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Preparation of [3α-^{3}H]3β-Hydroxy-18β- and 3α-Hydroxy-18(β and α)-glycyrrhetic Acid and Radioimmunoassay of Glycyrrhetic Acid[1)]

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[3α-^{3}H]3β-Hydroxy-18β-glycyrrhetic acid (**5**) for radioimmunoassay (RIA) of glycyrrhetic acid (GA) was prepared along with a small amount of the 3α-hydroxy compound (**6**) by the reduction of 3-oxo-18β-GA (**1**) with [^{3}H]sodium borohydride.

The ratios of 3β-hydroxy to 3α-hydroxy-GA (**7**:**8** and **9**:**10**) in the reduction products of 3-oxo-GA (**1** and **2**) with sodium borohydride, with hindered borohydride and with aluminum isopropoxide were determined by high-performance liquid chromatography. Sodium borohydride and potassium tri-*sec*-butyl borohydride allowed stereoselective reduction of the 3-oxo group to the 3β-hydroxy and 3α-hydroxy group, respectively, leaving the 11-oxo group untouched. Potassium-tri-*sec*-butyl borohydride was the most useful reducing agent for preparation of 3α-hydroxy-GA (**8** and **10**) from 3-oxo-GA.

Separation of the bound and free fraction for RIA of GA, in which anti-glycyrrhetyl-30-glycine-bovine serum albumin antiserum was used as the antibody, was carried out by a double antibody method using a goat antiserum to rabbit immunoglobulin G. A satisfactory standard curve for RIA of GA was obtained in the range of 2—200 ng/ml. The accuracy of this RIA of serum samples without extraction of GA from them is high, and should be satisfactory for application to pharmacodynamic studies of GA.

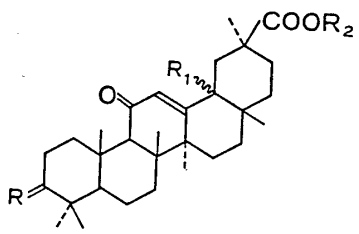
Keywords——[3α-^{3}H]3β-hydroxyglycyrrhetic acid; labeled antigen; glycyrrhetic acid radioimmunoassay; 3α-hydroxyglycyrrhetic acid; 3-oxoglycyrrhetic acid; stereoselective reduction; [^{3}H]-sodium borohydride; potassium-tri-*sec*-butyl borohydride; hindered borohydride

We have undertaken the enzyme immunoassay (EIA) of active components in oriental medicine as a preliminary to metabolic studies of these components in humans.

In the previous paper,[2)] we described the EIA of glycyrrhetic acid (GA), the aglycone of glycrrhizin (GL), which is one of the active components of Glycyrrhizae Radix. In this EIA of GA for serum and urine samples, a direct assay suffered from nonspecific interference with the antigen–antibody reaction of EIA. In order to avoid this interference, the assay was performed after extraction of GA from biological fluids with $CHCl_3$.[3)] Development of a highly specific and sensitive assay of GA was of great importance to elucidate the pharmacodynamics of GA. The sensitivity of radioimmunoassay (RIA), which uses a radioisotope instead of an enzyme (EIA) for labeling the antigen, seems to be higher than that of EIA because there is no interference in the competitive reaction of antigen and labeled antigen with antibody.

In this paper, we wish to report the preparation of [3α-^{3}H]3β-hydroxy-GA (**5**) for RIA of GA, and 3α-hydroxy-18(α and β)-GA (**8** and **10**) by the reduction of 3-oxo-18(β and α)-GA (**1** and **2**) with metal borohydrides, and RIA of GA.

