SYNTHESIS OF GLYCYRRHIZIC ACID CONJUGATES WITH AMINO-ACID METHYL ESTERS AND THEIR ABILITY TO STIMULATE ANTIBODY GENESIS IN MICE

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Conjugates of glycyrrhizic acid (GA) with methyl esters of *L*-amino acids (valine, methionine, and glutamic acid) containing the amino-acid residues in the carbohydrate moiety of the glycoside were synthesized using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The resulting GA conjugates at a dose of 2 mg/kg stimulated a primary immune response (production of antibody-forming cells, AFCs) in outbred mice by 1.6 – 3 times as compared with the control. The conjugate of GA with $Glu(OMe)_2$ stimulated antibody genesis in outbred mice 1.7 times more efficiently than *N*-acetylmuramyl dipeptide and showed a stimulating effect on AFC production in the spleen of CBA mice.

Keywords: glycyrrhizic acid, conjugates, synthesis, amino acids, methyl esters of amino acids, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, antibody-forming cells, mice.

Glycyrrhizic acid (GA, **I**) is the major constituent in extracts of licorice root (*Glycyrrhiza glabra* L. and *G. uralensis* Fisher). It is a leading natural triterpene glycoside that is promising as a platform for designing new immunomodulating and antiviral agents [1, 2]. GA stimulates proliferation of T- and B-lymphocytes and the formation of γ-interferon *in vitro* and *in vivo*, has a stimulating effect on secretion of interleukin-2 in peripheral lymphocyte culture, and is a promising stimulator of nonspecific immunity against viral infections [2, 3]. Chemical modification of GA opens new possibilities for producing semi-synthetic derivatives with improved properties [2, 4, 5]. We observed inhibitors of HIV-1 [1, 2, 6, 7], Epstein–Barr virus [8], SARS CoV [9], A/H1N1/pdm2009 influenza [10, 11], and Dengue 2 [12] and Zika flaviviruses [13] among GA derivatives. Several amino-acid and dipeptide conjugates of GA were low-toxicity immunomodulators [2, 14, 15]. Thus, GA conjugates with amino acids and their esters are a promising class of semi-synthetic biomolecules obtained from available plant glycosides for research and development of new immunotropic and antiviral agents.

I: R = OH
II: R = $(CH_3)_2CHCHCOOCH_3$ (ValOMe)
  |
  NH—
III: R = $CH_3SCH_2CH_2CHCOOCH_3$ (MetOMe)
  |
  NH—
IV: R = $CH_3OOC(CH_2)_2CHCOOCH_3$ $(Glu(OMe)_2)$
  |
  NH—
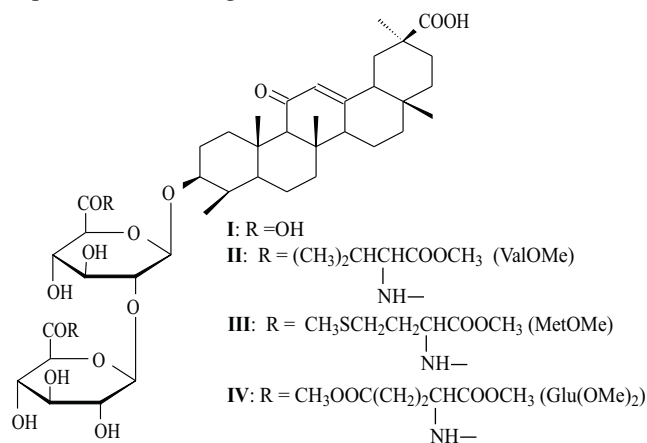
Methods for preparing amino-acid conjugates of GA containing three amino-acid residues or their esters using *N*-hydroxybenzotriazole (HOBt) and *N,N′*-dicyclohexylcarbodiimide (DCC) [16] or *N*-hydroxyphthalimide and DCC [17] were reported previously by us. Conjugates of GA containing amino-acid fragments in the diglucuronide part of GA were synthesized using *N*-hydroxysuccinimide (HOSu)

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and DCC [6, 13, 14]. A method for preparing conjugates of GA with amino-acid *tert*-butyl esters (followed by deblocking) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) that could selectively in one step introduce amino-acid fragments into the glycoside carbohydrate part was proposed [18].

