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Studies on the Enzyme Immunoassay of Bio-active Constituents Contained in Oriental Medicinal Drugs. II.[1] Enzyme Immunoassay of Glycyrrhizin[2]

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In order to develop an enzyme immunoassay of glycyrrhizin (GL), *N*-(glycyrrhizinyl)-ε-aminohexanoic acid and -*trans*-4-aminomethylcyclohexanecarboxylic acid were synthesized as haptens from GL *via* 6′,6″-di-*tert*-butyl GL (VI) as a key intermediate, which was obtained by the selective *tert*-butylation of GL with *O*-*tert*-butyl-*N*,*N*′-dicyclohexylisourea or by the hydrogenolysis of 6′,6″-di-*tert*-butyl-30-benzyl GL (V) over palladium carbon. Coupling of the hapten with bovine serum albumin (BSA) (carrier protein) and β-galactosidase (labelled enzyme) was carried out by the *N*-hydroxysuccinimide ester method. Anti-GL serum was elicited in rabbits by immunization with *N*-(glycyrrhizinyl)-ε-aminohexanoic acid-BSA conjugate (XIV). Separation of bound and free fractions was performed by a double antibody method using a goat antiserum to rabbit IgG. 7-β-D-Galactopyranosyl-4-methylcoumarin was used as the substrate for the fluorometric assay of β-galactosidase activity. A satisfactory standard curve for GL was obtained in the range of 0.2—20 ng/ml.

Keywords——enzyme immunoassay; glycyrrhizin; ε-aminohexanoic acid; *trans*-4-(aminomethyl)cyclohexanecarboxylic acid; *N*-hydroxysuccinimide ester method; *O*-*tert*-butyl-*N*,*N*′-dicyclohexylisourea; *O*-benzyl-*N*,*N*′-dicyclohexylisourea; β-galactosidase; 7-β-D-galactopyranosyloxy-4-methylcoumarin

We have undertaken the enzyme immunoassay (EIA) of bio-active constituents contained in oriental medicinal drugs as a preliminary to studies of the metabolism of these compounds in humans. In a previous paper,[2] we described the EIA of 18β-glycyrrhetic acid (GA), the aglycone of 18β-glycyrrhizin (GL), which is a principal constituent of Glycyrrhizae Radix. This EIA method was applied to determine GA and GL concentrations in human blood after intravenous and oral administration of GL.[3] However, the GL concentration was determined indirectly by means of EIA of GA, which was obtained by the hydrolysis of GL with dilute hydrochloric acid. This paper deals with the synthesis of *N*-glycyrrhizinylamino acids as haptens, preparation of the bovine serum albumin (BSA) conjugates, production and specificity of anti-GL antibody and the EIA of GL.

ε-Aminohexanoic acid and *trans*-4-(aminomethyl)cyclohexanecarboxylic acid were chosen as "chemical bridges" between GL and carrier protein for use in EIA. The GL molecule has three carboxyl groups at the 30, 6′ and 6″ positions. To introduce the chemical bridges at the carboxyl group of the 30 position, the other two carboxyl groups were protected with *tert*-butyl ester moieties as shown in Chart 1.

First, reaction of GL (I) with *O*-benzyl-*N*,*N*′-dicyclohexylisourea,[4] which can selectively esterify the carboxyl group of hydroxy acids, afforded 6′,6″-dibenzyl GL (II) and 6′,6″,30-tribenzyl GL (III) in 18.4 and 22.6% yields, respectively. In order to ascertain the locations of benzyl groups, II and III were each hydrolyzed with 20% H_2SO_4 in dioxane to give GA (XVI) from the former and 30-benzyl GA (XVII) from the latter. The two benzyl groups of II were located at the 6′ and 6″ positions of th diglucuronic acid moiety, and one of the three benzyl groups of III was located at the 30 position of the triterpene moiety. Partial hydrolysis of tribenzyl GL (III) with 5% potassium hydroxide in methanol afforded in 70% yield 30-benzyl

