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Glycyrrhetylamino Acids: Synthesis and Application to Enzyme Immunoassay for Glycyrrhetic Acid[1)]

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Glycyrrhetylamino acids (**5a**—**c**) were prepared by the condensation of glycyrrhetic acid (GA) with amino acids (glycine, γ-aminobutyric acid, and ε-aminohexanoic acid), which were selected for use as chemical bridges between the hapten and carrier protein in an enzyme immunoassay (EIA) for GA. The condensation was carried out in the presence of dicyclohexylcarbodiimide (method A), diphenyl phosphorazidate (method B) or diethyl phosphorocyanidate (method C), and method C gave the desired glycyrrhetylamino acids (**5a**—**c**) in the best yields. β-Galactosidase was used as the labeled enzyme and was conjugated with GA by the N-hydroxysuccinimide ester method. Separation of bound and free fractions was performed by a double antibody method using a goat antiserum to rabbit IgG. 7-β-D-Galactopyranosyloxy-4-methylcoumarin was used as substrate for the fluorometric assay of β-galactosidase activity. A satisfactory standard curve for GA was obtained in the range of 2.5—250 ng/ml.

Keywords——glycyrrhetic acid; glycyrrhetylamino acids; diphenyl phosphorazidate; diethyl phosphorocyanidate; enzyme immunoassay; N-hydroxysuccinimide ester method; double antibody method; β-galactosidase; 7-β-D-galactopyranosyloxy-4-methylcoumarin

Glycyrrhizin and its aglycone, 18β-glycyrrhetic acid (GA), are principal constituents of Glycyrrhizae Radix which is a well-known and very important crude drug in traditional oriental medicine. They are widely used in the treatment of gastric ulcer and allergic symptoms. However, the determination of their pharmacokinetics in man has been impeded by the lack of a suitable method for assay in serum and other biological materials. Although several methods (paper chromatography,[2)] thin-layer chromatography,[3)] gas chromatography,[4)] and high performance liquid chromatography[5)]) for the determination of GA have been reported, enzyme immunoassay (EIA) seems to be the most suitable analytical tool for quantitative analysis of GA in the presence of structural and functional analogs in biological fluids. Glycine, γ-aminobutyric acid and ε-aminohexanoic acid, which possess straight chains of three to seven atoms, were selected as "chemical bridges"[6)] between hapten and carrier protein. This paper deals with the condensation of GA with these amino acids, the preparation of labeled antigen, and the EIA procedure for GA.

Syntheses of Materials for EIA of GA

Four kinds of synthetic methods were examined to obtain glycyrrhetylamino acids (**5a**—**c**), as shown in Chart 1. Reaction of GA with methyl γ-aminobutyrate in the presence of dicyclohexylcarbodiimide gave mainly N-glycyrrhetyl-N,N′-dicyclohexylurea (**1d**) (44% yield) together with the desired methyl γ-(N-glycyrrhetylamino)butyrate (**3b**) in poor yield (18%) (Method A). Methyl N-glycyrrhetylamino acylates (**3a**—**c**) were obtained in 50—70% yield by condensing GA with methyl amino acylates (**2a**—**c**) in the presence of diphenyl phosphorazidate (DPPA). In these cases glycyrrhetylazide (**1e**) was afforded as a by-product in 4—20% yield (Method B). Methyl N-glycyrrhetylamino acylates (**3a**—**c**) were prepared without by-product formation in 80—87% yield by using diethyl phosphorocyanidate (DEPC) instead of DPPA in the above reaction (Method C). Methyl N-acetylglycyrrhetylamino acylates (**4a**—**c**) were obtained in 60—75% yield by condensing methyl amino acylates (**2a**—**c**) with

