[Chem. Pharm. Bull. 36(1) 8—14 (1988)]

Studies on the Enzyme Immunoassay of Bio-active Constituents Contained in Oriental Medicinal Drugs. IV.[1] Enzyme Immunoassay of Glycyrrhetic Acid

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(Received May 6, 1987)

Enzyme immunoassay of glycyrrhetic acid was developed by employing antisera elicited from in rabbits immunized with C-3 bridged glycyrrhetic acid–bovine serum albumin (BSA) conjugate (**15**, **16**) and C-3 bridged glycyrrhetic acid–β-galactosidase conjugate (**17**, **18**) as labeled antigens. Hemisuccinyl and hemiglutaryl groups, which were chosen as the chemical bridges, were introduced at the hydroxy group of *tert*-butyl glycyrrhetate (**2**). Immunoassay was performed with bridge heterologous combinations such as anti-glycyrrhetic acid–hemisuccinate–BSA (**15**) antiserum and glycyrrhetic acid–hemiglutarate–β-galactosidase conjugate (**18**) and the reverse combination (**16** and **17**). The sensitivity of enzyme immunoassays (EIA) expressed as the midpoint (logit $B/B_0=0$) was higher in the former combination (2.4 ng/tube) than the reverse combination (8.6 ng/tube). The cross reactivities of the anti-C-3 bridged glycyrrhetic acid antisera with C-3 substituted derivatives of glycyrrhetic acid were higher than those of the anti-C-30 bridged glycyrrhetic acid antiserum.

Keywords——enzyme immunoassay; antiserum; glycyrrhetic acid 3-hemisuccinate; glycyrrhetic acid 3-hemiglutarate; β-galactosidase; bridge heterology; double antibody method; labeled antigen

In the previous papers,[2,3] enzyme immunoassays (EIA) of 18β-glycyrrhetic acid (GA) and 18β-glycyrrhizin (GL) were developed by employing the antisera elicited in rabbits immunized with bovine serum albumin (BSA) conjugates of GA and GL derivatives which possessed a chemical bridge between their C-30 position and a carrier protein. Each antiserum showed high specificity. In this paper, we wish to report the preparation and specificity of antiserum elicited with C-3 bridged GA, and to compare the results with those obtained with antiserum to C-30 bridged GA.

As shown in Chart 1, to protect the carboxyl group of GA with a *tert*-butyl group, GA (**1**) was reacted with isobutene in the presence of concentrated H_2SO_4 to give a mixture of three compounds, *tert*-butyl glycyrrhetate (**2**), *tert*-butyl 3-*O*-*tert*-butyl-glycyrrhetate (**3**) and 3-*O*-*tert*-butyl-glycyrrhetic acid (**4**) in 14%, 19% and 7% yields, respectively. Reaction of **1** with *O*-*tert*-butyl-*N*,*N*′-dicyclohexylisourea afforded the desired compound **2** in 42% yield. As an alternative preparation of **2**, 3-*O*-acetylglycyrrhetic acid (**5**) was reacted with isobutene to give *tert*-butyl 3-*O*-acetyl-glycyrrhetate (**6**), which was hydrolyzed with KOH–MeOH to afford **2** in about 76% overall yield from **5**. On the other hand, the structure of **4** was confirmed by an alternative preparation, that is, the reaction of benzyl glycyrrhetate (**7**) with isobutene afforded benzyl 3-*O*-*tert*-butyl-glycyrrhetate (**8**), and hydrogenation over palladium–carbon then gave **4**.

