Synthesis and Antiviral Activity of Novel Glycyrrhizic Acid Conjugates with D-Amino Acid Esters

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Abstract—Glycyrrhizic acid (GA) conjugates with methyl and ethyl esters of D-amino acids (D-Trp, D-Phe, D-Tyr, D-Val, D-Leu) have been synthesized by the activated esters method using mixtures of N-hydroxybenzotriazole or N-hydroxysuccinimide with N,N'-dicyclohexylcarbodiimide. GA conjugate with D-Trp ethyl ester exhibited antiviral activity against influenza viruses A/H3N2, A/H1N1/pdm09, A/H5N1, B (SI > 10–29), and HRSV (SI > 25). GA conjugate with D-Trp methyl ester inhibited influenza virus A/H1N1/pdm09 (SI > 30).

Keywords: glycyrrhizic acid, amino acids, conjugates, antiviral activity

Glycyrrhizic acid (GA) (**I**) is the major triterpene glycoside in roots of common licorice *Glycyrrhiza glabra* L. and Ural licorice *Gl. uralensis* Fisher and a promising natural compound in the synthesis of new antiviral agents [1, 2]. GA is used to treat chronic hepatitis B and C infections in China and Japan (GMP F0115, Beijing Qinwutian Pharmaceutical Technology Co., Ltd). GA and its derivatives possess antiviral activity against the herpesviruses (the herpes simplex and varicella-zoster viruses) [3], Epstein-Barr virus [4], SARS-associated coronaviruses causing atypical pneumonia [5], human immunodeficiency virus [6], and influenza virus A/H1N1 [7].

GA is a natural stimulator of gamma-interferon; it inhibits viruses at early stages of the replicative cycle thus inducing no resistance. Highly purified GA is a lightly toxic compound (LD_{50} 5000 mg/kg); it is characterized by high bioavailability and is metabolized in the gastrointestinal tract and liver similar to corticosteroid hormones [1]. Chemical modification of GA and functional design of the derivatives are a promising approach to the preparation of new antiviral agents and immunomodulators [8].

RESULTS AND DISCUSSION

As part of our ongoing work on the synthesis of GA derivatives with antiviral properties of potential interest for medicine, we were the first to synthesize GA

conjugates with methyl and ethyl esters of D-amino acids (Scheme 1).

GA (**I**) carboxyl groups were activated with hydroxybenzotriazol (HOBt) or N-hydrosuccinimide (HONSu) and N,N'-dicyclohexylcarbodiimide (DCC) [9, 10]. Treatment of GA (**I**) with HOBt/DCC at GA–HOBt–DCC molar ratio 1 : 3.5 : 3.2 or HONSu/DCC at GA–HOSu–DCC molar ratio 1 : 5.2 : 3.2 in tetrahydrofuran/dioxane at 20–22°C resulted in activated tris(oxybenzotriazol) (**II**) and tris(oxysuccinimide) (**III**) esters and N,N'-dicyclohexylurea precipitate. Activated esters (**II**) and (**III**), after separation of the N,N'-dicyclohexylurea precipitate, were introduced into the reaction with D-amino acid ester hydrochlorides dissolved in the mixture of tetrahydrofuran–dioxane–DMF in the presence of N-ethylmorpholine (NEM) excess at room temperature (20–22°C) during 24 h. Individual (as evidenced by TLC) carboxyprotected conjugates (**IV–X**) were isolated by column chromatography on silica gel (SG) with 55–60% yields in case of HOBt and 45–50% in case of HONSu. Compound (**X**) was synthesized upon GA activation with HONSu/DCC at 0–5°C at GA–HONSu–DCC molar ratio 1 : 4.3 : 5.2.

Structures of the conjugates were confirmed by spectral techniques (IR, ^{1}H, and ^{13}C NMR). For example, in ^{13}C NMR spectra signals of C=O group carbons in amino acid esters were revealed by the additional signals in the weak field (174.9–170.0 ppm), and signals of OCH_3 groups, in the range 52.0–50.9 ppm. In ^{1}H NMR spectra of conjugates (**V–X**) signals of

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Table 1. Antiviral activity of GA conjugates against various influenza virus subtypes

Virus	(IV)			(V)		
	CTD_{50}	EC_{50}, µg/mL	SI	CTD_{50}	EC_{50}, µg/mL	SI
A/Pert/16/09 (H3N2)	620	74.4	8.3	>500	33	>15
A/California/07/09/(H1N1)pdm09	620	20.7	30.0	>500	44.5	>11
A/N1BRG/14/H5N1	–	–	–	>500	17.5	>29
B/Brisben/60/08	–	–	–	>500	50	>10

Table 2. Antiviral activity of GA conjugate (V) against the HRSV reference

The amount of conjugate (V) added to the virus, µg	Virus titer ($-\log TCID_{50}$)	Difference between the virus titers in control and experimental samples
0 (virus control)	5.0log	–
100	2.5log	2.5log
50	2.5log	2.5log
25	3.5log	1.5log

methoxycarbonyl groups were observed in the range 3.30–3.72 ppm. Aromatic amino acid residues (Trp, Phe, Tyr) produced a set of carbon atom signals in the range 138–111 ppm. In ^{13}C NMR spectrum of conjugate (X) carbon atom signal of free COOH group had a chemical shift value of 180.4 ppm, as in the GA spectrum [11].

