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SYNTHESIS AND CYTOTOXIC ACTIVITY OF OXIDIZED GALACTOMANNAN/ADR CONJUGATE

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ABSTRACT

Many polysaccharides are expected to apply as biomaterials because they generally show good biocompatibilities and biodegradabilities. It has recently been reported that the saccharides play important roles in biological recognition and the transmission of biological information on a cellar surface. Galactomannan (GalM) is a polysaccharide whose main chain is composed of β-1,4-linked mannose units only. It has some branching α-galactose residues at the C-6 position of mannose units. Therefore, it was of interest of us to use GalM as a drug carrier which was targeted to hepatocyte having a galactose receptor on its cellar surface. Dicarboxy-galactomannan (DC-GalM), which has reactive functional groups and is a carboxylic acid derivative of galactomannan, was prepared by IO^{4-}/ClO^{2-} oxidation of GalM. The obtained DC-GalM showed specific binding with maclura pomifera (MPA) [1] which has a specificity to α-galactose. Moreover, DC-GalM showed selective incorporation into hepatocyte. Adriamycine (ADR), which is one of the most prominent anticancer agents, was immobilized to DC-GalM. The DC-GalM/ADR conjugate showed specific cytotoxic activity against HepG2 human *hepatoma* cells which have a galactose receptor on the cell sur-

face, compared with Hela utrocervical *carcinoma* cells which have no galactose receptor.

INTRODUCTION

The receptor, which recognizes various biogenicities and species as an extrinsic factor, exists on the cell surface. The receptor which recognizes a specific saccharide is called a saccharide receptor. The liver parenchymal cells have a receptor which recognizes β-galactose, *N*-acetyl-β-galactosamine specifically [2]. Galactose was expected to apply as a targeting recognition factor because hepatocyte incorporated by endocytosis with recognition of these saccharides. Generally, the terminal saccharide is most important in saccharide recognition. Akaike et al. reported that a polystyrene derivative having lactose residues could recognize liver parenchymal cells, and there was the possibility that this polymer could be used as a cell culture matrix or liver-specific drug carrier [3, 4]. The technique of chemical syntheses of natural occurring branched saccharide chains leads to various sequences. Some natural polysaccharides have branched structures to a certain extent. The existence of a branched structure means that they have many terminal saccharide residues. The recognition ability of terminal residues of these branched polysaccharides invites attention. Galactomannan (GalM), such a branched polysaccharide, is a polymer of β-1,4-linked mannose, which has some monoresidual D-galactose at the C-6 position of D-mannose units in the main chain (Fig. 1). GalM is expected to exhibit a high affinity against liver parenchymal cells having the galactose receptor, which show specific binding with galactose, and are of interest for application as a liver-specific drug carrier. In this study we report the synthesis and cytotoxic activity of macromolecular prodrug immobilized ADR to GalM. In order to study the reactive functional groups which bind ADR, GalM was oxidized by IO^{4-}/ClO^{2-} oxidation to give DC-GalM which contains carboxylic acid groups. The interaction of lectin and DC-GalM was studied as a model reaction for biological recognition of branched polysaccharides by saccharide-specific receptors. In addition, the ability of DC-GalM to incorporate into hepatocyte was investigated. The conjugate of

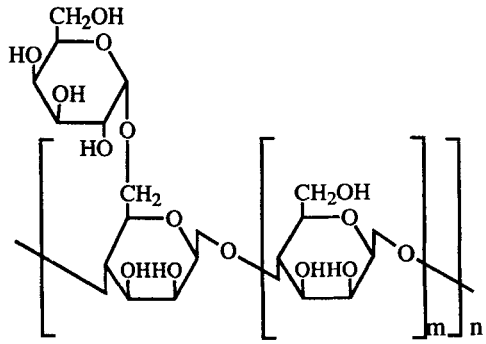


FIG. 1. Structure of galactomannan: $m = 1$ (guaran), $m = 2$ (taragalactomannan), $m = 3$ (carubin).

immobilized ADR to DC-GalM was synthesized. The DC-GalM/ADR conjugate obtained was evaluated for its specific cytotoxic activity against HepG2 human *hepatoma* compared with Hela utrocervical *carcinoma* cells, in vitro.

