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PHARMACOLOGICAL PROPERTIES OF NOVEL GLYCOPEPTIDES OF GLYCYRRHIZIC ACID

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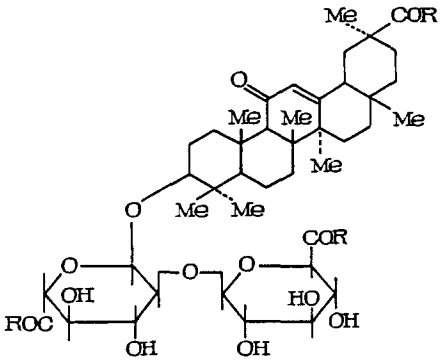
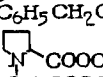
The pharmacological properties of a number of novel glycopeptides of glycyrrhizic acid containing fragments of methyl esters of L-amino acids and dipeptides were studied. The compounds were found to have a low toxicity with anti-inflammatory activity combined with an anti-ulcer effect. This distinguishes these compounds from known anti-inflammatory agents. The synthesis of three novel glycopeptides containing the fragments L-Glu(OMe)$_2$, L-Pro(OMe), and NHCH$_2$(CH)$_4$COOMe is described.

The main shortcoming of the popular anti-inflammatory steroidal and nonsteroidal drugs [ortofen, indomethacin, prednisolone, Butadion (phenylbutazone), Brufen (ibuprofen), etc.] is their ulcerogenic effect on the gastrointestinal tract [1]. This is why the search for novel anti-inflammatory drugs devoid of this undesirable property is urgent.

Glycerrhizic acid (GA) and its derivatives are known as anti-inflammatory and anti-ulcer compounds [2 – 6] having a low toxicity.



I: R=OH, IIa: R=CH$_3$CHNHCOOCH$_3$,
IIb: R=CH$_3$OOCCH$_2$CH$_2$CHNHCOOCH$_3$,
IIc: $\begin{matrix}CH_3\\CH_3\end{matrix}$>CHCH$_2$CHNHCOOH,
IId: R=C$_6$H$_5$CH$_2$CHNHCOOCH$_3$,
IIe: R=  ,
IIg: R=CH$_3$OOCCH$_2$CHNHCOOCH$_3$,
IIh: R=CH$_2$NHCH$_2$CONHCH$_2$COOCH$_3$,
IIf: R=NHCH$_2$CONHCH<$\begin{matrix}CH_3\\CH_3\end{matrix}$, (COOCH$_3$)
IIj: R=NHCH$_2$(CH$_2$)$_4$COOCH$_3$

To find novel chemical compounds in the series of GA derivatives having a high anti-inflammatory activity combined with an anti-ulcer effect, we studied the pharmacological properties of a number of novel glycopeptides of GA of the general formula (II) prepared from the glycoside (I) by the activated ester method in [7].

The synthesis of glycopeptides IIa, IIc, IId, IIh, and IIf was described in [7, 8]. The compounds IIb, IIe, IIg, and IIi were prepared by the activated ester method by treating the GA without preliminary protection of the hydroxyl groups of the hydrocarbon chain with N-hydroxysuccinimide (HOSu) and N,N′-dicyclohexylcarbodiimide (DCHC) in tetrahydrofuran or dioxane followed by condensation of the hydroxysuccinimide ester of GA formed with the methyl esters of L-glutamic acid, L-proline, and ε-aminocaproic acid as the hydrochlorides in the presence of an excess of triethylamine.

PHARMACOLOGICAL EXPERIMENTAL PART

The acute toxicity of the series of compounds was determined on white mongrel mice with a mass of 15 – 20 g using intraperitoneal injection. The toxicity parameters were calculated according to the technique of Litchfield and Wilcoxon [9]. The values of LD_{50} are given in Table 1.

On the basis of the decision of the State Committee of Standards of the USSR Council of Ministers of March 10, 1976 No. 579, the given compounds are assigned to class III – IV of low toxicity compounds.