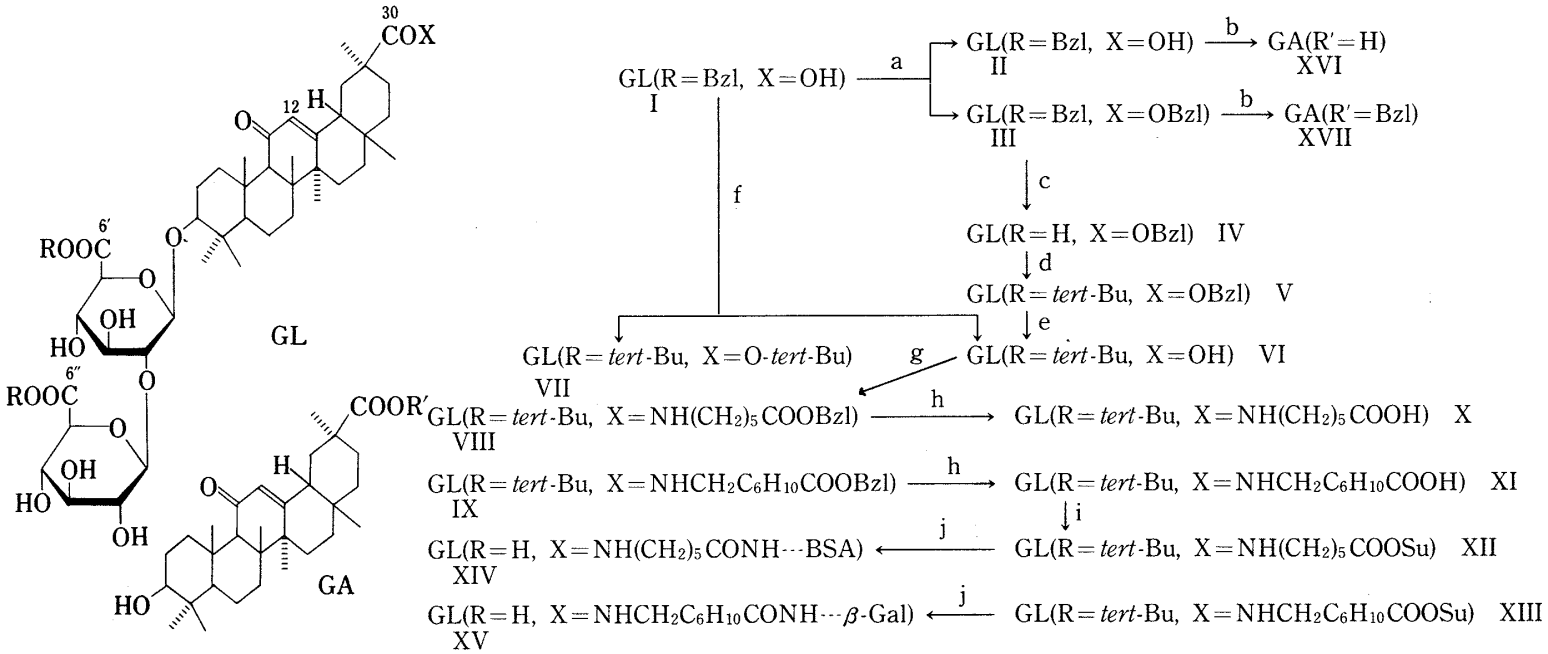
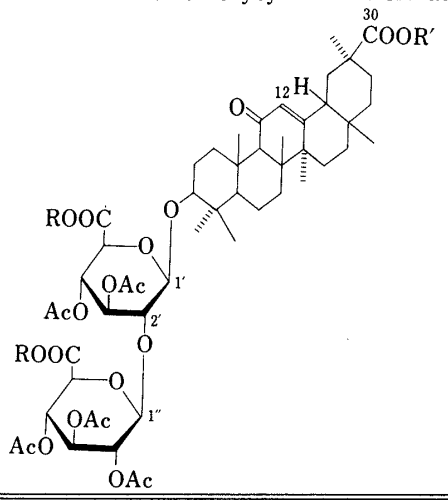


Chart 1. Synthetic Route to Glycyrrhizinylamino Acid and Protein Conjugate

(a) *O*-Benzyl-*N,N'*-dicyclohexylisourea; (b) 20% H_2SO_4-dioxane; (c) 5% KOH-MeOH; (d), (f) *O-tert*-butyl-*N,N'*-dicyclohexylisourea; (e), (h) Pd-C/H_2; (g) DEPC+$NH_2(CH_2)_5COOBzl$ or $NH_2CH_2C_6H_{10}COOBzl$; (i) HOSu+EDC; (j) 1) 50% TFA-CH_2Cl_2, 2) BSA or β-Gal in phosphate buffer (pH 7.3). Su: Succinimidly group.

TABLE I. ^{1}H-NMR Data for Glycyrrhizin Derivatives (in $CDCl_3$)



Compd. No.	R	R′	Anomeric H H-1′ (d)[a]	Anomeric H H-1″ (d)[a]	H-2′ (m)	H-12 (s)	Ph-H (5H) (s)		*tert*-Bu CH_3 (9H) (s)		CH_3 (s)
II′	$C_6H_5CH_2$	CH_3	4.51	4.76	3.87	5.68	7.37				0.74, 0.81, 0.97, 1.08, 1.11, 1.15, 1.34.
III′	$C_6H_5CH_2$	$C_6H_5CH_2$	4.50	4.74	3.85	5.56	7.38	7.39			0.73, 0.73, 0.95, 1.07, 1.09, 1.15, 1.32.
IV′	CH_3	$C_6H_5CH_2$	4.51	4.74	3.86	5.55	7.38				0.72, 0.81, 1.01, 1.09, 1.11, 1.15, 1.33.
V′	*tert*-Bu	$C_6H_5CH_2$	4.49	4.76	3.87	5.56	7.46		1.48	1.50	0.74, 0.84, 1.03, 1.10, 1.12, 1.16, 1.35.
VI′	*tert*-Bu	CH_3	4.48	4.75	3.86	5.67			1.46	1.50	0.80, 0.83, 1.02, 1.11, 1.12, 1.14, 1.35.
VII″	*tert*-Bu	*tert*-Bu	4.48	4.75	3.86	5.63			1.47 (18H)	1.51	0.79, 0.82, 1.01, 1.09, 1.10, 1.10, 1.32.