Antiviral activity of a number of synthesized GA conjugates was assessed in the culture of MDCK cells using various influenza viruses type A and B: influenza virus A/Pert/16//09(H3N2), pandemic influenza virus A/California/07/09(H1N1)pdm09, avian influenza virus A/NIBRG/14/H5N1, and influenza virus B/Brisben/60/08 (see Table 1).

For compounds under study, the toxic dose considerably exceeded 200 µg/mL, thus the median toxic dose, or dose causing 50% cell death (CTD_{50}), was evaluated as >200 µg/mL. Quantitative evaluation of antiviral activity was performed according to the selectivity index (SI), or therapeutic index, which is the product of CTD_{50} divided by effective concentration (EC_{50}) (a dose causing 50% decrease in the virus infectious titer): $SI = CTD_{50}/EC_{50}$.

In a screening study using the A/Pert/16/09(H3N2) strain, GA conjugates with methyl and ethyl esters of D-tryptophan (IV) and (V) were the most active: the decrease of infectious activity (virus titer expressed as a decimal logarithm of virus dose causing 50% cell death in a monolayer, $\log TCID_{50}$) under concentration safe for the cells in vitro (50 and 100 µg/mL) was 3.75log for compound (IV) and 4.3log for compound (V), which are very good values since compounds decreasing the virus infectious titer in vitro by −2log and beyond are considered promising candidates. Compounds (VI–X) did not exhibit significant antiviral activity. Antiviral activity of GA conjugates (IV) and (V) was studied against other influenza virus subtypes (Table 1). Conjugate (V) was shown to express considerable antiviral activity toward the pandemic influenza virus A/H1N1 (California, 2009), avian influenza virus A/H5N1(NIBRG), and influenza virus of group B (Brisben, 2008). The highest selectivity index (SI > 29) demonstrated by the compound was against the avian influenza virus A/H5N1.

Virus-inhibiting activity of compounds (IV) and (V) was studied in the MA-104 cell culture with respect to the standard human respiratory syncytial virus (HRSV). The results for compound (V) are presented in Table 2. Conjugate (V) was found to be active against the HRSV virus as well, efficiently inhibiting replication of the virus (EC_{50} 18.8 µg/mL, SI > 25). Compound (IV), even at a concentration of 6.3 µg/mL, caused degradation of cell monolayer in the given model.

Therefore, antiviral activity of this group of GA conjugates with respect to studied influenza virus and HRSV subtypes depended on the structure of amino acid residue and the ester group. Even for structurally related compounds, such as conjugates (IV) and (V) differing only by a single methylene group (methyl vs. ethyl ester), activity differed considerably.

EXPERIMENTAL

^{1}H and ^{13}C NMR spectra were registered on an Avance-III (Brüker, Germany) pulse spectrometer at a working frequency of 500.13 (^{1}H) and 125.47 (^{13}C) MHz and an AVX-300 (Brüker) spectrometer at working frequencies of 300 and 75.5 MHz (δ, ppm; spin–spin coupling constant, Hz); internal standard, tetramethylsilane. NMR spectral signals were referenced under common mode using the ACD LABS

software package and literature data for GA and its amino acid conjugates [6, 9–11].

IR spectra were recorded on a Prestige-21 (Shimadzu, Japan) IR spectrophotometer in a paste with paraffin oil. Optical activity was measured on a Perkin-Elmer 341 (Germany) polarimeter in a 1-dm tube at 20–22°C (λ_{Na} 546 nm). Melting points were determined on a Boetius apparatus. Elemental analysis data matched the calculated values.

TLC was performed on Sorbfil (Sorbopolimer, Russia) plates. Substances were detected with 5% H_2SO_4 in ethanol with further heating at 110–120°C for 2–3 min. Column chromatography was performed on the KSK (fraction 50–150, dry classification) silica gel by Sorbopolimer.

Solvents were purified according to techniques described in [12]. Solvents were vacuum dried at 50–60°C.

GA isolated from roots of *Glycyrrhiza uralensis* Fisher of Siberian populations according to [13] was used in the work. HOBt, HONSu, DCC, and D-amino acids were from Sigma-Aldrich.

General Method to Obtain D-Amino Acid Methyl Ester Hydrochlorides

To a suspension of 5 g of D-amino acid in 100 mL dry methanol at 0–5°C, 8–10 mL thionyl chloride were added dropwise, the mixture was stirred for 2 h and kept at 20–22°C for 24 h. The solvent was evaporated under vacuum at 30–40°C, the residue was mixed with dry ether, filtered off, dried, and recrystallized from methanol–ether mixture.

D-Valine methyl ester hydrochloride. Yield 75%, mp 155–156°C. $[\alpha]_{D^{20}}$ −15 ± 1° (*c* 1.0, EtOH). Literature data [14]: $[\alpha]_{D^{20}}$ −15.5 ± 2° (*c* 2.0, H_2O). IR (ν, cm^{-1}): 1730, 1575.

