Antiviral Activities of Glycyrrhizin and Its Modified Compounds against Human Immunodeficiency Virus Type 1 (HIV-1) and Herpes Simplex Virus Type 1 (HSV-1) *in Vitro*

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Received July 11, 1990

Chemically modified compounds of glycyrrhizin have been synthesized and evaluated for their inhibitory effect on the replication of human immunodeficiency virus type 1 (HIV-1) and herpes simplex virus type 1 (HSV-1). Among them, the 11-deoxo compound having a heteroannular diene structure at the C and D rings proved as active against HIV-1 as glycyrrhizin in MT-4 and MOLT-4 cells. It completely inhibited HIV-1-induced cytopathogenicity in both cell lines at a concentration of 0.16 mM. The compound was also effective against HSV-1 with a 50% inhibitory concentration of 0.5 μM.

Keywords glycyrrhizin analog; HIV-1; HSV-1; antiviral activity

It has been reported that glycyrrhizin, the major saponin isolated from licorice root (*Glycyrrhiza* spp.), is inhibitory to the replication of human immunodeficiency virus type 1 (HIV-1) *in vitro*, the etiologic agent of the acquired immune deficiency syndrome (AIDS).[1] This finding has been developed to the clinical use of a glycyrrhizin preparation, Stronger Neominophagen C (SNMC), to protect HIV-1 carriers from the crisis of AIDS.[2] In addition, it was demonstrated that glycyrrhizin inhibited the *in vitro* growth and cytopathogenicity of several deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) viruses including herpes simplex virus type 1 (HSV-1), Newcastle disease virus, vesicular stomatitis virus, and poliovirus type 1.[3] Baba and Shigeta, two of the present authors, reported the antiviral activity of glycyrrhizin against varicella–zoster virus (VZV) in cell culture.[4]

On the basis of these results, some chemically modified compounds of glycyrrhizin were investigated for their inhibitory effect on the replication of HIV-1 and HSV-1. The functional groups of glycyrrhetinic acid, the aglycone part of glycyrrhizin, at the 11 and 20 positions, and the carboxyl at the 5′ position of the sugar portion were modified. The αβ-unsaturated carbonyl system of the C-ring of the glycyrrhetinic acid part was converted into a heteroannular diene system at the C and D rings. By the above modifications, the following compounds (**3**—**7**) were prepared starting from glycyrrhizin (**1**) to test their antiviral activity.

Materials and Methods

Apparatus Melting points were taken on a Yanaco micro melting point apparatus and are uncorrected. Optical rotations were determined in MeOH with a JASCO model JZO ORD/CD polarimeter. Infrared (IR) spectra were recorded with a JASCO IR-700 spectrophotometer and ultraviolet (UV) spectra with a Hitachi 2-20 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL GX-400 NMR spectrometer using 10—20 mg/0.45 ml dimethyl sulfoxide (DMSO-d_6) as a solvent and tetramethylsilane (TMS) as an internal standard. Chemical shifts were reported in ppm downfield from this standard. Fast atom bombardment (FAB) mass spectral analysis was performed with a JEOL DX-300 spectrometer. A high performance liquid chromatograph (HPLC) consisting of a Spectra-Physics SP-8700 solvent pumping system with bellows damper (NBD-III), ERC-7520 (refraction index-detector) or SSC-Y-1000 (UV- detector) and a Rheodyne model 7125 injection value was used. Chromatograms were recorded on an SIC-7000B (System Instruments) integrator. Experiments were performed on a column (25 cm × 10 mm i.d.) (Senshu Scientific) packed with Nucleosil 100-5 C_{18} (octadecyl silica, 5 μm) (Macherey, Nagel and Co., Duren, G. F. R.). Column chromatography was performed using a Kieselgel 60 column (70—230 mesh, Merck). Thin layer chromatography (TLC) was conducted on precoated Kieselgel 60 F_{254} plated (0.20 mm thick, Merck) with $CHCl_3$–MeOH–H_2O (65 : 35 : 10, lower layer). The molecular composition of the compound given by the chemical formula was determined by elemental analysis.

Preparation of Test Compounds Commercially available glycyrrhizin (**1**) isolated from licorice root was used as the starting material for chemical modification.