The anti-inflammatory effect of glycopeptides of GA was studied on white mongrel mice using two inflammation models: induced by a 1% solution of carrageenan and a 3% solution of formalin. The inflammatory agents were injected into the aponeurosis of the foot of hind paw in a dose of 0.05 ml. The compounds under study were administered to the ani-

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TABLE 1. Acute Toxicity of Glycopeptides of GA*

Compound	LD_{50}, mg / kg
IIb	500 (350 – 600)
IId	135 (100 – 180)
IIe	1050 (± 92)
IIg	2000 (1666 – 2400)

* For LD_{50} of compound IIa see [9].

mals through a probe into the stomach in doses of 25, 50, and 100 mg / kg one hour before introducing the inflammatory agent and one and two hours after the formation of edema. The anti-inflammatory effect of the compounds was determined by the percent of increase in edema of the leg in comparison with a control animal. The value of ED_{50} was calculated by the technique of Litchfield and Wilcoxon [9]. Ortofen (Voltaren or diclofenac sodium) (LD_{50} 380 mg / kg, internally) and purified GA [1] (LD_{50} ~ 5000 mg / kg, internally) were used as the comparison compounds.

For the carrageenan model inflammation, all the glycopeptides of GA in the studied doses exhibit a pronounced anti-inflammatory effect in comparison with the control animals (Table 2). The glycopeptides IIa, IIb, and IId were the most active. They contain fragments of methyl esters of L-alanine, L-glutamic acid, and L-phenylalanine. Compound IId was more active when introduced in doses of 25 and 50 mg / kg. The anti-inflammatory effect of these GA derivatives is similar to that of ortofen (taken in ED_{50}) and more pronounced than the effect of GA. The glycopeptides IIc, IId (except for the dose of 100 mg / kg), IIg, IIf (except for the dose of 50 mg / kg), and IIh (except for the dose of 25 mg / kg) exhibit an anti-inflammatory effect similar to that of GA, but less effective than that of ortofen.

Elongation of the amino acid fragments lowers the anti-inflammatory activity (glycopeptide IIi).

We determined the effective doses (ED_{50}) of the series of glycopeptides and the glycoside itself for the carrageenan inflammation model (Table 3).

For inflammation produced by formalin, all the studied compounds inhibit the development of edema statistically significantly in comparison with the control animals (Table 4). The anti-inflammatory effect of glycopeptides IIa, IIc, IId, IIe, IIg, and IIh is not inferior to the effect of GA, while the glycopeptide IIi containing fragments of the Me ester of ε-aminocaproic acid is the least active in the given inflammation model.

The antiulcer effect of the GA derivatives was studied on white mongrel rats using models of experimental stomach ulcers caused by indomethacin and acetylsalicyclic acid. The disodium salt of glycyrrhetic acid (carbenoxolone) [10] and GA in equal doses were used as the comparison compounds. The animals were not fed for two days before producing the ulcers. The mucous membrane of the stomach was destroyed by the intraperitoneal injection of indomethacine in a dose of 20 mg / kg and intragastric administration of acetylsalicylic acid in a dose of 150 mg / kg twice a day. The day after simulating ulcers of the stomach, the animals were killed under chloroform narcosis, dissected, and the state of the mucous membrane of the stomach was studied. The anti-ulcer activity of the drugs was determined by the decrease in the number of destructions of the mucous membrane of the rats' stomachs.

TABLE 2. Anti-Inflammatory Activity of GA Glycopeptides for Carrageenan-Induced Inflammation

Compound(R)	Dose, mg/kg	Increase of paw edema, %	p
IIa L-AlaOMe	100	36.2 ± 1.7	< 0.001
	50	38.8 ± 2.7	< 0.001
	25	41.8 ± 4.3	< 0.001
IIb L-Glu$(OMe)_2$	100	38.8 ± 5.3	< 0.001
	50	37.8 ± 4.8	< 0.001
	25	31.3 ± 2.0	< 0.001
IIc L-LeuOMe	100	43.0 ± 4.4	< 0.001
	50	44.5 ± 3.3	< 0.002
	25	55.5 ± 4.6	< 0.05
IId L-PheOMe	100	50.0 ± 2.7	< 0.001
	50	34.8 ± 5.4	< 0.001
	25	35.6 ± 4.6	< 0.001
IIe L-ProOMe	100	58.8 ± 3.5	< 0.02
	50	53.7 ± 4.0	< 0.02
	25	48.2 ± 1.7	< 0.01
IIg L-Asp$(OMe)_2$	100	48.2 ± 4.8	< 0.001
	50	47.5 ± 3.0	< 0.001
	25	52.3 ± 4.4	< 0.02
IIh β-Ala-GlyOMe	100	45.5 ± 4.8	< 0.001
	50	52.6 ± 1.6	< 0.001
	25	61.0 ± 3.2	< 0.5
IIf Gly-L-ValOMe	100	43.0 ± 5.8	< 0.002
	50	57.5 ± 4.3	< 0.05
	25	53.1 ± 2.4	< 0.002
IIi $NHCH_2(CH_2)_4COOMe$	100	66.5 ± 2.6	< 0.05
	50	59.3 ± 3.4	< 0.01
	25	59.3 ± 2.0	< 0.01
GA	100	51.5 ± 3.9	< 0.001
Ortofen	8	39.3 ± 2.7	< 0.001
Control		74.92 ± 3.45	