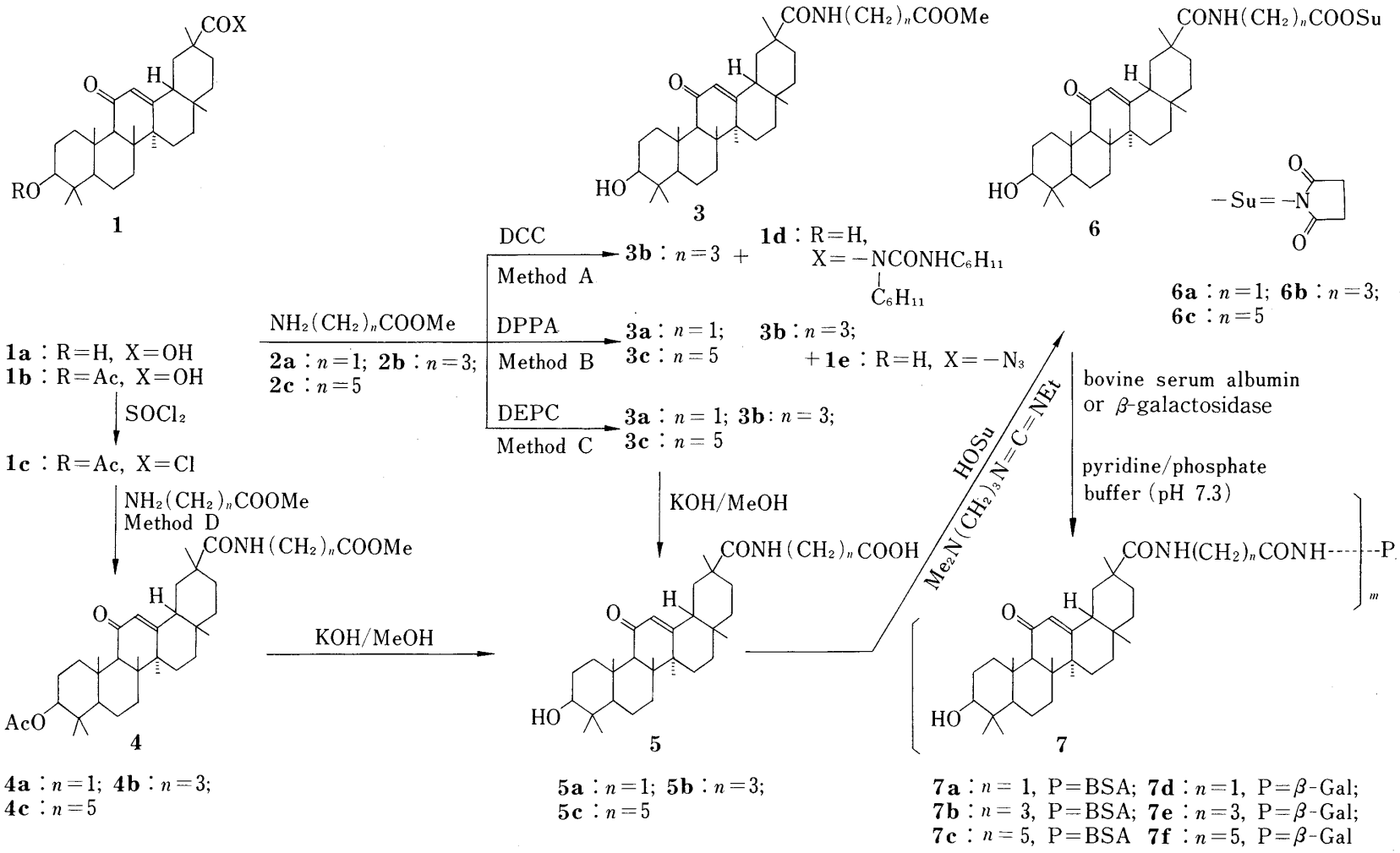

Chart 1

acetylglycyrrhetyl chloride (**1c**),[7] itself prepared in 53% yield by reacting acetylglycyrrhetyl acid (**1b**) with thionyl chloride (Method D).

In these condensation reactions DEPC was the most suitable coupling reagent.

Alkaline hydrolysis of methyl N-glycyrrhetylamino acylates (**3a**—**c**) and methyl N-acetylglycyrrhetylamino acylates (**4a**—**c**) afforded N-glycyrrhetylamino acids (**5a**—**c**).

