Synthesis of a new betulinic acid glycoconjugate with *N*-acetyl-D-galactosamine for the targeted delivery to hepatocellular carcinoma cells*

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A new promising conjugate of betulinic acid with *N*-acetyl-D-galactosamine was synthesized by the simple reaction sequence: esterification and copper-catalyzed azide-alkyne cycloaddition. The obtained glycoderivative exhibited high activity against hepatocarcinoma cell lines *in vitro*, selectivity of cytotoxic action, and excellent binding to the asialoglycoprotein receptor (ASGPR) of hepatocytes. Its affinity to the ASGPR was established by surface plasmon resonance spectroscopy and confirmed by molecular docking *in silico*. An original approach was proposed to enhance the cytotoxic properties of C-28 betulinic esters by introducing a hemioxalate fragment bearing free carboxyl group into the C(3) position of ring A.

Key words: glycoconjugate, betulinic acid, *N*-acetyl-D-galactosamine, hemiester, targeted delivery, liver, asialoglycoprotein receptor, hepatocellular carcinoma.

Hepatocellular carcinoma (HCC) ranks third in the number of fatal outcomes worldwide among human cancer diseases.[1] According to the statistics of World Human Organization (WHO), 782 500 new cases of HCC were recorded in 2012 and the mortality from HCC was 700 000 fatal cases.[2] The high fatality rate of HCC is first of all due to a long latency period, difficult early detection, and multiresistance to chemotherapy.[3] Severe adverse effects of anti-HCC medicines (systemic toxicity, anemia, mutagenicity, *etc*.) considerably restrict the wide applicability of chemotherapy. In addition, the production of these medicines includes large-scale organic synthesis, which inevitably results in a high cost of treatment for population. For these reasons, there is an obvious need to develop new anticancer drugs for the therapy of liver cancer with improved pharmacological profile and decreased cost of production.

The use of natural compounds and their semisynthetic derivatives is an important trend in the search of new and efficient drug substances.[4,5] Betulinic acid, a pentacyclic lupine-type triterpenoid which is known to exhibit cytotoxic activity against a wide range of cancer cells,[6] including HCC cells,[7] is of great interest for the design of anti-HCC drugs. However, drawbacks associated with its pharmacological profile and bioavailability restrict its application in clinical practice as the anticancer drug.[8] To solve current problems, betulinic acid (**1**) derivatives

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possessing enhanced activity against HCC were synthesized.[9,10] Various targeted delivery systems were proposed;[11] the prodrug approach to modify the structure of betulinic acid is widely used.[12] However, up to date none of synthesized compounds have passed clinical trials to give commercially available medicinal product. Thus, there remains an urgent need for medi cinal chemistry to further search for promising derivatives of betulinic acid capable of unveiling the full pharmacological potential of the natural triterpenoid for the therapy of HCC and other liver diseases.

In the present work, we synthesized a new betulinic acid conjugate with *N*-acetyl-D-galactosamine which can be targetedly delivered to hepatocytes and possesses high activity against hepatocellular carcinoma cells.