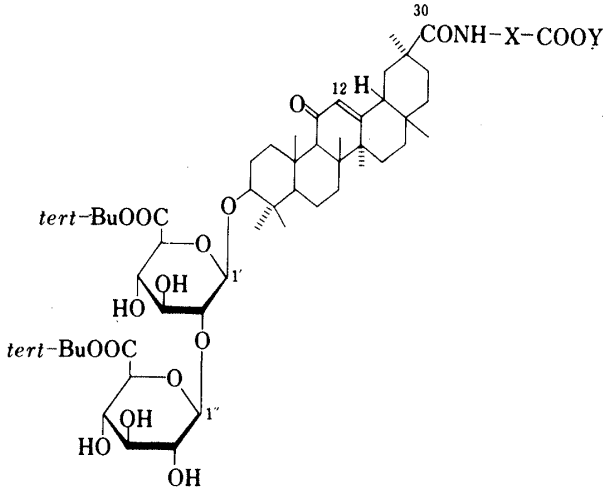
a) J=7 Hz.

GL (IV), whose benzyl group is resistant to alkaline hydrolysis.[5] *tert*-Butylation of IV with *O*-*tert*-butyl-*N*,*N*′-dicyclohexylisourea[6] gave 6′,6″-di-*tert*-butyl-30-benzyl GL (V) in 38% yield and hydrogenolysis over palladium carbon afforded 6′,6″-di-*tert*-butyl GL (IV) in 90% yield.

On the other hand, *tert*-butylation of GL (I) with *O*-*tert*-butyl-*N*,*N*′-dicyclohexylisourea gave 6′,6″-di-*tert*-butyl GL (VI) and 6′,6″, 30-tri-*tert*-butyl GL (VII) in 11.5 and 3% yields. This di-*tert*-butyl-ester (VI) was identical with the compound obtained from the above hydrogenolysis of di-*tert*-butyl-benzyl GL (V) by mixed mp examination and infrared (IR) (KBr) comparison. ^{1}H-Nuclear magnetic resonance (^{1}H-NMR) spectra data for the methyl and/or acetyl derivatives of compounds, II′-VII′, are shown in Table I. The spectra data showed the signals of seven methyl groups (s) and one vinyl proton (H-12, s) of the triterpene moiety, and two anomeric protons (H-1′, H-1″, d, J=7 Hz), and a methine proton (H-2′ m), of the glucuronic acid moiety. The H-1′ proton signal appeared at higher field than the H-1″ proton signal from the results of decoupling examination between anomeric protons and the H-2′ proton. The benzyl ester derivatives, II′—V′, exhibited the signals of aromatic protons (s), and the *tert*-butyl ester derivatives, V′—VII′, exhibited the signals of three methyls (s). These data substantiated the proposed structure II—VII.

Next, condensation of di-*tert*-butyl GL (VI) with benzyl ε-aminohexanoate and benzyl *trans*-4-(aminomethyl)cyclohexanecarboxylate by the use of diethyl phosphorylcyanide (DEPC) gave benzyl *N*-(di-*tert*-butyl-glycyrrhizinyl)aminohexanoate (VIII) and benzyl *N*-(di-*tert*-butyl-glycyrrhizinyl)aminomethylcyclohexanecarboxylate (IX) in 85.5 and 76.3% yields, respectively. Hydrogenolysis of VIII and IX over palladium carbon afforded *N*-(di-*tert*-

TABLE II. ^{1}H-NMR Data for Glycyrrhizinylamino Acid Dervatives (in $CDCl_3$)



Compd. No.	X	Y	Anomeric H H-1′ (d)[a]	Anomeric H H-1″ (d)[a]	H-12 (s)	-CHCO- (m)	-NHCH$_2$- (m)	*tert*-Bu CH$_3$ (9H) (s)	*tert*-Bu (9H) (s)	Su (4H) (s)
VIII	-$(CH_2)_5$-	$C_6H_5CH_2$	4.50	4.60	5.67	2.37 (2H)	3.40—3.84	1.47	1.50	
IX	-$CH_2C_6H_{10}$-	$C_6H_5CH_2$	4.48	4.56	5.64	2.75 (1H)	3.04—3.24	1.44	1.48	
X	-$(CH_2)_5$-	H	4.49	4.56	5.68	2.32 (2H)	3.46—3.86	1.49	1.51	
XI	-$CH_2C_6H_{10}$-	H	4.46	4.54	5.62	2.72 (1H)	3.04—3.24	1.46	1.48	
XII	-$(CH_2)_5$-	Su	4.48	4.57	5.64	2.32 (2H)	3.46—3.85	1.45	1.48	2.81
XIII	-$CH_2C_6H_{10}$-	Su	4.48	4.58	5.64	2.76 (1H)	3.06—3.30	1.46	1.49	2.80

a) J=7 Hz.

