Studies on the Enzyme Immunoassay of Bio-active Constituents Contained in Oriental Medicinal Drugs. V.[1] Preparation of Bovine Serum Albumin Conjugate and β-Galactosidase Labelled Antigen for Enzyme Immunoassay of 3β-(Monoglucuron-1′β-yl)-18β-glycyrrhetic Acid

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In order to prepare an immunogen for enzyme immunoassay of 3β-(monoglucuron-1′-β-yl)-18β-glycyrrhetic acid (3MGA), which was isolated from a patient with glycyrrhizin-induced pseudoaldosteronisms, benzyl glycyrrhetate (3) was allowed to react with an acetobromosugar (2) in the presence of silver carbonate to give benzyl 3β-(methyl 2′,3′,4′-triacetyl-glucuron-1′β-yl)-glycyrrhetate (5) and methyl 3′,4′-diacetyl-α-1′,2′-*O*-[1-(benzyl glycyrrhet-3β-yl)-ethylidene]-D-glucuronate (4). On the other hand, this reaction was carried out in the presence of mercuric cyanide in nitromethane to give compound 5, benzyl 3β-acetyl glycyrrhetate (6) and benzyl 11-oxo-A-neooleana-3(5),12-dien-3-oate (7). 4-Aminomethylcyclohexanecarboxylic acid and glycine were introduced as chemical bridges at C-30 of 3β-(*tert*-butylglucuron-1′β-yl)-glycyrrhetic acid (11) derived from compound 5. The former bridge was used to prepare an immunogenic conjugate with bovine serum albumin, and the latter bridge was used for antigen labelled with β-galactosidase.

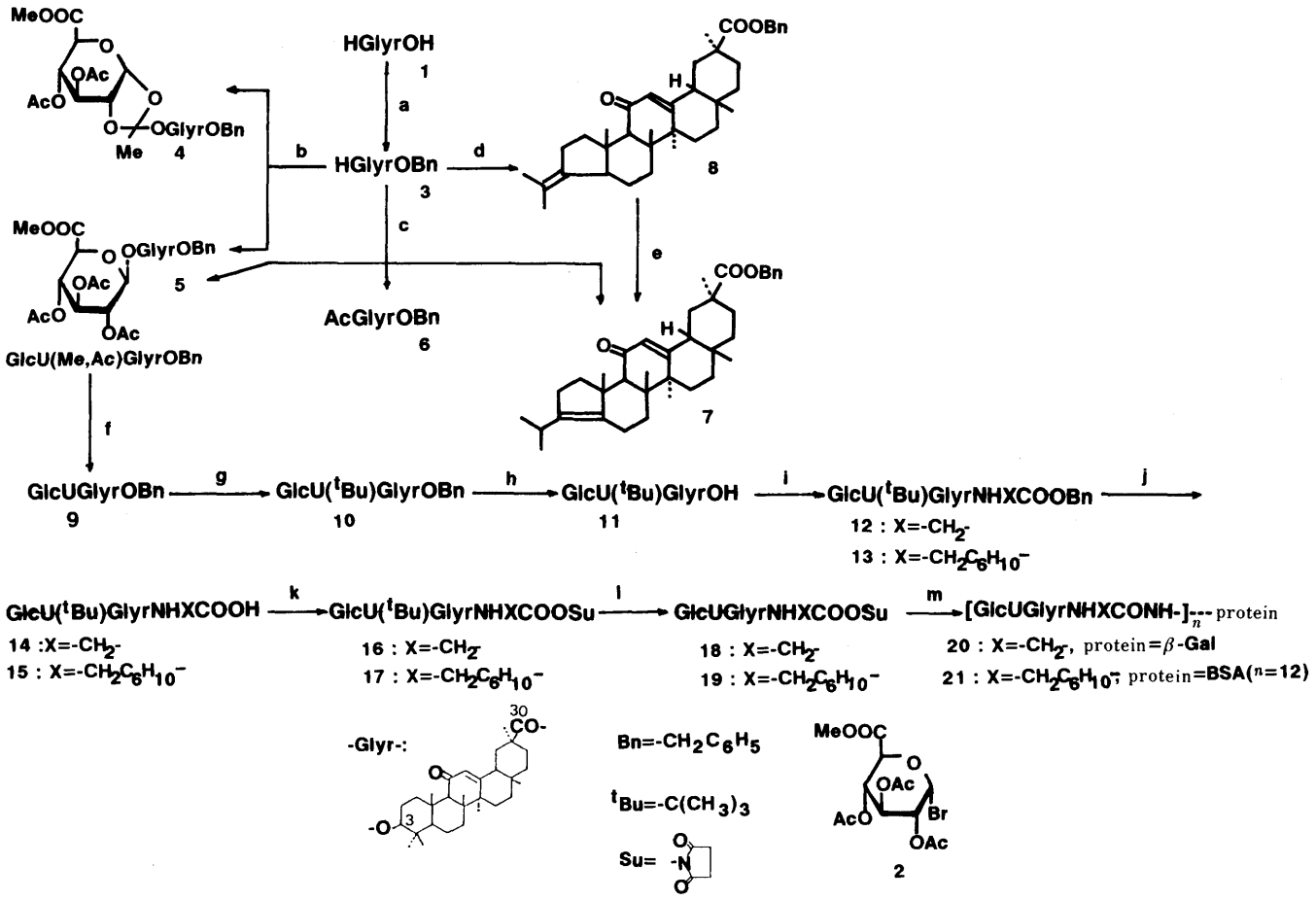
Keywords 3β-(glucuronyl)-18β-glycyrrhetic acid; bovine serum albumin conjugate; β-galactosidase labelled antigen; Koenigs–Knorr synthesis; benzyl 3β-(methyl 2′,3′,4′-triacetyl-glucron-1′β-yl)-glycyrrhetate; *ortho*-ester formation; glycyrrhetic acid A ring contraction; chemical bridge; glycine; aminomethylcyclohexanecarboxylic acid