Bovine serum albumin (BSA) and β-D-galactosidase (β-Gal) were used as the carrier protein and labeled enzyme for EIA, respectively, and were coupled with N-glycyrrhetylamino acids (**5a**—**c**) as shown in Chart 1. Reaction of **5a**—**c** with N-hydroxysuccinimide in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide gave N-hydroxysuccinimidyl glycyrrhetylamino acylates (**6a**—**c**), which were coupled with amino groups of BSA and β-Gal in pyridine-phosphate buffer (pH 7.3) to afford N-glycyrrhetylamino acid-BSA (**7a**—**c**) and β-Gal conjugates (**7d**—**f**), respectively. The number of GA molecules linked to a BSA molecule was determined by ultraviolet spectral analysis; 10.6, 9.8, and 10.2 molecules were incorporated in **7a**, **7b**, and **7c**, respectively.

EIA Procedure for GA

An antiserum for GA was obtained from a female rabbit immunized by the subcutaneous injection of glycyrrhetylglycine-BSA conjugate (**7a**) with complete Freund's adjuvant. EIA for GA was performed by a competitive binding procedure (double antibody method) with a goat antiserum to rabbit IgG. The enzyme activity was assayed according to the procedure of Kato *et al.*[8] with 7-β-D-galactopyranosyloxy-4-methylcoumarin as the substrate. We attempted EIA using three β-Gal conjugates (**7d**—**f**) against the GA-antiserum. In the case of **7d**, no inhibition occurred with GA. The two conjugates, **7e** and **7f**, were equal in binding capacity, but the former was more suitable than the latter as regards suppressibility by GA. Thus, we used **7e** in further experiments.

A typical standard curve for EIA of GA is shown in Fig. 1; the measurable range was 2.5—250 ng/ml. The specificity of anti-glycyrrhetylglycine-BSA serum was tested by cross-reaction studies with structural and functional analogs of GA in biological systems, and the results are shown in Table I. The GA antiserum reacted with two compounds, 18α-GA (66%) and sodium carbenoxolone (1.0%), and GA could be detected from a mixture with glycyrrhizin (0.1%). The procedure and results EIA of GA in blood of animals and man will be reported elsewhere.

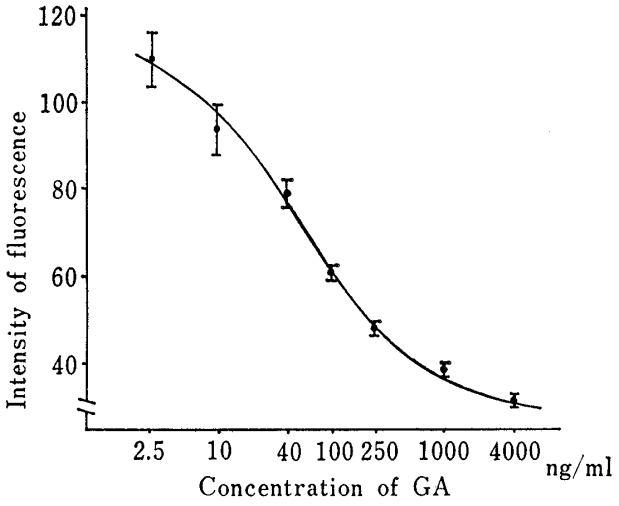

Fig. 1. Standard Curve for Enzyme Immunoassay of Glycyrrhetic Acid

Each point represents the mean±S.D. of 6 replicate determinations.

TABLE I. Specificity of Anti-glycyrrhetyl glycine-BSA Serum

Compound	Cross reaction (%)
18β-Glycyrrhetic acid	100
18α-Glycyrrhetic acid	66
Sodium carbenoxolone	1.0
Ammonium glycyrrhizinate	0.1
Sodium cholate	<0.02
Sodium deoxycholate	<0.02
Cholesterol	<0.1
Estradiol	<0.1
Aldosterone	<0.1
Hydrocortisone	<0.1
Progesterone	<0.1
Dihydrotestosterone	<0.1

Experimental

All melting points were taken on a microscopic hot stage (Yanagimoto melting point apparatus) and are uncorrected. Infrared (IR) spectra were measured with a JASCO IR-2 spectrometer. The specific rotations were measured with a JASCO DIP-4 polarimeter. Ultraviolet (UV) spectra were measured with a Beckman model 24 spectrometer. Preparative layer chromatography (PLC) was performed on silica gel (Merck, silicic acid PF_{254} containing $CaSO_4$). NMR spectra were taken at 90 MHz with a Varian EM 390 spectrometer using tetramethylsilane as an internal standard. The following abbreviations are used: singlet (s), doublet (d), multiplet (m), and broad (br).