A glance at Table 5 shows that for the indometacine ulcers model, the glycopeptides IIa, IId, and IIh lower the degree of damage to the mucous membrane of the rats' stomachs more than two-fold and do not differ in their anti-ulcer effect from carbenoxolone. The other compounds have low efficacy.

For the model of ulcers produced by acetylsalicylic acid, the studied compounds (except for compounds IIb and IIf) statistically significantly lower the degree of damage to the mucous membrane of the stomach, similar to the effect of carbenoxolone.

TABLE 3. Effective Doses of GA and Its Glycopeptides for Carrageenan-Induced Inflammation

Compound	ED_{50}, mg/kg
IIc	85 (47.8 – 98.8)
IIe	30 (16 – 55.5)
IIg	70 (56 – 87.5)
GA	82 (51 – 131)

The glycoside IIh and GA have the most pronounced anti-ulcer effect.

Consequently, the introduction of amino acid fragments into a glycoside molecule does not change its anti-inflammatory anti-ulcer properties appreciably, unlike other classes of GA derivatives [2 – 5].

CHEMICAL EXPERIMENTAL PART

Thin-layer chromatography was performed on Silufol plates (Czecho-Slovakia) using chloroform-alcohol systems of solvents, 7 : 1 (A), 4 : 1 (B), and chloroform-methanol-water, 45 : 10 : 1 (C). The spots for the compounds were detected with a 20% solution of phosphotungstic acid in alcohol with heating to 110 – 120°C for two or three minutes.

The IR spectra were recorded on a UR-20 spectrophotometer in a mull with vaseline oil. The electronic absorption spectra were obtained on a M-40 spectrophotometer in methanol. The optical activity was determined on a Perkin-Elmer 241MC polarimeter in a tube 1 dm long.

The compounds N,N′-dicyclohexylcarbodiimide from the Ferak company, L-amino acids from Reanal, and ε-aminocapronic acid from Chemapol were used for this work.

The methyl esters of L-glutamic and L-aspartic acids and L-proline were obtained as indicated in [7, 11].

1-O-[3β,20β]-11,30-dioxo-30-desoxy-30-(N-L-glutamic acid dimethyl ester)-olean-12-en-3-yl]-2-O-[β-D-6-oxo-6-desoxy-6-(N-L-g lutamic acid dimethyl ester)-glycopyranosyl]-α-D-6-oxo-6-desoxy-6-(N-L-glutamic acid dimethyl ester)-glucopyranoside (IIb). To a solution of 1.64 g (2 mmol) of glycyrrhizic acid in 50 ml of dry tetrahydrofuran at 0 – 5°C we added 1.15 g (10 mmol) of N-hydroxysuccinimide (HOSu) and 1.3 g (6 mmol) of N,N′-dicyclohexylcarbodiimide (DCC) and stirred the mixture at this temperature for 3 h and at room temperature (20 – 22°C) for 6 h, and held it for 12 h in a refrigerator at 4 – 8°C.

We filtered the precipitate of dicyclohexylcarbamide, and to the filtrate cooled to 0 – 5°C we added with stirring 1.4 g (6.5 mmole) of the hydrochloride of the dimethyl ester of L-glutamic acid, 1.2 ml (8.9 mmole) of triethylamine, and kept it at room temperature for 24 h with periodic stirring. We evaporated the solvent under vacuum, dissolved the residue in 200 ml of methylene chloride, and washed it with a 5% solution of HCl, water, a 5% solution of $NaHCO_3$, again with water, and dried it over $MgSO_4$. After evaporation of the solvent under vacuum, we obtained 2 g (76.9%) of the target glycoside (IIb) that was reprecipitated from the system chloroform-alcohol (5 : 1, v/v) with ester. $[\alpha]_D^{20} + 32°$ (c 0.06, MeOH), R_f 0.8 (B).

