

SYNTHESIS OF GLYCYRRHIZIC ACID CONJUGATES WITH S-BENZYL-L-CYSTEINE AND THEIR ANTIVIRAL ACTIVITY

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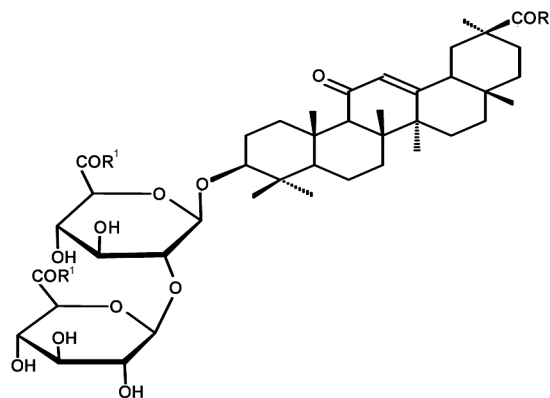
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A new method for the synthesis of glycyrrhizic acid (GA) conjugates with S-benzyl-L-cysteine using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide is proposed. It is established that 3-O-{2-O-[N-(β-D-glucopyranosyluronyl)-L-cysteine-S-benzyl]-N-(β-D-glucopyranosyluronyl)-L-cysteine-S-benzyl}-(3β,20β)-11-oxo-30-(N-carbonyl-L-cysteine-S-benzyl)-30-norolean-12-ene is superior to GA in inhibiting the accumulation of HIV-1 virus-specific protein p24 (viral antigen) in MT-4 cell culture (IC₅₀ 3 μg/mL, SI 90) and is 50 – 55 times less toxic to cells than azidothymidine.

Keywords: glycyrrhizic acid, S-benzyl-L-cysteine, conjugates, synthesis, anti-HIV activity.

The development of new drugs for treatment and prevention of viral infections of various etiologies is a key problem in chemistry and medicine because of the ubiquitous spread of HIV infections and viral hepatitis B and C and the emergence of new viral infections (A/H1N1, bird and swine flus, Ebola fever, chikungunya, Zika, West Nile, etc.) [1, 2]. Currently, the search for new antiviral agents is associated with the discovery of compounds with new structures and unique mechanisms of action and with chemical modification of already known antiviral compounds to produce derivatives with improved antiviral properties [3]. Use of natural lead compounds with established antiviral activity is a promising approach to the search for new antiviral agents [4, 5]. Glycyrrhizic acid (GA) (1) is such a compound. It is the main triterpene glycoside from licorice roots (*Glycyrrhiza glabra* L., *G. uralensis* Fisher) [6-8]. Japanese researchers discovered in 1987-1988 the ability of GA to inhibit HIV *in vitro* and *in vivo* [9, 10]. GA was successfully employed clinically to treat AIDS patients [11, 12]. The main drawbacks of GA are its low efficacy as an inhibitor of HIV reverse transcriptase and the high doses that are necessary to suppress HIV reproduction [13, 14].

Previously, several GA conjugates with amino acids and dipeptides were shown to possess potent antiviral activity against HIV-1 [15, 16], coronavirus SARS CoV [17], Ep-



- I: R = R' = OH;
 II: R = R' = C₆H₅CH₂SCH₂CHNH- (CysSBn-OBu^t);
 III: R = R' = C₆H₅CH₂SCH₂CHNH- (CysSBn);
 IV: R = OH, R' = C₆H₅CH₂SCH₂CHNH-;
 V: R = OH, R' = C₆H₅CH₂SCH₂CHNH-;

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stein-Barr virus [18], and A/H1N1/pdm2009 influenza virus [19]. Cysteine-containing GA conjugates turned out to be inhibitors of SARS-associated coronaviruses [17] and immunostimulants [20]. Methods for preparing GA conjugates with various *L*- and *D*-amino acid esters via activation of GA carboxylic acids using *N*-hydroxybenzotriazole (HOBt)-*N,N'*-dicyclohexylcarbodiimide (DCC) [21], *N*-hydroxyphthalimide–DCC [22], or *N*-hydroxysuccinimide (HOSu)–DCC [15, 16] were proposed. Use of DCC to activate GA was accompanied by release of *N,N'*-dicyclohexylurea, which formed side *N*-acylureas and contaminated the target products [23]. Water-soluble carbodiimides, in particular 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (DEC), which does not form dicyclohexylurea, are more attractive as condensing agents [24]. The goal of the present work was to develop an improved method using DEC to prepare GA conjugates with *S*-benzyl-*L*-cysteine that are interesting for medicine and as antiviral agents.