18β-Olean-12-ene-3β,11β-diol-30-oic Acid 3-*O*-β-D-Glucuronopyranosyl-(1→2)β-D-glucuronopyranoside (11β-Hydroglycyrrhizin) (2) To a mixture of $NaBH_4$ (7.56 g, 0.2 mol) dissolved in aq 1N NaOH (50 ml) and tetrahydrofuran (THF) (100 ml) at 80 °C, glycyrrhizin (**1**) (8.23 g, 0.01 mol) dissolved in THF (100 ml) was added dropwise and allowed to react for 24 h under reflux in a H_2O bath. The reaction mixture was stirred with acetone (50 ml) at room temperature (r.t.), and dil. HCl was added to adjust to pH 9—10. The filtrate of the reaction mixture was concentrated to 100 ml, and then precipitated by the addition of acetone (700 ml) and MeOH (300 ml). The crude product was refluxed with boiling 75% EtOH (200 ml) to remove insoluble substances. From the filtrate 11β-hydroglycyrrhizin Na salt (7.6 g) was separated (yield 85%). 11β-Hydroglycyrrhizin (**2**) was obtained by treating the Na-salt with Dowex 50 W-X2 Type II in H_2O. mp 271—277 °C (dec.), $[\alpha]_D$ +80.0° (MeOH), $C_{42}H_{64}O_{16}\cdot 2H_2O$. IR ν_{max}^{KBr} cm^{-1}: 1050 (C–OH), 1080 (C–O–C), 1710 (COOH), 2950 (CH_3), 3450 (OH). (−)FAB MS *m/z*: 823 $(M-1)^+$. ^{13}C-NMR δ: 179.0 (s, C-30), 170.5 (s), 170.0 (s, C′-6, C″-6), 148.5 (s, C-13), 121.5 (d, C-12), 104.1 (d), 103.3 (d, C′-1, C″-1), 61.2 (d, C-11).

Olean-11,13(18)-diene-3β-ol-30-oic Acid 3-*O*-β-D-Glucuronopyranosyl-(1→2)β-D-glucuronopyranoside (3) 11β-Hydroglycyrrhizin Na salt (5 g) dissolved in H_2O (100 ml) was treated with dil. HCl to adjust to pH 7—8. The solution was heated at 100 °C for 6 h. After cooling, the reaction mixture was acidified to pH 2—3 and then extracted twice with *n*-BuOH (100 ml). On evaporation, compound **3** was obtained. Yield: 4.2 g (92.7%); mp 247—250 °C (dec.), $[\alpha]_D$ −120.0° (MeOH), $C_{42}H_{62}O_{15}\cdot 2H_2O$. UV λ_{max}^{MeOH} nm: 243, 251, 260. IR ν_{max}^{KBr} cm^{-1}: 1040 (C–OH), 1080 (C–O–C), 1640 (C=C–C=C), 1720 (COOH), 2930 (CH_3), 3400 (OH). (−)FAB MS *m/z*: 805 $(M-1)^+$. ^{1}H-NMR (in CD_3OD) δ (TMS): 6.35 (1H, dd J=16 Hz, vinyl-H), 5.59 (1H, d, J=12 Hz, vinyl-H). ^{13}C-NMR δ: 178.9 (s, C-30), 170.2 (s), 169.9 (s, C′ 6. C″-6), 135.1 (s, C-30), 134.4 (s) (C-13, C-18), 127.8 (d), 126.3 (d, C-11, C-12), 104.6 (d), 103.5 (d, C′-1, C″-2), 88.5 (d, C-3). Kitagawa *et al.*[5] isolated compound **3**, mp 249—251 °C, $[\alpha]_D$ −120.0° (MeOH), from Dong-bei licorice (Glycyrrhiza uralensis root) as one of the minor saponins and called licorice saponin C-2.

Olean-11,13(18)-diene-3β,30-diol-3-*O*-β-D-glucopyranosyl(1→2)β-D-glucopyranoside (4) Trimethyl ester penta-acetate of compound **3** (500 mg) dissolved in dry THF (20 ml) was added to a solution of $NaAlH_2(OCH_2CH_2OCH_3)_2$ (70% in toluene) (2.9 ml) in dry THF (20 ml) under vigorous stirring at 60 °C in an N_2 atmosphere. The reaction was