The present work reports the synthesis of GA conjugates with *L*-amino-acid methyl esters (valine, methionine, and glutamic acid, **II-IV**) with immunostimulant activity using EDC as the condensing reagent.

EXPERIMENTAL CHEMICAL PART

IR spectra were taken in Vaseline oil mulls on a Prestige-21 IR spectrophotometer (Shimadzu). PMR and ^{13}C NMR spectra were recorded in CD_3OD on a Bruker Avance-III pulsed spectrometer at operating frequency 500.13 (^{1}H) and 125.47 MHz (^{13}C). Chemical shifts were given in ppm vs. TMS internal standard. Optical activity was measured in a 1-dm tube at 20 – 22°C on a PerkinElmer 341 polarimeter (λ_{Na} 546 nm).

TLC used Sorbfil plates (Sorbpolimer) and solvent systems C_6H_6–EtOH (5:1) and $CHCl_3$–EtOH (5:1). Spots of compounds were detected by H_2SO_4 solution (5%) in EtOH followed by heating at 110 – 120°C for 2 – 3 min. Column chromatography used KSK silica gel (50 – 150 fraction, dry classification) (Sorbpolimer).

HPLC analysis of GA used a Shimadzu LC-20 liquid chromatograph (Japan) with a Vydac 218TP C18 reversed-phase column (250 × 4.6 mm) (Supelco, USA), MeOH mobile phase at flow rate 1 mL/min, and a spectrophotometric diode-array detector at λ = 254 nm.

GA with 92 ± 1% purity obtained from the monoammonium salt of GA drug substance [19], 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Roth, Germany), and *L*-amino-acid methyl ester hydrochlorides (Reanal, Hungary) were used in the work.

General method for preparing conjugates II-IV

A solution of GA (0.82 g, 1 mmol) in DMF (20 mL) was stirred; treated with the amino-acid methyl ester hydrochloride (2.5 – 3.0 mmol), EDC (2.5 – 3.0 mmol), and Et_3N (7 – 8 mmol); held at 20 – 22°C for 24 h with periodic stirring; and diluted with a mixture of cold H_2O acidified with citric acid to pH ~3 – 4. The precipitate was filtered off, rinsed with H_2O, dried, and chromatographed over a column of silica gel with elution by $CHCl_3$–EtOH (400:10, 300:10, 200:10, 100:10, 50:10, v/v) with TLC monitoring.

3-*O*-{2-*O*-[*N*-(β-*D*-Glucopyranosyluronoyl)-*L*-valine methyl ester]-*N*-(β-*D*-glucopyranosyluronoyl)-*L*-valine methyl ester}-(3β,20β)-11-oxo-30-norolean-12-ene (II). Yield 56% (amorphous substance). R_f 0.52 ($CHCl_3$ — EtOH, 5:1); $[\alpha]_D^{20}$ + 65° (s 0.04; EtOH); 97.3% pure by HPLC, τ 2.77 min (Fig. 1). IR spectrum (ν, cm^{-1}): 3600 – 3200 (OH, NH); 1740, 1660, 1540 (CONH). PMR spectrum (500 MHz,