EXPERIMENTAL

Materials

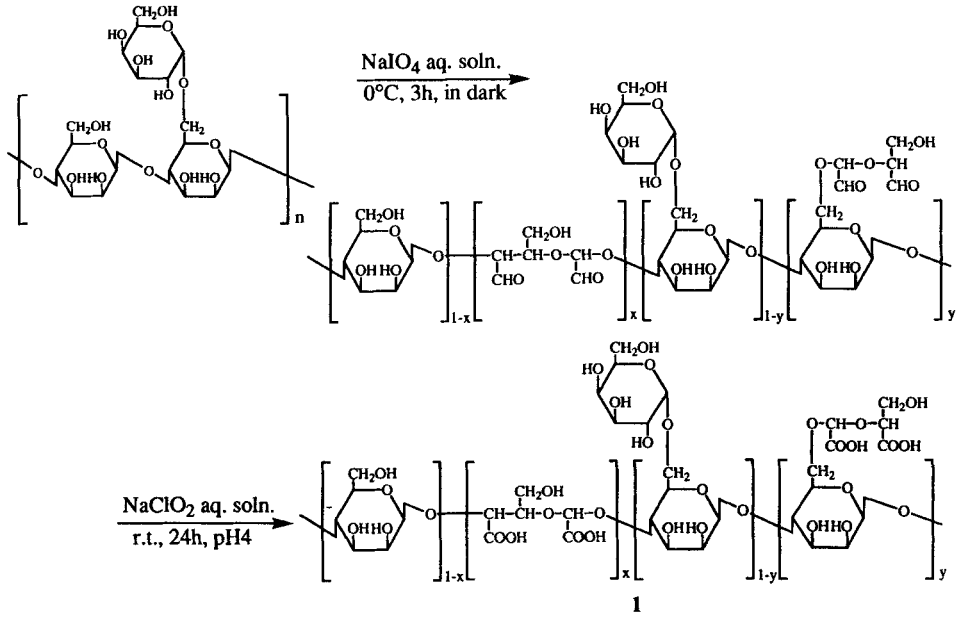
Galactomannan is divided into the following three main groups: guaran, tara-galactomannan, and carubin. Guaran ($m = 1$) from guarseeds was used as a galactomannan (GalM), and it was supplied by Taiyo Chemical Co. The content ratio of D-mannose to D-galactose of GalM was determined to be approximately 1/2.1 by gas chromatography analysis after acidic hydrolysis of GalM, which agrees fairly well with Ref. 5. Maclura pomifera (MPA) labeled with tetramethylrhodamine isothiocyanate (TRITC) was purchased from E·Y Laboratories, Inc. Adriamycine hydrochloride (ADR/HCl) was obtained from Meiji Seika Kaisha Ltd. *N,N'*-Dimethylformamide (DMF) and the other organic solvents were purified by the usual distillation methods. The other reagents were of commercial grade and used without further purification.

Tumor Cell Line

HepG2 human *hepatoma* and Hela utrocervical *carcinoma* cell line of solid tumor cell were maintained in Dulbecco's modified Eagle's minimum essential medium (D-MEM) containing 10% heat inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, and 18 mmol/L sodium bicarbonate.

Oxidation of GalM

Preparation of DC-GalM was carried out according to Ref. 6 with minor modifications, and is described in Scheme 1. GalM (3.0 g) was dissolved in 150.0 mL or 0.049 M aqueous sodium periodate and stirred at 0°C for 1 hour in the dark. The reaction solution was dialyzed in distilled water using cellulose tubing for 3 days and was freeze-dried to give dialdehyde-galactomannan in 2.2 g yield (73.8 wt%) as a white powder. Generation of the aldehyde group was confirmed by precipitation formation after treated with phenylhydrazine [7] and the appearance of the C=O group in IR spectra (1650 cm^{-1}) and ^{13}C-NMR spectra ($\delta = 185$ ppm). The obtained dialdehyde-galactomannan (1.5 g) was dissolved in 65 mL of 1.03 M aqueous sodium chlorite. The pH of the solution was adjusted to 4 by acetic acid. The reaction mixture was stirred at room temperature for 24 hours, and then nitrogen was passed through the solution until a colorless solution was obtained. The pH of the solution was raised to 9 with 1 N aqueous NaOH. The solution was dialyzed in distilled water for 7 days. The obtained solution, which was a sodium salt of DC-GalM, was passed through a column packed with a cation-exchange resin (Amberlite 120B H^+ type) and freeze-dried to give DC-GalM in 1.40 g yield (93.7 wt%) as a white powder. The degree of introduction of the carboxylic acid group (DCA) as measured by a neutralization titration method [8] was 44 mol%/sugar unit, which meant the molar content of the opened ring was 22 mol%/sugar unit.



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SCHEME 1.

The number-average molecular weight (M_n) of the DC-GalM was determined to be 1.6×10^4, and the ratio of the weight-average molecular weight (M_w) to M_n was determined to be 1.5 by gel-permeation chromatography (GPC). The content ratio of D-mannose to D-galactose of DC-GalM was determined by gas-chromatography analysis after acidic hydrolysis of galactomannan. DC-GalM (30 mg) was dissolved in 1 N HCl, and the solution was refluxed with stirring at 100°C to afford monomeric mannose and galactose. The reaction mixture was evaporated. The dried sample was dissolved in water and then freeze-dried to give the monosaccharide of DC-GalM. The obtained monosaccharide (10 mg) was dissolved in 1 mL pyridine, and hexamethyldisilazane (0.2 mL) and trimethylchlorosilane (0.1 mL) [9] were added to the solution. The solution was stirred vigorously for 1 minute and allowed to stand for 10 minutes. The supernatant solution was analyzed by gas chromatography. The following spectral date of DC-GalM confirmed the structure. IR (KBr disk): absorptions at 3363 (OH), 2934 (CH_2), 1736 (COOH), and 1027 cm^{-1} (C—O—C). ^{13}C-NMR (D_2O): δ 62.2–64.5 (CH_2), 70.2–77.5 (CHOH, $CH(CH_2O)$), 100.2–104.1 (OCHO), and 172.6–178 ppm (COOH).