Methyl Glycyrrhetylamino Acylate (3a—c)——Method A (Methyl γ-(N-glycyrrhetylamino)butyrate (**3b**)): A solution of dicyclohexylcarbodiimide (103 mg, 0.5 mmol) in $CHCl_3$ (1 ml) was added to a stirred mixture of glycyrrhetic acid (235 mg, 0.5 mmol), methyl γ-aminobutyrate [prepared from 85 mg (0.6 mmol) of the hydrochloride with 0.08 ml of Et_3N] and $CHCl_3$ (10 ml) at 0°. The mixture was stirred for 2 hr at the same temperature and then at room temperature overnight. The resulting precipitate was filtered off. The filtrate was washed with 1 N HCl, H_2O, and 10% Na_2CO_3, dried ($MgSO_4$), and concentrated to give a syrup, which was purified by PLC using 5% acetone–$CHCl_3$ as a developing solvent. The zone with *Rf* 0.2 gave 50 mg (18% yield) of **3b**. The zone with *Rf* 0.7 gave 160 mg (44% yield) of **1d**.

N-Glycyrrhetyl-N,N′-dicyclohexylurea (**1d**): Colorless needles from CH_2Cl_2–hexane. mp 178—180°. IR ν_{max}^{KBr} cm^{-1}: 1690, 1660 (sh), 1650. MS *m/z*: 676 (M^+). *Anal.* Calcd for $C_{43}H_{68}N_2O_4$: C, 76.28; H, 10.12; N, 4.14. Found: C, 75.92; H, 10.12; N, 4.37.

Method B (General Procedure): Et_3N (0.17 ml, 1.2 mmol) was added to a mixture of glycyrrhetic acid (235 mg, 0.5 mmol), methyl amino acylate hydrochloride (**2a—c**) (0.55 mmol), diphenyl phosphorazidate (158 mg, 0.55 mmol) and dimethyl formamide (DMF) (3 ml) under stirring at 5°. After 1 hr, the mixture was stirred for 20 hr at room temperature. After addition of water (10 ml), the mixture was extracted with AcOEt (100 ml). The extract was washed with 1 N HCl, H_2O, and 10% Na_2CO_3, dried ($MgSO_4$), and concentrated *in vacuo*. The product was purified by PLC using 5% acetone–$CHCl_3$ as a developing solvent. The zone with *Rf* 0.8 gave glycyrrhetylazide (**1e**) in 4—20% yield. The zone with *Rf* 0.2 gave methyl N-glycyrrhetylamino acylate (**3a**, 70%; **3b**, 54%; **3c**, 56% yield).

Glycyrrhetylazide (1e): Colorless needles from CH_2Cl_2–hexane. mp 275—278°. IR ν_{max}^{KBr} cm^{-1}: 2260, 2150. NMR ($CDCl_3$) δ: 3.3 (1H, m, CHOH), 5.7 (1H, s, CH=C=). MS *m/z*: 495 (M^+), 467 (M^+-N_2). The parent ion was detected by FD-MS.

Method C (General Procedure): The same procedure as Method B but with diethyl phosphorocyanidate instead of diphenyl phosphorazidate gave rise to the same products (**3a** 82%, **3b** 87%, and **3c** 80% yield).

Methyl N-Glycyrrhetylglycinate (**3a**): Colorless needles from isopropyl ether. mp 265—266°. $[\alpha]_D^{25}$ +150.8° (*c*=1, $CHCl_3$). MS *m/z*: 541 (M^+). *Anal.* Calcd for $C_{33}H_{51}NO_5 \cdot 1/2H_2O$: C, 71.96; H, 9.52; N, 2.54. Found: C, 72.15; H, 9.60; N, 2.66. NMR ($CDCl_3$) δ: 3.23 (1H, m, CHOH), 3.73 (3H, s, OCH_3), 4.06 (2H, d, *J*=6 Hz, $NHCH_2$), 5.7 (1H, s, CH=C=), 6.23 (1H, br, NH).