It has been reported by Carmeron[4)] that the reduction of 3-oxo-18β-GA (**1**) with a



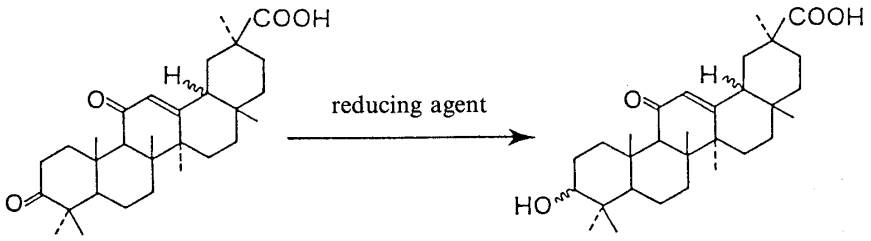
1: R=O, R_1=H-, R_2=H
2: R=O, R_1=H····, R_2=H
3: R=O, R_1=H-, R_2=CH_3
4: R=O, R_1=H····, R_2=CH_3
5: R=HO(3H), R_1=H-, R_2=H
6: R=HO(3H), R_1=H-, R_2=H
7: R=HO(H), R_1=H-, R_2=H
8: R=HO(H), R_1=H-, R_2=H
9: R=HO(H), R_1=H····, R_2=H
10: R=HO(H), R_1=H····, R_2=H
11: R=HO(H), R_1=H-, R_2=CH_3
12: R=HO(H), R_1=H-, R_2=CH_3
13: R=HO(H), R_1=H····, R_2=CH_3
14: R=HO(H), R_1=H····, R_2=CH_3

Chart 1

solution of [^{3}H]sodium borohydride in NaOH containing ethanol gave only [3α-^{3}H]3β-hydroxy-18β-GA (**5**). We examined this reduction in a solution of NaOH containing isopropyl alcohol to afford a mixture of [3α-^{3}H]3β-hydroxy (**5**) and [3β-^{3}H]3α-hydroxy-18β-GA (**6**) in 94% and 6% yields, respectively. The former, separated from the reaction mixture by preparative thin layer chromatography (PTLC), exhibited a single peak in high-performance liquid chromatography (HPLC). When [^{3}H]$NaBH_4$ (100 or 25 mCi) was used on the reduction, the specific activity of [3α-^{3}H]3β-hydroxy-18β-GA (**5**) was 2.8 or 0.72 Ci/mmol, respectively; these materials were used for the RIA of GA.

Hattori *et al.* reported that 3α-hydroxy-18β-GA (**8**) was obtained in 6% yield along with 3β-hydroxy-18β-GA (**7**) in 20% yield by reduction of 3-oxo-18β-GA (**1**) with an intestinal bacterial mixture,[5] and 3α-hydroxy-18α-GA (**10**) was obtained in 5.4% yield[6] from 3-oxo-18α-GA (**2**) by the action of *Clostridium innocum* (Es24-06). On the other hand, the reduction of methyl 3-oxo-glycyrrhetate (**3**) with aluminum isopropoxide (Meerwein–Ponnodorf–Verley reduction), reported by Tolstikov *et al.*,[7] afforded methyl 3β- (**11**) and 3α-hydroxy-18β-glycyrrhetate (**12**) in 40% and 60% yields. In connection with these reports, the reduction of cyclic ketones using hindered borohydrides, especially lithium (L-selectride) and potassium tri-*sec*-butyl borohydride (K-selectride) has been shown to occur with a high degree of stereoselectivity by attack from the equatorial side.[8] Among these reducing agents, metal borohydrides and aluminum isopropoxide were applied to the reduction of 3-oxo-GA (**1** and **2**). The reductions were carried out with 2 eq of sodium borohydride in a solution of NaOH at room temperature, with 2 eq of hindered borohydride in tetrahydrofuran (THF) at −78 °C, and with aluminum isopropoxide in toluene at 110 °C, respectively. The ratio of 3β-hydroxy-GA to 3α-hydroxy-GA (**7**:**8** and **9**:**10**) in the reduced products was determined by HPLC (Table I). As shown in Table I, sodium borohydride and K-selectride allowed stereoselective reduction of the 3-oxo group to the 3-β and 3-α hydroxy group, respectively, leaving the 11-

TABLE I. Ratio of 3β-Hydroxy to 3α-Hydroxy Products from the Reduction of 3-Oxoglycyrrhetic Acid with Reducing Agents



Reducing agent	Reaction Temp. (°C)	Reaction Time (h)	3-Oxo-GA 18β Ratio of products (%) 3β : 3α	3-Oxo-GA 18α Ratio of products (%) 3β : 3α
$NaBH_4$	r.t.	2	94 : 6	92 : 8
$LiB[CH(CH_3)C_2H_5]_3H$	−78	24	65 : 35	56 : 44
$KB[CH(CH_3)C_2H_5]_3H$	−78	24	5 : 95	28 : 72
$LiB[CH(CH_3)CH(CH_3)_2]_3H$	−78	24	71 : 29	65 : 35
$Al(O\text{–iso-}C_3H_7)_3$	110	2	31 : 69	37 : 63

r.t.: room temperature.