EXPERIMENTAL CHEMICAL PART

PMR and ^{13}C NMR spectra were recorded with TMS internal standard on a Bruker AM-300 spectrometer at operating frequency 300 and 75.5 MHz. IR spectra were taken from Vaseline-oil mulls on a Prestige-21 IR spectrophotometer (Shimadzu). Optical density was measured in a 1-dm tube at 20–22°C (λ_{Na} 546 nm) on a PerkinElmer 341 polarimeter.

TLC used Sorbfil plates (Sorbpolimer). Spots of compounds were detected by H_2SO_4 (5%) in EtOH followed by heating at 110–120°C for 2–3 min. Column chromatography used KSK silica gel (50–150 fraction, Sorbpolimer).

GA (96% pure) prepared as before [25]; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma-Aldrich, Germany); and *S*-benzyl-*L*-cysteine *tert*-butyl ester hydrochloride (Reanal, Hungary) were used in the work. Et_3N was stored for 1 d over KOH and distilled. Solvents were purified using standard methods [26].

3-O-{2-O-[*N*-(β-*D*-Glucopyranosyluronoyl)-*L*-cysteine-*S*-benzyl]-*N*-(β-*D*-glucopyranosyluronoyl)-*L*-cysteine-*S*-benzyl}-(3β,20β)-11-oxo-30-(*N*-carbonyl-*L*-cysteine-*S*-benzyl)-30-norolean-12-ene (III). A solution of GA (0.82 g, 1 mmol) in DMF (20 mL) was treated with HOBt (0.48 g, 3.5 mmol), *S*-benzyl-*L*-cysteine *tert*-butyl ester hydrochloride (1.2 g, 4.0 mmol), DEC (0.67 g, 3.5 mmol), and Et_3N (0.8 mL, 8 mmol); stirred at 20–22°C for 10–12 h, diluted with H_2O , and acidified to pH ~ 3–4 with citric acid. The precipitate was filtered off, rinsed with H_2O , dried, and reprecipitated from aqueous EtOH to afford carboxy-protected conjugate **II** (1.40 g). IR spectrum, ν , cm^{-1} : 3500–3200 (OH, NH), 1739 (COOBu^t), 1662 (C=O), 1621 (C_6H_5), 1520 (CONH), 1505 (C_6H_5). Conjugate **II** (0.68 g, 0.5 mmol) was worked up with CF_3COOH solution in CH_2Cl_2 (2 mL/20 mL) at 20–22°C, evaporated to dryness, and chromatographed over a column of silica gel with elu-

tion by CHCl_3 –MeOH– H_2O (200:10:1, 100:10:1, 50:10:1, 25:10:1; vol%). Yield 0.35 g (58%) (amorphous compd.); $[\alpha]_D^{20} +35^\circ$ (c 0.05, MeOH); lit. [20]: $[\alpha]_{20}^D +34^\circ$ (s 0.02; MeOH). IR spectrum, ν_{max} , cm^{-1} : 3600–3200 (OH, NH), 1710 (COOH), 1670 (C=O), 1550 (CONH), 1510 (Ph). ^{13}C NMR spectrum (DMF- d_7), δ , ppm: 199.1 (C11), 177.2 (C30), 171.3, 170.7 (C6'', C6'), 169.3 (C13), 127.7 (C12), 103.7 (C1''), 102.9 (C1'), 88.1 (C3), 80.1 (C2'), 75.3 (C5'', C5'), 74.8 (C3'), 74.1 (C3'), 73.7 (C3''), 72.6 (C2''), 71.7 (C4'', C4'), 61.0 (C9), 54.3 (C5), 47.8 (C18), 45.7 (C8), 44.6 (C20), 42.8 (C14), 42.5 (C19), 40.4 (C4), 38.8 (C1), 38.4 (C22), 36.1 (C10), 32.2 (C7), 31.9 (C17), 31.1 (C21), 29.5 (C29), 28.6 (C23), 28.3 (C28), 27.8 (C2), 27.3, 27.1 (C15, C16), 22.3 (C27), 17.7 (C26), 16.6 (C6), 15.7 (C24), 15.5 (C25); 3Cys(SBn): 173.0 (COOH), 172.4 (COOH), 172.1 (COOH), 137.8 (C arom.), 137.6 (2C arom.), 130.0 (C arom.), 128.9 (2C arom.), 128.5 (2C arom.), 128.4 (2C arom.), 128.1 (C arom.), 127.9 (2C arom.), 127.8 (C arom.), 127.2 (C arom.), 126.8 (C arom.), 126.4 (2C arom.), 51.3 (CH), 50.8 (CH), 50.5 (CH), 35.7 (CH_2), 35.5 (CH_2), 35.3 (CH_2), 26.8 (CH_2), 26.7 (CH_2), 26.6 (CH_2). Found, %: N 2.8, S 6.7. $\text{C}_{57}\text{H}_{89}\text{N}_3\text{O}_{19}$. Calc., %: N 3.0, S 6.8. $M = 1402.7$.