Measurement of Interaction of DC-GalM with Lectin

The interaction of DC-GalM with Maclura pomifera (MPA) was investigated by the fluorescence depolarization method [10]. MPA-labeled TRITC (50 μL) dissolved in 0.01 M phosphate buffer solution (2950 μL, pH 7.4) was added to the cuvette. The cuvette was placed in a thermostated spectrophotometer with a polarizer and analyzer at 37°C, and the fluorescence depolarization index, $1/P$ value, was measured (excitation at 550 nm and emission at 573 nm) every 1 minute. After

3 minutes the 0.01 M phosphate buffer solution (200 μL, 5 mg/mL) of DC-GalM was added and the fluorescence depolarization index was measured. After a further 3 minutes the 0.01 M phosphate buffer solution (200 μL) of D-galactose was added and the fluorescence depolarization index was again measured.

Measurement of Cytotoxicity of DC-GalM

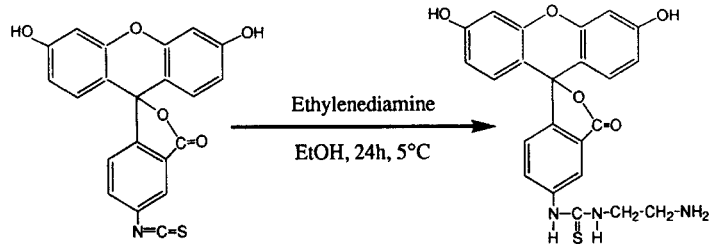
The in vitro cytotoxicity of DC-GalM against Hela utrocervical *carcinoma* cells was measured according to methods described previously [11]. The tumor cell suspension (200 μL) containing 5.0×10^4 cells in a culture medium containing 10% FCS was distributed in a 96-wells multiplate (Corning 25860MP) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 48 hours. The culture medium containing 10% FCS (150 μL) was exchanged, and then the PBS solution (50 μL) with DC-GalM was distributed and incubated in the same condition at 37°C for 48 hours. The number of viable cells was determined by means of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] enzyme assay [12] using a microplate reader (MTP-120, Corona Electric Co.). The cytotoxicity was calculated by the following equation:

$$\text{Cytotoxicity (in \%)} = (C - T)/C \times 100$$

where C = number of viable cells after 48 hours incubation without DC-GalM
T = number of viable cells after 48 hours incubation with DC-GalM

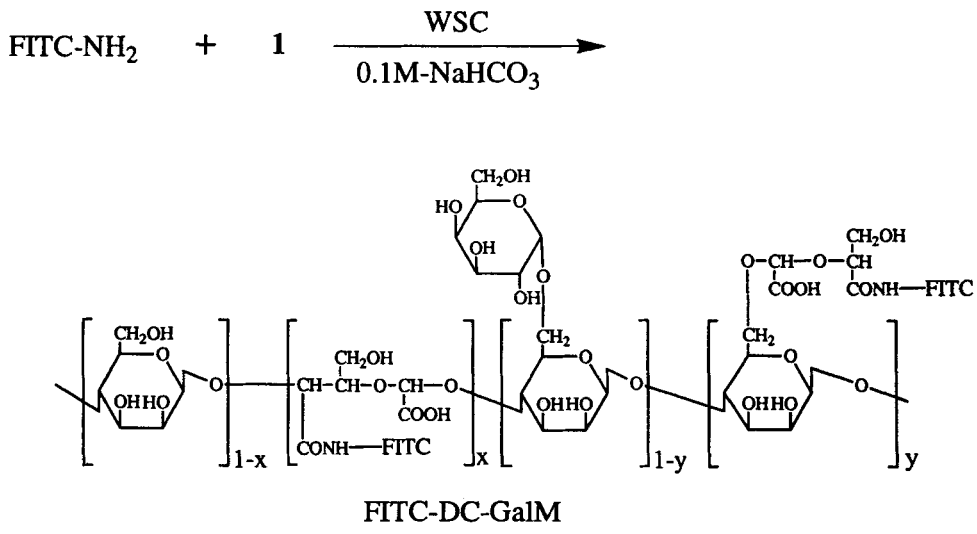
Measurement of Incorporation into Cells of DC-GalM

The incorporation into a cell of DC-GalM was measured by fluorescence measurement with DC-GalM-labeled fluorescence isothiocyanate (FITC). Synthesis of FITC-DC-GalM was carried out according to Schemes 2 and 3. FITC (200 mg) was suspended in 2 mL of ethanol, and an ethanol solution (240 mL) of ethylenediamine (ED) (17.4 mL) was added to the solution and stirred at 5°C for 24 hours. Then the excess ED and ethanol were removed in vacuo. The residue was dissolved in methanol and reprecipitated into diethylether. The precipitate was dissolved in water and freeze-dried to give FITC-ED in 258 mg yield. DC-GalM (70 mg) was dissolved in 0.1 M sodium hydrogen carbonate aqueous solution ($NaHCO_3$ aqueous solution), and FITC-ED (30 mg) was added to the ice-cooled solution, followed by



Fluorescence isothiocyanate(FITC) FITC-ED

SCHEME 2.