In the previous paper,[2] we reported the isolation of 3β-(monoglucuron-1′β-yl)-18β-glycyrrhetic acid (3MGA) from serum of a patient with glycyrrhizin-induced pseudoaldosteronism who had received large doses of glycyrrhizin (GL) for the treatment of liver diseases, and we described its synthesis. Although the concentration of 3MGA in the serum of this patient was measured by high-performance liquid chromatography (HPLC), the development of an enzyme immunoassay (EIA) of 3MGA, which would be more sensitive than HPLC, was required to assay samples of biological fluid.

In this paper, we wish to report the preparation of 3MGA–bovine serum albumin (BSA) conjugate and –β-galactosidase (β-Gal) conjugate, for use as the immunogen and labelled antigen, respectively, in the EIA of 3MGA. 4-Aminomethyl-cyclohexanecarboxylic acid (Amcha) and glycine were introduced at the C-30 position of 3MGA as chemical bridges between the hapten and carrier protein. The former bridge was used for the immunogenic conjugate and the latter for the labelled antigen.

In order to introduce a glucuronyl group at the C-3 position of 18β-glycyrrhetic acid (**1**; GA), the carboxyl group at the C-30 position was protected with a benzyl group, which was introduced with *O*-benzyl-*N*,*N*′-dicyclohexylisourea to give benzyl glycyrrhetate (**3**) as shown in Chart 1. Compound **3** reacted with methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-D-α-glycopyranuronate (acetobromosugar) (**2**)[3] in the presence of silver carbonate under Koenigs–Knorr conditions to afford methyl 3′,4′-diacetyl-α-1′,2′-*O*-[1-(benzyl glycyrrhet-3β-yl)-ethylidene]-D-glucuronate (**4**) and benzyl 3β-(methyl 2′,3′,4′-triacetyl-glucuron-1′β-yl)-glycyrrhetate (**5**) in 12.5% and 46.4% yields, respectively. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of compound **5** exhibited a doublet signal at δ 4.64 (1H, J=8 Hz) due to the β-anomeric proton. The ^{1}H-NMR spectrum of compound **4** revealed the presence of a methyl group[4] on an ethylidene moiety as a singlet signal at δ 1.75 (3H). On the other hand, compound **3** reacted with the acetobromosugar (**2**) in the presence of mercuric cyanide in nitromethane to give compound **5**, benzyl 3β-acetyl glycyrrhetate (**6**) and benzyl 11-oxo-A-neooleana-3(5),12-dien-30-oate (**7**), which is a dehydrated and contracted derivative in the A ring of compound **3**, in 25.4%, 14.6% and 19.5% yields, respectively. The ^{1}H-NMR spectrum of compound **7** indicated the presence of an isopropyl group with two doublet signals at δ 0.93 and 1.00 (each 3H, J=7 Hz), and a septet signal at δ 2.66 (1H, J=7 Hz). In order to confirm the structure of compound **7**, the dehydration of compound **3** with phosphorus pentachloride[5] in the presence of anhydrous sodium acetate in benzene was carried out to give benzyl 11-oxo-A-neooleana-3,12-dien-30-oate (**8**), which exhibited two three-proton singlet signals at δ 1.61 and 1.75 due to methyl groups of the isopropylidene group. The isopropylidene group of compound **8** was converted to an isopropyl group by treatment with hydrochloric acid[6] to afford compound **7**, whose physical data were identical with those of the sample obtained under the Koenigs–Knorr conditions using mercuric cyanide.