3-O-{2-O-[*N*-(β-*D*-Glucopyranosyluronoyl)-*L*-cysteine-*S*-benzyl]-*N*-(β-*D*-glucopyranosyluronoyl)-*L*-cysteine-*S*-benzyl}-(3β,20β)-11-oxo-30-norolean-12-ene (V). A solution of GA (0.82 g, 1 mmol) in dioxane (20 mL) was treated with HOSu (0.57 g, 5 mmol), DEC (0.57 g, 3 mmol), and Et_3N (0.5 mL), stirred at 20–22°C for 5 h; treated with *S*-benzyl-*L*-cysteine *tert*-butyl ester hydrochloride (0.76 g, 2.5 mmol) and Et_3N (0.3 mL), stored with periodic stirring at 20–22°C for 24 h, diluted with cold H_2O , and acidified with citric acid to pH ~ 4. The precipitate was filtered off, rinsed with H_2O , and dried to afford protected conjugate **IV** (1.1 g) that was worked up without further purification with CF_3COOH in CH_2Cl_2 as above, evaporated to dryness, and chromatographed over a column of silica gel with elution by CHCl_3 –MeOH– H_2O (200:10:1, 100:10:1, 50:10:1, vol%). Yield 0.72 g (60%); $[\alpha]_D^{20} +55^\circ$ (c 0.04; MeOH). Lit. [20]: $[\alpha]_D^{20} +52^\circ$ (s 0.02; MeOH). IR spectrum, ν_{max} , cm^{-1} : 3500–3200 (OH, NH), 1730 (COOH), 1657 (C=O), 1530 (CONH), 1500 (Ph). ^{13}C NMR spectrum (DMF- d_7), δ , ppm: 199.7

TABLE 1. Quantitative Characteristics of HIV-1 Protein p24 Inhibition by **III** in MT-4 Cell Culture as Compared to GA

Compound	CD ₅₀ , μg/mL*	ID ₅₀ , μg*	ID ₉₀ , μg*	SI (ND ₅₀ /ID ₅₀)
III	270	3	100	90
GA [28, 29]	1950	125	950	15.6

* CD₅₀, ID₅₀, and ID₉₀ values are averages of three measurements ($n = 3$) ± 0.7–1.0.

(C11), 178.3 (C30), 170.4, 170.3, 170.1 (C6'', C6', C13), 127.5 (C12), 105.2 (C1''), 104.3 (C1'), 89.0 (C3), 82.4 (C2'), 77.0 (C5''), 76.9 (C5'), 76.0 (C3'), 75.9 (C3''), 74.9 (C2''), 73.4, 73.0 (C4', C4''), 62.2 (C9), 55.5 (C5), 49.0 (C18), 45.8 (C8), 44.0 (C20), 43.8 (C14), 41.7 (C19), 39.9 (C4), 39.6 (C1), 37.3 (C22), 36.6 (C10), 33.7 (C7), 33.0 (C17), 32.3 (C21), 29.5 (C29), 28.8 (C23), 28.3 (C28), 27.9 (C2), 27.0, 26.0 (C16, C15), 23.5 (C27), 18.9 (C26), 17.8 (C6), 16.8 (C24), 16.7 (C25); 2Cys(SBn): 172.5 (COOH), 172.4 (COOH), 139.2 (C arom.), 138.1 (C arom.), 131.0 (C arom.), 130.0 (C arom.), 129.6 (2C arom.), 129.1 (C arom.), 129.0 (2C arom.), 128.9 (C arom.), 128.4 (C arom.), 128.3 (C arom.), 52.8 (CH), 52.6(CH), 35.8 (CH₂), 35.5 (CH₂), 26.7 (2CH₂). Found, %: N 2.30, S 5.22. C₆₂H₈₄N₂O₁₈S₂. Calc., %: N 2.32, S 5.30. M = 1209.42.

EXPERIMENTAL BIOLOGICAL PART

The cytotoxicity and anti-HIV-1 activity of **III** were studied using the traditional model of primary HIV-infected MT-4 lymphoid cells and HIV-1/EVK strain as described before [13 – 16]. The reference drug was GA (96% pure) [25].