D-Leucine methyl ester hydrochloride. Yield 85%, mp 148–150°C. $[\alpha]_{D^{20}}$ −14 ± 1° (*c* 1.0, H_2O). Literature data [14]: $[\alpha]_{D^{20}}$ −(12–16)° (*c* 2.0, H_2O). IR (ν, cm^{-1}): 1745, 1584.

D-Phenylalanine methyl ester hydrochloride. Yield 84%, mp 158–160°C. $[\alpha]_{D^{20}}$ − 35 ± 1° (*c* 1.0, EtOH). Literature data [14]: mp 160°C, $[\alpha]_{D^{20}}$ − 37° (*c* 2.0, EtOH). IR (ν, cm^{-1}): 1747, 1585.

D-Tryptophan methyl ester hydrochloride. Yield 78%, mp 216–218°C. $[\alpha]_{D^{20}}$ +16 ± 1° (*c* 1.0, MeOH). Literature data [14]: mp 218–220°C, $[\alpha]_{D^{20}}$ +18° (*c* 5.0, EtOH). IR (ν, cm^{-1}): 1748, 1578, 1502, 1458.

D-Tryptophan ethyl ester hydrochloride. To a suspension of 5 g (43.4 mmol) D-tryptophan in 100 mL absolute ethanol, 8 mL of thionyl chloride were added dropwise under ice-bath cooling and the mixture was stirred for 2 h till the precipitate dissolved. The mix-

ture was kept at 20–22°C for 24 h with intermittent mixing, the solvent and excess thionyl chloride were dried, and the residue was treated with dry ether and dried again. Yield 80%. $[\alpha]_{D^{20}}$ + 9 ± 1° (*c* 1.0, H_2O). Literature data [14]: $[\alpha]_{D^{20}}$ +10 ± 1° (*c* 2.0, H_2O). IR (ν, cm^{-1}): 3283, 1746, 1575, 1513, 1504.

General Procedure to Obtain GA Conjugates Using N-Hydroxybenzotriazol–N,N′-Dicyclohexylcarbodiimide (Method A)

To a solution of 0.82 g (1 mmol) GA in 20 mL dioxane, 0.47 g (3.5 mmol) *N*-hydroxybenzotriazol and 0.70 g (3.2 mmol) DCC were added at 0–5°C; the mixture was stirred at the same temperature for 1 h and at 22–24°C, for 5 h. The dicyclohexylurea precipitate was filtered off; 4.0 mmol amino acid ester hydrochloride, 5 mL DMF, and 0.7 mL (6 mmol) *N*-ethylmorpholine were added to the filtrate. The mixture was kept under intermittent stirring for 24 h at 20–22°C, diluted with cold water, and acidified with citric acid to pH of 3–4; the precipitate was filtered off, washed with water, and dried. The product was isolated on a silica gel column with $CHCl_3$–MeOH–H_2O mixtures 300 : 10 : 1, 200 : 10 : 1, 100 : 10 : 1, and 50 : 10 : 1 (v/v, step gradient) under TLC control. Individual (according to TLC) fractions were joined and vacuum-dried.

General Procedure to Obtain GA Conjugates Using N-Hydroxysuccinimide–N,N′-Dicyclohexylcarbodiimide (Method B)

To a solution of 0.82 g (1 mmol) GA in 20 mL tetrahydrofuran, 0.60 g (5.2 mmol) *N*-HONSu and 0.70 g (3.2 mmol) DCC were added at 0–5°C; the mixture was stirred at the same temperature for 1 h and at 20–22°C, for 6 h. The *N,N*′-dicyclohexylurea precipitate was filtered off; 4.0 mmol amino acid ester hydrochloride, 5 mL DMF, and 0.7 mL (6 mmol) *N*-ethylmorpholine were added to the filtrate. The mixture was kept under intermittent stirring for 24 h at 20–22°C, diluted with cold water, and acidified with citric acid to pH of 3–4; the precipitate was filtered off, washed with water, and dried. The product was isolated on a silica gel column with $CHCl_3$–MeOH–H_2O mixtures 300 : 10 : 1, 200 : 10 : 1, 100 : 10 : 1, and 50 : 10 : 1 (v/v, step gradient) under TLC control. Individual (according to TLC) fractions were joined and vacuum-dried.

3-*O*-{2-*O*-[*N*-(β-D-glucopyranosyluronoyl)-D-tryptophan methyl ester]-*N*-(β-D-glucopyranosyluronoyl)-D-tryptophan methyl ester}-(3β,20β)-11-oxo-30-(*N*-carbonyl-D- tryptophan methyl ester)-30-norolean-12-ene (IV). Yield 0.85 g (60%) (method A) (amorphous powder). R_f 0.44 (benzene–ethanol, 5 : 1). $[\alpha]_{D^{20}}$ +45 ± 1° (*c* 0.06, EtOH). Found, %: 65.52; H 7.10; N 5.75. $C_{78}H_{98}N_6O_{19}$. Calcd., %: C 65.81; H 6.94; N 5.90. (M_r 1423.36). ^{1}H NMR (300 MHz, ace-