continued for 1 h, and after cooling, the reaction mixture was decomposed with 10% HCl to adjust pH 3—4 and added with aq. *n*-BuOH. The organic layer of the filtered solution was washed with H_2O 3 times. The solvent was evaporated to obtain a residue which was chromatographed on a silica gel (solvent: $CHCl_3$–MeOH (9 : 1)) to give a main fraction. The fraction was separated on HPLC using MeOH–H_2O (8 : 2) to afford compound **4**. Yield: 273 mg; mp 267—270 °C, $[\alpha]_D$ −41.6° (MeOH), $C_{42}H_{68}O_{12}\cdot 4H_2O$. UV λ_{max}^{MeOH} nm: 243, 252, 260. IR ν_{max}^{KBr} cm^{-1}: 1030 (C–OH), 1080 (C–O–C), 1640 (C=C–C=C), 2920 (CH_3), 3400 (O–H). (−)FAB MS *m/z*: 763 $(M-1)^+$. ^{13}C-NMR δ: 135.2 (s), 134.6 (s, C-13, C-18 or reversed), 127.5 (d), 127.0 (d, C-11, C-12), 104.7 (d), 103.5 (d, C′-1, C″-1), 64.2 (t, C-30), 61.0 (t), 60.8 (t, C′-6, C″-6).

18β-Olean-12-ene-11-oxo-3β,30-diol-3-*O*-β-D-glucuronopyranosyl(1→2)β-D-glucuronopyranoside (5) Penta-acetate of glycyrrhizin dimethyl ester at the di-glucuronide moiety was prepared according to the method of Sasaki *et al.*[6] The selective reduction of glycyrrizin 30-COOH was performed according to Yamada's method.[7] A solution of ethyl chloroformate (0.55 g) in THF (2.5 ml) was added to a solution of dimethyl ester pentaacetate of glycyrrhizin (5.3 g) and triethylamine (0.51 g) in THF (25 ml) during the course of 30 min at −5 °C, and the mixture was stirred for 30 min at the same temperature. The precipitate was filtered off and washed with THF, and the combined filtrate and washings were added during 30 min to a solution of $NaBH_4$ (0.47 g) in H_2O (5 ml) at 10—15 °C on cooling. Then the reaction mixture was stirred at r.t. for 4 h and made acidic with HCl, when the reaction mixture was separated into two layers. The organic layer washed with 10% aq. NaOH and H_2O was dried over anhydr. Na_2SO_4. The solvent was evaporated to obtain a residue which was chromatographed on a silica gel (solvent: $CHCl_3$–MeOH (100 : 1)) to give a main product (1.25 g). One percent aq. KOH solution (14 ml) was added to the solution of the main product (200 mg), and the mixture was stirred at r.t. for 20 min. After deionization by treatment with Amberlite IRA-120B, the product was purified by HPLC (solvent: CH_3CN–3% aq. AcOH (4 : 6)) to afford compound **5**. Yield: 133 mg; mp 241—245 °C, $[\alpha]_D$ +54.0° (MeOH), $C_{42}H_{64}O_{15}\cdot 3H_2O$. UV λ_{max}^{MeOH} nm: 248. IR ν_{max}^{KBr} cm^{-1}: 1050 (C–OH), 1090 (C–O–C), 1650 (C=O), 1720 (COOH), 2920 (CH_3), 3420 (O-H). (+)FAB MS *m/z*: 809 $(M+1)^+$. ^{13}C-NMR δ: 199.5 (s, C-11), 170.6 (s), 170.4 (s, C′-6, C″-6), 170.2 (s, C-13), 127.5 (d, C-12), 104.8 (d), 103.6 (d, C′-1, C″-1), 64.3 (t, C-30).

18β-Olean-12-ene-3β,11ξ,30-triol-3-*O*-β-D-glucopyranosyl(1→2)β-D-glucopyranoside (6) Treatment of glycyrrhizin (500 mg) with diazomethane in MeOH at r.t. for 4 h yielded trimethyl ester. On acetylation glycyrrhizin trimethyl ester gave pentaacetate. Penta-acetylglycyrrhizin trimethyl ester dissolved in dry THF (20 ml) was added to a solution of $NaAlH_2(OCH_2CH_2OCH_3)_2$ (70% in toluene) (2.9 ml) in dry THF (20 ml) under vigorous stirring at 60 °C in an N_2 atmosphere. The reaction was continued for 1 h. After cooling, the reaction mixture was decomposed with 10% HCl to adjust to pH 3—4 and extracted with aq. *n*-BuOH (50 ml). The organic layer was washed with H_2O 3 times. The solvent was evaporated to obtain a residue which was chromatographed on silica gel (solvent: $CHCl_3$–MeOH–H_2O (40 : 10 : 1)) to give a main fraction. The fraction was separated on HPLC using a solvent system MeOH–H_2O (8 : 2) to afford compound **6**. Yield: 208 mg; mp 216—218 °C, $[\alpha]_D$ +51.2° (MeOH), $C_{42}H_{70}O_{13}\cdot 4H_2O$. IR ν_{max}^{KBr} cm^{-1}: 1030 (C–OH), 1080 (C–O–C), 2940 (CH_3), 3400 (O–H). (−)FAB MS *m/z*: 781 $(M-1)^+$. ^{13}C-NMR δ: 144.7 (s, C-13), 127.4 (d, C-12), 103.8 (d), 103.6 (d, C′-1, C″-1), 64.3 (d, C-11), 64.3 (t, C-30), 61.0 (t), 60.9 (t, C′-6, C″6).