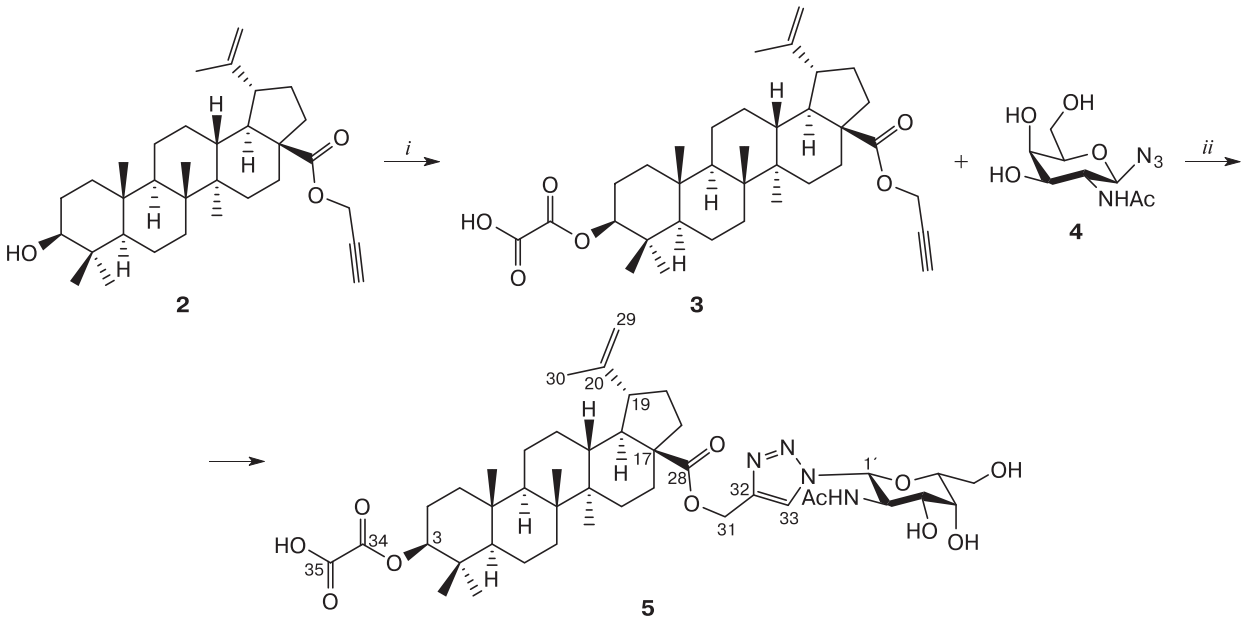
Results and Discussion

At the present time, it is considered that the targeted delivery to biological targets in cells (receptors, antigens, glycoproteins, *etc*.) allows one to reduce adverse effects and systemic toxicity of medicines, as well as to decrease the therapeutic dose of drug substance.[13] Researchers gives considerable attention to the search of optimum ways for delivery of therapeutic agents to liver,[3] especially to hepatocytes, which constitute more than 80% of the total number of liver cells and are directly related to the progress of HCC.[14] Human hepatocytes express on their surface a lectin specific for these cells, asialoglycoprotein receptor (ASGPR), which is involved in metabolic processes and recognizes galactose and *N*-acetyl-D-galactosamine (GalNAc) residues.[15] Upon binding of galactose residues to ASGPR, hepatocytes can uptake glycoconjugated molecules through receptor-mediated endocytosis. Cumulatively, all of this makes ASGPR an important target for the targeted therapy of HCC. Earlier, we have synthesized conjugates of triterpenoids with GalNAc as molecules with targeted properties for targeting to hepatocytes.[16] Taking into account a great potential of triterpenoids for the design of new anticancer drugs as low-molecular-weight conjugates,[17,18] in the present work we synthesized a new derivative of betulinic acid (**1**) and GalNAc, as well as carried out the primary assessment of its pharmacological properties, cytotoxicity, and binding to ASGPR.

When developing an original synthesis method, we took propargyl betulinate **2** as a basis (Scheme 1). Earlier, we have shown that modification of betulinic acid through the carboxyl function in the C(28) position of the hydrocarbon skeleton leads to a loss in the initial cytotoxicity against HepG2 and Huh7 hepatocarcinoma cell lines.[19] However, in this work we found that the presence of a free carboxy group in the form of hemioxalate of the hydroxy group at the C(3) position of betulinic acid allows one to keep the *in vitro* cytotoxicity of the conjugate.

The carboxy group was introduced to ring A of the betulinic acid derivative **2**[20] containing a triple bond through a short oxalic fragment by acylation of alcohols with acid chlorides. The acylating agent was oxalyl

Scheme 1



Reagents and conditions: *i.* 1) $(COCl)_2$, CH_2Cl_2, 24 h, ~20 °C; 2) 1,4-dioxane—H_2O/H^+; *ii.* **4**, $CuSO_4$, NaAsc, DMF, Ar, ~20 °C, 24 h.

chloride, which can react with the hydroxy group at the position 3 of triterpenoids.[21] Then, the intermediate acid chloride was dissolved in the minimum amount of 1,4-dioxane and subjected to acid hydrolysis in water. After completion of the hydrolysis, compound **3** was isolated by reversed-phase chromatography, since the product showed a strong increase in the polarity after the free COOH group has been introduced. The use of normal-phase chromatography was restricted by the need in strongly polar eluent, such as MeOH, which result in partial re-esterification of the free carboxy group in compound **3**. After purification, the product was isolated as a white powder in yield of 48%.

The structure of hemioxalate **3** was established by ^{1}H NMR spectroscopy. A downfield shift of the signal for the H(3) atom (δ 4.65) compared to that for compound **2** (δ 3.18) suggests the formation of ester. The ^{13}C NMR spectrum of compound **3** displayed signals at δ 157.33 and 157.05 typical of oxalic carbons.