tone-d_6): 0.84, 0.93, 1.08, 1.13, 1.26, 1.37, 1.47 (21 H, all s, 7 CH$_3$), 1.50−3.50 (m, CH, CH$_2$), 3.60, 3.63, 3.72 (9H, all s, 3 OCH$_3$), 3.90−5.10 (m, H1'-H6', H1"-H6"), 5.63 (1H, s, H12), 7.00−7.65 (m, H ar.), 10.1, 10.5 (3H, br. s, 3NH). ^{13}C NMR (75.5 MHz, acetone-d_6): 199.0 (C11), 174.0 (C30), 170.6 (C6"), 170.5 (C6'), 169.5 (C13), 128.1 (C12), 103.7 (C1"), 102.4 (C1'), 89.5 (C3), 77.9 (C2'), 76.4 (C5"), 76.3 (C5'), 74.4 (C3',3"), 73.8 (C2"), 72.6 (C4"), 72.4 (C4'), 61.4 (C9), 54.8 (C5), 47.8 (C18), 45.1 (C8), 43.4 (C20), 43.0 (C14), 41.0 (C19), 39.3 (C4), 38.5 (C1), 37.2 (C22), 36.6 (C10), 32.4 (C7), 31.6 (C17), 30.8 (C21), 27.7 (C29), 27.3 (C28), 27.0 (C23), 26.3 (C16), 26.1 (C15), 25.4 (C2), 22.8 (C27), 18.2 (C26),17.1 (C6), 16.3 (C25), 15.8 (C24); D-TrpOMe: 172.0, 171.8, 171.4, 136.6, 136.6, 136.4, 128.1, 128.0, 124.0, 123.8, 121.3, 118.8, 118.6, 118.1, 117.9, 111.6, 111.4, 53.0, 52.5, 51.6, 51.5, 50.9.

3-O-{2-O-[N-(β-D-glucopyranosyluronoyl)-D-tryptophan ethyl ester]-N-(β-D-glucopyranosyluronoyl)-D-tryptophan ethyl ester}-(3β,20β)-11-oxo-30-(N-carbonyl-D-tryptophan ethyl ester)-30-norolean-12-ene (V). Yield 0.86 g (58%) (method A); 0.70 g (48%) (method B) (yellowish amorphous powder). R_f 0.58 (chloroform−ethanol, 5 : 1). [α]$_D^{20}$ +40 ± 1° (c 0.06, EtOH). Found, %: C 66.20; H 7.32; N 5.58. C$_{81}$H$_{104}$N$_6$O$_{19}$. Calcd., %: C 66.37; H 7.15; N 5.73 (M_r 1465.7). IR (ν, cm^{-1}): 3400−3200 (OH, NH), 1740 (COOEt), 1655 (C^{11}=O), 1651 (Ph), 1529 (CONH). ^{1}H NMR (300 MHz, CD$_3$OD): 0.84, 1.03, 1.07, 1.10, 1.27, 1.36, 1.42 (21H, all s, 7 CH$_3$), 1.50−3.20 (m, CH, CH$_2$), 3.31, 3.63, 3.67, 3.71 (15H, all s, 3 CH$_2$CH$_3$), 3.80−4.70 (m, H1'-H6', H1"- H6"), 5.64 (1H, s, H12), 7.05, 7.07, 7.12, 7.29, 7.31, 7.36, 7.39, 7.47, 7.50, 7.53, 7.55, 7.88 (15H, m, H ar.). ^{13}C NMR (75.5 MHz, CD$_3$OD): 202.6 (C11), 175.9 (C30), 171.9 (C13), 171.8 (C6"), 171.7 (C6'), 129.2 (C12), 105.2 (C1"), 103.9 (C1'), 91.1 (C3), 83.0 (C2), 79.5 (C5"), 79.3 (C5'), 77.8 (C3'), 77.6 (C3"), 75.7 (C2"), 74.9 (C4"), 73.5 (C4'), 63.0 (C9), 56.3 (C5), 46.8 (C8), 44.9 (C20), 44.6 (C14), 42.5 (C19), 40.7 (C4), 39.9 (C1), 38.6 (C22), 38.0 (C10), 33.8 (C7), 33.0 (C17), 32.2 (C21), 29.7 (C29), 28.9 (C28), 28.5 (C23), 27.6 (C16), 27.5 (C15), 26.6 (C2), 23.9 (C27), 19.4 (C26), 18.4 (C6), 17.5 (C25), 17.0 (C24); D-TrpOEt: 174.4, 173.4, 172.5, 138.3, 138.0, 128.9, 128.3, 126.0, 124.8, 122.6, 122.5, 120.0, 119.3, 119.0, 112.8, 112.6, 110.0, 109.6, 54.8, 54.1, 53.0, 52,4, 52.2, 36.5, 35.8.