18β-Olean-12-ene-3β,30-diol-3-*O*-β-D-glucopyranosyl(1→2)β-D-glucopyranoside (7) A solution of compound **6** (300 mg) in dry EtOH (30 ml) was stirred with Pd/C (10%, 1 g) for 12 h under an H_2 stream. The reaction mixture was filtered and evaporated *in vacuo* to obtain compound **7**. Yield: 164 mg; mp 222—224 °C, $[\alpha]_D$ +36.8° (MeOH), $C_{42}H_{70}O_{12}\cdot 3H_2O$. IR ν_{max}^{KBr} cm^{-1}: 1030 (C–OH), 1080 (C–O–C), 2940 (CH_3), 3380 (O–H). (−)FAB MS *m/z*: 765 $(M-1)^+$. ^{13}C-NMR δ: 144.4 (s, C-13), 121.6 (d, C-12), 103.8 (d), 103.6 (d, C′-1, C″-1), 64.3 (t, C-30), 61.0 (t), 60.9 (t, C′-6, C″-6), 23.0 (t, C-11).

Biological Experiments A. Cells and Viruses MT-4[8] and MOLT-4 (clone 8)[9] were used for anti-HIV-1 assays. The cells were cultured and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml of penicillin G, and 100 μg/ml streptomycin. The HIV-1 was obtained from the culture supernatant of MOLT-4/HTLV-III_B cells.[10] For anti-HSV-1 test, we employed a KOS strain of HSV-1 and human embryonic fibroblast (HEF) cells growing in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated newborn calf serum (NCS) and antibiotics.

B. Anti-HIV-1 Assay The activity of the compounds against HIV-1 replication in MT-4 was based on the inhibition of virus-induced cytopathogenicity, as previously described.[11] Briefly, MT-4 cells were suspended at 2×10^5 cells per ml and infected with HIV-1 at 1000 50% cell culture infective doses per ml. Immediately after infection, 100 μl of the cell suspension was put into each well of a flat-bottomed microtiter tray containing various concentrations of the test compounds. After 5 d of incubation at 37 °C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method, as previously described.[12]

The activity was also monitored by the viability and HIV-1 antigen expression in MOLT-4 (clone 8) cells infected with HIV-1 in the absence or presence of the test compounds. In this experiment, MOLT-4 (clone 8) cells were infected with HIV-1 at a multiplicity of infection of 0.002 and incubated at 37 °C. On day 3 after infection, viral antigen expression was measured by indirect immunofluorescence using a polyclonal antibody. For the viability test, half of the culture medium was replaced by fresh culture medium containing appropriate concentrations of the compounds. The cells were incubated for another 2 d, and the number of viable cells was counted microscopically in a hematocytometer by the trypan blue exclusion method. The cytotoxicity of the compounds was determined by measuring the viability of mock-infected MT-4 and MOLT-4 (clone 8) cells in parallel with the virus-infected cells.

C. Reverse Transcriptase (RT) Assay The effect of the compounds on HIV-1 RT activity was evaluated with the enzyme obtained from disrupted virions which had been partially purified and concentrated from the supernatant of MOLT-4/HTLV-III_B cells. The assay was performed at 37 °C for 30 min in a 50 μl reaction mixture containing 50 mM Tris–HCl (pH 8.4), 2 mM dithiothreitol, 100 mM KCl, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 μCi of [methyl-^{3}H]-dTTP (30 Ci/mmol), 0.01 OD unit of poly(rA). oligo (dT), test compound and *ca.* 0.1 U of enzyme. The reaction was stopped with 200 μl of 5% trichloroacetic acid, and the precipitated material was analyzed for radioactivity.