Next, compound **5** was hydrolyzed with potassium hydroxide in methanol to give benzyl 3β-(glucuron-1′β-yl)-glycyrrhetate (**9**) as an amorphous powder. *tert*-Butyl esterification of compound **9** with *O*-*tert*-butyl-*N*,*N*′-dicyclohexylisourea gave benzyl 3β-(*tert*-butylglucuron-1′β-yl)-glycyrrhetate (**10**). Removal of the benzyl group from compound **10** with palladium–carbon afforded 3β-(*tert*-butylglucuron-1′β-yl)-glycyrrhetic acid (**11**). Compound **11** was condensed with benzyl glycinate and benzyl Amcha by using diethylphosphorocyanidate (DEPC) to afford benzyl *N*-[3β-(*tert*-butylglucuron-1′β-yl)-glycyrrhetyl]-glycinate (**12**) and benzyl *N*-[-3β-(*tert*-butylglucuron-1′β-yl)-glycyrrhetyl]-Amcha (**13**), respectively. Removal of the benzyl group from **12** and **13** by hydrogenolysis with palladium–carbon gave *N*-[3β-(*tert*-butylglucuron-1′β-yl)-glycyrrhetyl]-glycine (**14**) and *N*-[3β-(*tert*-butylglucuron-1′β-yl)-glycyrrhetyl]-Amcha (**15**), respectively. Compound **14** and



a) C_6H_{11}–N=C($OCH_2C_6H_5$)–NH–C_6H_{11}
b) (**2**)+Ag_2CO_3 in $ClCH_2CH_2Cl$
c) (**2**)+$Hg(CN)_2$ in CH_3NO_2
d) PCl_5+AcONa in benzene
e) HCl in $CHCl_3$
f) 5% KOH–MeOH
g) C_6H_{11}–N=C($O^{tert}Bu$)–NH–C_6H_{11}
h) Pd–C/H_2
i) $NH_2CH_2COOCH_2C_6H_5$·TsOH or $NH_2CH_2C_6H_{10}COOCH_2C_6H_5$·TsOH+DEPC
j) Pd–C/H_2
k) *N*-hydroxysuccinimide+EDC
l) 50% TFA–CH_2Cl_2
m) protein in phosphate buffer pH 7.3

Chart 1

15 were condensed with *N*-hydroxysuccinimide by the use of ethyl dimethylaminopropyl carbodiimide hydrochloride (EDC) to afford succinimidyl *N*-[3β-(*tert*-butylglucuron-1′β-yl)-glycyrrhetyl]-glycinate (**16**) and -Amcha (**17**), respectively. The ^{1}H-NMR spectra of **16** and **17** exhibited the signals of two methylene groups of the succinimidyl group as singlets at δ 2.71 (4H) and 2.84 (4H), respectively. After removal of the *tert*-butyl group from **16** and **17** with trifluoroacetic acid, the former product (**18**) was coupled with β-Gal and the latter (**19**) was coupled with BSA in phosphate buffer (pH 7.3) to give 3-MGA–glycine–β-Gal (**20**) and 3MGA– Amcha–BSA (**21**), respectively. The BSA-conjugate (**21**) as used for immunization of rabbits after purification by dialysis. About twelve molecules of 3MGA were incorporated per BSA molecule in the BSA-conjugate as judged by ultraviolet (UV) spectral analysis.[7] The β-Gal conjugate (**20**) was used as a labelled antigen for EIA after purification on on a Sepharose 6B column.

Experimental

All melting points were taken on a microscopic hot stage (Yanagimoto melting point apparatus) and uncorrected. Optical rotation was measured with a JASCO DIP-4 polarimeter. Spectra were obtained with the following instruments: ^{1}H-NMR on JNM-GX 270 and 400 spectrometers [solvent, $CDCl_3$ unless otherwise indicated: internal standard, tetramethylsilane (TMS); chemical shift, δ (ppm): abbreviations, s (singlet), d (doublet), t (triplet), br (broad), sept (septet). Thin-layer chromatography (TLC) was performed on precoated silica gel plates 0.25 mm thick (Kieselgel F_{254}, Merck) or 2 mm thick for preparative TLC (PTLC), and spots were visualized by spraying with 1% $Cs(SO_4)_2$ in 10% H_2SO_4 followed by heating, or under UV light (254 nm).