3-O-{2-O-[N-(β-D-glucopyranosyluronoyl)-D-phenylalanine methyl ester]-N-(β-D-glucopyranosyluronoyl)-D-phenylalanine methyl ester}-(3β,20β)-11-oxo-30-(N-carbonyl-D-phenylalanine methyl ester)-30-norolean-12-ene (VI). Yield 0.72 g (55%) (method A). R_f 0.45 (benzene−ethanol, 5 : 1). [α]$_D^{20}$ +25 ± 1° (c 0.06, EtOH). Found, %: C 65.86; H 7.22; N 3.14. C$_{72}$H$_{95}$N$_3$O$_{19}$. Calcd., %: C 66.19; H 7.33; N 3.22. (M_r

1306.49). IR (ν, cm^{-1}): 3600−3200 (OH, NH), 1744 (COOMe), 1651 (C^{11}=O), 1520 (CONH), 1498. ^{1}H NMR (300 MHz, DMSO-d_6): 0.61, 0.71, 0.84, 0.90, 0.93, 1.22, 1.30 (21H, all s, 7 CH$_3$), 1.50−3.20 (m, CH, CH$_2$), 3.38, 3.58, 3.61 (9H, all s, 3 OCH3), 3.90−5.50 (m, H1'-H6', H1"-H6"), 5.70 (1H, s, H12), 7.20−8.30 (m, H ar.). ^{13}C NMR (75.5 MHz, DMSO-d_6): 198.9 (C11), 175.3 (C30), 169.2 (C13), 168.4 (C6"); 168.1 (C6'), 127.4 (C12), 103.4 (C1"), 103.3 (C1'), 88.3 (C3), 80.2 (C2'), 76.0 (C5"), 75.9 (C5'), 74.9 (C3"), 74.4 (C3', C2"), 71.4 (C4"), 71.2 (C4'), 61.0 (C9), 54.3 (C5), 47.4 (C18), 47.0 (C8), 44.6 (C14), C20), 42.7 (C19), 37.0 (C4), 36.4 (C1), 36.2 (C22), 35.7 (C10), 33.2 (C7), 32.0 (C17), 31.1 (C21), 28.2 (C29), 28.1 C28), 27.1 (C23), 25.8 (C16), 25.5 (C15), 25.2 (C2), 22.8 (C27), 18.2 (C26), 16.8 (C6), 15.9 (C24, C25); D-PheOMe: 172.1, 171.3, 171.1, 137.8, 136.7, 136.6, 129.0, 128.9, 128.8, 128.3, 128.1, 128.0, 126.4, 126.1, 53.3, 53.1, 53.0, 51.7, 51.6.

3-O-{2-O-[N-(β-D-glucopyranosyluronoyl)-D-thyrosine methyl ester]-N-(β-D-glucopyranosyluronoyl)-D-thyrosine methyl ester}-(3β,20β)-11-oxo-30-(N-carbonyl-D-thyrosine methyl ester)-30-norolean-12-ene (VII). Yield 0.61 g (45%) (method B). R_f 0.60 (chloroform−methanol−water, 45 : 10 : 1). [α]$_D^{20}$ +45 ± 1° (c 0.04, EtOH). Found, %: C 63.57; H 7.23; N 2.95. C$_{72}$H$_{95}$N$_2$O$_{22}$. Calcd., %: C 63.84; H 7.07; H N 3.10. (M_r 1354.49). IR (ν, cm^{-1}): 3600−3200 (OH, NH), 1736 (COOMe), 1652 (C^{11}=O), 1520 (CONH), 1500 (Ph). ^{1}H NMR (300 MHz, CD$_3$OD.): 0.86, 1.04, 1.14, 1.22, 1.31, 1.42, 1.45 (21H, s, 7CH$_3$), 1.40−3.20 (m, CH, CH$_2$), 3.30, 3.68, 3.74 (9H, all s, 3OCH$_3$), 3.80−4.90 (m, H1'-H6', H1"-H6"), 5.72 (1H, s, H12), 6.40−7.35 (m, H ar.). ^{13}C NMR (75.5 MHz, CD$_3$OD): 201.2 (C11), 174.0 (C30), 171.8 (C13), 171.4 (C6', C6"), 128.2 (C12), 105.2 (C1"), 104.2 (C1'), 90.9 (C3), 80.1 (C2'), 77.6 (C5"), 77.1 (C5'), 75.7, 75.3 (C2", C3', C3"), 73.5 (C4'), 73.1 (C4"), 63.1 (C9), 56.4 (C5), 47.5 (C18), 46.8 (C8), 44.9 (C20), 43.7 (C14), 42.4 (C19), 40.8 (C4), 40.3 (C1), 38.6 (C22), 38.1 (C10), 33.76 (C7), 33.0 (C17), 32.2 (C21), 29.2 (C29), 28.9 (C28), 28.2 (C23), 27.6 (C16), 27.4 (C15), 26.6 (C2), 23.9 (C27), 19.4 (C26), 18.5 (C6), 17.1 (C24), 17.4 (C25); D-TyrOMe: 173.0, 172.0, 171.8, 131.5, 131.3, 129.2, 129.0, 116.6, 116.5, 68.1, 67.7, 67.6, 55.3, 54.9, 52.9, 52.8.