Methyl γ-Glycyrrhetylaminobutyrate (**3b**): Semicrystalline. MS *m/z*: 569 (M^+). NMR ($CDCl_3$) δ: 2.3 (2H, m, CH_2CO), 3.1—3.5 (3H, m, $NHCH_2$, CHOH), 3.66 (3H, s, OCH_3), 5.63 (1H, s, CH=C=), 6.1 (1H, br, NH).

Methyl ε-(N-Glycyrrhetylamino)hexanoate (**3c**): Semicrystalline. MS *m/z*: 597 (M^+). NMR ($CDCl_3$) δ: 2.3 (2H, m, CH_2CO), 3.1—3.5 (3H, m, CH_2NH, CHOH), 3.6 (3H, s, OCH_3), 5.6 (1H, s, CH=C=), 5.7 (1H, br, NH).

Methyl N-Acetylglycyrrhetylamino Acylates (4a—c)——Method D (General Procedure): A solution of methyl amino acylate [prepared from 0.55 mmol of the hydrochloride with 0.08 ml of Et_3N] in CH_2Cl_2 (50 ml) was added to a solution of 3-acetylglycyrrhetyl chloride[7] (**1c**) (244 mg, 0.5 mmol) in CH_2Cl_2 (2 ml). The mixture was stirred for 20 hr at room temperature, treated with H_2O (10 ml), and extracted with CH_2Cl_2 (50 ml). The extract was washed with 1 N HCl and H_2O, dried ($MgSO_4$) and concentrated *in vacuo* to give methyl N-acetylglycyrrhetylamino acylate. (**4a**, 60%; **4b**, 75%; **4c**, 70% yield).

Methyl N-Acetylglycyrrhetylglycinate (**4a**): Colorless needles from CH_2Cl_2–hexane. mp 244—245°. $[\alpha]_D^{25}$ +135.7° (*c*=1, $CHCl_3$). MS *m/z*: 583 (M^+). *Anal.* Calcd for $C_{35}H_{53}NO_6 \cdot 1/2H_2O$: C, 70.91; H, 9.18; N, 2.36. Found: C, 71.16; H, 9.27; N, 2.42. NMR ($CDCl_3$) δ: 3.8 (3H, s, OCH_3), 4.0 (2H, d, *J*=6.0 Hz, CH_2NH), 4.5 (1H, m, CHOAc), 5.7 (1H, s, CH=C=), 6.2 (1H, br t, NH).

Methyl γ-(N-Acetylglycyrrhetylamino)butyrate (**4b**): Semicrystalline. MS *m/z*: 611 (M^+). NMR ($CDCl_3$) δ: 2.3 (2H, m, CH_2CO), 3.3 (2H, m, CH_2NH), 3.6 (3H, s, OCH_3), 4.3 (1H, m, CHOAc), 5.7 (1H, s, CH=C=), 6.0 (1H, br, NH).

Methyl ε-(N-Acetylglycyrrhetylamino)hexanoate (**4c**): Colorless needles from CH_2Cl_2–hexane. mp 224—225°. $[\alpha]_D^{16}$ +115.6° (*c*=1, $CHCl_3$). MS *m/z*: 639 (M^+). *Anal.* Calcd for $C_{39}H_{61}NO_6 \cdot 1/2H_2O$: C, 72.18; H, 9.63; N, 2.16. Found: C, 72.48; H, 9.74; N, 2.04. NMR ($CDCl_3$) δ: 2.3 (2H, m, CH_2CO), 3.3 (2H, m, CH_2NH), 3.7 (3H, s, OCH_3), 4.3 (1H, m, CHOAc), 5.7 (1H, s, CH=C=), 5.9 (1H, br, NH).