Methyl 2,3,4-Tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate (2) This compound was prepared from D-glucuronolactone by the method of Bollenback *et al.*[3]

Benzyl Glycyrrhetate (3) *O*-Benzyl *N,N′*-dicyclohexylisourea (4.7 g, 0.015 mol) was added to a solution of glycyrrhetic acid (4.7 g, 0.01 mol) in $CHCl_3$ (50 ml) and refluxed for 5 h. The mixture was filtered, and the filtrate was concentrated to syrup, which was extracted with AcOEt (100 ml). The extract was washed successively with 5% $NaHCO_3$ and brine, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The residue was recrystallized from CH_2Cl_2–hexane (1 : 1) to give compound **3** (5.9 g, 99%). mp 131—133 °C (colorless needles). $[\alpha]_D^{26}$ +137.5° (c=1, $CHCl_3$). ^{1}H-NMR δ: 0.73, 0.80, 1.00, 1.11, 1.13, 1.16, 1.35 (each 3H, s, CH_3×7), 3.22 (1H, m, H-3), 5.14 (2H, ABq, J=12 Hz, $CH_2C_6H_5$), 5.55 (1H, s, H-12), 7.37 (6H, s, C_6H_5). *Anal.* Calcd for $C_{37}H_{52}O_4$: C, 79.24; H, 9.35. Found C, 79.03; H, 9.43.

Reaction of Benzyl Glycyrrhetate (3) with the Acetobromosugar (2) in the Presence of Silver Carbonate Compound **3** (1.8 g, 3.2 mmol) and the acetobromosugar (**2**) (3.2 g, 8 mmol) were allowed to react in the presence

of Fetizon reagent (9.1 g, containing 16 mmol of Ag_2CO_3) and anhydrous $CaSO_4$ (10 g) in dichloroethane (50 ml) according to the method of Breiskorn and Lang.[8] The reaction mixture was filtered and the filtrate was concentrated *in vacuo* to a syrup, which was chromatographed over silica gel (80 g) (2.5 × 34 cm). The first eluate with 50% hexane–$CHCl_3$ (150 ml) was concentrated *in vacuo* and the residue was purified by PTLC with $CHCl_3$ as the developing solvent. The zones of *Rf* 0.7 and 0.6 gave methyl 3′,4′-diacetyl-α-1′,2′-*O*-[1-(benzyl glycyrrhet-3β-yl)-ethylidene]-D-glucuronate (**4**) (350 mg) and benzyl 3β-(methyl 2′,3′,4′-triacetyl-glucuron-1′β-yl)-glycyrrhetate (**5**) (440 mg). The second eluate with 50% hexane–$CHCl_3$ (300 ml) gave compound **5** (630 mg), and the third eluate with 50% hexane–$CHCl_3$ (250 ml) was purified by PTLC with 3% acetone–$CHCl_3$ as a developing solvent in the same manner as described above to give compound **5** (230 mg) and the starting material (**3**) (610 mg, 33.8%). The total yields of products were: compound **4** (0.35 g, 12.5%); compound **5** (1.3 g, 46.4%).

Compound **4**: mp 105—107 °C. $[\alpha]_D^{25}$ +73.8° (*c*=0.64, $CHCl_3$). *Anal.* Calcd for $C_{50}H_{68}O_{13}$: C, 68.47; H, 7.82. Found: C, 68.23; H, 7.78. ^{1}H-NMR δ: 0.73, 0.78, 0.93, 1.10, 1.13, 1.16, 1.35, 1.75 (each 3H, s, CH_3 × 8), 2.09, 2.10 (each 3H, s, CH_3CO × 2), 3.24 (1H, br t, H-3), 3.77 (3H, s, OCH_3), 4.29 (1H, br t, H-2′), 4.35 (1H, d, *J*=10 Hz, H-5′), 5.13—5.22 (2H, m, H-3′, H-4′), 5.18 (2H, s, ABq, *J*=12 Hz, $C\underline{H}_2C_6H_5$), 5.54 (1H, s, H-12), 5.86 (1H, d, *J*=5 Hz, H-1′), 7.38 (5H, s, C_6H_5).

Compound **5**: mp 134—136 °C (colorless needles from CH_2Cl_2–hexane). $[\alpha]_D^{24}$ +75.3° (*c*=0.73, $CHCl_3$). *Anal.* Calcd for $C_{50}H_{68}O_{13}$: C, 68.47; H, 7.82. Found: C, 68.54; H, 7.79. ^{1}H-NMR δ: 0.76, 0.79, 0.95, 1.12, 1.15, 1.18, 1.35 (each 3H, s, CH_3 × 7), 2.05, 2.05, 2.07 (each 3H, s, CH_3CO × 3), 3.17 (1H, br t, H-3), 3.80 (3H, s, OCH_3), 4.05 (1H, d, *J*=10 Hz, H-5′), 4.64 (1H, d, *J*=8 Hz, H-1′), 5.29 (2H, ABq, *J*=12 Hz, $C\underline{H}_2C_6H_5$), 5.04—5.38 (3H, m, H-2′, H-3′, H-4′), 5.59 (1H, s, H-12), 7.41 (5H, s, C_6H_5).