CD_3OD, δ, ppm): 7.88 (2H, s, 2NH), 5.57 (^{1}H, s, H12), 4.77 (1H, d, J 7.7 Hz, H1″), 4.58 (1H, d, J 7.4 Hz, H1'), 4.44 – 4.42 (2H, m, H3″, H3'), 3.48 – 3.81 (3H, m, H4″, H4″, H3), 3.74 (6H, s, 2OCH$_3$), 3.66 – 3.61 (3H, m), 3.54 – 3.44 (3H, m), 3.35 – 3.18 (3H, m), 2.86 (^{1}H, s, H18), 2.58 – 2.52 (3H, m), 2.24 (^{1}H, s, H9), 2.21 – 2.14 (5H, m), 2.04 – 1.58 (7H, m), 1.45 (^{1}H, s), 1.40 (3H, s, CH$_3$), 1.36 (2H, s), 1.27 – 1.24 (2H, m), 1.16 (2H, s), 1.13 (6H, s, 2CH$_3$), 1.05 (3H, s, CH$_3$), 1.00 (3H, s, CH$_3$), 0.98 – 0.92 (15H, m, 5CH$_3$), 0.85 (2H, m), 0.82 (3H, s, CH$_3$), 0.80 – 0.76 (2H, m). ^{13}C NMR spectrum (500 MHz, CD_3OD, δ, ppm): 202.25 (C$_{11}$), 180.16 (C30), 171.67 (C6″), 171.37 (C6'), 170.97 (C13), 128.85 (C12), 105.29 (C1″), 104.80 (C1'), 90.37 (C$_3$), 82.11 (C2'), 77.50 (C5″), 77.23 (C5'), 76.44 (C3″), 75.97 (C3'), 75.35 (C2″), 73.17 (C4'), 73.05 (C4″), 62.96 (C9), 56.32 (C5), 49.74 (C18), 46.63 (C8), 44.77 (C20), 44.49 (C14), 42.30 (C19), 40.55 (C4), 40.14 (C1), 38.91 (C22), 37.97 (C10), 33.71 (C7), 32.87 (C17), 32.23 (C21), 29.19 (C29), 28.75(C23), 28.41 (C28), 27.51 (C16), 27.39 (C15), 27.30 (C2), 23.88 (C27), 18.26 (C26), 19.32(C6), 18.74 (C24), 18.11 (C25), 2ValOMe: 172.99 (COOMe), 172.87 (COOMe), 58.69 (α-CH), 58.28 (α-CH), 52.71 (OCH$_3$), 52.58 (OCH$_3$), 31.98 (2CH), 19.50 (CH$_3$), 19.40 (CH$_3$), 17.05 (CH$_3$), 16.99 (CH$_3$). Found, %: C 61.73, H 7.95, N 2.58. $C_{54}H_{84}N_2O_{18}$. Calc., %: C 61.81, H 8.07, N 2.67. M = 1049.22.

3-*O*-{2-*O*-[*N*-(β-*D*-Glucopyranosyluronoyl)-*L*-methionine methyl ester]-*N*-(β-*D*-glucopyranosyluronoyl)-*L*-methionine methyl ester}-(3β,20β)-11-oxo-30-norolean-12-ene (III). Yield 55% (amorphous substance). R_f 0.52 ($CHCl_3$–EtOH, 5:1). $[\alpha]_D^{20}$ + 58° (s 0.04, MeOH); 95.8% pure by HPLC, τ 2.64 min (Fig. 2). Lit. [20]: $[\alpha]_D^{20}$ + 56° (s 0.02, MeOH). IR spectrum (ν, cm^{-1}): 3500 – 3200 (OH, NH); 1739, 1656, 1534 (CONH). Found, %: N 2.46; S 5.64. $C_{54}H_{84}O_{18}N_2S_2$. Calc., %: N 2.52; S 5.75. PMR (500 MHz) and ^{13}C NMR spectra (125 MHz) agreed with those published earlier [20].