N-Glycyrrhetylamino Acids (5a—c)——General Procedure: Methyl N-glycyrrhetylamino acylate (**3a—c**) (100 mg) was treated with 5% KOH–MeOH (3 ml) and the mixture was refluxed on a water bath for 30 min. The mixture was acidified with 4 N HCl and extracted with CH_2Cl_2 (100 ml). The extract was washed with H_2O, dried ($MgSO_4$), and concentrated *in vacuo* to give N-glycyrrhetylamino acid (**5a—c**) in 80% yield.

The same procedure using methyl N-acetylglycyrrhetylamino acylates (**4a**—**c**) instead of methyl N-glycyrrhetylamino acylates (**3a**—**c**) gave rise to the same products (**5a**—**c**) in 80% yield.

N-Glycyrrhetylglycine (**5a**): Colorless needles from isopropyl ether. mp 255—257°. $[\alpha]_D^{25}$ +158.6° (c=0.5, 50% MeOH–$CHCl_3$) MS m/z: 527 (M^+). *Anal.* Calcd for $C_{32}H_{49}NO_5 \cdot 1/2H_2O$: C, 71.60, H, 9.39; N, 2.61. Found: C, 71.22; H, 9.21; N, 2.53. NMR (CD_3OD) δ: 3.2 (1H, m, CHOH), 4.0 (2H, m, CH_2NH), 5.7 (1H, s, CH=C=), 6.9 (1H, br, NH).

γ-(N-Glycyrrhetylamino)butyric Acid (**5b**): Colorless needles from isopropyl ether–CH_2Cl_2. mp 208—210°. $[\alpha]_D^{25}$ +146.4° (c=1, 50% MeOH–$CHCl_3$). MS m/z: 555 (M^+). *Anal.* Calcd for $C_{34}H_{53}NO_5 \cdot 1/2H_2O$: C, 72.30; H, 9.64; N, 2.48. Found: C, 71.93; H, 9.15; N, 2.27. NMR (CD_3OD) δ: 2.3 (2H, m, CH_2CO), 3.1—3.6 (3H, m, CH_2NH, CHOH), 5.7 (1H, s, CH=C=), 6.7 (1H, br, NH).

ε-(N-Glycyrrhetylamino)hexanoic Acid (**5c**): Colorless needles from isopropyl ether. mp 275—277°. $[\alpha]_D^{25}$ +123.2° (c=1.4, $CHCl_3$). MS m/z: 583 (M^+). *Anal.* Calcd for $C_{36}H_{57}NO_5 \cdot 1/2H_2O$; C, 72.93; H, 9.86; N, 2.36. Found: C, 72.69; H, 10.15; N, 2.13. NMR ($CDCl_3$) δ: 2.3 (2H, m, CH_2CO), 3.1—3.8 (3H, m, CH_2NH, CHOH), 5.7 (1H, s, CH=C=), 5.9 (1H, br, NH).

N-Hydroxysuccinimidyl Glycyrrhetylamino Acylate (6a—c)——General Procedure: 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (0.8 mmol) was added to a stirred mixture of N-glycyrrhetylamino acid (**5a**—**c**) (0.5 mmol), N-hydroxysuccinimide (0.5 mmol) and 80% dioxane (5 ml) at 15° and the mixture was stirred for 20 hr at room temperature. After addition of H_2O, the mixture was extracted with AcOEt (150 ml). The extract was washed with H_2O, dried ($MgSO_4$) and concentrated *in vacuo*. The product was purified, if necessary, by PLC.

N-Hydroxysuccinimidyl Glycyrrhetylglycinate (**6a**): Semicrystalline. NMR ($CDCl_3$) δ: 2.8 (4H, s, succinimidyl) 4.0 (2H, d, CH_2NH), 3.2 (1H, m, CHOH), 5.7 (1H, s, CH=C=), 6.6 (1H, br, NH).

N-Hydroxysuccinimidyl Glycyrrhetyl-γ-aminobutyrate (**6b**): Semicrystalline. NMR ($CDCl_3$) δ: 2.6 (2H, m, CH_2CO), 2.8 (4H, s, succinimidyl), 3.2—3.7 (3H, m, CH_2NH, CHOH), 5.6 (1H, s, CH=C=), 6.6 (1H, br, NH).