TABLE 4. Anti-Inflammatory Activity of GA Glycopeptides for Formalin-Induced Inflammation

Compound	Dose, mg/kg	Increase of paw edema, %	p
IIa	100	35.6 ± 2.4	< 0.001
IIb	100	48.0 ± 3.3	< 0.001
IIc	85*	45.5 ± 2.2	< 0.001
IId	100	39.0 ± 2.8	< 0.001
IIe	39*	44.2 ± 1.4	< 0.001
IIg	70*	44.6 ± 3.6	< 0.001
IIh	100	38.3 ±2.1	< 0.001
IIf	100	41.5 ± 2.4	< 0.001
IIi	100	51.0 ± 1.4	< 0,001
GA	100	41.5 ± 3.9	< 0.001
Ortofen	8	35.2 ± 3.2	< 0.001
Control		70.0 ± 2.7	

* The anti-inflammatory activity of the glycopeptides was assayed in doses corresponding to the values of ED_{50}.

TABLE 5. Anti-Ulcer Activity of Glycopeptides of GA When Introduced in a Dose of 100 mg/kg

Compound	Average number of stomach destructions caused by			
	indomethacin	p	acetylsalicylic acid	p
IIa	6.3 ± 0.9	< 0.002	4.1 ± 0.8	< 0.05
IIb	10.3 ± 1.0	0.05	5.8 ± 0.9	> 0.5
IId	5.8 ± 0.6	< 0.001		
IIh	5,8 ± 0.6	< 0.002	3.5 ± 0.4	< 0.002
IIf	16.6 ± 2.0	> 0.05	4.8 ± 0.6	> 0.5
IIi	12.3 ± 1.7	> 0.5	4.2 ± 1.0	< 0.05
GA	12.1 ± 1.5	> 0.5	3.1 ± 0.8	< 0.002
Carbenoxolone	5.8 ± 0.4	< 0.001	3.6 ± 0.4	< 0.002
Control	14.0 ± 1.8		7.0 ± 0.9	

IR spectrum, ν_{max}, cm^{-1}: 3600 – 3200 (OH, NH), 1740 (COOMe), 1660 (C_{11}–O), 1540 (CONH), 1210 (C–O–C ester).

UV spectrum, λ_{max}, nm (log ε) (MeOH): 250.5 (3.95). $C_{63}H_{99}N_3O_{25}$. The results of elemental analysis correspond to the calculated values.

1-O-[(3β,20β)-11,30-dioxo-30-(N-L-proline methyl ester)-olean-12-en-3-yl)-2-O-[β-D-6-oxo-6-desoxy-6-(N-L-pro line methyl ester)-glucopyranosyl]-β-D-6-oxo-6-desoxy-6-(N-L-proline methyl ester)-glucopyranoside (IIe). To a solution of 1.64 (2 mmole) of glycyrrhizic acid in 50 ml of dry dioxane at 0 – 5°C we added 1.2 g (10.4 mmole) of HOSu, 1.4 g (6.5 mmole) of DCC and stirred the mixture with cooling for 1.5 h, at room temperature for 6 h, let the mixture stand overnight in a refrigerator, and filtered off the

precipitate of dicyclohexylcarbamide. To the filtrate, we added 1.6 g (12.2 mmole) of the hydrochloride of the methyl ester of L-proline in 20 ml of dry dimethylformamide and 1.8 ml (13.1 mmole) of triethylamine and held the mixture with periodic stirring for 48 h at room temperature. We diluted the mixture with water, acidified it with citric acid, separated the viscous precipitate, washed it with water by decanting, and dried it.

We chromatographed the dry residue (1 g) in a column of silica gel L (40/100 μm), eluting with a chloroform-alcohol mixture in the ratio of 25 : 1 to 1 : 1 (v/v). With a mixture of 4 : 1 to 2 : 1, we washed out 0.6 g (26%) of a glycopeptide (IIe) in the form of an amorphous cream-colored substance, $[\alpha]_D^{20}$ + 50° (c 0.02, EtOH). R_f 0.58 (C).

IR spectrum, ν_{max}, cm^{-1}: 3690 – 3200 (OH), 1740 (COOMe), 1650 (C_{11}–O), UV-spectrum, λ_{max}, nm (log ε) (EtOH): 248 (4.10). $C_{60}H_{89}N_3O_{19}$. The results of elemental analysis correspond to the calculated values.