TABLE II. ^{13}C-NMR Spectral Data for **12** and **14**

Carbon	Compound 12	Compound 14	Carbon	Compound 12	Compound 14	Carbon	Compound 12	Compound 14
1	33.5	33.7	11	200.4	199.9	21	31.2	31.9
2	25.5	25.4	12	128.6	124.2	22	37.8	28.5
3	75.9	75.9	13	169.1	165.6	23	28.4[a)]	28.4
4	37.6	37.5	14	45.7	45.0	24	22.4	22.4
5	48.5	48.5	15	26.5	26.7	25	16.3	16.5
6	17.5	17.5	16	26.5	26.7	26	18.8	18.6
7	32.7	33.5	17	31.9	35.5	27	23.6	20.8[a)]
8	43.3	44.1	18	48.5	40.4	28	28.5[a)]	16.0
9	61.8	60.7	19	41.2	36.0	29	28.6	21.0[a)]
10	37.3	37.0	20	44.1	42.6	30	177.0	177.0
						OCH_3	51.8	51.9

The spectra were taken at 100 MHz in $CDCl_3$ with TMS an internal standard. Values are in ppm. *a*) Assignment may be interchanged in each column.

oxo group untouched. K-Selectride was the most useful reducing agent for the preparation of 3α-hydroxy-GA (**8** and **10**) from 3-oxo-GA (**1** and **2**). The other reducing agents were less stereoselective. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra of methyl 3α-hydroxy-18β- (**12**) and -18α-glycyrrhetate (**14**), which were obtained by the reduction of 3-oxo-GA (**1** and **2**) with K-selectride followed by methylation with diazomethane, were measured (Table II). The carbon atoms were assigned by means of the distortionless enhancement by polarization transfer (DEPT) technique as well as by comparison of these spectra with those of methyl 3β-hydroxy-18β- (**11**) and -18α-glycyrrhetate (**13**).[9] The chemical shift value of carbon atoms in the A-ring and of the methyl carbon atom of C-24 were similar to those of the relevant carbon atoms of dammarane-type[10] and lanostane-type triterpenoids.[11]

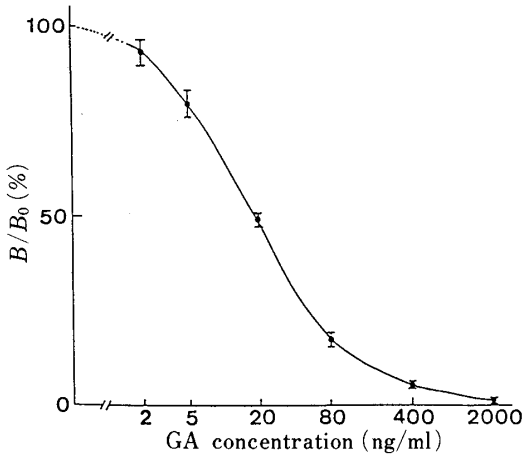


Fig. 1. Standard Curve for RIA of Glycyrrhetic Acid

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

TABLE III. Recovery of GA Added to Sera from 4 Patients Receiving GL

GA added (ng)		Patient A	Patient B	Patient C	Patient D	Average recovery of added GA (%)[a]
		GA (ng/ml)				
		8.1	1.2	2.5	3.8	
2	Calcd	10.1	3.2	4.5	5.8	
	Found	9.8	4.6	4.9	5.9	
	Recovery (%)	97.0	144.0	108.9	101.7	112.9 ± 18.4
10	Calcd	18.1	11.2	12.5	13.8	
	Found	19.5	9.6	11.0	12.6	
	Recovery (%)	107.7	85.7	88.0	91.3	93.2 ± 8.6
40	Calcd	48.1	41.2	42.5	43.8	
	Found	41.0	46.0	45.0	42.0	
	Recovery (%)	85.2	111.6	105.6	95.9	99.6 ± 10.0
100	Calcd	108.1	101.2	102.5	103.8	
	Found	105.0	115.0	100.0	110.0	
	Recovery (%)	97.1	113.6	97.6	106.0	103.6 ± 6.8

a) Mean ± S.D.