3-*O*-{2-*O*-[*N*-(β-*D*-Glucopyranosyluronoyl)-*L*-glutamic acid dimethyl ester]-*N*-(β-*D*-glucopyranosyluronoyl)-*L*-glutamic acid dimethyl ester}-(3β,20β)-11-oxo-30-norolean-12-ene (V). Yield 52% (amorphous substance). R_f 0.70 ($CHCl_3$–EtOH, 5:1); $[\alpha]_D^{20}$ + 46° (s 0.10; MeOH); 98.1% pure by HPLC, τ 2.64 min (Fig. 3). Lit. [21]: $[\alpha]_D^{20}$ +43 ± 2° (s 0.03, MeOH). IR spectrum (ν, cm^{-1}): 3500 – 3200 (OH, NH), 1742, 1660, 1545 (CONH). PMR spectrum (500 MHz, CD_3OD, δ, ppm): 7.85 (2H, s, 2NH), 5.57 (1H, s, H12), 4.78 (1H, d, J 7.9 Hz, H1″), 4.63 (1H, d, J 8.0 Hz, H1'), 4.58 – 4.50 (3H, m, H3″, H3', H3), 4.23 – 4.11 (2H, m, H2″, H2'), 3.85 – 3.79 (2H, m, H4″, H4'), 3.77, 3.74, 3.73 (12H, all s, 4OCH$_3$), 3.66 – 3.39 (10H, m), 3.34 (1H, s), 3.20 (^{1}H, s), 3.31 – 3.27 (2H, m), 2.86 (1H, s, H18), 2.68 – 2.60 (2H, m), 2.42 (1H, s H9), 2.20 – 1.59 (12H, m), 1.43, 1.41 (9H, s, 3CH$_3$), 1.36 – 1.22 (3H, m), 1.17 (3H, s, CH$_3$), 1.13 (6H, s,
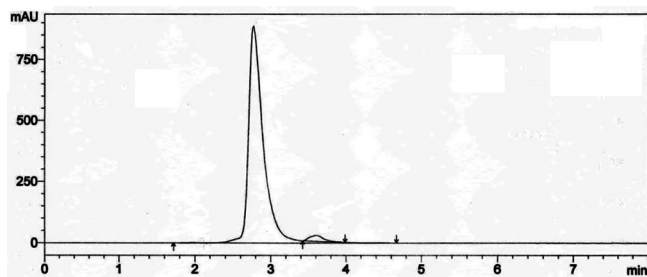
Fig. 1. HPLC of GA conjugate with ValOMe (**II**). Vydac TP C18 column; MeOH mobile phase; flow rate 1 mL/min; retention time (τ) 2.77 min; UV detector at λ = 254 nm; 97.3 $\pm$ 0.8% pure.
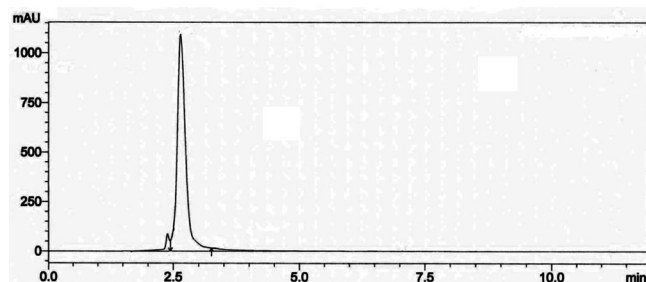

Fig. 2. HPLC of GA conjugate with MetOMe (**III**). Vydac TP C18 column; MeOH mobile phase; flow rate 1 mL/min; retention time (τ) 2.64 min; UV detector at λ = 254 nm; 95.8 $\pm$ 0.8% pure.