1-O-[(3β,20β)-11,30-dioxo-30-(N-L-aspartic acid dimethyl ester)-olean-12-en-3-yl]-2-O-[β-D-6-oxo-6-desoxy-6-(N-L-a spartic acid dimethyl ester)-glucopyranosyl]-β-D-6-oxo-6-desoxy-6-(N-L-aspartic acid dimethyl ester)-glucopyranoside (IIg). To a solution of 1.64 g (2 mmole) of glycyrrhizic acid in 50 ml of dry dioxane at 0°C we added 1.2 g (10.4 mmole) of N-hydroxysuccinimide in 1.3 g (6 mmole) of dicyclohexylcarbodiimide and stirred the mixture at this temperature for 2.5 – 3 h, at room temperature for 6 h, left the mixture overnight in a refrigerator, filtered off the precipitate of dicyclohexylcarbamide, added 1.7 g (7 mmole) of the hydrochloride of the dimethyl ester of L-aspartic acid and 1.4 ml (10.2 mmole) of triethylamine, and kept the mixture at room temperature with periodic stirring for 24 h. We evaporated the solution under vacuum, dissolved the solvent in methylene chloride (200 ml), washed it with 5% HCl, water, 5% $NaHCO_3$, and water, and dried it over $MgSO_4$. By evaporating the solvent under vacuum, we obtained 1.9 g (76%) of the glycopeptide (IIg). We chromatographed it in a column of silica gel L (100/250 μ) and eluted it with a mixture of chloroform-methanol in the ratio of 50 : 1, 25 : 1, and 10 : 1 (v/v).

We used a mixture of 50 : 1 and 25 : 1 to wash out 1.5 g (60%) of the analytically pure product (IIg) in the form of an amorphous yellowish substance. $[\alpha]_D^{20}$ + 60° (c 0.02, MeOH). R_f 0.5 (C).

IR spectrum, ν_{max}, cm^{-1}: 3600 – 3200 (OH, NH); 1740 (COOR); 1660 (C_{11}–O); 1540 (CONH).

UV spectrum, λ_{max}, nm (log ε) (MeOH): 248 (4.07). $C_{60}H_{89}N_3O_{25}$. The results of elemental analysis correspond to the calculated values.

1-O-[(3β,20β)-11,30-dioxo-30)-(N-L-ε-aminocaproic acid methyl ester)-olean-12-en-3-yl]-2-O-[β-D-oxo-6-desoxy-6-(N-L- 28Me-aminocaproic acid methyl ester)-glucopyranosyl]-β-D-6-oxo-6-desoxy-6-(N-L-ε-aminocap roic acid methyl ester)-glucopyranoside (IIi).

(a) Methyl ester of L-ε-aminocaproic acid. To 5 g of ε-aminocaproic acid in 200 ml of methanol at 0°C, we added dropwise 20 ml of distilled thionyl chloride and stirred the mixture for 1 h with cooling, 1 h at room temperature until dissolution, and left it overnight. We evaporated the solvent under vacuum at 40°C, ground the residue with dry ester, filtered off the precipitate, and recrystallized it from the system methanol-ester. The yield was 5.9 g (91%). The melting point was 128 – 130°C.

(b) To a solution of 1.64 g (2 mmole) of glycyrrhizic acid in 50 ml of dry dioxane at 0 – 5°C, we added 1.2 g (10.5 mmole) of N-hydroxysuccinimide and 1.3 g (3.2 mmole) of dicyclohexylcarbamide and stirred the mixture for 1 h with cooling, 6 h at room temperature, and left it overnight in a cooler. We filtered off the precipitate of dicyclohexylcarbamide, to the filtrate we added 1.7 g (10 mmole) of the hydrochloride of the methyl ester of L-ε-aminocaproic acid and 1.5 ml (6.3 mmole) of tributylamine, and held it with periodic stirring for 48 h. We diluted the solution with cold water, acidified it with citric acid to pH 3, filtered off the white precipitate of the glycopeptide, washed it with water, and dried it (1.66 g) (68%). After reprecipitation from the system chloroform-alcohol (5 : 1) with ester, we obtained 0.95 g (35.2%) of the glycopeptide (IIi), homogeneous after thin layer chromatography (a white powder). $[\alpha]_D^{20}$ + 27° (c 0.022, EtOH, R_f 0.45 (C), 0.3 (A).

IR spectrum, ν_{max}, cm^{-1}: 3600 – 2300 (OH, NH), 1730 (COOMe), 1660 (C_{11}–O), 1530 (CONH).

UV spectrum, λ_{max}, nm (log ε) (EtOH): 249 (3.99). $C_{60}H_{108}N_3O_{22}$. The results of elemental analysis correspond to the calculated values.

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