TABLE III. Specificity of Anti-glycyrrhizinyl-ε-aminohexanoic Acid-BSA Serum

Compound	Cross reaction (%)
Ammonium 18β-glycyrrhizinate	100
Ammonium 18α-glycyrrhizinate	47
Liquiritic acid diglucuronide	9.6
18β-Glycyrrhetic acid	< 0.03
Sodium carbenoxolone	< 0.02
Cholesterol	< 0.02
Sodium deoxycholate	< 0.02
Sodium cholate	< 0.02
Paeoniflorin	< 0.02
Albiflorin	< 0.02
Benzoyl paeoniflorin	< 0.02
Saikosaponin b_3	< 0.02
Saikosaponin c	< 0.02
Sodium D-glucuronate	< 0.003

butyl-glycyrrhizinyl)aminohexanoic acid (X) and *N*-(di-*tert*-butyl-glycyrrhizinyl)aminomethylcyclohexanecarboxylic acid (XI) in 70.6 and 75.8% yields, respectively. Compounds X and XI were condensed with *N*-hydroxysuccinimide by the use of ethyl dimethylaminopropylcarbodiimide hydrochloride (EDC) to give hydroxysuccinimidyl *N*-(di-*tert*-butyl-glycyrrhizinyl)amino acylates, (XII) and (XIII), respectively. ^{1}H-NMR spectral data for VIII—XIII are given in Table II. The spectra showed two anomeric proton signals (d, J=7 Hz) of the diglucuronic acid moiety, one vinyl proton (s) of the triterpene moiety, two methylene protons (each m)

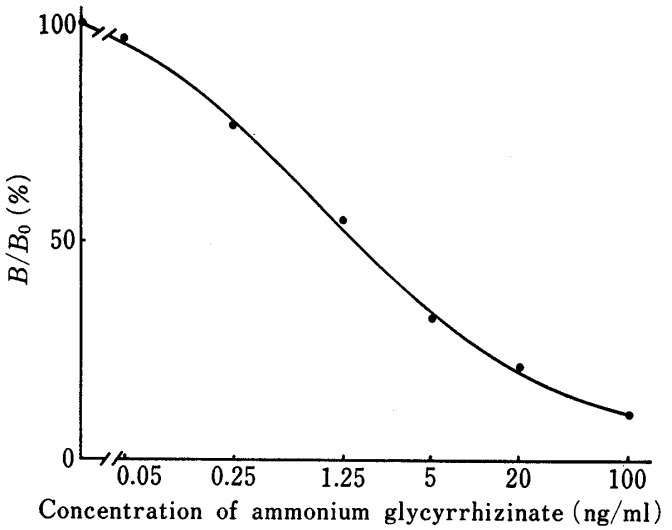


Fig. 1. Standard Curve for EIA of Glycyrrhizin

adjacent to the amino and the carbonyl groups of the amino acid moiety, and methyl protons (s) of the *tert*-butyl groups. *N*-Hydroxysuccinimidyl esters, (XII) and (XIII), exhibited ethyene proton signals (s). These data substantiated the proposed structures VIII—XIII.

Removal of the *tert*-butyl groups from hydroxysuccinimidyl *N*-(di-*tert*-butyl-glycyrrhizinyl)amino acylates, (XII) and (XIII), was performed with 50% trifluoroacetic acid in dichloromethane to afford *N*-hydroxysuccinimidyl *N*-(glycyrrhizinyl)amino acylates. These hydroxysuccinimidyl esters were used for the next step without purification. Hydroxysuccinimidyl *N*-(glycyrrhizinyl)aminohexanoate was coupled with BSA in phosphate buffer (pH 7.3) to afford *N*-(glycyrrhizinyl)aminohexanoic acid-BSA conjugate (XIV), which was used for immunization after purification by dialysis. The incorporation of the hapten in the conjugate was examined by ultraviolet (UV) spectral analysis. Hydroxysuccinimidyl *N*-(glycyrrhizinyl)-aminomethylcyclohexanecarboxylate was coupled with β-galactosidase (β-Gal) and purified on a Sepharose 6B column to give *N*-(glycyrrhizinyl)aminomethylcyclohexanecarboxylic acid-β-Gal conjugate (XV), which was used as the labeled antigen for EIA.

Rabbits were immunized with GL–BSA conjugate (XIV) emulsified with complete Freund's adjuvant. The antibody was tested at intervals several weeks to determnie the 50% binding amounts of GL-β-Gal conjugate (XV). An antiserum obtained from the rabbits immunized with the GL–BSA conjugate for four months showed remarkably increased activity towards GL. The bound and free GL-β-Gal conjugates were separated by a double antibody method with a goat antiserum to rabbit IgG, and the enzyme activity of the immune precipitate was determined fluorometrically with 7-β-D-galactopyranosyloxy-4-methylcoumarin as a substrate. A typical standard curve is shown in Fig. 1; the measurable range was 0.2—20 ng/ml. The cross reactivity of anti-GL–BSA serum with some GL-related compounds and constituents of Chinese Paeony root, which is often prescribed with Glycyrrhizae Radix in Kanpo remedies, was tested and the results are shown in Table III. The antiserum reacted with two compounds, 18α-GL (47%) and liquiritic acid diglucuronide (9.6%), and GL could be determined in a mixture with 18β-GA (0.03%). The procedure and results of EIA for GL in the blood of animals and man will be reported elsewhere.