The proton nuclear magnetic resonance (^{1}H-NMR) spectra of compounds **3**, **4** and **8**

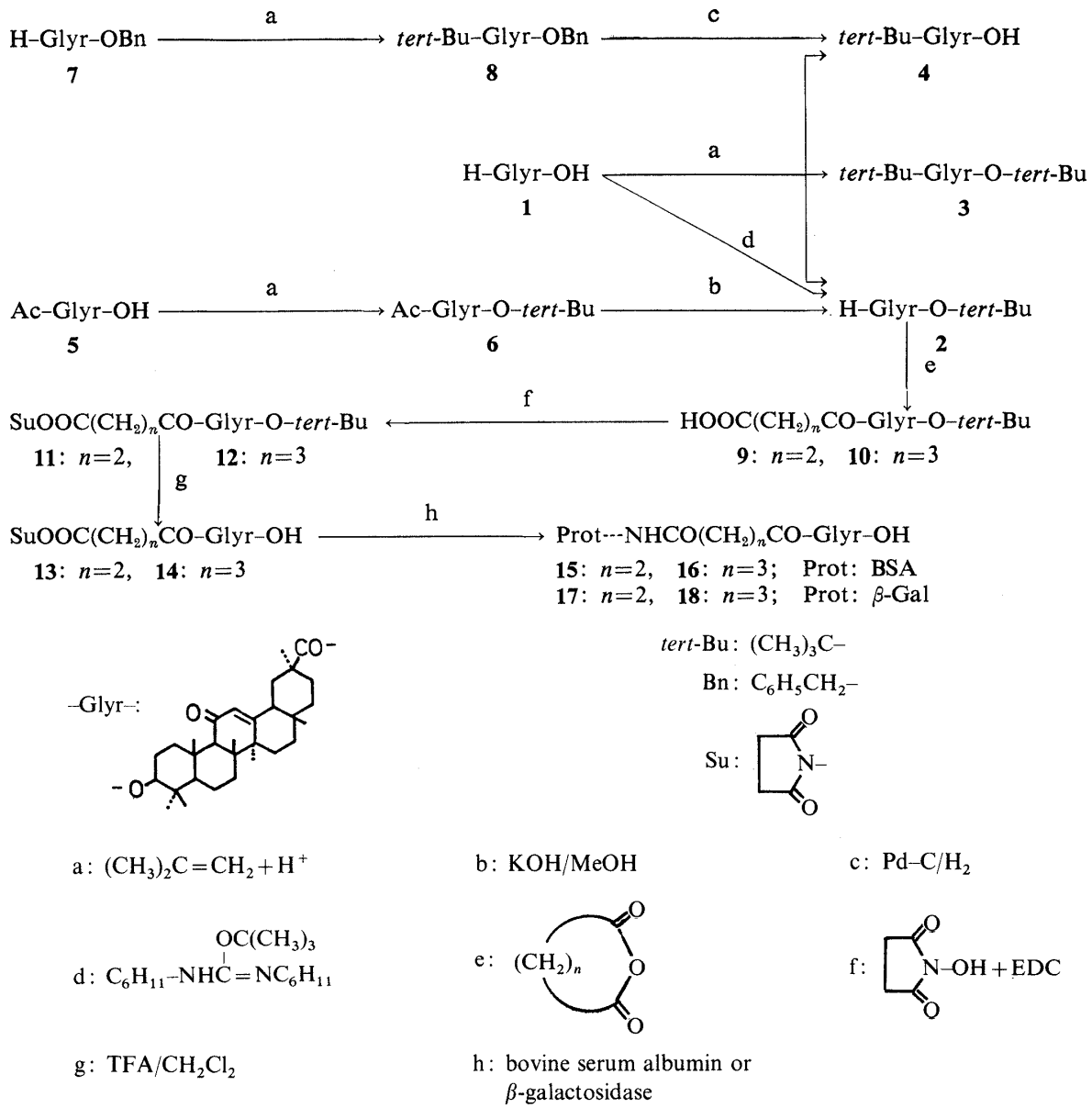
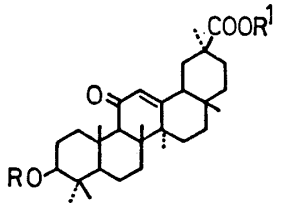


Chart 1. Preparation of Glycyrrhetic Acid–Hemisuccinate– and Glycyrrhetic Acid–Hemiglutarate–Protein Conjugates for Enzyme Immunoassay of Glycyrrhetic Acid

exhibited a 9H singlet due to three methyls of the *tert*-butyl ether group at δ 1.18—1.19 ppm and those of compounds **2**, **3** and **6** exhibited a 9H singlet due to the *tert*-butyl ester group at δ 1.46 ppm (Table I).