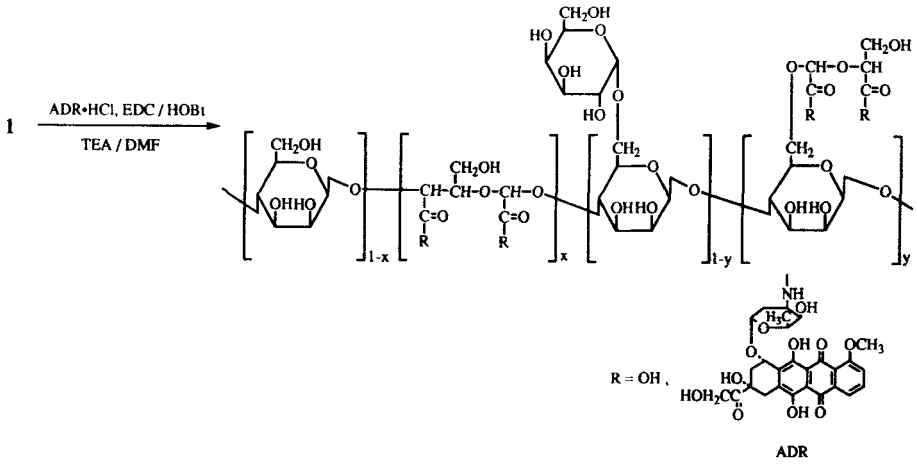
FITC-DC-GalM

SCHEME 3.

water-soluble carbodiimide (WSC) (18.6 mg), and stirred at 0°C for 2 hours and then at 35°C for 22 hours. The reaction mixture was reprecipitated into methanol and dialyzed in distilled water. Then the solution was freeze-dried to give FITC-DC-GalM in 64 mg yield. The degree of introduction of FITC in mol% per sugar unit (DFITC) of FITC-DC-GalM was 1.17, which was estimated from UV absorbance at 491 nm in 0.1 M $NaHCO_3$ aqueous solution using the ϵ_{491} value (7.8×10^4 $mol^{-1} \cdot dm^3 \cdot cm^{-1}$) of FITC as a standard. The incorporation of DC-GalM was measured against HepG2 and HLE human *hepatoma* cells in vitro. A tumor cell suspension (1 mL) containing 5.0×10^4 cells in a culture medium containing 10% FCS was distributed in a 24-wells multiplate and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 48 hours. A culture medium containing 10% FCS (900 mL) was exchanged, then the PBS solution (50 μL, 30 μg/mL) with FITC-DC-GalM was distributed, and the PBS solution of galactose or mannose was added to each well to give a concentration of 1000 μg/mL per well. Moreover, the PBS solution (50 μL, 30 μg/mL) with FITC-DC-GalM was distributed and incubated in the same condition at 37°C for 24 hours. After centrifuging, the supernatant solution (200 μL) of culture medium was taken out, and PBS (2 mL, pH 7.4) was added to the solution. The solution was added to the cuvette. The cuvette was placed in a thermostated spectrophotometer with a polarizer and analyzer at 37°C, and the fluorescence was measured (excitation at 493 nm and emission at 518 nm) to give the quantity of FITC-DC-GalM incorporated into the cells.

Synthesis of DC-GalM/ADR Conjugate

Synthesis of DC-GalM/ADR conjugate was carried out according to Scheme 4. DC-GalM (50 mg, opened ring = 22%) was dissolved in a small amount of water, diluted with dimethylformamide (DMF), and then concentrated in vacuo. Water-soluble carbodiimide (WSC) (78.1 mg) and hydroxybenzotriazole (HOBt)



SCHEME 4.

(55.1 mg) dissolved in DMF were added to the ice-cooled solution. Then ADR (10 mg) dissolved in 2 mL of DMF (which had been kept in the dark) and 3.51 μL of triethylamine (TEA) were mixed. The solution was added dropwise to the solution containing DC-GalM. The reaction mixture was stirred and kept in the dark at 0°C for 2 hours and then at room temperature for 12 hours. After evaporation of solvents, the residue was subjected to gel-filtration chromatography (column: Sephadex LH-20, 1.0 × 100 cm, eluent: DMF). The high-molecular weight fraction was isolated and evaporated. The red solid obtained was dissolved in water and freeze-dried to give a DC-GalM/ADR conjugate. The characterization of the obtained conjugate was carried out by measurement of UV, IR, TLC (solution: 1-butanol-acetic acid-water (4:1:1, v/v), R_f = 0.0) and HPLC analysis. The degree of introduction of ADR in mol% per sugar unit (DADR) of the conjugate was estimated from UV absorbance at 495 nm in water using the ϵ_{495} value (11700 $mol^{-1} \cdot dm^3 \cdot cm^{-1}$) of ADR/HCl as the standard. Yield: 45.0 mg. IR (KBr): 3398 (OH), 1632 (CONH), 1585 (C=O, quinone), 1263, 1020, 873 cm^{-1} (aromatic ring of ADR).