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotation was measured with a JASCO DIP-4 polarimeter. Spectra were obtained with the following machines: UV on a Beckman model 24, ^{1}H-NMR on Varian XL 200 and EM 390 Spectrometers (solvent, $CDCl_3$; internal standard, tetramethylsilane; chemical shifts, δ (ppm); abbreviations are s, singlet; d, doublet; m, multiplet.), FD–MS on a Hitachi M-80 spectrometer. Fluorimetry was performed on a Shimadzu RF-503 recording spectrofluorophotometer. 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, and detection was achieved by UV irradiation (254 nm) or by spraying 1% $Ce(SO_4)_2$ in 10% H_2SO_4 followed by heating. Column chromatography was performed on Kieselgel 60 (70—230 mesh, Merck) and Mallinckrodt silica gel (100 mesh, Merck). Buffer A: 0.02 M phosphate-buffered saline containing 0.1% BSA, 0.1% NaN_3, 0.001% $MgCl_2$.

Benzylation of 18β-Glycyrrhizin(6′,6″-Dibenzyl-GL (II) and 6′,6″30-Tribenzyl-GL (III))——A solution of *O*-benzyl-*N*,*N*′-dicyclohexylisourea[4] (4.6 g, 14.6 mmol) in $CHCl_3$ (8 ml) was added to a solution of GL (4 g, 4.8 mmol) in dimethylformamide (DMF) (5 ml) under stirring at room temperature. Stirring was continued for 3 d, and the precipitate (dicyclohexylurea) formed was filtered off. The filtrate was washed with 10% citric acid and water, dried ($MgSO_4$) and concentrated to give a syrup. This syrup was chromatographed on silica gel (140 g). Stepwise elution gave the following results: crude tribenzyl-GL with 3% MeOH–$CHCl_3$, crude dibenzyl GL with 5% MeOH–$CHCl_3$. Crude tribenzyl GL was purified by preparative TLC with 10% MeOH–$CHCl_3$ and recrystallized from $CHCl_3$+isopropyl ether to give III, 1.2 g (22.6%), mp 137°C $[\alpha]_D^{24}$ +70.0° (c=1, $CHCl_3$). FD–MS m/z: 1092 (M^+, $C_{68}H_{80}O_{16}$). *Anal.* Calcd for $C_{63}H_{80}O_{16}\cdot H_2O$: C, 68.09; H, 7.44. Found: C, 68.22; H, 7.46. Pentaacetate (III′): III (50 mg) was acetylated with Ac_2O (0.5 ml) and pyridine (0.5 ml) at room temperature as usual to give III′, which was recrystallized from $CHCl_3$+hexane, mp 118—121°C, $[\alpha]_D^{26}$ +37.5° (c=1, $CHCl_3$), *Anal.* Calcd for $C_{73}H_{90}O_{21}\cdot H_2O$: C, 66.35; H, 7.02. Found: C, 66.30; H, 7.15.

Crude dibenzyl GL was purified by preparative TLC with 20% MeOH–$CHCl_3$ and recrystallized from 10% MeOH–$CHCl_3$+isopropyl ether to give II, 0.9 g (18.4%), mp 176—180°C, $[\alpha]_D^{25}$ +58.6° (c=1.2, 10% MeOH–$CHCl_3$), FD–MS m/z: 1003 [$(M+1)^+$, $C_{56}H_{74}O_{16}$]. *Anal.* Calcd for $C_{56}H_{74}O_{16}\cdot H_2O$: C, 65.86; H, 7.50. Found: C, 66.10; H, 7.71. Pentaacetate of 6′,6″-dibenzyl-30-methyl GL (II′): A solution of II (50 mg) in a small amount of MeOH was methylated with ethereal diazomethane and then acetylated as usual to give II′, which was recrystallized from $CHCl_3$+hexane, mp 130°C, $[\alpha]_D^{25}$ +40.8° (c=0.9, $CHCl_3$), *Anal.* Calcd for $C_{67}H_{86}O_{21}\cdot H_2O$: C, 64.61; H, 7.12. Found: C, 64.43; H, 7.38.

Hydrolysis of II and III——A solution of III (50 mg) in dioxane (3 ml) was treated with 20% H_2SO_4 (2 ml) and the reaction mixture was warmed on a water bath for 4 h. After cooling, the mixture was concentrated *in vacuo* and the residue was dissolved in AcOEt (100 ml). The AcOEt solution was washed with water, dried ($MgSO_4$), and concentrated *in vacuo* to give 30-benzyl GA (XVI), 16 mg (62.5%), which was identified by direct comparison with an authentic sample.

II (50 mg) was hydrolyzed in the same manner as described for XVI to give 18β-glycyrrhetic acid (XVII), 16 mg (68.3%), which was identified by direct comparison with an authentic sample.

30-Benzyl GL (IV)——A stirred solution of III (1.17 g, 1.07 mmol) in MeOH (40 ml) was treated with 3% KOH–MeOH (4 ml) at room temperature. After 4 h, the reaction mixture was diluted with MeOH (80 ml) and neutralized with Dowex 50W ×8 (H^+ form), then the filtrate was concentrated *in vacuo* to give 30-benzyl GL (IV), 0.75 g (76.5%), which was recrystallized from MeOH, colorless needles, mp 215°C, $[\alpha]_D^{24}$ +72.4° (c=0.55, 50% MeOH–$CHCl_3$). *Anal.* Calcd for $C_{49}H_{68}O_{16}\cdot H_2O$: C, 63.21; H, 7.58. Found: C, 62.9; H, 7.87.