Next, hemisuccinyl and hemiglutaryl groups, which were chosen as the bridges between GA and carrier protein, were introduced at the 3-hydroxyl group of GA by the reaction of compound **2** with succinic anhydride and glutaric anhydride in pyridine to give *tert*-butyl 3-*O*-hemisuccinyl-(**9**) and 3-*O*-hemiglutaryl-glycyrrhetate (**10**) in 44% and 48% yields, respectively. The ^{1}H-NMR spectra of compounds **9** and **10** showed the signals of two methylene groups adjacent to carbonyl of the hemisuccinyl and hemiglutaryl groups as a broad singlet at δ 2.68 ppm (4H) and a multiplet at δ 2.43 ppm (4H), respectively. Compounds **9** and **10** were condensed with *N*-hydroxy-succinimide by the use of ethyl dimethylaminopropyl carbodiimide hydrochloride (EDC) to afford *tert*-butyl 3-*O*-(succinimidooxycarbonyl)propionyl-(**11**) and 3-*O*-(succinimidooxycarbonyl)butyryl-glycyrrhetate (**12**). The ^{1}H-NMR spectra of **11** and **12**

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



Compd. No.	R	R^1	CH_3 (s) 3H	*tert*-Bu CH_3 (s) 9H	H-3 (m) 1H	H-12 (s) 1H	$-CH_2CO-$	
2	H	*tert*-Bu	0.81, 0.81, 1.00, 1.10, 1.12, 1.13, 1.36	1.46	3.24	5.64		
3	*tert*-Bu	*tert*-Bu	0.79, 0.81, 0.93, 1.12, 1.14, 1.15, 1.37	1.19 1.46	3.04	5.62		
4	*tert*-Bu	H	0.79, 0.83, 0.92, 1.12, 1.14, 1.23, 1.37	1.18	3.02	5.62		
6	Ac	*tert*-Bu	0.81, 0.88, 0.88, 1.12, 1.13, 1.17, 1.36	1.46	4.54	5.64		$-CH_2C_6H_5$
8	*tert*-Bu	Bzl	0.73, 0.79, 0.92, 1.09, 1.14, 1.16, 1.35	1.18	3.02	5.55		5.12 (2H, ABq) 7.39 (5H, s)
9	$HOOC(CH_2)_2CO$	*tert*-Bu	0.80, 0.87, 0.87, 1.11, 1.21, 1.25, 1.36	1.45	4.57	5.64	2.68 (4H, br s)	
10	$HOOC(CH_2)_3CO$	*tert*-Bu	0.81, 0.88, 0.88, 1.12, 1.13, 1.17, 1.37	1.46	4.56	5.64	2.43 (4H, m)	
11	$SuOOC(CH_2)_2CO$	*tert*-Bu	0.80, 0.87, 0.89, 1.11, 1.13, 1.16, 1.36	1.46	4.59	5.64	2.75, 2.97 (each 2H, m)	Su 2.84 (4H, s)
12	$SuOOC(CH_2)_3CO$	*tert*-Bu	0.81, 0.87, 0.88, 1.12, 1.13, 1.16, 1.36	1.46	4.57	5.64	2.47, 2.71 (each 2H, m)	Su 2.85 (4H, s)

Chemical shifts are given on the δ (ppm) scale with tetramethylsilane as an internal standard.