3-O-{2-O-[N-(β-D-glucopyranosyluronoyl)-D-valine methyl ester]-N-(β-D-glucopyranosyluronoyl)-D-valine methyl ester}-(3β,20β)-11-oxo-30-(N-carbonyl-D-valine methyl ester)-30-norolean-12-ene (VIII). Yield 0.55 g (48%) (method B). R_f 0.47 (benzene−ethanol, 5 : 1). [α]$_D^{20}$ +40 ± 1° (c 0.04, EtOH). Found, %: C 60.75; H 7.70; N 3.50. C$_{60}$H$_{93}$N$_3$O$_{19}$. Calcd., %: C 60.96; H 7.46; N 3.61 (M_r 1162.43). IR (ν, cm^{-1}): 3600−3200 (OH, NH), 1739 (COOMe), 1662 (C^{11}=O), 1528 (CONH). ^{1}H NMR (300 MHz,

DMSO-d_6): 0.65, 0.75, 0.84, 0.86, 0.91, 0.96, 1.01, 1.08, 1.23, 1.33 (39H, all s, 7 CH$_3$, 6 CH$_3$ Val), 1.40–3.20 (m, CH, CH$_2$), 3.37, 3.62 (9H, s, OCH$_3$), 3.70–5.40 (m, H1'-H6', H1"-H6"), 5.64 (1H, s, H12). ^{13}C NMR (75.5 MHz, DMSO-d_6): 199.1 (C11), 177.7 (C30), 169.8 (C13), 168.4 (C6"), 168.1 (C6'), 127.3 (C12), 104.5 (C1"), 103.4 (C1'), 88.2 (C3), 82.0 (C2'), 76.3 (C5"), 76.1 (C5'), 75.6 (C3"), 74.9 (C3'), 74.5 (C2'), 71.4 (C4"), 71.0 (C4'), 61.2 (C9), 54.4 (C5), 48.1 (C18), 44.9 (C8), 43.5 (C20), 43.1 (C14), 42.9 (C19), 37.5 (C22), 36.4 (C10), 32.2 (C7), 31.6 (C17,C21), 28.4 (C29), 27.9 (C28), 27.3 (C23), 26.1 (C16), 25.9 (C2, C16), 22.9 (C27), 19.4 (C26), 18.0 (C6), 16.1 (C25), 15.9 (C24); D-ValOMe: 171.8, 170.0, 57.1, 51.8, 51.6, 30.6, 30.1, 18.9, 18.4, 16.9.

3-O-{2-O-[N-(β-D-glucopyranosyluronoyl)-D-leucine methyl ester]-N-(β-D-glucopyranosyluronoyl)-D-leucine methyl ester}-(3β,20β)-11-oxo-30-(N-carbonyl-D-leucine methyl ester)-30-norolean-12-ene (IX). Yield 0.60 g (50%) (method B). R_f 0.42 (benzene–ethanol, 5 : 1). [α]$_{D^{20}}$ +55 ± 1° (c 0.04, EtOH). Found, %: C 62.46; H 8.62; H N 3.35. C$_{63}$H$_{101}$N$_3$O$_{19}$. Calcd., %: C 62.62; H 8.45; N 3.49 (M_r 1204.45). IR (ν, cm^{-1}): 3600–3200 (OH, NH), 1741 (COOMe), 1662 (C^{11}=O), 1532 (CONH). ^{1}H NMR (500 MHz, CD$_3$OD): 0.84, 0.89, 0.91, 0.94, 0.96, 1.06, 1.13, 1.25, 1.41 (39H, all s, 7CH$_3$, 6CH$_3$ Leu), 1.50–3.25 (m, CH, CH$_2$), 3.30 (3H, s, OCH$_3$), 3.40–3.56 (m, CH, CH$_2$), 3.40–3.64 (m, CH, CH$_2$), 3.68, 3.70 (6H, both s, 2OCH$_3$), 3.74–4.76 (m, H1'-H6', H1"-H6"), 5.60 (1H, S, H12). ^{13}C NMR (125 MHz, CD$_3$OD): 202.47 (C11), 176.45 (C30), 172.67 (C6"), 172.01 (C6'), 171.39 (C13), 129.04 (C12), 105.27 (C1"), 105.04 (C1'), 90.50 (C3), 82.05 (C2'), 77.65 (C5"), 77.26 (C5'), 76.19 (C3'), 76.58 (C3"), 76.03 (C2"), 73.32 (C4"), 73.17 (C4'), 63.07 (C9), 56.39 (C5), 49.85 (C18), 46.70 (C8), 44.98 (C20), 44.53 (C14), 42.48 (C19), 40.63 (C4), 40.27 (C1), 38.68 (C22), 38.03 (C10), 33.73 (C7), 32.94 (C17), 32.11 (C21), 28.94 (C29), 28.56 (C28), 28.43 (C23), 27.58 (C16), 27.38 (C15), 25.91 (C2), 23.84 (C27), 19.29 (C26), 18.49 (C6), 17.05 (C24), 17.25 (C25); D-LeuOMe: 174.87, 174.29, 174.04, 52.87, 52.81, 51.97, 51.85, 51.79, 41.90, 41.44, 30.49, 23.41, 23.33, 22.22, 22.12, 21.82.