2CH$_3$), 1.06 – 0.86 (4H, m), 0.83 (3H, s, CH$_3$), 0.81 – 0.73 (2H, m). ^{13}C NMR spectrum (125 MHz, CD$_3$OD, δ, ppm): 202.50 (C11), 180.31 (C30), 171.24 (C6″), 171.14 (C6′), 170.67 (C13), 128.93 (C12), 106.27 (C1″), 105.27 (C1′), 90.91 (C3), 83.99 (C2′), 77.32 (C5″), 77.07 (C5′), 76.15 (C3″), 75.78 (C3′), 73.35 (C2″), 72.94 (C4″), 72.81 (C4′), 63.11 (C9), 56.50 (C5), 49.87 (C18), 46.73 (C8), 44.87 (C20), 44.60 (C14), 42.41 (C19), 40.55 (C4), 40.24 (C1), 39.00 (C22), 38.02 (C10), 33.82 (C7), 32.96 (C17), 32.01 (C21), 29.68 (C29), 29.22 (C23), 28.79 (C28), 27.59 (C16), 27.39 (C15), 26.58 (C2), 23.91 (C27), 19.35 (C26), 18.42 (C6), 17.92 (C24), 17.02 (C25), 2Glu(OMe)$_2$: 174.10 (COOMe), 174.00 (COOMe), 172.60 (2COOMe), 53.05 (α-CH), 53.00 (α-CH), 52.87 (OMe), 52.73 (OMe), 28.36 (CH$_2$), 28.20 (CH$_2$), 27.04 (CH$_2$), 27.00 (CH$_2$). Found, %: C 58.85, H 7.50, N 2.40. C$_{56}$H$_{84}$O$_{22}$N$_2$. Calc., %: C 59.14, H 7.44, N 2.46. M = 1137.24.

EXPERIMENTAL PHARMACOLOGICAL PART

Animals were kept under standard vivarium conditions with access *ad libitum* to feed and water. Experiments with animals were conducted in compliance with international rules (*European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes*, No. 123, Strasbourg, 1986; *Protocol of Amendment to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes*, Strasbourg, Jun. 22, 1998) and were approved by The Commission for Biomedical Ethics, Ufa Institute of Chemistry, Branch of UFRC, RAS.

Immunomodulating activity

The effect of **II-IV** on the primary immune response was studied in 48 outbred and 18 CBA mice (18 – 20 g) using the method of Jerne and Nordin [22] as modified by Cunningham [23]. Animals were immunized intraperitoneally (i.p.) with a suspension of sheep erythrocytes four days before the tests. One day after immunization, the animals were injected once i.p. with **II** and **III** at a dose of 2 mg/kg and with **IV** at doses of 0.5 and 2 mg/kg. The number of hemolysis zones corresponding to the amount of antibody-forming cells (AFCs) in the whole spleen was counted after incubation in a thermostat at 37°C for 2.5 h for five days after immunization. Then, the AFCs were recalculated per 10^6 splenocytes. The reference drug was the known glycopeptide immunostimulant *N*-acetylmuramyl-*L*-alanyl-*D*-isoglutamine (MDP, CAS No. 53678-77-6, Sigma-Aldrich) at a dose of 2 mg/kg [24]. Statistical analysis used the Statistica 10 program. Data

TABLE 1. Effect of Compounds on AFC Production in Spleen of Outbred Mice (Number of Mice in Group n = 8)

Compound, R	Dose, mg/kg	Number of AFC per 10^6 splenocytes	p^{**}
Control*	–	1623 $\pm$ 618	–
II R = ValOMe	2	3090 $\pm$ 225	< 0.01
III R = MetOMe	2	2624 $\pm$ 313	< 0.02
IV R = Glu(OMe)$_2$	0.5	3780 $\pm$ 1135	< 0.05
IV R = Glu(OMe)$_2$	2	4912 $\pm$ 1262	< 0.05
MDP	2	2836 $\pm$ 287	< 0.01

* Control, normal saline; **statistically significant vs. control group for p < 0.05

TABLE 2. Effect of GA Conjugate **IV** on AFC Production in Spleen of CBA Mice (Number of Animals in Group n = 6)

Compound	Dose, mg/kg	Number of AFC per 10^6 splenocytes	p^{**}
Control*	–	530 $\pm$ 183	-
Conjugate **IV**	2	1327 $\pm$ 247	< 0.02
Conjugate **IV**	5	1498 $\pm$ 360	< 0.02