exhibited the signals of two methylene groups of the succinimidyl group as a singlet at δ 2.84 (4H) and 2.85 (4H), respectively, and the C-3 methine proton adjacent to the acyloxy group showed a broad multiplet signal at δ 4.56—4.59, as did *tert*-butyl 3-*O*-acetyl-glycyrrhetate (**6**) (δ, 4.54), while the C-3 methin proton of 3-*tert*-butyl ether derivatives such as **3**, **4** and **8** showed a broad multiplet signal at δ 3.02—3.04 (Table I). After removal of the *tert*-butyl group from **11** and **12** with trifluoroacetic acid, they were coupled with BSA and β-galactosidase (β-Gal) in phosphate buffer (pH 7.3) to give GA–3-hemisuccinate–(**15**) and GA–3-hemiglutarate–BSA (**16**), GA–3-hemisuccinate–(**17**) and GA–3-hemiglutarate–β-Gal (**18**) conjugates. BSA-conjugates, **15** and **16**, were used for immunization after purification by dialysis. About ten molecules of GA were incorporated per BSA molecule in the BSA-conjugates as judged by UV spectral analysis. β-Gal conjugates, **17** and **18**, were used as labeled antigens for EIA after purification on a Sepharose 6B column.

Antisera to GA were elicited in rabbits immunized with BSA-conjugate, **15** and **16**, in the same manner as previously described.[3] EIA of GA was performed by a competitive binding procedure with goat anti-rabbit immunoglobulin G (IgG) as the second antibody and the bound enzyme activity in the immune precipitate was determined fluorometrically with 7-β-D-galactopyranosyloxy-4-methylcoumarin as the substrate. The EIA of GA was carried out with a bridge heterologous combination of the antiserum and the labeled antigen such as anti-GA–3-hemisuccinate–BSA antiserum and GA–3-hemiglutarate–β-Gal, or anti-GA–3-hemiglutarate–BSA antiserum and GA–hemisuccinate–β-Gal.

Optimum dilution of the antisera determined by the previous method[4] was 8000—10000-fold. Typical standard curves for EIA of GA are shown as linearized logit–log plots[5] in Fig. 1. The measurable range was 0.4—400 ng/tube. The sensitivity of EIA expressed as the midpoint,[6] logit $B/B_0=0$, was 2.4 ng/tube with the combination of anti GA–glutarate–BSA antiserum and GA–hemisuccinate–β-Gal, and 8.6 ng/tube with the reverse combination. The sensitivity of the former combination was greater than that of the latter. The cross reactivities of anti-C-3 bridged GA–BSA (**15** and **16**) antisera are compared with those of anti-C-30 bridged-GA–BSA antiserum, reported previously,[2] in Table II. The cross reactivities of the former antisera with C-3 substituted derivatives of GA such as GL (54%, 57%) and carbenoxolone (215%, 114%) were higher than those of the latter antiserum (0.1%, 1.0%). These results suggested that the specificity of binding of free hapten to antiserum was determined by chemical groups on the hapten far from the point of attachment of the original

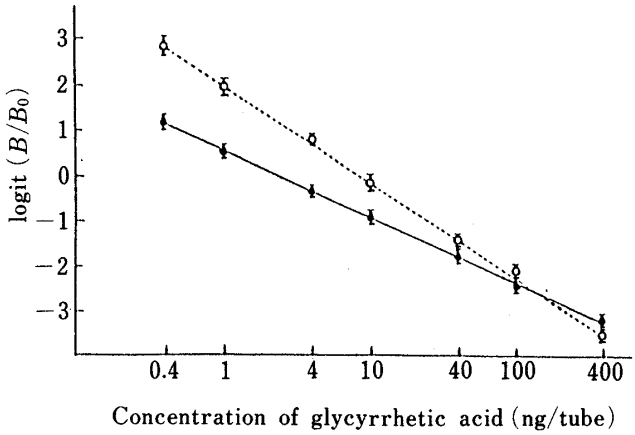


Fig. 1. Standard Curve for Enzyme Immunoassay of Glycyrrhetic Acid

Each point shows the mean ± S.D. of 6 replicate determinations in a linearized logit–log plot. B_0 = % binding in the absence of GA. B = % binding in the presence of GA. logit$(B/B_0)=\ln[B/B_0/(100-B/B_0)]$. ○---○, anti-GA–3-hemisuccinate–BSA antiserum with GA–3-hemiglutarate–β-Gal; ●—●, anti-GA–3-hemiglutarate–BSA antiserum with GA–3-hemisuccinate–β-Gal.