The anti-C-3 bridged-bovine serum albumin (BSA) antisera for immunoassays of carbenoxolone[12] and GA[13] could not distinguish clearly between GA and its C-3 derivatives. For RIA of GA, therefore, we chose anti-C-30 bridged GA antisera which were obtained from rabbits immunized with glycyrrhetyl-30-glycine–BSA conjugate in the same manner as described previously.[1] RIA of GA was performed by a competitive binding procedure with goat antiserum to rabbit immunoglobulin G (IgG) as the second antibody. After dissolving the immune precipitate in 1 N NaOH, its radioactivity was measured with a liquid scintillation counter. Optimum dilution of antisera determined by the reported method[14] was 300-fold. A typical standard curve for RIA of GA is shown in Fig. 1. The measurable range was approximately the same as that of EIA,[1] 2—200 ng/ml.

As mentioned above, EIA of GA in samples of biological fluid was carried out by the extraction of GA from these samples. To test the accuracy of this RIA of serum samples without extraction of GA, the recoveries of various amounts (2—100 ng) of GA, which was added to sera of four patients who had received glycyrrhizin, were determined by this RIA,

and all recoveries were approximately 100%, as shown in Table III. This RIA of GA was superior to the EIA of GA for the assay of biological fluids, and further details will be reported elsewhere.

Experimental

All melting points were taken on a microscopic hot stage (Yanagimoto melting point apparatus) and are uncorrected. Optical rotations were measured with a JASCO DIP-4 polarimeter. Spectra were obtained with the following machines. Ultraviolet (UV) on a Shimadzu UV-260 spectrophotometer. NMR on JEOL 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)]. TLC was performed on precoated silica gel plates 0.25 mm thick (Kieselgel F_{254}, Merck) or 2 mm thick for PTLC, and spots were visualized by spraying with 1% $Cs(SO_4)_2$ in 10% H_2SO_4 followed by heating, or detected under UV light (254 nm).

18β-GA, purchased from Tokyo Kasei Ltd. (Tokyo), was recrystallized from EtOH. 18α-GA was purchased from Sigma Chemical Company (U.S.A.). Solution (1 M) of potassium and lithium tri-*sec*-butyl borohydride (K-selectide and L-selectride) and lithium tri-siamyl borohydride (LS-selectride) in THF were obtained from Aldrich Chemical Company (U.S.A.). [^{3}H]Sodium borohydride was obtained from Daiichi Kagaku Yakuhin Co., Ltd. (Tokyo).

3-Oxo-18β-glycyrrhetic Acid (1)——Jones reagent (2.8 M; 4 ml) was added to a solution of 18β-GA (**7**) (4.7 g, 10 mmol) in acetone (200 ml) under stirring for 30 min at 0 °C and the reaction mixture was poured into ice-H_2O (500 ml). The resulting precipitate was filtered off and then dissolved in $CHCl_3$ (100 ml). The $CHCl_3$ solution was washed with H_2O, dried over anhydrous $MgSO_4$ and evaporated *in vacuo*. The residue was recrystallized from EtOH (200 ml) to give **1** (4.0 g, 85.5%) as colorless leaflets, mp <300 °C. $[\alpha]_D^{24}$ +178° ($c=1$, $CHCl_3$). *Anal.* Calcd for $C_{30}H_{44}O_4$: C, 76.88: H, 9.46. Found: C, 76.63; H, 9.54. IR (KBr): 3300 (OH), 1725 (COOH), 1670 (C=O), 1640 (conj. C=O), 1610 (conj. C=C) cm^{-1}. ^{1}H-NMR (δ): 0.86, 1.07, 1.11, 1.17, 1.23, 1.28, 1.38 (each 3H, s, CH_3), 5.75 (1H, s, H-12). UV λ_{max}^{MeOH} nm (ε): 244 (11770). MS *m/z*: 468 (M^+, 30%), 453 (10%), 303 (100%), 262 (95%), 135 (93%).