* Control, normal saline; ** statistically significant vs. control group for p < 0.05.
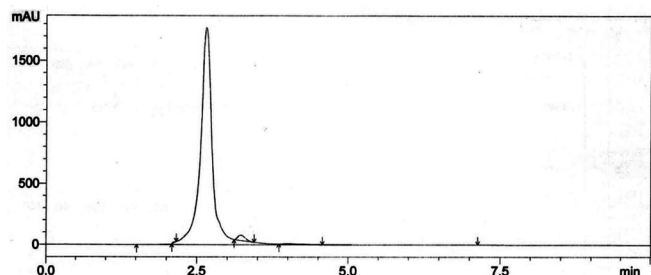
Fig. 3. HPLC of GA conjugate with Glu(OMe)$_2$ (**IV**). Vydac TP C18 column; MeOH mobile phase; flow rate 1 mL/min; retention time (τ) 2.65 min; UV detector at $\lambda = 254$ nm; $98.1 \pm 0.8\%$ pure.

were given as means (*M*) and their standard errors (± *SEM*). The statistical significance of intergroup differences was evaluated using the Student *t*-criterion. Differences were considered statistically significant for $p < 0.05$.

Determination of acute toxicity

The acute toxicity of conjugate **IV** was determined using 36 white outbred female mice (18 – 20 g) (six animals in each group) with a single i.p. injection of doses from 100 to 600 mg/kg. Animals were observed for 14 d, noting premature deaths, general condition, locomotor activity, and demand for feed and water. The toxicity parameters were calculated by the Litchfield and Wilcoxon method [25].

RESULTS AND DISCUSSION

The condensation of GA with the amino-acid methyl esters (amino component, AC) as the hydrochlorides was performed in DMF at GA/EDC/AC ratios of 1/2.5 – 3.0/2.5 – 3.0 mmol at room temperature in the presence of an excess of Et$_3$N. Target conjugates **II-IV** were isolated by column chromatography (CC) over silica gel (SG) in 52 – 60% yields. Compounds **III** and **IV** were synthesized previously using HOSu and DCC [20, 21]. The structures of the synthesized compounds were confirmed by spectral methods (IR, PMR, and ^{13}C NMR spectroscopy). Thus, ^{13}C NMR spectra of conjugates **II-IV** showed resonances for carbonyl C atoms of amino-acid residue COOMe groups at 174.2 – 172.5 ppm. The resonance of the aglycon C30-OOH C atom remained about the same as that of GA (180.3 – 180.2 ppm) [26], which indicated that the CO–NH bonds formed at the COOH groups of the diglucuronide part of the glycoside, which had absorption bands at 1540 – 1530 cm^{-1} in the IR spectra. The purity of the compounds was monitored by HPLC and was 95% and greater (Figs. 1 – 3).

The effects of conjugates **II-IV** on the primary immune response (AFC production) were studied in 48 outbred mice. The influence of a particular compound on the humoral component of the immune system was inferred from the amount of AFCs per 10^6 splenocytes. Table 1 presents the test results.

TABLE 1 shows that conjugates **II-IV** had pronounced effects on stimulation of antibody genesis, increasing the number of AFCs in spleens of the mice by 1.6 – 3.0 times as compared to the control. Conjugate **IV** was the most active compound at a dose of 2 mg/kg, stimulating AFC production in spleens of outbred mice by 1.7 times more efficiently than MDP upon injection at equal doses.

The influence of conjugate **IV** on the humoral component of the immune response was also studied in 18 CBA mice (18 – 20 g) at doses of 2 and 5 mg/kg. Table 2 presents the test results. It was found that conjugate **IV** at the studied doses stimulated AFC production in spleens of the CBA mice by 2.5 – 2.8 times more efficiently than the control.

The acute toxicity of **IV** was determined in white outbred mice. The LD$_{50}$ value of **IV** was 500 (350 – 600) mg/kg, which allowed it to be assigned to class III moderately hazardous compounds [27].

Thus, the studied GA conjugates **II-IV** were stimulators of antibody genesis in mice. The most active compound **IV** had higher immune-stimulating activity than MDP and was of interest for expanded pharmacological studies as an immunomodulator.

ACKNOWLEDGMENTS

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