TABLE II. Specificity of Anti-GA–3-Hemisuccinate– and Anti-GA–3-Hemiglutarate–BSA Antisera

Compounds	Cross reaction (%)		
	Anti-GA–3-hemi-succinate–BSA	Anti-GA–3-hemi-glutarate–BSA	Anti-GA–30-glycine–BSA[a]
18β-Glycyrrhetic acid	100	100	100
18α-Glycyrrhetic acid	1.0	1.6	66
Carbenoxolone	215	114	1.0
Ammonium glycyrrhizinate	54	57	0.1
Estradiol	0	0	<0.1
Progesterone	0	0	<0.1
Hydrocortisone	0	0	<0.1

a) M. Kanaoka, S. Yano, H. Kato, and N. Nakano, *Chem. Pharm. Bull.*, **29**, 1533 (1981).

compound to the protein immunogen (BSA).[7] These anti-C-3 bridged GA–BSA antisera, therefore, were not available for the EIA of GA in the presence of GL and carbenoxolone.

Experimental

All melting points were taken on a microscopic hot stage (Yanagimoto melting point apparatus) and are uncorrected. Optical rotation was measured with JASCO DIP-4 polarimeter. Spectra were obtained with the following instruments: ultraviolet spectrum (UV) on a Beckman model 24; ^{1}H-NMR on Varian XL 200 and JEOL JNM-GX 270 spectrometers [(solvent, $CDCl_3$ unless otherwise indicated; internal standard, tetramethylsilane; chemical shift, δ (ppm); abbreviations; s (singlet), d (doublet), m (multiplet), br (broad)]. Fluorometry was performed on a Shimadzu RF-503 recording spectrofluorophotometer. Thin-layer chromatography (TLC) was performed on precoated silica gel plates 0.25 mm thick (Silicagel 70 F_{254} Wako) or 2 mm thick for preparative TLC (Kieselgel 60 F_{254} Merck), and spots were visualized by spraying 1% $Ce(SO_4)_2$ in 10% H_2SO_4 followed by heating, or detected under UV light (254 nm). Buffer A: 0.02 M phosphate-buffered saline containing 0.1% BSA, 0.1% NaN_3, 0.001% $MgCl_2$. Buffer B: 0.02 M phosphate-buffered saline containing 0.1% γ-globulin, 0.1% NaN_3, 0.001% $MgCl_2$.

***tert*-Butyl Glycyrrhetate (2)**——(i) Reaction of GA with Isobutene: Isobutene (20 ml) and few drops of concentrated H_2SO_4 were added to a solution of GA (1 g, 2.13 mmol) in CH_2Cl_2 (20 ml) under cooling, and the mixture was stirred at room temperature overnight. After evaporation of the solvent at room temperature *in vacuo*, the residue was dissolved in AcOEt (60 ml). The AcOEt solution was washed successively with aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and evaporated to leave a crystalline solid, which was purified by preparative TLC with 3% acetone–$CHCl_3$ as a developing solvent. The zone with *Rf* 0.9 gave *tert*-butyl 3-*O*-*tert*-butyl-glycyrrhetate (**3**) (236 mg, 19%). The zone with *Rf* 0.5 gave *tert*-butyl glycyrrhetate (**2**) (157 mg, 14%). The zone with *Rf* 0.3 gave 3-*O*-*tert*-butyl-glycyrrhetic acid (**4**) (78 mg, 7%).