Pentaacetate of 6′,6″-Dimethyl-30-benzyl GL (IV′): Methylation and acetylation of IV (30 mg) in the manner described for II′ gave IV′, 15 mg (40%), which was recrystallized from $CHCl_3$+hexane, colorless needles, mp 140—145°C, $[\alpha]_D^{24}$ +37.7° (c=0.9, $CHCl_3$). *Anal.* Calcd for $C_{61}H_{82}O_{21}\cdot H_2O$: C, 62.65; H, 7.24. Found: C, 62.41; H, 7.42.

6′,6″-Di-*tert*-butyl-30-benzyl GL (V)——A solution of *O*-*tert*-butyl-*N*,*N*′-dicyclohexylisourea containing CuCl [prepared form *tert*-butyl alcohol (222 mg, 3 mmol),dicyclohexylcarbodiimide (618 mg, 3 mmol) and CuCl (6 mg) by the method of Vowinkel[6]] in $CHCl_3$ (3 ml) was added to a solution of IV (0.91 g, 1 mmol) in DMF at room temperature under stirring. After 3 d, the precipitate formed was filtered off and washed with $CHCl_3$ (50 ml). The combined filtrate and washing were washed with 10% citric acid and water, dried ($MgSO_4$) and concentrated *in vacuo*. The yellow oily residue was chromatographed on silica gel with 3% MeOH–$CHCl_3$ to give V (380 mg, 38%), which was recrystallized from $CHCl_3$+hexane, colorless needles, mp 182—185°C, $[\alpha]_D^{24}$ +69.3° (c=1.18, $CHCl_3$). *Anal.* Calcd for $C_{57}H_{84}O_{16}\cdot H_2O$: C, 65.62; H, 8.31. Found: C, 65.45; H, 8.53.

Pentaacetate (V′): Acetylation of V in the manner described for III′ gave V′, which was recrystallized from isopropyl ether+hexane, colorless needles, mp 139—142°C, $[\alpha]_D^{25}$ +44.7° (c=0.87, $CHCl_3$). *Anal.* Calcd for $C_{67}H_{94}O_{21}\cdot H_2O$: C, 64.20; H, 7.72. Found: C, 64.66; H, 7.71.

6′,6″-Di-*tert*-butyl GL (VI)——A solution of V (170 mg, 0.16 mmol) in AcOEt (20 ml) was catalytically hydrogenated over 5% Pd-carbon (80 mg) at atmospheric pressure. The residual solid obtained from the filtrate by evaporation of the solvent was recrystallized from 20% MeOH–$CHCl_3$+isopropyl ether to give VI (139 mg, 90%), colorless needles, mp 214—216°C, $[\alpha]_D^{28}$ +57.3° (c=1, 10% MeOH–$CHCl_3$). *Anal.* Calcd for $C_{50}H_{78}O_{16}\cdot H_2O$: C, 63.00; H, 8.46. Found: C, 62.83; H, 8.66.

Pentaacetate of 6′,6″-Di-*tert*-butyl-30-methyl GL (VI′): Methylation and acetylation of VI (30 mg) in the manner described for II′ gave VI′ (27 mg, 73%), colorless plates, mp 163—165°C, $[\alpha]_D^{25}$ +38.5° (c=0.8, $CHCl_3$). *Anal.* Calcd for $C_{61}H_{90}O_{21}\cdot H_2O$; C, 62.22; H, 7.88. Found: C, 61.91; H, 8.01.

***tert*-Butylation of 18β-Glycyrrhizin (6′,6″-Di-*tert*-butyl-GL (VI) and 6′,6″,30-Tri-*tert*-butyl-GL (VII))**——A solution of *O*-*tert*-butyl-*N*,*N*′-dicyclohexylisourea containing CuCl [prepared from *tert*-butyl alcohol (1.8 g, 24.3 mmol), dicyclohexylcarbodiimide (5 g, 24.3 mmol) and CuCl (18 mg) by the method of Vowinkel[6]] in $CHCl_3$ (3 ml) was added to a solution of GL (4 g, 2.86 mmol) in DMF (8 ml) at room temperature under stirring. After 3 d, the precipitate formed was filtered off and washed with 10% MeOH–$CHCl_3$ (100 ml). The combined filtrate and washing were concentrated *in vacuo*, and the residue was extracted with AcOEt

(200 ml). The AcOEt extract was washed with water, dried ($MgSO_4$), and concentrated to leave syrup, which was purified by silica gel column chromatography with 5% MeOH–$CHCl_3$ to give VI (520 mg, 11.5%) and VII (132 mg, 3%). VII: Recrystallized from $CHCl_3$+isopropyl ether, colorless prisms, mp 214—216°C, $[\alpha]_D^{26}$ +54.7° (c=1, $CHCl_3$). *Anal.* Calcd for $C_{54}H_{86}O_{16}\cdot H_2O$: C, 65.49; H, 8.49. Found: C, 65.33; H, 8.63.