Reaction of Benzyl Glycyrrhetate (3) with the Acetobromosugar (2) in the Presence of Mercuric Cyanide A stirred solution of compound **3** (0.9 g, 1.6 mmol) in 40 ml of nitromethane–benzene (5 : 3) was evaporated until most of the benzene had been removed and then cooled to room temperature. Mercuric cyanide (0.33 g, 1.3 mmol), compound **2** (0.52 g, 1.3 mmol) and $CaSO_4$ (0.9 g) were added, and the reaction mixture was refluxed for 12 h with exclusion of moisture. Additional mercuric cyanide (0.33 g, 1.3 mmol) and compound **2** (1.3 mmol) were added, and refluxing was continued for 6 h. After cooling, the mixture was filtered and the residue was washed with $CHCl_3$ (100 ml). The filtrate and washings were combined, washed successively with 5% $NaHCO_3$, and brine, dried over anhydrous $MgSO_4$, and concentrated *in vacuo* to a syrup, which was chromatographed on a silica gel column (2 × 24 cm). The column was eluted with hexane–$CHCl_3$ (1 : 4), 5 ml fractions being collected. Fractions 9—16 were combined and concentrated *in vacuo*. The residue was recrystallized from hexane–CH_2Cl_2 (2 : 1) to give benzyl 11-oxo-neooleana-3(5),12-dien-30-oate (**7**) (120 mg). Fractions 17—19 gave a mixture of compound **7** and benzyl 3β-acetyl-glycyrrhetate (**6**), which were separated by PTLC using 2% acetone–$CHCl_3$ to afford compound **7** (50 mg) and compound **6** (36 mg). Fractions 20 and 21 gave compound **6** (88 mg). Fractions 22—24 gave compound **6** (18 mg) and compound **5** (74 mg). Fractions 25—27 gave compound **5** (154 mg). Fractions 28—41 gave compound **5** (130 mg) and the starting material (**3**) (230 mg, 25.5%). Total yields of products were: compound **5** (358 mg, 25.4%); compound **6** (142 mg, 14.6%) and compound **7** (170 mg, 19.5%).

Compound **7**: mp 174—176 °C. $[\alpha]_D^{26}$ +182.3° (*c*=1, $CHCl_3$). *Anal.* Calcd for $C_{37}H_{50}O_3$: C, 81.87; H, 9.29. Found: C, 81.85; H, 9.44. ^{1}H-NMR δ: 0.77, 1.09, 1.16, 1.19, 1.28 (each 3H, s, CH_3 × 5), 0.93, 1.00 (each 3H, d, *J*=7 Hz, $(C\underline{H}_3)_2CH$), 2.66 (1H, sept, *J*=7 Hz, $(CH_3)_2C\underline{H}$), 5.15 (2H, ABq, *J*=12 Hz, $C\underline{H}_2C_6H_5$), 5.65 (1H, s, H-12), 7.37 (5H, m, C_6H_5).

Alternative Preparation of Compound 7[5] A stirred solution of compound **3** (1 g, 1.78 mmol) in 100 ml of benzene–petroleum ether (1 : 1) was added to phosphorus pentachloride (0.9 g) at −10 °C for 20 min. The mixture was poured into ice-water (50 ml), and the organic layer was washed with 5% $NaHCO_3$ and brine, dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. The residue was recrystallized from CH_2Cl_2–MeOH to give benzyl 11-oxo-A-neooleana-3,12-dien-30-oate (**8**) (690 mg, 71%), mp 210—213 °C. $[\alpha]_D^{26}$ +185.5° (*c*=1, $CHCl_3$). *Anal.* Calcd for $C_{37}H_{50}O_3$: C, 81.87; H, 9.29. Found: C, 81.44; H, 9.38. ^{1}H-NMR δ: 0.76, 0.84, 1.09, 1.16, 1.34, 1.61, 1.75 (each 3H, s, CH_3 × 7), 5.15 (2H, ABq, *J*=12 Hz, $C\underline{H}_2C_6H_5$), 5.61 (1H, s, H-12), 7.36 (5H, m, C_6H_5).

A solution of compound **8** (60 mg) in $CHCl_3$ (4 ml) saturated with hydrogen chloride was stirred at room temperature for 1 d. The reaction mixture was washed successively with brine, 5% $NaHCO_3$, and brine, dried over anhydrous $MgSO_4$ and then evaporated *in vacuo*. The residue was recrystallized from CH_2Cl_2–hexane as colorless needles (52 mg, 86%) whose mp (174—176 °C) and physical data were identical with those of compound **7** described above.