Methyl 3-Oxo-18β-glycyrrhetate (3)——A solution of diazomethane in Et_2O[15] was added to a solution of **1** (200 mg) in MeOH (5 ml) until it showed a persistent yellow color and the mixture was allowed to stand overnight. The resultant precipitate was recrystallized from CH_2Cl_2 + hexane to give **3** (178 mg, 86%) as colorless leaflets. mp 254—256 °C. $[\alpha]_D^{24}$ +170° ($c=1$, $CHCl_3$). *Anal.* Calcd for $C_{31}H_{46}O_4$: C, 77.13; H, 9.61. Found: C, 77.36; H, 9.43. IR (KBr): 2930 (OH), 1720 ($COOCH_3$), 1700 (C=O), 1645 (conj. C=O), 1610 (conj. C=C) cm^{-1}. MS *m/z*: 482 (M^+, 46%), 467 (11%), 317 (60%), 276 (45%), 135 (97%).

3-Oxo-18α-glycyrrhetic Acid (2)——A solution of 18α-GA (**9**) (4.7 g, 10 mmol) in THF (150 ml) was treated in the same manner as described above to give **2** (4.2 g, 89.7%) as colorless prisms, mp <300 °C. $[\alpha]_D^{24}$ +116.4° ($c=1$, $CHCl_3$). *Anal.* Calcd for $C_{30}H_{44}O_4$: C, 76.88; H, 9.46. Found: C, 76.77; H, 9.41. IR (KBr): 3250 (OH), 1720 (COOH), 1670 (C=O), 1650 (conj. C=O), 1610 (conj. C=C) cm^{-1}. ^{1}H-NMR (δ): 0.75, 1.07, 1.10, 1.18, 1.27, 1.34, 1.34 (each 3H, s, CH_3), 5.56 (1H, d, $J=1.7$ Hz, H-12).[16] UV λ_{max}^{MeOH} nm (ε): 244 (10600). MS *m/z*: 468 (M^+, 16%) 453 (16%), 303 (100%), 262 (17%), 135 (62%).

Methyl 3-Oxo-18α-glycyrrhetate (4)——A solution of **2** (300 mg) in MeOH (9 ml) was treated in the same manner as described above to give **4** (268 mg, 87%) as colorless leaflets (recrystallized from CH_2Cl_2 + hexane) mp 260—263 °C. $[\alpha]_D^{24}$ +108° ($c=1$, $CHCl_3$). *Anal.* Calcd for $C_{31}H_{46}O_4$: C, 77.13; H, 9.61. Found: C, 77.13, H, 9.52. IR (KBr): 2925 (OH), 1720 ($COOCH_3$), 1700 (C=O), 1640 (conj. C=O), 1610 (conj. C=C) cm^{-1}. MS *m/z*: 482 (M^+, 15%), 467 (10%), 317 (100%), 276 (10%), 135 (48%).

[3α-^{3}H]3β-Hydroxyglycyrrhetic Acid (5)——A solution of **1** (7.2 mg, 15.38×10^{-3} mmol) in 0.1 N NaOH–isopropyl alcohol (2 : 1) (0.3 ml) was added to a solution of 100 mCi of [^{3}H]$NaBH_4$ (15 Ci/mmol, 6.66×10^{-3} mmol) in 0.01 N NaOH–isopropyl alcohol (9 : 1) (0.5 ml) under stirring with ice cooling. After being stirred at room temperature for 2 h, the reaction mixture was warmed at 40 °C for 1 h, and acidified with 0.1 N HCl to pH 2.0 under ice cooling. The mixture was extracted with $CHCl_3$ (30 ml). The extract was washed with brine, dried over anhydrous $MgSO_4$ and then evaporated *in vacuo*. The residue (7 mg, 97%) was separated by PTLC using 5% MeOH–$CHCl_3$ as the developing solvent into three zones, F_1 (*Rf* 0.7, 0.1 mg), F_2 (*Rf* 0.55, 0.2 mg) and F_3 (*Rf* 0.4, 6 mg, 83%). From the result of identification of each zone by HPLC, F_1 was the starting material and a small amount of a mixture of **5** and **6**, F_2 was a mixture of **5** and **6**, and F_3 was **5** having a specific activity of 2.8 mCi/mmol. When **1** (4 mg, 8.5×10^{-3} mmol) was reduced with 25 mCi of [^{3}H]$NaBH_4$ (7.9 Ci/mmol, 3.16×10^{-3} mmol) in the same manner as described above, the specific activity of **5** was 0.72 mCi/mmol. Compound **5** thus obtained was used for RIA of GA without further purification.