Pentaacetate (VII′): Acetylation of VII (100 mg) in the manner described for III′ gave VII′, which was recrystallized from $CHCl_3$+MeOH, colorless needles, mp 153—155°C, $[\alpha]_D^{26}$ +10.9° (c=1, $CHCl_3$). *Anal.* Calcd for $C_{64}H_{94}O_{21}\cdot H_2O$: C, 63.03; H, 8.10. Found: C, 62.88; H, 8.38.

Compound VI was identical with the substance obtained by the hydrogenolysis of V over Pd carbon.

Benzyl *N*-(Di-*tert*-butyl-glycyrrhizinyl)-ε-aminohexanoate (VIII)——A stirred solution of VI (50 mg, 0.053 mmol) and benzyl ε-amino hexanoate tosylate (39 mg, 0.1 mmol) in DMF (0.5 ml) was treated with diethyl phosphorylcyanide (16.3 mg, 0.1 mmol) and triethylamine (0.014 ml, 0.1 mmol) at 5°C. After 1 h, the reaction mixture was stirred at room temperature for 12 h and then dissolved in AcOEt (100 ml). The solution was washed with 10% citric acid, Na_2CO_3 and water, dried ($MgSO_4$), and concentrated to leave a colorless solid (52 mg, 85.5%). The solid was recrystallized from $CHCl_3$+isopropyl ether to give VIII, mp 155—156°C, $[\alpha]_D^{25}$ +53.8° (c=1, $CHCl_3$). *Anal.* Calcd for $C_{63}H_{95}NO_{17}\cdot H_2O$: C, 65.43; H, 8.46. Found: C, 65.19; H, 8.72.

Benzyl *N*-(Di-*tert*-butyl-glycyrrhizinyl)-*trans*-4-aminomethylcyclohexanecarboxylate (IX)——A stirred solution of VI (120 mg, 0.13 mmol) and benzyl *trans*-4-aminomethylcyclohexanecarboxylate tosylate (108 mg, 0.26 mmol) in DMF (1.5 ml) was treated with diethyl phosphorylcyanide (42 mg, 0.26 mmol) and triethylamine (0.036 ml, 0.26 mmol) at 5°C. After 1 h, the reaction mixture was stirred at room temperature for 12 h and then dissolved in AcOEt (200 ml). The solution was washed with 10% citric acid, 10% Na_2CO_3 and water, dried ($MgSO_4$), and concentrated to give a colorless solid (190 mg, 76.3%). The solid was recrystallized from isopropyl ether to give IX, colorless needles, mp 185°C, $[\alpha]_D^{25}$ +56.5° (c=1, $CHCl_3$). *Anal.* Calcd for $C_{65}H_{97}NO_{17}\cdot H_2O$: C, 66.02; H, 8.44. Found: C, 65.92; H, 8.62.

***N*-(Di-*tert*-butyl-glycyrrhizinyl)-ε-aminohexanoic Acid (X)**——A solution of VIII (60 mg, 0.052 mmol) in 20% MeOH–AcOEt (20 ml) was catalytically hydrogenated over 5% Pd-carbon (60 mg) at atmospheric pressure for 5 h. The residual solid obtained from the filtrate by evaporation of the solvent was recrystallized from MeOH–isopropyl ether to give X (39 mg, 70.6%), colorless needles, mp 211—214°C, $[\alpha]_D^{25}$ +61.6° (c=1, 50% MeOH–$CHCl_3$). *Anal.* Calcd for $C_{56}H_{89}NO_{17}\cdot H_2O$: C, 63.07; H, 8.60. Found: C, 62.88; H, 8.87.

***N*-(Di-*tert*-butyl-glycyrrhizinyl)-*trans*-4-aminomethylcyclohexanecarboxylic Acid (XI)**——A solution of IX (100 mg, 0.086 mmol) in 10% MeOH–AcOEt (20 ml) was catalytically hydrogenated over 5% Pd-carbon (100 mg) at atmospheric pressure for 2.5 h. The residue obtained from the filtrate by evaporation of the solvent was recrystallized from isopropyl ether to give XI (70 mg, 75.8%), colorless needles, mp 218°C, $[\alpha]_D^{25}$ +69.0° (c=1, 10% MeOH–$CHCl_3$). *Anal.* Calcd for $C_{58}H_{91}NO_{17}\cdot H_2O$: C, 63.77; H, 8.58. Found: C, 63.43; H, 8.87.

Hydroxysuccinimidyl *N*-(Di-*tert*-butyl-glycyrrhizinyl)-ε-aminohexanoate (XII)——A solution of X (100 mg, 0.093 mmol) and *N*-hydroxysuccinimide (20 mg, 0.174 mmol) in pyridine (0.5 ml) was treated with ethyl dimethylaminopropylcarbodiimide·HCl (3.3 mg, 0.174 mmol) at 0°C. After 1 h, the mixture was stirred overnight at room temperature and dissolved in AcOEt (150 ml). The AcOEt solution was washed successively with 10% citric acid, water and 5% $NaHCO_3$, dried ($MgSO_4$), and concentrated *in vacuo*. The residue was purified by preparative TLC with 10% MeOH–$CHCl_3$ to give XII (30 mg, 27.5%) and the starting material (25 mg). XII: Recrystallized from $CHCl_3$+isopropyl ether, colorless prisms, mp 200—202°C. *Anal.* Calcd for $C_{60}H_{92}N_2O_{19}\cdot H_2O$: C, 61.94; H, 8.14. Found: C, 62.18; H, 7.95.