Benzyl 3β-(Glucuron-1′β-yl)-glycyrrhetate (9) A stirred solution of compound **5** (718 mg 0.8 mmol) in 5% KOH–MeOH (4 ml) was refluxed for 1 h. The reaction mixture was diluted with MeOH (80 ml) and adjusted to pH 6.0 by adding Dowex 50 (H^+) resin. After filtration to remove the resin, the filtrate was concentrated *in vacuo* to syrup, which was recrystallized from isopropyl ether–MeOH (510 mg, 82%) to give a semisolid. $[\alpha]_D^{26}$ +74.6° (*c*=1, 10% MeOH–$CHCl_3$). ^{1}H-NMR (10% CD_3OD–$CDCl_3$) δ: 0.74, 0.86, 1.04, 1.10, 1.13, 1.17, 1.35 (each 3H, s, CH_3 × 7), 3.40—3.80 (4H, m, H-2′, H-3′, H-4′, H-5′), 4.41 (1H, d, *J*=8 Hz, H-1′), 5.18 (2H, ABq, *J*=12 Hz, $C\underline{H}_2C_6H_5$), 5.75 (1H, s, H-12), 7.20 (5H, s, C_6H_5).

Benzyl 3β-(*tert*-Butylglucuron-1′β-yl)-glycyrrhetate (10) *O*-*tert*-Butyl-*N*,*N*′-dicyclohexylisourea (270 mg, 1.35 mmol) was added to a solution of compound **9** (500 mg, 0.68 mmol) in dimethylformamide (DMF) (1 ml) with stirring for 3 d. The reaction mixture was filtered and the residue was washed with AcOEt (50 ml). The AcOEt solution was washed with brine, 5% $NaHCO_3$, and brine, then dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was purified by PTLC using 5% MeOH–$CHCl_3$ as a developing solvent. The zone with *Rf* 0.7 gave compound **10** (238 mg, 44%), which was recrystallized from CH_2Cl_2–isopropyl ether, as colorless needles mp 218—219 °C. $[\alpha]_D^{26}$ +73.9° (*c*=1, $CHCl_3$). *Anal.* Calcd for $C_{47}H_{68}O_{10}$: C, 71.18; H, 8.64. Found: C, 71.33; H, 8.82. ^{1}H-NMR δ: 0.75, 0.88, 1.03, 1.12, 1,16, 1.16, 1.35 (each 3H, s, CH_3 × 7), 1.51 (9H, s, $C(CH_3)_3$), 3.47—3.84 (4H, m, H-2′, H-3′, H-4′, H-5′), 4.4 (1H, d, *J*=8 Hz, H-1′), 5.19 (2H, ABq, $C\underline{H}_2C_6H_5$), 5.57 (1H, s, H-12), 7.41 (5H, s, C_6H_5).

3β-(*tert*-Butylglucuron-1′β-yl)-glycyrrhetic Acid (11) A solution of compound **10** (120 mg, 0.15 mmol) in AcOEt (10 ml) was hydrogenated over 10% Pd–C (60 mg) at atmospheric pressure for 2 d. The catalyst was removed by filtration and washed with AcOEt. The filtrate and washings were combined and concentrated *in vacuo* to leave an amorphous powder (94 mg, 88%). $[\alpha]_D^{25}$ +61.2° (*c*=1, 10% MeOH–$CHCl_3$). ^{1}H-NMR (CD_3OD) δ: 0.84, 0.88, 1.07, 1.15, 1.15, 1.18, 1.44 (each 3H, s, CH_3 × 7), 1.51 (9H, s, $C(CH_3)_3$), 3.21—3.70 (4H, m, H-2′, H-3′, H-4′, H-5′), 4.39 (1H, d, *J*=8 Hz, H-1′), 5.62 (1H, s, H-12).

Benzyl *N*-[3β-(*tert*-Butylglucuron-1′β-yl)-glycyrrhetyl]-glycinate (12) DEPC (20 mg, 0.12 mmol) was added to a stirred mixture of compound **11** (70 mg, 0.1 mmol) and benzyl glycinate *p*-toluenesulfonate (35 mg, 0.12 mmol) in DMF (1 ml). The reaction mixture was adjusted to pH 7.0 by adding triethylamine at 0 °C for 3 h. The solution was diluted with H_2O (15 ml) and extracted with AcOEt (70 ml). The extract was washed with 1 N citric acid, brine, 5% $NaHCO_3$, and brine, and dried over anhydrous $MgSO_4$. After removal of the solvent, the residue was recrystallized from CH_2Cl_2 + isopropyl ether to give compound **12** (74 mg, 87%), mp 154—158 °C. *Anal.* Calcd for $C_{49}H_{71}NO_{11}$: C, 69.23; H, 8.42. Found: C, 69.32; H, 8.60. $[\alpha]_D^{26}$ +66.3° (*c*=1, $CHCl_3$). ^{1}H-NMR δ: 0.81, 0.86, 1.04, 1.13, 1.14, 1.16, 1.37 (each 3H, s, CH_3 × 7), 1.48 (9H, s, $(CH_3)_3C$), 3.40—3.85 (4H, m, H-2′, H-3′, H-4′, H-5′), 4.10 (2H, br t, *J*=5 Hz, N-CH_2CO), 4.38 (1H, d, *J*=8 Hz, H-1′), 5.10 (2H, s, $C\underline{H}_2C_6H_5$), 5.73 (1H, s, H-12), 6.10 (1H, br t, N$\underline{H}$-CH_2), 7.37 (5H, s, C_6H_5).