D. Anti-HSV-1 Assay Assay of the compound for HSV-1 replication was carried out in confluent HEF monolayer cells in a flat-bottomed microtiter tray. The HEF cells were infected with 50 plaque forming units (PFU) of HSV-1 and incubated at 37 °C in the presence of various concentrations of the test compounds. After 5—7 d, HSV-1-induced cytopathogenicity was recorded microscopically.

Results and Discussion

When the inhibitory effect of glycyrrhizin (**1**) and its derivatives (**3**—**7**) was examined for HIV-1 replication in MT-4 cells, glycyrrhizin (**1**) and compound **3** were found to have achieved more than 80% protection of the cells from the virus-induced destruction at a concentration of 0.62 and 0.16 mM, respectively (Table I). In particular, compound **3** almost completely suppressed HIV-1-induced cytopathogenicity at this concentration. Compounds **4** and **5** were slightly inhibitory to HIV-1 replication at subtoxic concentrations (Table I), yet other compounds (**6** and **7**) did not show any activity at their non-toxic concentrations (data not shown).

In MOLT-4 (clone 8) cells, compound **3** also exhibited the selective inhibition at a concentration between 0.16 and 0.31 mM (Fig. 1). The compound slightly reduced the number of viable mock-infected cells at a concentration of 1.24 mM. The HIV-1 antigen expression in MOLT-4 (clone 8) was suppressed by compounds **1** and **3** at concentrations higher than 0.16 mM (Fig. 2). In contrast, compounds **4** and **5** were totally inactive against the antigen expression. When we examined the effect of compound **3** on HIV-1 RT activity, no inhibition was observed at the concentrations active against HIV-1 induced cytopathogenicity in MOLT-4 cells.

In the next experiment, we investigated the inhibitory effect of glycyrrhizin and its homologs on HSV-1 replication in HEF cells. As shown in Table III, glycyrrhizin and compound **3** proved inhibitory to HSV-1 replication, their 50% inhibitory concentrations (IC_{50}) for HSV-1 cytopatho-

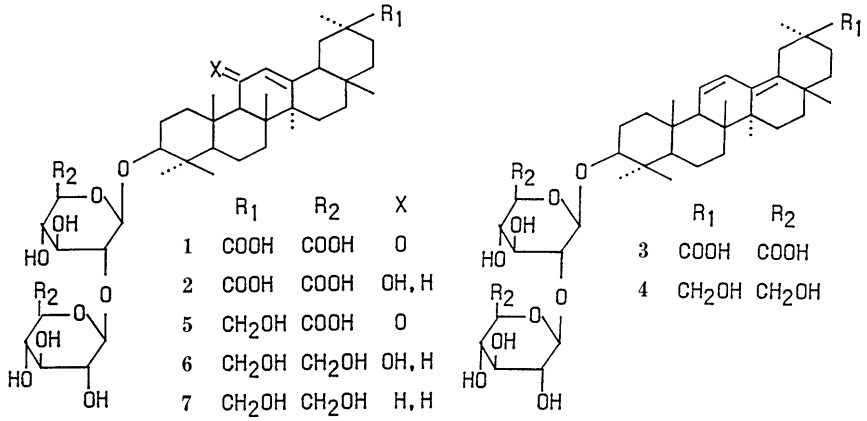

Chart 1

TABLE I. Inhibitory Effect of Glycyrrhizin and its Modified Compounds on HIV-1 Replication in MT-4 Cells

Concentration (mM)	Number of viable cells[a] (%)			
	1	3	4	5
2.48	0	0	N.D.	N.D.
1.24	30.7	0	N.D.	N.D.
0.62	81.9	0	N.D.	N.D.
0.31	39.1	60.9	N.D.	17.5
0.16	8.4	92.8	56.7	57.4
0.08	8.1	11.6	20.4	7.0
0.04	4.5	1.7	9.6	10.2

a) Number of viable cells is determined by the MTT method and expressed as the percentage of mock-infected control. N.D., not determined.

