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) 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 immunotropc and antiviral agents.

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-hydroxysuccinimide 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)
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)carbo-diimide 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 13C NMR spectra were recorded in CD3OD on a Bruker Avance-III pulsed spectrometer at operating frequency 500.13 (1H) and 125.47 MHz (13C). 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=546 nm).

TLC used Sorbfil plates (Sorbpolimer) and solvent systems C6H6–EtOH (5:1) and CHCl3–EtOH (5:1). Spots of compounds were detected by H2SO4 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 Et3N (7 – 8 mmol); held at 20 – 22°C for 24 h with periodic stirring; and diluted with a mixture of cold H2O acidified with citric acid to pH 3 – 4. The precipitate was filtered off, rinsed with H2O, dried, and chromatographed over a column of silica gel with elution by CHCl3–EtOH (400:10, 300:10, 200:10, 50:10, v/v) with TLC monitoring.

**3-O-[2-O-N-β-D-Glucopyranosyluronoyl]-L-valine methyl ester]-N-(β-D-glucopyranosyluronoyl)-L-methionine methyl ester)-(3β,20β)-11-oxo-30-norolean-12-ene (III)**. Yield 55% (amorphous substance). Rf. 0.52 (CHCl3–EtOH, 5:1); [α]D20 + 58° (s 0.04; MeOH); 95.8% pure by HPLC, τ = 2.64 min (Fig. 2). Lit. [20]: [α]D20 + 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. C53H84O13N2S2. Calcd.: %: N 2.52; S 5.75. PMR spectrum (500 MHz) and 13C NMR spectrum (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 (IV)**. Yield 52% (amorphous substance). Rf. 0.70 (CHCl3–EtOH, 5:1); [α]D20 + 46° (s 0.10; MeOH); 98.1% pure by HPLC, τ = 2.64 min (Fig. 3). Lit. [21]: [α]D20 + 43 ± 2° (s 0.03, MeOH). IR spectrum (ν, cm–1): 3500 – 3200 (OH, NH); 1742, 1660, 1545 (CONH). PMR spectrum (500 MHz, CD3OD, δ, ppm): 7.85 (2H, s, 2NH), 5.57 (1H, s, H12), 4.77 (1H, d, J 7.7 Hz, H11), 4.58 (1H, d, J 7.4 Hz, H11), 4.44 – 4.42 (2H, m, H3′, H3′′), 3.48 – 3.81 (3H, m, H4′, H4′′, H5), 3.74 (6H, s, 2OCH3), 3.66 – 3.61 (3H, m), 3.54 – 3.44 (3H, m), 3.35 – 3.18 (3H, m), 2.86 (1H, s, H18), 2.58 – 2.52 (3H, m), 2.24 (1H, s, H9), 2.21 – 2.14 (5H, m), 2.04 – 1.98 (7H, m), 1.45 (1H, s), 1.40 (3H, s, CH3), 1.36 (2H, s), 1.27 – 1.24 (2H, m), 1.16 (2H, s), 1.13 (6H, s, 2CH3), 1.05 (3H, s, CH3), 1.00 (3H, s, CH3), 0.98 – 0.92 (15H, m, 5CH3), 0.85 (2H, m), 0.82 (3H, s, CH3), 0.80 – 0.76 (2H, m). 13C NMR spectrum (500 MHz, CD3OD, δ, ppm): 202.25 (C11), 180.16 (C30), 171.67 (C6′), 171.37 (C6′′), 170.97 (C13), 128.85 (C12), 105.29 (C1′), 104.80 (C1′′), 90.37 (C1′′′), 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 (C14), 46.63 (C8), 44.77 (C20), 44.49 (C14), 42.30 (C19), 40.55 (C4), 40.14 (C14), 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 (COCOMe), 172.87 (COOME), 58.69 (α-CH), 58.28 (α-CH), 52.71 (OCH3), 52.58 (OCH3), 31.98 (2CH), 19.50 (CH3), 19.40 (CH3), 17.05 (CH3), 16.99 (CH3). Found, %: N 6.18; H 7.95, N 2.58. C53H84O13N2S2. Calcd.: %: N 2.52; S 5.75. PMR spectrum (500 MHz) and 13C NMR spectrum (125 MHz) agreed with those published earlier [20].
2CH₃), 1.06 – 0.86 (4H, m), 0.83 (3H, s, CH₃), 0.81 – 0.73 (2H, m). ¹³C NMR spectrum (125 MHz, CD₃OD, δ, ppm): 202.50 (C₁₁), 180.31 (C₃₀), 171.24 (C₆/c₁₇₈, ppm), 171.14 (C₆/c₁₆₂), 170.67 (C₁₃), 128.93 (C₁₂), 106.27 (C₁’, 105.27 (C₁/c₁₆₂), 90.91 (C₃), 83.99 (C₂/c₁₆₂), 77.32 (C₅/c₁₇₈), 77.07 (C₅/c₁₆₂), 76.15 (C₃/c₁₇₈), 75.78 (C₃/c₁₆₂), 73.35 (C₂/c₁₇₈), 72.94 (C₄/c₁₇₈), 72.81 (C₄/c₁₆₂), 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 (C₁), 19.35 (C₂₆), 18.42 (C₆), 17.92 (C₂₄), 17.02 (C₂₅), 2Glu(OMe): 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 (CH₂), 28.20 (CH₂), 27.04 (CH₂), 27.00 (CH₂). Found, %: C 58.85, H 7.50, N 2.40. C₅₆H₈₄O₂₂N₂. Calc., %: C 59.14, H 7.44, N 2.46. M=1 137.24.

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 10⁶ splenocytes. The reference drug was the known glycopeptide immuno-stimulant 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 10⁶ splenocytes | p**   |
|------------|------------|----------------------------------|------|
| Control*   | –          | 1623 ± 618                       | –    |
| II R = ValOMe | 2          | 3090 ± 225                       | < 0.01 |
| III R = MetOMe | 2          | 2624 ± 313                       | < 0.02 |
| IV R = Glu(OMe)₂ | 0.5      | 3780 ± 1135                      | < 0.05 |
| IV R = Glu(OMe)₂ | 2          | 4912 ± 1262                      | < 0.05 |
| MDP       | 2          | 2836 ± 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 10⁶ splenocytes | p**   |
|----------|------------|----------------------------------|------|
| Control* | –          | 530 ± 183                        | –    |
| Conjugate IV | 2          | 1327 ± 247                      | < 0.02 |
| Conjugate IV | 5          | 1498 ± 360                      | < 0.02 |

* Control, normal saline; ** statistically significant vs. control group for p < 0.05.
Determination of acute toxicity of Et$_3$N. Target conjugates 3.0 mmol at room temperature in the presence of an excess formed in DMF at GA/EDC/AC ratios of 1/2.5 – 3.0/2.5 –

RESULTS AND DISCUSSION

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 food and water. The toxicity parameters were calculated by the Litchfield and Wilcoxon method [25].

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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.

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