Benzyl *N*-[3β-(*tert*-Butylglucuron-1′β-yl)-glycyrrhetyl]-Amcha (13) DEPC (52 mg, 0.32 mmol) was added to a stirred mixture of compound **11** (190 mg, 0.27 mmol) and benzyl aminomethylcyclohexylcarboxylate *p*-toluenesulfonate (134 mg, 0.32 mmol) in DMF (1.5 ml) and the reaction mixture was treated in the same manner as above to give compound **13** (214 mg, 93%). mp 165—168 °C. *Anal.* Calcd for $C_{55}H_{81}NO_{11}$: C, 70.86; H, 8.76. Found: C, 70.92; H, 8.83. $[\alpha]_D^{24}$ +53.3° (*c*=1, $CHCl_3$). ^{1}H-NMR δ: 0.81, 0.87, 1.04, 1.12, 1.16, 1.37 (each 3H, s, CH_3 × 7), 1.50 (9H, s, $C(CH_3)_3$), 3.21 (2H, m, $CONHC\underline{H}_2$), 3.47—3.83 (4H, m, H-2′, H-3′, H-4′, H-5′), 4.40 (1H, d, *J*=8 Hz, H-1′), 5.14 (2H, s, $C\underline{H}_2C_6H_5$), 5.67 (1H, m, NH–), 5.70 (1H, s, H-12), 7.38 (5H, s, C_6H_5).

***N*-[3β-(*tert*-Butylglucuron-1′β-yl)-glycyrrhetyl]-glycine (14)** A solution of compound **12** (74 mg, 0.087 mmol) in 10% MeOH–AcOEt (10 ml) was hydrogenated over 10% Pd–C (50 mg) at atmospheric pressure for 6 h. The catalyst was removed by filtration and washed with MeOH. The filtrate and washings were combined and concentrated *in vacuo* to leave an amorphous powder, which was recrystallized from MeOH–isopropyl ether to give **14** (53 mg, 80%). $[\alpha]_D^{26}$ +55.5° (*c*=0.6, 10% MeOH–$CHCl_3$). ^{1}H-NMR (10% CD_3OD–$CDCl_3$) δ: 0.81, 0.86, 1.04, 1.12, 1.14, 1.37 (each 3H, s, CH_3 × 7), 1.48 (9H, s, $C(CH_3)_3$), 3.85 (1H, dd, *J*=18, 4 Hz, CONH–C$\underline{H}$), 4.16 (1H, dd, *J*=18, 6 Hz, CONH–C$\underline{H}$), 4.35 (1H, d, *J*=8 Hz, H-1′), 5.75 (1H, s, H-12), 6.55 (1H, br m, NH).

***N*-[3β-(*tert*-Butylglucuron-1′β-yl)-glycyrrhetyl]-Amcha (15)** A solution

of compound **13** (100 mg, 0.1 mmol) in AcOEt (20 ml) was hydrogenated in the same manner as above to give **15**, mp 222—226 °C. Colorless needles. $[\alpha]_D^{27}$ +66.5° ($c=1$, 10% MeOH–$CHCl_3$). *Anal.* Calcd for $C_{48}H_{75}NO_{11}$: C, 68.46: H, 8.98. Found: C, 68.31; H, 9.01. ^{1}H-NMR (10% CD_3OD–$CDCl_3$) δ: 0.80, 0.85, 1.03, 1.12, 1.12. 1.14. 1.37 (each 3H, s, $CH_3\times 7$), 1.49 (9H, s, $C(CH_3)_3$), 4.37 (1H, d, $J=8$ Hz, H-1′), 5.65 (1H, s, H-12), 6.18 (1H, m, NH).