Measurement of Cytotoxicity of the Conjugate

The cytotoxicity was evaluated by two methods.

The cytotoxicity of the conjugate was measured against HepG2 human *hepatoma* or Hela utrocervical *carcinoma* cells in vitro. The tumor cell suspension (100 μL) containing 5.0 × 10^4 cells in a culture medium containing 10% FCS was distributed in a 96-wells multiplate (Corning 25860MP) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 48 hours. The culture medium containing 10% FCS (100 μL) was exchanged, and then the PBS solution (20 μL) with conjugate and free ADR were distributed and incubated in the same condition at 37°C for 48 hours. The number of viable cells was determined by means of MTT assay [12] using a microplate reader (MTP-120, Corona Electic Co.). The cytotoxicity was calculated by the following equation:

$$\text{Cytotoxicity}\,(\%) = (C - T)/C \times 100$$

where C = number of viable cells after 48 hours incubation without drug
T = number of viable cells after 48 hours incubation with drug

Moreover, the inhibition effect by galactose addition on the cytotoxic activity of the conjugate and free ADR against HepG2 human *hepatoma* or Hela utrocervical *carcinoma* cells was measured in vitro. The tumor cell suspension (100 μL) containing 5.0×10^4 cells in a culture medium containing 10% FCS was distributed in a 96-wells multiplate (Corning 25860MP) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 48 hours. The culture medium containing 10% FCS (100 μL) was exchanged, and then the PBS solution (20 μL) with conjugate and galactose (3.0×10^{-4} mol/L and 3.6×10^{-2} mol/L, respectively) were distributed and incubated at 4°C for 2.5 hours. Then the cells were washed with a culture medium containing 10% FCS, and the culture medium containing 10% FCS was distributed and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 48 hours. The number of viable cells was determined by means of MTT assay [12] using a microplate reader (MTP-120, Corona Electric Co.). The cytotoxicity was calculated by the above equation.

Measurement

Molecular weight of polymers was measured by gel-permeation chromatography (GPC) (column: Shodex OHpack SB-803, Showa Denko K. K., eluent: 1/15 M phosphate buffer, standard: pullulan). IR spectra were measured on a Perkin-Elmer 1600 Series FT-IR spectrometer. ^{1}H- and ^{13}C-NMR spectra were measured with a Jeol GSX-400 spectrometer using DSS as the reference.

RESULTS AND DISCUSSION

Preparation of DC-GalM

We prepared DC-GalM by means of IO^{4-}/ClO^{2-} oxidation of galactomannan. The results of the IO^{4-}/ClO^{2-} oxidation of galactomannan are shown in Table 1. DCA values could be controlled by varying the concentration of sodium periodate at the first reaction step. The M_n of the DC-GalM was about 1.6×10^4. When the reaction time of the first step was longer (3 hours), DC-GalM with a high DCA value was obtained. However, its M_n became relatively low. This is because the degradation reaction was dependent on the reaction time. When the concentration of sodium periodate was high, units of both mannose and galactose of GalM were oxidized nonselectively to afford DC-GalM with a relatively large ratio of M_w to M_n. On the contrary, when the concentration of sodium periodate was low, the mannose units of GalM were oxidized selectively to give DC-GalM with a relatively small ratio of M_w to M_n.

Binding of DC-GalM with MPA

Some kinds of cell are known to have receptors for specific saccharides. It was expected that DC-GalM would have an affinity for saccharide-receptor positive cells because it has branched galactose. We therefore investigated the interaction of

TABLE 1. Synthesis of Dicarboxy-galactomannan (DC-GalM)

	First reaction step[a]			Second reaction step[b]				
Run	Time, h	Concentration of $NaIO_4$, mol/L	Yield, wt%	Yield, wt%	DCA,[c] mol%/ sugar unit	M_n[d]	M_w/M_n[d]	Man/Gal[e]
1	1	0.528	71.3	89.2	44	1.12×10^4	3.67	2.1/1
2	1	0.100	64.5	93.5	42	1.31×10^4	1.98	1.7/1
3	1	0.084	60.5	91.3	30	1.34×10^4	2.09	1.8/1
4	1	0.042	66.0	96.5	12	1.17×10^4	2.04	2.0/1
5	3	0.049	73.8	93.7	42	1.61×10^4	1.58	1.3/1

[a]Oxidation of GalM to dialdehyde-GalM by $NaIO_4$ aqueous solution.
[b]Oxidation of dialdehyde-GalM to DC-GalM by $NaClO_2$ aqueous solution.
[c]Degree of introduction of carboxylic acid group per sugar unit.
[d]Estimated by GPC.
[e]Molar ratio of mannose to galactose estimated by gas chromatography.

DC-GalM with a lectin (MPA) as a model of interaction of DC-GalM with galactose-receptor positive cells. The measurement of binding DC-GalM with MPA was observed by the fluorescence depolarization method using MPA labeled with TRITC. The fluorescence depolarization index, $1/P$, was given by the following equations:

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$$

$$1/P = 1/P_0 + (1/P_0 - 1/3) \cdot RT\tau/V \cdot \eta$$

$I_{\parallel}$ = fluorescence intensity in parallel direction, $I_{\perp}$ = fluorescence intensity in perpendicular direction, P = observed value of fluorescence polarization, P_0 = a constant (maximal value of P obtained in a rigid medium, i.e., P extrapolated to $T/\eta = 0$), R = gas constant, T = absolute temperature, η = viscosity (poise), τ = relaxation time of fluorescence excitation(s), V = molecular volume.