N-Hydroxysuccinimidyl ε-(Glycyrrhetylamino)hexanoate (**6c**): Semicrystalline. NMR ($CDCl_3$) δ: 2.6 (2H, m, CH_2CO), 2.8 (4H, s, succinimidyl), 3.1—3.7 (3H, m, CH_2NH, CHOH), 5.6 (1H, s, CH=C=), 5.8 (1H, br, NH).

Preparation of Glycyrrhetylamino Acid-BSA Conjugates (7a—c)——General Procedure: A solution of the N-hydroxysuccinimidyl glycyrrhetylamino acid ester (**6a**—**c**) (3×10^{-8} mol) in pyridine (0.5 ml) was added to a phosphate buffer (pH 7.3, 0.7 ml) solution of BSA (1.5×10^{-9} mol) and the mixture was stirred at 5° for 24 hr. The resulting turbid solution was dialyzed for 5 days against distilled water with two changes a day. The dialysate was further purified by chromatography on a Sephadex G-25 column. The protein fraction was then lyophilized and stored until use for immunization.

Determination of the Number of GA Molecules linked to a BSA Molecule——The analyses were performed by comparing the absorbances of the conjugates with those of N-hydroxysuccinimidyl glycyrrhetylaminoacylates; the ε values at 250 nm were 11000 (**6a**), 14000 (**6b**) and 13000 (**6c**). The protein contents of the conjugate solutions were determined by the method of Lowry *et al.*[9]

Preparation of Antiserum for GA——The glycyrrhetylglycine-BSA conjugate (**7a**) (2 mg) was dissolved in sterile isotonic saline (1 ml) and emulsified with the same amount of complete Freund's adjuvant (Difco, Detroit, Mich., U.S.A.). The emulsion was injected into domestic albino female rabbits subcutaneously and intramuscularly at multiple sites on the back and legs. Booster injections with half the initial amount of immunogen were administered once every two weeks for two months and monthly thereafter. The blood was collected by puncture of the ear vein 10 to 14 days after the last booster injection. The serum was separated by centrifugation for 15 min and was stored at −20° until use.

Preparation of Glycyrrhetyl-β-D-galactosidase Conjugates (7d—f)——A solution of N-hydroxysuccinimide ester (**6a**—**c**) (2×10^{-8} mol) in pyridine (1 μl) was added to a solution of β-Gal (1×10^{-9} mol) in 0.05 M phosphate buffer (0.5 ml, pH 7.3) and was stirred at 0° for 7 hr. The mixture was directly chromatographed on a Sepharose 6B column (1.5 cm × 30 cm) with 0.02 M phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% BSA, and 0.1% NaN_3 (buffer A). The peak fractions were pooled at 4° until use.

Assay Procedure——A mixture of the standard solution of GA (1 mg/ml in EtOH), γ-glycyrrhetylaminobutyric acid-β-Gal conjugate, and anti-GA serum was diluted with buffer A. Sample or standard solution of GA (100 μl) was added to 20000-fold-diluted antiserum (100 μl) and 120 μU of β-Gal conjugate (50 μl). The mixture was incubated at room temperature for 2 hr, then 20 μl of a 100-fold-diluted solution of normal rabbit serum and 50 μl of a 10-fold-diluted solution of goat antiserum to rabbit IgG were added. After further incubation at 4° for 12 hr, the reaction mixture was washed with buffer A and centrifuged twice.

Measurement of β-D-galactosidase Activity——The precipitates were incubated with 1×10^{-4} M 7-β-D-galactopyranosyloxy-4-methylcoumarin (150 μl) at 30° for 30 min. After incubation, 2 ml of 0.1 M glycine–NaOH buffer (pH 10.3) was added to the reaction mixture, and the fluorescence intensity of 7-hydroxy-4-methylcoumarin was measured at 365 and 448 nm for excitation and emission, respectively, with a Shimadzu RF-503 spectrofluorophotometer.

Specificity of the Antiserum——The immune reactivities of some glycyrrhetic acid-related compounds or phenolic and neutral steroids toward anti-glycyrrhetylglycine-BSA serum were assayed by using γ-glycyrrhet-

ylaminobutyric acid-β-Gal conjugate according to the assay procedure described above, except that GA was replaced by an analog or steroid. The results are shown in Table I.

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