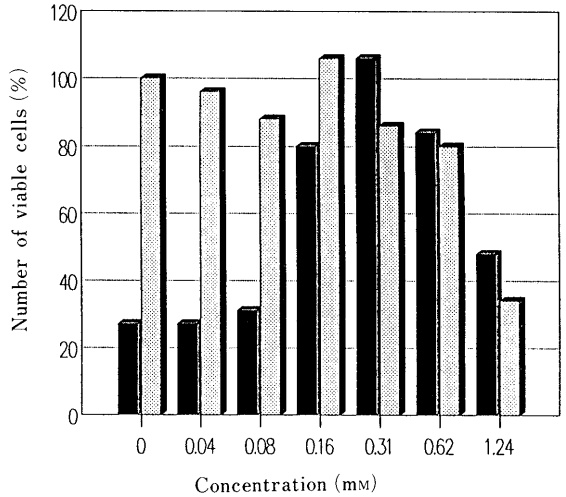

Fig. 1. Inhibitory Effect of Compound **3** on HIV-1 Replication in MOLT-4 Cells

The viability of HIV-1-infected cells (■) and mock-infected cells (▨) was assessed by the trypan blue exclusion method. The number of viable cells is expressed as the percentage of the mock-infected control.

genicity being 3.6 and 0.5μM, respectively. Other compounds (**4**—**7**) did not affect the replication of HSV-1 nor the cell morphology of mock-infected HEF cells (data not shown).

Based on the anti-HIV-1 activity of glycyrrhizin *in vitro*,[1] the glycyrrhizin preparation, SNMC has been clinically applied to HIV-1 carriers by i.v. administration, and indeed, their immunological parameters have been improved.[2] The present study indicates that a glycyrrhizin analog compound

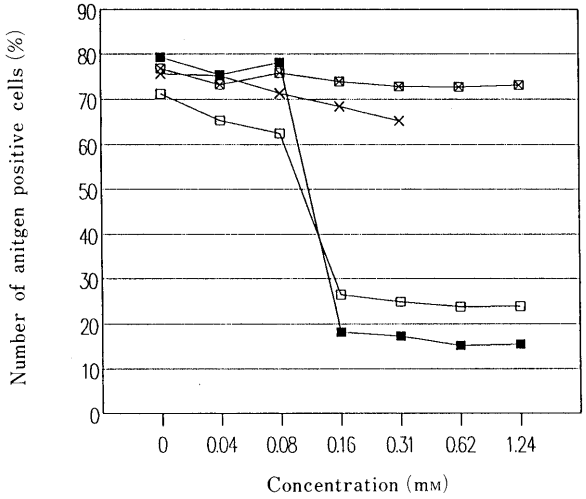

Fig. 2. Inhibitory Effect of Glycyrrhizin (■) and Compounds **3** (□), **4** (⊠) and **5** (×) on HIV-1 Antigen Expression in MOLT-4 Cells

HIV-1 antigen positive cells were detected by indirect immunofluorescence using polyclonal antibody on day 3 after infection.

3 also seems applicable to the treatment of AIDS. Furthermore, compound **3** might be safer than glycyrrhizin in long-term administration because, it is less capable of exerting pseudoaldosteronism, as suggested by our earlier investigations.[13]

The RT assay suggests that, like glycyrrhizin,[1] the anti-HIV-1 activity of compound **3** is not due to the inhibition of RT activity. The mechanism of action of the compound remains to be elucidated. It has been demonstrated that glycyrrhizin partially prevents the adsorption of HIV-1 particles to $CD4^+$ cells.[14] Inhibition of protein kinase C (PKC) activity by glycyrrhizin has also been discussed in relation to its anti-HIV-1 effect.[15] However, it is still not known whether the inhibition of virus adsorption and the inhibition of PKC activity are definitely related, though the binding of HIV-1 to $CD4^+$ lymphocytes induces rapid phosphorylation of CD4 receptors which involve PKC.[15] On the other hand, comparative study with other glycyrrhizin analogs (**4**—**7**) has revealed that the presence of a carboxyl group at the 20-position in compound **3** is essential for the anti-HIV-1 activity, while the hetero-annular diene C/D ring system in compound **3** is convertible with the $\alpha\beta$-unsaturated carbonyl structure in C-ring of glycyrrhizin. These results suggest

that the carboxyl group at 20-position may play a crucial role in inhibiting the virus adsorption.

Considering the current extreme prevalence of AIDS, the finding of effective chemotherapeutic agents is highly desired. In this regard, compound **3** should be further pursued for its potential as an anti-AIDS agent.

Acknowledgements The authors thank Dr. A. Sato, Mr. S. Mori and Mr. K. Konno, Fukushima Medical College, for their excellent technical assistance, and Messrs. T. Takeuchi, N. Nagata, T. Okai and M. Abe of Minophagen Pharmaceutical Co. for their useful support. Their appreciation is also extended to Prof. I. Nishioka, Kyushu University, and members of the Central Analytical Laboratory, Meiji College of Pharmacy, for FAB-MS, NMR, IR and UV spectral analyses.

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