Hydroxysuccinimidyl *N*-(Di-*tert*-butyl-glycyrrhizinyl)-*trans*-aminomethylcyclohexanecarboxylate (XIII)
A stirred solution of XI (56 mg, 0.052 mmol) and *N*-hydroxysuccinimide (11 mg, 0.097 mmol) in DMF (0.5 ml) was treated with ethyl dimethylaminopropylcarbodiimide·HCl (18.5 mg, 0.097 mmol) at 0°C. After 1 h, the reaction mixture was stirred overnight at room temperature and dissolved in AcOEt (100 ml). The AcOEt solution was washed with 10% citric acid, water and 5% $NaHCO_3$, dried ($MgSO_4$), and concentrated *in vacuo*. The residue was purified by preparative TLC with 10% MeOH–$CHCl_3$ to give XIII (22 mg, 36%), and the starting material (15 mg). XIII: Recrystallized from $CHCl_3$+isopropyl ether, colorless needles, mp 171—173°C. *Anal.* Calcd for $C_{62}H_{94}N_2O_{19}\cdot H_2O$: C, 62.60; H, 8.14. Found: C, 62.42; H, 8.31.

***N*-(Glycyrrhizinyl)aminohexanoic Acid–BSA Conjugate (XIV)**——XII (25 mg, 0.022 mmol) was dissolved in 50% trifluoroacetic acid–CH_2Cl_2 (2 ml) under stirring at 0°C. After being stirred for 2 h at room temperature, the reaction mixture was concentrated *in vacuo* below room temperature, and the residue was washed with dried ether 3 times and then dried over P_2O_5 to give hydroxysuccinimidyl *N*-(glycyrrhizinyl)aminohexanoate (20 mg, 88.6%) as a semisolid. A solution of the crude product (18.5 mg, 0.018 mmol) in pyridine (0.5 ml) was added to a phosphate buffer (pH 7.3, 0.5 ml) solution of BSA (20 mg, 0.003 mmol) and the mixture was stirred at 5°C for 24 h. The resulting turbid solution was dialyzed successively for 5 d against 50, 25, 15 and 10% pyridine–H_2O and H_2O. The dialysate was further purified by chromatography on a Sephadex G-25 column.

Determination of the Number of GL Molecules linked to One BSA Molecule——The UV spectrometric analysis was performed by comparing the absorbance at 258 nm of the conjugate (XIV) with those of BSA and hydroxysuccinimidyl *N*-(glycyrrhizinyl)aminohexanoate as controls in 0.05 M phosphate buffer (pH 7.3)

and using the following constants: ε value for BSA 23450, for hapten 13390. The protein contents of the conjugate solution were determined by the method of Lowry *et al.*[7] The number of hapten molecules coupled to one BSA molecule was determined to be 26.3.

Preparation of Antiserum to GL—The GL-aminohexanoic acid–BSA conjugate (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 d after the last booster injection. The serum was separated by centrifugation for 15 min and was stored at −20°C until use.

Preparation of Glycyrrhizinyl-β-D-Galactosidase Conjugate (XV)——XIII (25 mg, 0.021 mmol) was dissolved in 50% trifluoroacetic acid–CH_2Cl_2 (2 ml) under stirring at 0°C. After being stirred for 2 h at room temperature, the reaction mixture was concentrated *in vacuo* below room temperature, and the residue was triturated with dried ether and dried over P_2O_5 *in vacuo* to give hydroxysuccinimidyl *N*-(glycyrrhizinyl)-aminomethylcyclohexanecarboxylate (20 mg, 88%) as a semisolid. This active ester (50.3 μg, 47×10^{-9} mol) [5 μl of a solution of the ester (10.06 mg) in pyridine (1 ml) was pipetted off] was added to a solution of β-galactosidase (1 mg, 2×10^{-9} mol) in 0.05 M phosphate buffer (pH 7.3, 0.5 ml) and the mixture was stirred at 0°C for 7 h. The 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.

Assay Procedure——Sample or standard solution of GL (100 μl) was added to 30000-fold-diluted antiserum (100 μl) and 120 U of β-Gal conjugate (50 μl). The mixture was incubated at room temperature for 2 h, and 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°C for 12 h, the reaction mixture was washed with buffer A and centrifuged twice.

Measurement of β-D-Galactosidase Activity——The precipitate was incubated with 1×10^{-4} M 7-β-D-galactopyranosyloxy-4-methylcoumarin (150 μl) at 30°C 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 (365 and 448 nm for excitation and emission, respectively).

Specificity of the Antiserum——The immune reactivities of thirteen kinds of GL-related compounds toward anti-glycyrrhizinyl-aminohexanoic acid–BSA serum were assayed by using glycyrrhizinyl-aminomethyl-cyclohexanecarboxylic acid-β-Gal conjugate according to the assay procedure described above. Reaction ratio (%) = GL concentration required to induce 50% inhibition of antiserum binding/sample concentration required to induce 50% inhibition of antiserum binding. The results are shown in Table III.

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