -(k_2+k_3)(EGM_b) \quad (2)$$
$$dM_b/dt = k_2 \times (EGM_a) - k_1 M_b \quad (3)$$

These are kinetic equations based on scheme (B), and EGM_b and M_b are the concentrations of E-glu–MMC and MMC at time, t, in the incubation of E-glu–MMC.

$$d(EBGM_c)/dt = -(k_4+k_5+k_6)(EBGM_c) \quad (4)$$
$$d(EGM_c)/dt = k_5 \times (EBGM_c) - (k_2+k_3)(EGM_c) \quad (5)$$
$$dM_c/dt = k_4 \times (EBGM_c) + k_2 \times (EGM_c) - k_1 M_c \quad (6)$$

These kinetic equations are based on scheme (C), and $EBGM_c$, EGM_c and M_c are the concentrations of EB-glu–MMC, E-glu–MMC and MMC at time, t, in the incubation of EB-glu–MMC.

The equations for the time–concentration profiles of MMC, E-glu–MMC and EB-glu–MMC in the incubation media were obtained by solving the above kinetic equations, and are shown in Table 1.

The rate constants were determined by fitting the profiles calculated from the equations in Table 1 to the observed curves by the least squares technique, in which the non-linear least squares program, MULTI,[23] was used. Namely, first, the k_1 value was obtained by the least squares technique for the decomposition profile of MMC in the incubation of MMC, when the initial estimate was obtained by graphic analysis. The obtained k_1 value was fixed in the following procedure. Next, the values of k_2 and k_3 were determined by the least squares technique for the profiles of E-glu–MMC degradation and MMC regeneration in the incubation of E-glu–MMC, when graphic analysis of the profile of E-glu–MMC degradation was utilized to obtain the initial estimates of k_2 and k_3. The obtained values of k_2 and k_3 as well as the k_1 value were fixed in fitting the calculated profiles to the data for the incubation of EB-glu–MMC. The values of k_4, k_5 and k_6 were determined by the least squares technique for the conversion profiles of EB-glu–MMC, E-glu–MMC and MMC in the incubation of EB-glu–MMC, when graphic analysis of the profile of EB-glu–MMC degradation was utilized to obtain the initial estimates of k_4, k_5 and k_6. The variable parameters were well converged in all cases except the incubation of EB-glu–MMC at pH 11.0. In the determination of k_4, k_5 and k_6 at pH 11.0, the value of k_6 was further fixed to 0, and then the values of k_4 and k_5 were calculated from the curve fitting by the least squares technique. Figure 6 shows the values of k_1-k_6 determined from the above procedures. The lack of symbols means that the rate constant at the corresponding pH is zero. MMC decomposed quickly at pH 4.0, while at pH 4.0 E-glu–MMC and EB-glu–MMC decomposed

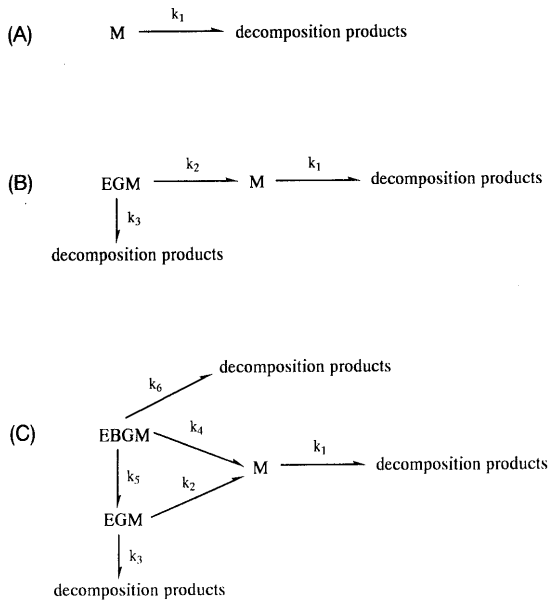

Fig. 5. Conversion Schemes for MMC (A), E-glu–MMC (B) and EB-glu–MMC (C)

M, MMC; EGM, E-glu–MMC; EBGM, E-glu–MMC. k_1–k_6 are pseudo-first order rate constants for the depicted reactions.