Compound **3**: mp 255—257 °C (colorless prisms from hexane–CH_2Cl_2). $[\alpha]_D^{24}$ +118° (c=1, $CHCl_3$). MS *m/z*: 582 (M^+), 567 (M^+-15), 526 (M^+-56), 470 (M^+-112). *Anal.* Calcd for $C_{38}H_{62}O_4$: C, 78.30; H, 10.72. Found: C, 78.37; H, 10.63.

Compound **2**: mp 228—230 °C (colorless needles from hexane–CH_2Cl_2). $[\alpha]_D^{24}$ +137° (c=1, $CHCl_3$). MS *m/z*: 526 (M^+), 511 (M^+-15), 470 (M^+-56). *Anal.* Calcd for $C_{34}H_{54}O_4$: C, 77.52; H, 10.33. Found: C, 77.79; H, 10.24.

Compound **4**: mp 275—277 °C (dec.) (colorless needles from hexane–CH_2Cl_2). $[\alpha]_D^{24}$ +143° (c=1, $CHCl_3$). MS *m/z*: 526 (M^+), 511 (M^+-15), 470 (M^+-56). *Anal.* Calcd for $C_{34}H_{54}O_4$: C, 77.52; H, 10.33. Found: C, 77.58; H, 10.40.

(ii) Reaction of GA with *O*-*tert*-Butyl *N,N'*-Dicyclohexylisourea: A solution of GA (940 mg, 2 mmol) and *O*-*tert*-butyl *N,N'*-dicyclohexylisourea (616 mg, 2.2 mmol) in dimethylformamide (DMF) (2 ml) was stirred at room temperature for 4 d. The reaction mixture was dissolved in AcOEt (150 ml) and washed successively with aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and evaporated *in vacuo* to leave a crystalline solid, which was purified by preparative TLC with 3% acetone–$CHCl_3$ as a developing solvent. The zone with *Rf* 0.5 gave **2** (443 mg, 42%). This product was identical with compound **2** described above on basis of mixed melting point measurement and comparison of the MS and ^{1}H-NMR spectra.

(iii) *Via tert*-Butyl 3-Acetyl-glycyrrhetate (**6**): Isobutene (50 ml) and few drops of concentrated H_2SO_4 were added to a solution of 3-*O*-acetyl-glycyrrhetic acid (**5**) (1.96 g, 3.82 mmol) in CH_2Cl_2 (50 ml) under cooling, and the mixture was stirred at room temperature for 2 d, then washed successively with aqueous $NaHCO_3$ and brine, dried

over $MgSO_4$, and evaporated *in vacuo* to leave a crystalline solid. This was recrystallized from CH_2Cl_2 + isopropyl ether to give *tert*-butyl 3-*O*-acetyl glycyrrhetate (**6**) (1.8 g, 83%), mp 263—265 °C, colorless needles. $[\alpha]_D^{24}$ +121° (c=1, $CHCl_3$). MS *m/z*: 568 (M^+), 553 (M^+-15), 512 (M^+-56). *Anal.* Calcd for $C_{36}H_{56}O_5$: C, 76.01; H, 9.92. Found: C, 75.93; H, 9.76.

Hydrolysis of Compound **6**: Compound **6** (1.8 g, 3.17 mmol) was added to 2.5% KOH–MeOH (30 ml) and warmed at 60 °C for 2 h. After removal of the solvent *in vacuo*, the residue was recrystallized from hexane + CH_2Cl_2 to give **2** as a colorless needles (1.53 g, 92%). This product was identical with compound **2** described above on the basis of mixed melting point measurement and comparison of MS and ^{1}H-NMR spectra.