The increase of molecular volume (i.e., molecular weight) should be observed as the decrease of $1/P$. If DC-GalM can bind with MPA labeled with TRITC, the $1/P$ value of TRITC should be decreased. The results of interaction of DC-GalM with MPA are shown in Fig. 2. DC-GalM could bind specifically with MPA lectin, and it was suggested from the results in vitro that a part of them was incorporated via the saccharide receptor into the cell.

The Cytotoxicity of DC-GalM

The cytotoxicity of the DC-GalM was investigated against Hela utrocervical *carcinoma* cells in vitro and compared with GalM. The results are shown in Fig. 3. DC-GalM showed low cytotoxicity in each of its concentration. Any difference in opened ring values of galactomannan had no effect on the cytotoxicity. The cytotoxicity of DC-GalM was found to increase slightly compared with that of galactomannan.

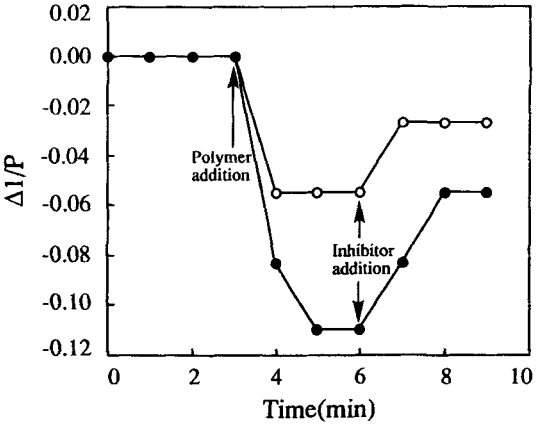


FIG. 2. Interaction DC-GalM with MPA monitored by fluorescence depolarization index ($1/P$). (●) GalM, (○) DC-GalM; molar content of opened ring (OP) = 15 mol%/sugar unit.

The Incorporation into Cells of DC-GalM

FITC-DC-GalM in which a minimum of more than one FITC unit per sugar chain was introduced was synthesized according to the reaction steps shown in Schemes 2 and 3. The results of incorporation of DC-GalM into HepG2 and Hela cells are shown in Fig. 4. The incorporation of DC-GalM into cells was decreased by the addition of galactose as an inhibitor. On the other hand, the addition of mannose had no effect on the incorporation of DC-GalM into cells. Therefore, it was considered that a part of DC-GalM was incorporated into hepatocyte cells through galactose-receptor-mediated endocytosis.

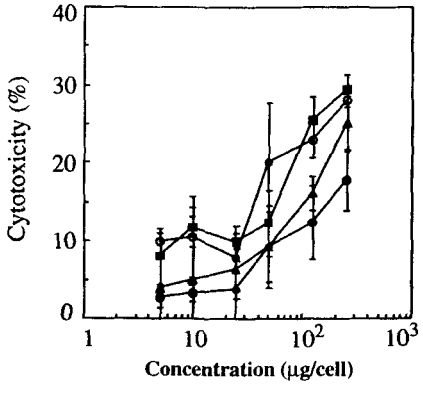


FIG. 3. Cytotoxicity of DC-GalM against Hela human utrocervical *carcinoma* cells in vitro. (●) GalM, (○) DC-GalM; OP = 6 mol%/sugar unit, (▲) DC-GalM; OP = 15 mol%/sugar unit, (■) DC-GalM; OP = 21 mol%/sugar unit.

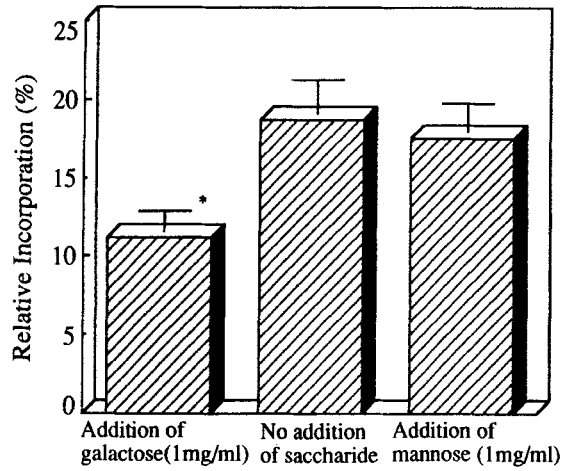


FIG. 4. Incorporation of FITC-labeled DC-GalM into Hep G2 human *hepatoma* cells after 24 hours of incubation in vitro. The asterisk indicates $P < .05$ by Student's t test.