Table 1. Concentration Equations of MMC, E-glu–MMC and EB-glu–MMC Based on the Kinetic Models

Model[a]	Equation[b]
A	$M_c(t) = M_a(0) \times \exp(-k_1 t)$
B	$EGM_b(t) = EGM_b(0) \times \exp(-(k_2+k_3)t)$ $M_b(t) = (EGM_b(0) \times k_2/(k_1-(k_2+k_3))) \times (\exp(-(k_2+k_3)t) - \exp(-k_1 t)) + M_b(0) \times \exp(-k_1 t)$
C	$EBGM_c(t) = EBGM_c(0) \times \exp(-(k_4+k_5+k_6)t)$ $EGM_c(t) = (EBGM_c(0) \times k_5/((k_2+k_3)-(k_4+k_5+k_6))) \times (\exp(-(k_4+k_5+k_6)t) - \exp(-(k_2+k_3)t)) + EGM_c(0) \times \exp(-(k_2+k_3)t)$ $M_c(t) = h_1 \times (\exp(-(k_4+k_5+k_6)t) - \exp(-k_1 t)) - h_2 \times (\exp(-(k_2+k_3)t) - \exp(-k_1 t)) + h_3 \times (\exp(-(k_2+k_3)t) - \exp(-k_1 t)) + h_4 \times (\exp(-(k_4+k_5+k_6)t) - \exp(-k_1 t)) + M_c(0) \times \exp(-k_1 t)$

a) A, B and C correspond to the conversion models, A, B and C in Fig. 5, respectively. *b*) $M_a(t)$ means the concentration of MMC at time, t, for model A. $EGM_b(t)$ and $M_b(t)$ represent the concentrations of E-glu–MMC and MMC, respectively, at time, t, for model B. $EBGM_c(t)$, $EGM_c(t)$ and $M_c(t)$ mean the concentrations of EB-glu–MMC, E-glu–MMC and MMC, respectively, at time, t, for model C. $h_1 = EBGM_c(0) \times k_2 \times k_5/(((k_2+k_3)-(k_4+k_5+k_6))(k_1-(k_4+k_5+k_6)))$; $h_2 = EBGM_c(0) \times k_2 \times k_5/(((k_2+k_3)-(k_4+k_5+k_6))(k_1-(k_2+k_3)))$; $h_3 = EGM_c(0) \times k_2/(k_1-(k_2+k_3))$; $h_4 = EBGM_c(0) \times k_4/(k_1-(k_4+k_5+k_6))$.

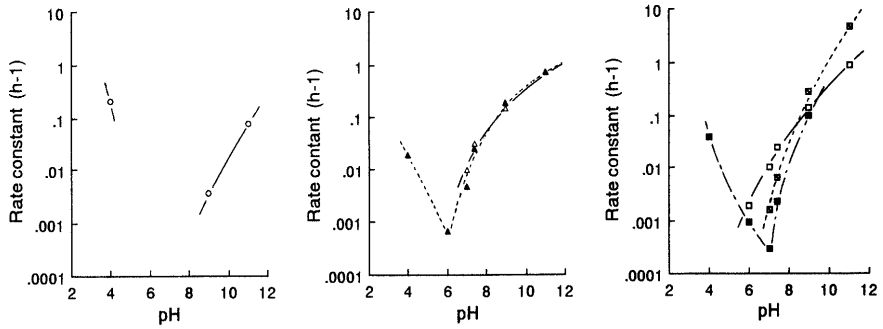

Fig. 6. pH Profiles of Rate Constants, k_1—k_6

k_1—k_6 are the same as shown in Fig. 5. k_1, ○; k_2, △; k_3, ▲; k_4, □; k_5, ⧅; k_6, ■.

directly without conversion to MMC at a much slower rate than MMC. The rate constant, k_4, for the direct regeneration from EB-glu–MMC to MMC was similar to the rate constant, k_2, from E-glu–MMC to MMC at the tested pH range. Every rate constant was very small at a very weakly acidic—neutral pH, and each compound was stable in this pH range. The pH-rate constant profiles suggested that the effect of pH on the conversion rate from EB-glu–MMC to E-glu–MMC tended to be greater than that of pH on the regeneration rates of MMC from both compounds at the neutral—weakly basic pH.

These experiments have clarified the stability characteristics of EB-glu–MMC and E-glu–MMC in the soluble form in an aqueous 50% PG solution of various pH. Since EB-glu–MMC and E-glu–MMC are water-insoluble, they must be dissolved in an aqueous solution such as the buffered solution containing 50% (v/v) PG described above if they are to be administered at the dosage form of solution. This *in vitro* stability study suggested that EB-glu–MMC and E-glu–MMC should be very stable in aqueous 50% (v/v) PG near neutral pH. Their drug release characteristics and their solubility in the body are believed to influence the antitumor effect. Their antitumor properties in soluble and suspended dosage forms will be determined in the following studies.

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