Reduction of 3-Oxoglycyrrhetic Acid (1 and 2) with K-Selectride——3α-Hydroxy-18β-GA (**8**): Reduction of **1**: A solution of **1** (94 mg, 0.2 mmol) in dry THF (4 ml) under an argon atmosphere was cooled in a dry ice–acetone bath (−78 °C) and a 1 M solution of K-selectride in THF (0.4 ml, 0.4 mmol) was added with a syringe. After cooling at the same temperature for 2 h, the reaction mixture was left to stand overnight at room temperature. The reaction was

quenched with 1 N HCl to pH 2.0 under ice cooling, and extracted with $CHCl_3$ (50 ml). The extract was washed with brine, dried over anhydrous $MgSO_4$, and evaporated *in vacuo* to give a crude product (87 mg, 92%). A part of the product was subjected to HPLC to determine the ratio of **7** to **8** (given in Table I). The remaining residue was separated by PTLC using 5% MeOH–$CHCl_3$ as the developing solvent into two zones, F_1 (*Rf* 0.55, 80 mg, 85%) and F_2 (*Rf* 0.4, 1 mg). From the results of examination of each zone by HPLC, F_1 contained **8** and F_2 contained a mixture of **7** and **8**. Recrystallization of F_1 from CH_2Cl_2 + MeOH gave **8** as colorless prisms, mp <300 °C. $[\alpha]_D^{26}$ +134° ($c=1$, 10% MeOH–$CHCl_3$). *Anal.* Calcd for $C_{30}H_{46}O_4$: C, 76.55; H, 9.85. Found: C, 76.54; H, 9.80. IR (KBr): 3500 (OH), 1710 (COOH), 1635 (conj. C=O) 1615 (conj. C=C) cm^{-1}. ^{1}H-NMR (δ): 0.831, 0.868, 0.966, 1.13, 1.15, 1.23, 1.39 (each 3H, s, CH_3), 3.42 (1H, t, $J=3$ Hz, H-3) 5.69 (1H, s, H-12). UV λ_{max}^{MeOH} nm (ε): 249 (12060). MS *m/z*: 470 (M^+, 28%), 303 (90%), 262 (88%), 175 (42%), 135 (57%). This sample was shown to be identical with an authentic sample[4] by comparison of the IR spectrum, and TLC and HPLC behavior.

Methyl 3α-Hydroxy-18β-glycyrrhetate (**12**): A solution of diazomethane in Et_2O was added to a solution of **8** (20 mg) in MeOH (2 ml) until a persistent yellow color was seen and then allowed to stand overnight. The resultant precipitate was recrystallized from CH_2Cl_2 + hexane to give **12** (14 mg, 67%) as colorless needles, mp 215—217 °C (Lit.[7] 217—218 °C). *Anal.* Calcd for $C_{31}H_{48}O_4$: C, 76.81; H, 9.98. Found: C, 76.87; H, 9.97. IR (KBr): 3475 (OH), 1725 ($COOCH_3$), 1640 (conj. C=O) 1610 (conj. C=C) cm^{-1}. ^{1}H-NMR (δ): 0.857, 0.920, 1.02, 1.16, 1.20, 1.20, 1.43 (each 3H, s, CH_3), 3.47 (1H, t, $J=3$ Hz, H-3), 3.73 (3H, s, OCH_3), 5.59 (1H, s, H-12). MS *m/z*: 484 (M^+, 44%), 317 (92%), 276 (97%), 175 (100%), 135 (98%).