Synthesis of DC-GalM/ADR Conjugate

GalM/ADR conjugate was synthesized according to the reaction shown in Scheme 4. The coupling reaction of the amino group of the daunosamine sugar moiety of ADR and the carboxyl group of DC-GalM was carried out by using the WSC/HOBt method. The formation of conjugate was monitored by TLC. Free ADR showed a red spot at $R_f = 0.3$, but in the case of the conjugate the red spot due to the anthracycline ring of ADR did not move from the starting point ($R_f = 0.0$). The conjugate was easily separated from nonbound free ADR and adriamycinon, which is the degradation product of ADR, by gel-filtration chromatography. A typical elution profile of gel-filtration chromatography (Sephadex LH-20, DMF) monitored by UV at 495 nm for the reaction mixture of conjugate is shown in Fig. 5. Fractions 1, 2, and 3 in Fig. 5 were attributed to the conjugate, ADR, and adriamycinon, respectively. As shown in Fig. 6, the shape of the UV absorption spectrum for ADR residues of the obtained conjugate was the same as that for free ADR. This means that ADR was not impaired through the coupling reaction and purification. The purity of the conjugate was also confirmed by HPLC analysis [column: TSK gel ODS-120T, eluent: acetonitrile/0.01 M $NH_4H_2PO_4$ aqueous solution, 65:35 (v/v), fluorescence detector with excitation at 480 nm and emission at 590 nm]. The HPLC elution profile for the conjugate showed a single peak. The degree of introduction of ADR in mol% per sugar unit (DADR) of the conjugate was 3.1 mol%.

Cytotoxic Activity of the Conjugate in Vitro

The cytotoxic activity of the DC-GalM/ADR conjugate was investigated against HepG2 or Hela cells in vitro compared with free ADR. The result is shown in Fig. 7. The cytotoxic activity of the DC-GalM/ADR conjugate was very low compared with that of free ADR. This is because of the difference of incorporation

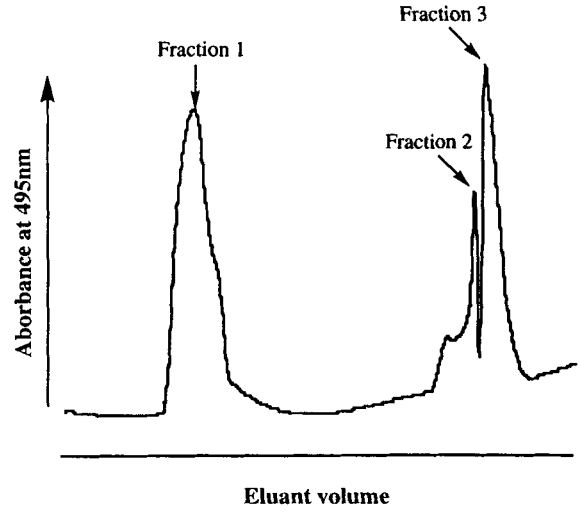


FIG. 5. Elution profile of gel-filtration chromatography for the reaction mixture of ADR/HCl and DC-GalM monitored by UV absorbance at 495 nm (column: Sephadex LH-20, 1 × 100 cm; eluent: DMF).

into the cells of the drugs. It is believed that free ADR is incorporated into cells by diffusion, while the conjugate is taken up through endocytosis.

The Inhibition Effect by Galactose Addition on the Cytotoxic Activity of the Conjugate

The cytotoxic activity of the conjugate in the absence or presence of galactose against HepG2 or Hela cells compared with free ADR is shown in Figs. 8 and 9. The cytotoxic activity of free ADR was about the same in the absence and presence of

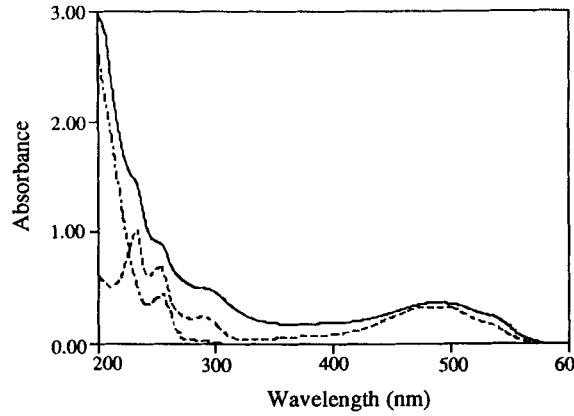


FIG. 6. UV/VIS spectra of DC-GalM (— · —), ADR/HCl (- - -), and DC-GalM/ADR conjugate (——) in water.

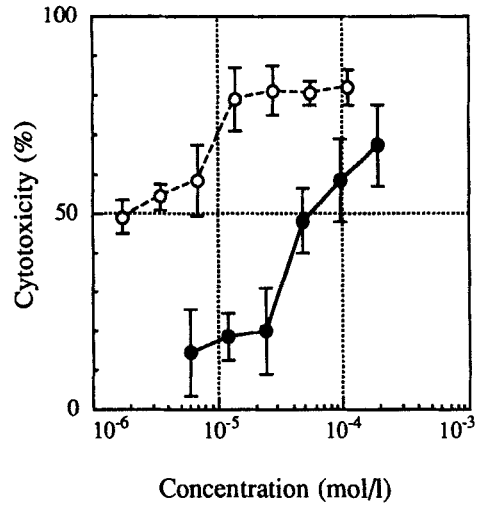


FIG. 7. Cytotoxicity of DC-GalM/ADR conjugate and free ADR against HepG2 cells after 48 hours of incubation in vitro. (○) ADR/HCl, (●) DC-GalM/ADR conjugate.

galactose. On the other hand, the cytotoxic activity of the conjugate decreased with the addition of galactose. Moreover, the cytotoxic activity of the conjugate against Hela cells (which do not have a galactose receptor) was at about the same level in the absence and presence of galactose, while that against HepG2 cells was decreased by the addition of galactose. These results suggest that the conjugate is incorporated into cells through receptor-mediated endocytosis. These results indicate that guaran (GalM), which is a sort of galactomannan, will find useful application as a liver-specific drug carrier.