3-O-{2-O-[N-(β-D-glucopyranosyluronoyl)-D-valine methyl ester]-N-(β-D-glucopyranosyluronoyl)-D-valine methyl ester}-(3β,20β)-11-oxo-30-norolean-12-en-30-ic acid (X). To a solution of 0.82 g (1 mmol) GA in 20 mL tetrahydrofuran at 0–5°C, 0.50 g (4.3 mmol) HONSu and 0.54 g (2.5 mmol) DCC were added and the mixture was stirred at the same temperature for 1 h and left overnight in a refrigerator. On the next day, the N,N'-dicyclohexylurea sediment was filtered off, 0.42 g (2.5 mmol) amino acid ester hydrochloride and 0.6 mL (5.0 mmol) N-ethylmorpholine were added at 0–5°C. The mixture was stirred at the same temperature for 3 h and kept under intermittent

stirring at 20–22°C for 20 h; then cold water was added and the solution was acidified with citric acid to pH 3–4. The precipitate was filtered off, washed with water, dried, and separated on a silica gel column with the CHCl$_3$–MeOH–H$_2$O mixtures 300 : 10 : 1, 200 : 10 : 1, 100 : 10 : 1, 50 : 10 : 1 (v/v, step gradient) under TLC control. Yield 0.48 g (46%). R_f 0.40 (benzene–ethanol, 5 : 1). [α]$_{D^{20}}$ +65 ± 1° (c 0.06, EtOH). Found, %: C 61.60; H 7.85; N 2.52. C$_{54}$H$_{84}$N$_2$O$_{18}$. Calcd., %: C 61.81; H 8.07; N 2.67. (M_r 1049.22). IR (ν, cm^{-1}): 3600–3200 (OH, NH), 1739 (COOMe), 1659 (C=O), 1532 (CONH). ^{1}H NMR (300 MHz, CD$_3$OD): 0.82, 0.83, 0.95, 0.96, 1.04, 1.14, 1.17, 1.42 (33H, all s, 7CH$_3$, 4CH$_3$ Val), 1.50–2.70 (m, CH, CH$_2$), 3.31, 3.74 (6H, both s, 2 OCH$_3$), 3.74–4.68 (m, H1'-H6', H1"-H6"), 5.57 (1H, s, H12). ^{13}C NMR (75.5 MHz, CD$_3$OD): 202.6 (C11), 180.4 (C30), 171.5 (C13), 171.0 (C6', C6"), 129.0 (C12), 105.5 (C1"), 105.0 (C1'), 90.8 (C3), 82.4 (C2'), 77.8 (C5"), 77.4 (C5'), 76.2 (C2"), 75.7 (C3'), 75.7 (C3"), 73.1 (C4', C4"), 63.2 (C9), 56.5 (C5), 46.8 (C8), 40.8 (C4), 40.2 (C1), 39.1 (C22), 38.1 (C10), 44.9 (C20), 44.6 (C14), 42.5 (C14), 33.8 (C7), 33.0 (C17), 32.1 (C21), 29.2 (C29), 28.8 C28), 28.3 (C23), 27.6 (C16), 27.4 (C15), 26.6 (C2), 23.9 (C27), 19.4 (C26), 18.2 (C6), 17.1 (C24, C25); D-ValOMe: 173.2, 172.9, 58.9, 58.6, 52.8, 52.6, 18.8, 18.5.

Study of Toxicity and Antiviral Activity

Viruses and cells. Studies were conducted on a primary culture of dog kidney cells MDCK for all influenza viruses and MA-104 cells, for human respiratory syncytial virus (HRSV). For screening of antiviral activity of the conjugates, reference virus A/Pert/16/09(H3N2) was used. Antiviral effect of the most active compounds was studied on international standard influenza virus strains of various subtypes: A/California/07/09(H1N1)pdm09, avian influenza virus A/NIBRG/14/H5N1, influenza virus B/Brisben/60/08, as well as the HRSV standard.