3α-Hydroxy-18α-GA (**10**): Reduction of **2**: Reduction of **2** (94 mg, 0.2 mmol) with K-selectride in the same manner as described above gave a crude product (82 mg, 87%) A part of the crude product was subjected to HPLC to determine the ratio of **9** to **10** (given in Table I). The other residue was separated by PTLC using 7% MeOH–$CHCl_3$ as the developing solvent into two zones, F_1 (*Rf* 0.54, 54 mg, 57%) and F_2 (*Rf* 0.4, 18 mg). From the results of examination of each zone by HPLC, F_1 contained **10**, and F_2 contained a mixture of **9** and **10**. Recrystallization of F_1 from CH_2Cl_2 + di-isopropyl ether gave **10** as colorless needles, mp >300 °C. $[\alpha]_D^{24}$ +74.4° ($c=1$, 10%, MeOH–$CHCl_3$). *Anal.* Calcd for $C_{30}H_{46}O_4$: C, 76.55; H, 9.85. Found: C, 76.31; H, 9.77. IR (KBr): 3575 (OH), 1705 (COOH), 1640 (conj. C=O) 1610 (conj. C=C) cm^{-1}. ^{1}H-NMR (δ): 0.737, 0.868, 0.962, 1.14, 1.21, 1.27, 1.36 (each 3H, s, CH_3), 3.46 (1H, t, $J=3$ Hz, H-3) 5.59 (1H, d, $J=1.8$ Hz, H-12).[16] UV λ_{max}^{MeOH} nm (ε): 244 (8650). MS *m/z*: 470 (M^+, 12%), 303 (100%), 262 (15%), 175 (10%), 135 (47%).

Methyl 3α-Hydroxy-18α-glycyrrhetate (**14**): Methylation of **10** (20 mg) with diazomethane in the same manner as described above gave **14** (14 mg, 67%) as colorless leaflet (recrystallized from CH_2Cl_2 + hexane), mp 222—225 °C. *Anal.* Calcd for $C_{31}H_{48}O_4$: C, 76.81; H, 9.98. Found: C, 76.77; H, 10.03. IR (KBr): 3580 (OH), 1710 ($COOCH_3$), 1640 (conj. C=O), 1610 (conj. C=C) cm^{-1}. ^{1}H-NMR (δ): 0.711, 0.869, 0.961, 1.14, 1.210, 1.22, 1.34 (each 3H, s, CH_3), 3.42 (1H, t, $J=3$ Hz, H-3), 3.69 (3H, s, OCH_3), 5.57 (1H, br d, $J=1.8$ Hz, H-12).[16] MS *m/z*: 484 (M^+, 14%), 317 (100%), 276 (13%), 175 (10%), 135 (46%).

Reduction of 3-Oxoglycyrrhetic Acid (1 and 2) with L-Selectride——Reduction of **1**: Reduction of **1** (94 mg, 0.2 mmol) with L-selectride in the same manner as described above gave a crude product (84 mg, 89%). The crude products was subjected to HPLC to determine the ratio of **7** to **8** as shown in Table I.

Reduction of **2**: Reduction of **2** (94 mg, 0.2 mmol) with L-selectride (0.4 mmol) in the same manner as described above gave a crude product (92 mg, 97%). The crude product was subjected to HPLC to determine the ratio of **9** to **10** as shown in Table I.

Reduction of 3-Oxoglycyrrhetic Acid (1 and 2) with LS-Selectride——Reduction of **1**: Reduction of **1** (94 mg, 0.2 mmol) with LS-selectride (0.4 mmol) in the same manner as described above gave a crude product (81 mg). From the result of HPLC, the crude product contained the starting material (22%) together with **7** and **8** in the ratio shown in Table I.

Reduction of **2**: Reduction of **2** (94 mg, 0.2 mmol) with LS-selectride (0.4 mmol) in the same manner as described above gave crude product (86 mg) which contained the starting material (17%), together with **9** to **10** in the ratio given in Table I.

Reduction of 3-Oxoglycyrrhetic Acid (1 and 2) with Sodium Borohydride——Reduction of **1**: A solution of **1** (94 mg, 0.2 mmol) in 1 N NaOH–isopropyl alcohol (2 : 1) (1 ml) was added to a solution of $NaBH_4$ (3.8 mg, 0.1 mmol) in 0.1 N NaOH–isopropyl alcohol (9 : 1) (0.5 ml) under stirring at 0 °C. After being stirred at room temperature for 2 h, the reaction mixture was acidified with 1 N HCl to pH 2.0 and extracted with $CHCl_3$ (15 ml). The extract was washed with brine, dried over anhydrous $MgSO_4$, and evaporated *in vacuo* to leave a crude product (91 mg, 96%). The crude product was subjected to HPLC to determine the ratio of **7** to **8** as shown in Table I.