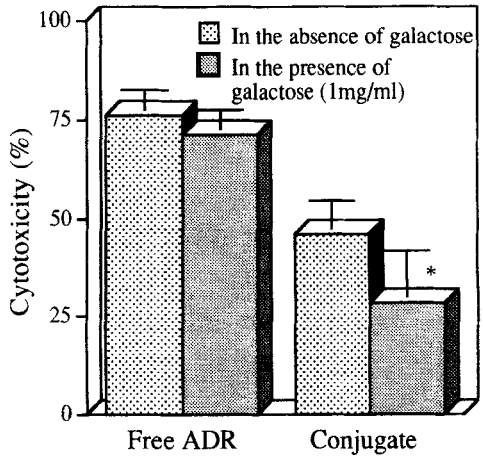


FIG. 8. Effect of the addition of galactose on the cytotoxicity of free ADR and DC-GalM/ADR conjugate against HepG2 cells for 2.5 hours in vitro. [ADR] = 5.0×10^{-5} mol/L. The asterisk indicates $P < .02$ by Student's t test.

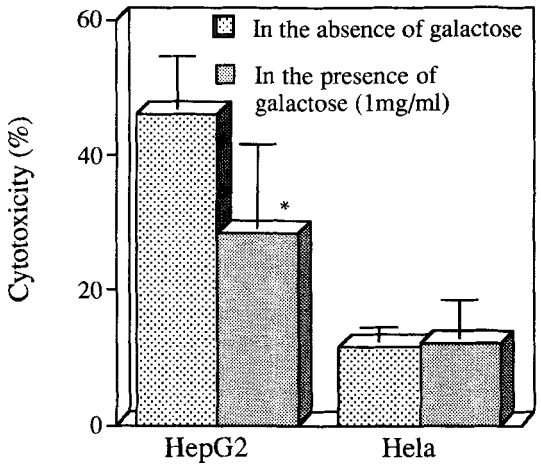


FIG. 9. Effect of the addition of galactose on the cytotoxicity of DC-GalM/ADR conjugate against HepG2 and Hela cells in vitro. Incubated at 4°C for 2.5 hours with conjugate in the absence or presence of free galactose. [ADR] = 5.0×10^{-5} mol/L. The asterisk indicates $P < .02$ by Student's t test.

CONCLUSIONS

Dicarboxy-galactomannan (DC-GalM) was prepared by IO^{4-}/ClO^{2-} oxidation of guaran. DC-GalM showed the specific binding with maclura pomifera (MPA) which has a specificity to α-galactose and selective incorporation into hepatocyte. The DC-GalM/ADR conjugate showed specific cytotoxic activity against HepG2 human *hepatoma* cells having galactose receptors on the cellular surface.

Thus, guaran was concluded to be suitable as a liver-specific drug carrier.

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REFERENCES

[1] P. Z. Allen, *Infect. Immunol., 47*, 90 (1985).
[2] R. L. Hudgin and G. Ashwell, *J. Biol. Chem., 249*, 7369 (1974).
[3] T. Akaike, A. Kobayashi, K. Kobayashi, and H. Sumitomo, *J. Bioact. Compat. Polym., 4*, 51 (1989).
[4] M. Goto, A. Kobayashi, K. Kobayashi, K. Saito, and T. Akaike, *Drug Deliv. Syst. (Jpn), 7*, 173 (1992).
[5] R. L. Whistler and D. F. Durso, *J. Am. Chem. Soc., 74*, 5140 (1952).
[6] S. Matsumura, M. Nishioka, and S. Yoshikawa, *Makromol. Chem., Rapid Commun., 12*, 89 (1991).

[7] V. C. Barry and P. W. Mitchell, *J. Chem. Soc.*, p. 4020 (1954).
[8] S. M. Neal and W. A. Springfellow, *Trans. Faraday Soc., 33*, 881 (1937).
[9] C. C. Sweeley, R. Bentley, M. Makita, and W. W. Wells, *J. Am. Chem. Soc., 85*, 2497 (1963).
[10] Y. Ohya, K. Ihara, J. Murata, T. Sugitou, and T. Ouchi, *Carbohyd. Polym., 25*, 123 (1994).
[11] Y. Ohya, K. Nonomura, and T. Ouchi, *J. Bioact. Compat. Polym., 10*, 223 (1995).
[12] T. Mosmann, *J. Immunol. Methods, 65*, 55 (1983).

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