To study **toxicity**, as well as antiviral activity, the conjugates were dissolved in DMSO at a concentration of 20 mg/mL thus forming a standard solution. Immediately before the experiment, the standard solution was diluted with cell incubation medium 100-fold, so that the final DMSO concentration did not exceed 1% (maximum concentration affording unaffected cell viability and metabolism). Therefore, the maximum working concentration of the compounds was 200 μg/mL. Toxicity of GA conjugates (**IV**) and (**V**) was studied at higher concentrations (>500 μg/mL). Further on, a series of six two-fold dilutions was prepared (to produce solutions of 100, 50, 25, 12.5, 6.25, and 3.13 μg/mL, respectively) with MDCK cell growth medium (α-MEM, Biolot, Russia), which were used to evaluate the conjugate toxicity. The compounds were introduced into a one-day MDCK culture grown in 96-well plates, then the plates were incubated for
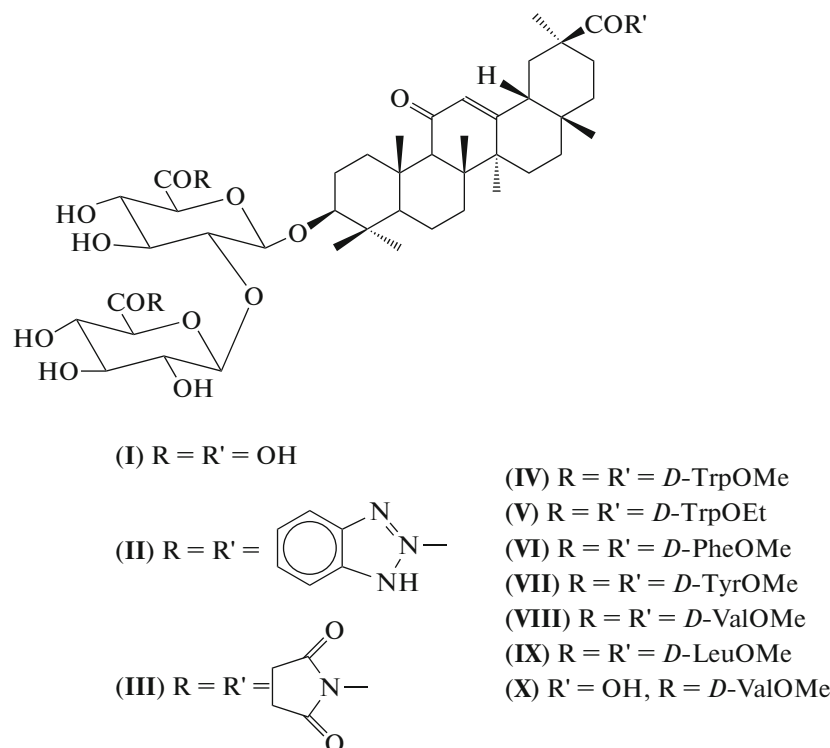
72 h at 37°C in the presence of 5% CO_2. After visual evaluation of cytotoxicity by the level of monolayer integrity, toxicity was determined quantitatively by measuring the residual ability of cell in culture to reduce a tetrazole dye MTT (thiazolyl blue) by mitochondrial and partially cytoplasmic dehydrogenases of cells. The intensity of the reducing dye color restoration correlates with cell viability rate. The test is often used in virology for evaluation of cytopathogenic effect of viruses on cells [15]. Its results can be interpreted as the level of cell stability towards the effect of viruses. The microtetrazolium test is also widely used to evaluate the effects of the toxicants, pharmacological preparations, and unfavorable environmental factors on cells [16]. The MTT reduction was assayed on a Varioscan (ThermoFischer) microplate reader at 550 nm. Median toxic concentration (CTD_{50}) was determined by photometry analysis of the results by using linear regression in Excel 2010 software.

Evaluation of the Conjugate Antiviral Activity In Vitro

Antiviral activity of each compound was screened at a single maximum concentration (in two repeats), at which 100% monolayer cells survived. A one-day MDCK culture grown in a 96-well plate was inspected visually in an inverted microscope to verify the monolayer integrity. Further, 10-fold virus dilutions in supporting growth medium supplemented with trypsin

were performed: dilution logarithm ($\log c$) was in the range from −1 to −7. Plates with cell monolayers were washed with serum-free medium twice and then the agents were introduced into relevant wells of the plate in the volume of 50 µL. Control wells were filled with equal volume of growth medium. The plates were incubated for 60 min at 37°C in the presence of CO_2. Then virus was introduced into cells at relevant dilution. Each concentration of a conjugate was analyzed thrice for each of the virus dilutions. Control wells were filled with the same volume of growth medium. Some wells were used to verify the absence of toxicity by evaluation of the monolayer cell integrity compared to cell control and the rate of cytopathic effect of the virus on cells.

Virus reproduction was evaluated in the blood agglutination reaction (BAR) and expressed as titer of hemagglutination ($-\log TCID_{50}/50$ µL) compared to control (intact virus 10-times more active compared to the control (intact virus at decimal dilutions). Cytopathic effect of the virus was evaluated quantitatively using the above-mentioned technique (MTT) and expressed as $\Delta \log TCID_{50}/50$ µL compared to control virus. Table 1 provides the mean value obtained in two tests. A compound or conjugate typically is considered a promising antiviral agent candidate if in vitro $\Delta \log TCID_{50} \geq 2.0$ [17].

(I) R = R' = OH

(II) R = R' =

(III) R = R' =

(IV) R = R' = *D*-TrpOMe
(V) R = R' = *D*-TrpOEt
(VI) R = R' = *D*-PheOMe
(VII) R = R' = *D*-TyrOMe
(VIII) R = R' = *D*-ValOMe
(IX) R = R' = *D*-LeuOMe
(X) R' = OH, R = *D*-ValOMe

Scheme 1.

The decrease in the infectious titer of a virus evidenced antiviral activity of the compounds. Virus titer is the decimal logarithm of the virus suspension dilutions causing 50% monolayer cell ($-\log TCID_{50}$) death. Using the data obtained we calculated the 50-% efficiency concentration (EC_{50}) which caused decrease in the virus titer twice (by $0.3 \log TCID_{50}$) and the selectivity index (SI) was calculated as the CTD_{50}/EC_{50} ratio.

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