Reduction of **2**: **2** (94 mg, 0.2 mmol) was reduced with $NaBH_4$ (3.8 mg, 0.1 mmol) in the same manner as described above to give a crude product (86 mg, 91%). The crude product was subjected to HPLC to determine the ratio of **9** to **10** (given in Table I).

Reduction of 3-Oxoglycyrrhetic Acid (1 and 2) with Aluminum Isopropoxide——Reduction of **1**: A mixture of **1** (30 mg, 0.064 mmol) and freshly prepared aluminum isopropoxide (50 mg) in toluene (2 ml) was heated under reflux for 2 h. After cooling to room temperature, the reaction mixture was treated with 1 N HCl (5 ml) to decompose the aluminum complex and extracted with $CHCl_3$ (30 ml). The extract was washed with brine, dried over anhydrous

$MgSO_4$, and evaporated *in vacuo*. The residue (29 mg, 96%) was subjected to HPLC to determine the ratio of **7** to **8** (given in Table I).

Reduction of **2**: Reduction of **2** (50 mg, 0.11 mmol) with aluminum isopropoxide (80 mg) in the same manner as described above gave a crude product (48 mg, 96%). The crude product was subjected to HPLC to determine the ratio of **9** to **10** as (given in Table I).

HPLC Conditions——A Shimadzu model LC-6A equipped with an SPD 6A UV-VS spectrophotometric detector for peak detection (254 nm), and with a model CR-3A data processor for the calculation of peak area, was used. Conditions: column, Nucleosil $7C_{18}$ (250 × 4.5 mm i.d.), $5C_{18}$ (150 × 4.6 mm i.d.); column temperature, 40 °C; flow rate, 1.0 ml/min; mobile phase, CH_3CN–2% AcOH (A, 63%; B, 73%), MeOH–H_2O (C, 80%, D, 83%) containing 5 mM tetra-*n*-butylammonium hydroxide and adjusted to pH 6.0 by adding 20% H_3PO_4. Retention times (min) were as follows. Mobile phase A: **1** (19.793), **2** (18.9), **7** (16.248), **8** (17.732) **9** (15.523), **10** (17.15). B: **1** (8.23), **2** (8.055), **7** (6.332), **8** (7.062), **9** (6.215), **10** (6.957). C: **1** (13.137), **2** (12.588), **7** (13.59), **8** (15.255), **9** (12.953), **10** (14.913). D: **1** (9.247), **2** (8.922), **7** (9.347), **8** (10.448), **9** (9.027), **10** (10.233).

HPLC Samples: The crude product of the reduction was dissolved in 50 ml of $CHCl_3$ and diluted 100-fold with the same solvent, then 5 μl of this solution was injected into the HPLC column.

Procedure of RIA——A mixture of the standard solution of GA or GA sample (50 μl), 300-fold diluted anti-glycyrrhetyl-30-glycine-BSA antiserum (100 μl) and [3α-^{3}H]GA (50 μl, 5 nCi/tube) was incubated at room temperature for 2 h, and then 10-fold diluted goat antiserum to rabbit IgG was added to the incubation mixture, and the mixture was allowed to stand at 4 °C overnight. After addition of buffer B (1 ml, 0.96 M phosphate buffer solution containing 0.01 M ethylenediaminetetraacetic acid (EDTA) and 0.1% BSA), the mixture was centrifuged at 3000 rpm for 20 min. The supernatant was removed by decantation and the immune precipitate was washed with buffer B (1 ml) and recentrifuged. The precipitate was dissolved in 1 N NaOH (100 μl) to measure the radioactivity by liquid scintillation counting.

Recovery in RIA of Serum Sample——A recovery test was carried out by adding known amounts of GA (2, 10, 40 and 100 ng) to sera of 4 patients receiving glycyrrhizin in which the GA concentration were 1.2, 2.5, 3.8 and 8.1 ng/ml. Aliquots of 50 ml of the 10-fold diluted samples were assayed by the procedure described above.

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References and Notes

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