Succinimidyl *N*-[3β-(*tert*-Butylglucuron-1′β-yl)-glycyrrhetyl]-glycinate (16) EDC (6 mg, 0.03 mmol) was added to a stirred mixture of compound **14** (20 mg, 0.026 mmol) and *N*-hydroxysuccinimide (4 mg, 0.03 mmol) in DMF (0.5 ml) under ice cooling. The mixture was stirred at room temperature overnight and poured into ice-water. The precipitate was collected by filtration, washed with H_2O, dissolved in CH_2Cl_2 (0.5 ml) and reprecipitated with hexane to give compound **16** (11 mg, 49%). Amorphous powder. $[\alpha]_D^{26}$ +75.5° ($c=0.2$, 10% MeOH–$CHCl_3$). ^{1}H-NMR δ: 0.81, 0.86, 1.04, 1.12, 1.14, 1.15, 1.37 (each 3H, s, $CH_3\times 7$), 1.48 (9H, s, $C(CH_3)_3$), 2.71 (4H, s, Su), 3.40—3.90 (4H, m, H-2′, H-3′, H-4′, H-5′), 3.85 (1H, dd, $J=18$, 4 Hz, CONH–CH), 4.17 (1H, dd, $J=18$, 6 Hz, CONH–CH), 4.36 (1H, d, $J=7.0$ Hz, H-1′), 5.72 (1H, s, H-12).

Succinimidyl *N*-[3β-(*tert*-Butylglucuron-1′β-yl)-glycyrrhetyl]-Amcha (17) A solution of compound **15** (18 mg, 0.021 mmol), EDC (6 mg 0.031 mmol) and *N*-hydroxysuccinimide (4 mg, 0.031 mmol) in DMF was worked up as described above to give compound **17** (13 mg, 65%) as a semisolid. $[\alpha]_D^{23}$ +55.6° ($c=1.3$, $CHCl_3$). ^{1}H-NMR δ: 0.81, 0.86, 1.02, 1.12, 1.13, 1.15, 1.36 (each 3H, s, $CH_3\times 7$), 1.48 (9H, s, $C(CH_3)_3$), 2.84 (4H, s, Su), 3.43—3.78 (4H, m, H-2′, H-3′, H-4′, H-5′), 4.20 (1H, d, $J=8$ Hz, H-1′), 5.67 (1H, s, H-12), 5.67 (1H, m, NH).

Preparation of 3MGA–Amcha–BSA Conjugate (21) Compound **17** (10 mg, 10.6 μmol) was dissolved in 50% trifluoroacetic acid–CH_2Cl_2 (1 ml) under stirring at 0 °C. After being stirred for 1.5 h at the same temperature, the reaction mixture was concentrated *in vacuo* at below room temperature. The residue was washed with dried ether three times and then dried over P_2O_5 *in vacuo* to give succinimidyl *N*-[(glucuronyl)-glycyrrhetyl]-Amcha (**19**) (7 mg, 74%) as an amorphous powder. A solution of the crude product (7 mg, 7.9×10^{-3} mmol) in pyridine (0.2 ml) was added to a solution of BSA (26 mg, 3.9×10^{-4} mmol) in 0.05 M phosphate buffer (pH 7.3, 0.5 ml) and the mixture was stirred at 5 °C for 24 h. The resulting turbid solution was diluted with water to make a total volume of 3 ml and dialyzed successively for 6 d against 7%, 4%, 2%, 1% pyridine–H_2O and H_2O to give 3MGA–Amcha–BSA (**21**).

Determination of Number of 3MGA Molecules Linked to One BSA Molecule in the BSA-Conjugate (21) The UV spectrometric analysis was performed by comparing the absorbance at 255 nm of the conjugate (**21**) with those of BSA and 3MGA[2] as controls in 0.05 M phosphate buffer (pH 7.3) and by using the following constants: molecular weight of BSA 67000; ε value for BSA, 17400; ε value for 3MGA, 12200. The protein content of the conjugate **21** was determined by the method of Lowry *et al.*[9] The number of 3MGA moieties coupled to one BSA moiety was determined to be 12.3.

Preparation of 3MGA–Glycine–β-Galactosidase Conjugate (20) Compound **16** (10 mg, 11.6 μmol) was treated with 50% trifluoroacetic acid (0.5 mol) in the same manner as described above to give succinimidyl [3β-(glucuronyl)-glycyrrhetyl]-glycinate (**18**) (6.8 mg, 73%) which was used in the next step without further purification. Compound **18** (140 μg, 17.5×10^{-8} mol) [14 μl of a solution of compound **18** (1 mg) in pyridine (100 μl) was pipetted off] was added to a solution of β-galactosidase (4.5 mg, 87.5×10^{-10} mol) in 0.05 M phosphate buffer (pH 7.3, 0.5 ml) and the mixture was stirred at 0 °C overnight. The reaction mixture was directly chromatographed on a Sepharose 6B column (1.5 × 30 cm) with buffer A. The peak fractions were pooled at 4 °C until use. The amount of enzyme conjugate was expressed as units of enzyme activity, one unit of enzyme activity being defined as the amount that hydrolyzed 1 μmol of 7-β-D-galactopyranosyl-oxy-4-methylcoumarin per min.

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