3-*O*-*tert*-Butyl-glycyrrhetate (4)——Isobutene (5 ml) and 2 drops of concentrated H_2SO_4 were added to a solution of benzyl glycyrrhetate (**7**) (280 mg, 0.5 mmol) in CH_2Cl_2 (5 ml) under cooling, and the mixture was stirred at room temperature for 3 d. After removal of excess of isobutene *in vacuo* at room temperature, the residue was dissolved in AcOEt (50 ml). The AcOEt solution was washed successively with aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and evaporated *in vacuo* to leave a crystalline solid, which was purified by preparative TLC with 3% acetone–$CHCl_3$ as the developing solvent. The zone with *Rf* 0.7 gave benzyl 3-*tert*-butyl-glycyrrhetate (**8**) (37 mg, 12%). The zone with *Rf* 0.3 gave the starting material (100 mg). Compound **8**: mp 163—165 °C. Colorless leaflets from CH_2Cl_2 + isopropyl ether. $[\alpha]_D^{24}$ +133° (c=1, $CHCl_3$). MS *m/z*: 616 (M^+), 601 (M^+-15), 560 (M^+-56). *Anal.* Calcd for $C_{41}H_{60}O_4$: C, 79.82; H, 9.80. Found: C, 79.77; H, 9.72.

Hydrogenolysis of Compound **8**: A solution of compound **8** (30 mg) in AcOEt (5 ml) was hydrogenated over 10% Pd–C (10 mg) at atmospheric pressure for 2 h. The catalyst was removed by filtration and washed with AcOEt. The filtrate and washings were combined and concentrated *in vacuo* to leave a crystalline solid, which was recrystallized from hexane–CH_2Cl_2 to give **4** (12 mg, 46.8%). This product was identical with the authentic sample described above on the basis of mixed melting point measurement and comparison of the MS and ^{1}H-NMR spectra.

***tert*-Butyl 3-Hemisuccinyl-glycyrrhetate (9)**——A solution of compound **2** (105 mg, 0.2 mmol) and succinic anhydride (22 mg, 0.22 mmol) in pyridine (1 ml) was warmed at 70—75 °C for 2 d, and then poured into ice-water. The precipitates were collected by filtration and washed with H_2O to give a crystalline solid, which was purified by preparative TLC with 10% MeOH–$CHCl_3$ as a developing solvent. The zone with *Rf* 0.2 gave **9** (55 mg, 44%), mp 189—190 °C. Colorless needles (from hexane + CH_2Cl_2). $[\alpha]_D^{25}$ +107° (c=0.97, $CHCl_3$). MS *m/z*: 626 (M^+), 611 (M^+-15), 570 (M^+-56). *Anal.* Calcd for $C_{38}H_{58}O_7$: C, 72.81; H, 9.33. Found: C, 72.75; H, 9.18.

***tert*-Butyl 3-Hemiglutaryl-glycyrrhetate (10)**——A solution of compound **2** (105 mg, 0.2 mmol) and glutaric anhydride (25 mg, 0.22 mmol) in pyridine was worked up as described above to give compound **10** (60 mg, 47%): mp 207—210 °C. Colorless needles from hexane + CH_2Cl_2. $[\alpha]_D^{25}$ +97.7° (c=1, $CHCl_3$). MS *m/z*: 640 (M^+), 625 (M^+-15), 584 (M^+-56). *Anal.* Calcd for $C_{39}H_{60}O_7$: C, 73.09; H, 9.44. Found: C, 72.94; H, 9.35.

***tert*-Butyl 3-*O*-(Succinimidooxycarbonyl)propionyl-glycyrrhetate (11)**——EDC (21 mg, 0.11 mmol) was added to a solution of compound **9** (44 mg, 0.07 mmol) and *N*-hydroxysuccinimide (13 mg, 0.11 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 and washed with H_2O to give a crystalline solid, which was recrystallized from hexane–CH_2Cl_2 to give **11** (23 mg, 45%). mp 145—147 °C. Colorless needles. $[\alpha]_D^{26}$ +90.3° (c=1, $CHCl_3$), MS *m/z*: 723 (M^+), 708 (M^+-15), 667 (M^+-56). *Anal.* Calcd for $C_{42}H_{61}NO_9$: C, 69.68; H, 8.49; N, 1.94. Found: C, 69.43; H, 8.46; N, 2.00.