Cytotoxicity was determined by dissolving **III** in DMSO. Aliquots of appropriate dilutions (three for each dilution) were placed into wells of a 96-well plate for cell inoculation. The inoculation concentration was 0.5×10^6 cells/mL. Cells were cultivated in 96-well plates (Costar, USA) in growth medium [RPMI-1640 with added fetal bovine serum (10%), *L*-glutamine (0.06%), and gentamycin (100 µg/mL)] at 37°C and 5% CO₂ for 4 d. When the incubation was finished, the fraction of viable cells was counted in a Goryaev chamber after staining with trypan blue. A dose-dependent curve was constructed. The compound concentration causing the death of 50% of the cells (CD₅₀) was determined.

The anti-HIV activity of **III** was assessed by infecting MT-4 cells (concentration 2×10^6 cells/mL) with HIV-1/EVK strain at infection multiple 0.2 – 0.5 infection units per cell for 1 h at 37°C. Infected and control cells (without virus) were diluted with growth medium to an inoculation concentration of $5 (10^5)$ cells/mL and placed into wells of 96-well plates. Then, solutions of the tested compound (three wells for each dilution) were placed into the corresponding wells. Then, the plates were cultivated as above. The final concentration of the tested drugs in the cell suspension varied from 0.1 to 100 µg/mL. The inhibitory effect of **III** was assessed on day 4 of cultivation by measuring the amount of virus antigen p24 using immunoenzyme assay. Furthermore, the fraction of viable cells was determined after staining with trypan blue by counting in a Goryaev chamber. Dose-dependent curves were constructed using the experimental data. The quantitative inhibition characteristics were determined as ID₅₀, the compound concentration suppressing virus production by 50% or providing 50% protection of

cells from death by infection; ID₉₀, the compound concentration suppressing virus production by 90% or providing 90% protection of cells from death by infection; SI, the selectivity index or ratio of toxic dose CD₅₀ to its effective dose ID₅₀. The standards were azidothymidine (0.1 µg/mL) and GA (100 µg/mL). The quantitative characteristics were determined by processing the results of three measurements using the Excel 2010 program.

RESULTS AND DISCUSSION

Carboxy-protected conjugate **II** was synthesized by condensation of GA with *S*-benzyl-*L*-cysteine *tert*-butyl ester hydrochloride using HOBt–DEC at room temperature (20 – 22°C) in DMF in the presence of an excess of Et₃N. Treatment of conjugate **II** with a solution of CF₃COOH in CH₂Cl₂ at 20 – 22°C produced target product **III** with three SBnCys moieties in 58% yield after column chromatography (CC) over silica gel (SG). Compound **IV** with an SBnCysOBu^t moiety in the carbohydrate part was synthesized using HOSu–DEC in dioxane. The resulting carboxy-protected GA conjugate **IV** was deprotected without further purification by CF₃COOH to afford conjugate **V** in 60% yield after CC over SG.

Cytotoxicity and antiviral activity of **III** were studied using the traditional model of primary HIV-infected MT-4 lymphoid cells (HIV-1/EVK strain) as before [13, 15, 16]. The reference drug was GA (96% pure) [25] at a concentration of 100 µg/mL and the well-known anti-HIV drug azidothymidine (AZT) at the therapeutic dose of 0.1 µg/mL [27, 28]. Table 1 presents the quantitative characteristics for inhibition of HIV-1 virus-specific protein p24.

The 50% cytotoxic dose CD₅₀ of the studied compound that caused the death of 50% of the cells was 270 µg/mL. Compound **III** exhibited pronounced anti-HIV-1 activity, inhibiting with high efficacy the accumulation of virus-specific protein p24 with ID₅₀ = 3 µg/mL or 2.2 µM (compound concentration suppressing virus production by 50%) and ID₉₀ = 100 µg/mL (90% suppression of virus reproduction). The selectivity index (SI, ratio of toxic dose CD₅₀ to effective dose ID₅₀) for inhibition of HIV-1 p24 was 90 for **III**, which was substantially greater than that for GA, the SI of which varies from 4.4 to 24 depending on the measurement parameters and purity [13, 28]. However, 90% suppression of virus reproduction was attained only with **III** at high concentration (100 µg/mL). It is noteworthy that **III** in the studied concentration range did not show noticeable protection from cytopathogenic activity of the virus. Compound **III**, in contrast to AZT, was nontoxic for HIV-infected cells and was 50 – 55 times less toxic than AZT (CD₅₀ = 3.5 µM) [27, 28].

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