***tert*-Butyl 3-*O*-(Succinimidooxycarbonyl)butyryl-glycyrrhetate (12)**——A solution of compound **10** (48 mg, 0.075 mmol), EDC (21 mg, 0.11 mmol) and *N*-hydroxysuccinimide (13 mg, 0.11 mmol) in pyridine was worked up as described above to give compound **12** (23 mg, 42%). mp 149—152 °C. Colorless needles from hexane–CH_2Cl_2. $[\alpha]_D^{26}$ +92.0° (c=1, $CHCl_3$). MS *m/z*: 737 (M^+), 722 (M^+-15), 681 (M^+-56). *Anal.* Calcd for $C_{43}H_{63}NO_9$: C, 69.98; H, 8.64; N, 1.90. Found: C, 69.88; H, 8.71; N, 2.10.

Preparation of GA–3-Hemisuccinate–(15) and GA–3-Hemiglutarate–BSA Conjugate (16)——Compound **11** (13 mg, 0.018 mmol) was dissolved in 50% trifluoroacetic acid–CH_2Cl_2 (1 ml) under stirring at 0 °C. After being stirred for 2.5 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 3-*O*-(succinimidooxycarbonyl)propionyl-glycyrrhetic acid (**13**) (9 mg, 75%) as an amorphous powder. A solution of the crude product (3.7 mg, 5.6×10^{-3} mmol) in pyridine (0.2 ml) was added to a solution of BSA (18.5 mg, 0.28×10^{-3} 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% and 1% pyridine–H_2O and H_2O to give **15**. Compound **12** was also worked up according to the procedure described above to give the BSA-conjugate (**16**).

Determination of Number of GA Molecules Linked to One BSA Molecule——The UV spectrometric analysis was performed by comparing the absorbance at 252 nm of the conjugates **15** and **16** with those of BSA and 3-hemisuccinyl-GA(carbenoxolone) and 3-hemiglutaryl GA 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, 2600; for carbenoxolone, 14521; for 3-hemiglutaryl GA, 27532. The protein contents of the conjugate **15** and **16** were determined by the method of Lowry *et al.*[8] The numbers of hemisuccinyl GA and hemiglutaryl GA moieties coupled to one BSA moiety were determined to be 9.8 and 10.5, respectively.

Preparation of GA–3-Hemisuccinate- (17) and GA–3-Hemiglutarate-β-D-galactosidase (18) Conjugates——Compound **13** (54 μg, 8×10^{-8} mol) (27 μl of a solution of **13** (2 mg) in pyridine (1 ml) was pipetted off) was added to a solution of β-D-galactosidase (2 mg, 4×10^{-9} 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. Compound **14** was worked up according to the procedure described above to give β-D-Gal-conjugate (**18**).

Assay Procedure——A sample or standard solution of GA (100 μl) was added to 8000—10000-fold diluted anti-GA-antiserum (100 μl) and GA–β-Gal conjugate (**15**, **16**; 50 μl). The mixture was incubated at room temperature for 2 h and then 100-fold diluted normal rabbit serum (20 μl) and 10-fold diluted goat antiserum to rabbit IgG (50 μl) were added. After further incubation at 4 °C overnight, the resulting immune precipitate was incubated with 1×10^{-4} M 7-β-galactopyranosyloxy-4-methylcoumarin (150 μl) at 30 °C 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 (365 nm and 448 nm for excitation and emission, respectively).

Specificity of the Anti-GA-Antisera——The cross reactivities of GA related compounds with the anti-GA-antisera were examined by using GA–β-Gal conjugates in a bridge heterologous combination. Reaction ratio (%) = GA 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 II.

Acknowledgement The authors are indebted to Mr. M. Morikoshi of this university for measurements of MS.

References and Notes

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