At the final step, the GalNAc residue was introduced into the triterpenoid structure by copper-catalyzed azide-alkyne [3+2] cycloaddition (CuAAc). 2-Acetamido-2-deoxy-β-D-galactopyranosyl azide (**4**) was obtained by the earlier described method.[22] The reaction between the betulinic acid alkynyl derivative **3** and azide **4** was carried out in a classical manner[23] in the presence of $CuSO_4$ and sodium ascorbate (NaAsc). After completion of the reaction, the product was purified on reversed-phase silica gel column, which was due to a high polarity of the final conjugate and the presence of free COOH group. As a result, compound **5** was obtained in 81% yield.

The structure of conjugate **5** was established by NMR spectroscopy and its composition and molecular formula were confirmed by high-resolution mass spectrometry (ESI HRMS). The ^{1}H NMR spectrum displays a downfield singlet at δ 7.97 typical of the aromatic 1,2,3-triazole ring (H(33)). At the same time, the ^{13}C NMR spectra contained signals for the 1,2,3-triazole ring carbons at δ 148.26 and 121.57. The β-configuration of the amino monosaccharide moiety in the structure of conjugate **5** remained unchanged after the reaction. The ^{1}H NMR spectral analysis showed that the spin-spin coupling constant of the signal for the anomeric H(1′) atom was equal to J = 10 Hz, which suggests the *trans*-orientation of substituents at the C(1′) and C(2′) atoms.[24]

Thus, a short sequence of simple chemical reactions afforded a new betulinic acid conjugate **5** with *N*-acetyl-D-galactosamine. The synthesized compound was studied for *in vitro* cytotoxicity and binding to ASGPR. The cytotoxicity was determined against HepG2 and Huh7 hepatocarcinoma cells and PC3 prostate cancer cells as the control by the MTT test[25] (Table 1).

The reference sample was doxorubicin. Simultaneously, the values of cytotoxic effect were determined for the starting betulinic acid (**1**). As it is seen from Table 1, compound **5** exhibited activity against HepG2 hepatocarcinoma cells with IC_{50} = 6.4 μmol L^{-1}, which is comparable with that of the starting natural triterpenoid (IC_{50} = 3.4 μmol L^{-1}). The achieved selectivity exceeded that for betulinic acid. For example, conjugate **5** was twofold more active against HepG2 cells than against Huh7 and PC3 ones. The HepG2 cell culture is known to abundantly express ASGPR, while this lectin is present in Huh7 cells in much iower amounts.[26] The PC3 prostate cancer cell line is ASGPR-negative and was used as the control of action selectivity.

Table 1. Cytotoxicity of betulinic acid glycoconjugate **5**

Compound	IC_{50}/μmol L^{-1}		
	HepG2	Huh7	PC3
5	6.4±1.8	12.4±1.8	12.9±2.4
Betulinic acid	3.4±0.2	4.9±0.4	5.0±0.5
Doxorubicin	0.38±0.07	0.15±0.04	0.11±0.03
DMSO	—*	—	—

* No activity.

Thus, the presence of hemioxalate as a part of conjugate **5** kept the initial cytotoxicity level of betulinic acid, while the GalNAc moiety provided the targeted delivery to hepatocytes. To estimate the binding of compound **5** to ASGPR, we determined the dissociation constant (K_D) of the ligand—receptor complex by surface plasmon resonance (SPR) spectroscopy. The obtained value of K_D was 0.21 nmol L^{-1}, which is significantly lower than that for the reference ligand, *N*-acetyl-D-galactosamine (K_D > 100 nmol L^{-1}).

We assume that, in this case, the presence of a hydrophobic hydrocarbon skeleton of betulinic acid, which favors additional interactions with the receptor, plays a key role in high binding to ASGPR. The 1,2,3-triazole ring of conjugate **5** also comes into hydrophobic contacts with aromatic amino acids of the ligand-binding pocket of ASGPR and forms hydrogen bonds through the nitrogen atoms.[27]

In addition, we performed *in silico* molecular docking of compound **5** using the validated ASGPR model. The analysis of docking data revealed interactions between the glycoside moiety of conjugate **5** and the corresponding carbohydrate-recognition binding site of ASGPR. The amino monosaccharide formed hydrogen bonds with the Asp241, Glu252, and Asn264 residues, as well as interacted with calcium ion, which is a prerequisite for *in vivo* binding to ASGPR.[28] In addition, the carbon backbone of conjugate **5** came into hydrophobic contacts with Pro237 (Fig. 1). The ester bond with a linker in the C(28) position formed a hydrogen bond with Arg236.

The calculated energy of binding E^{score} was 4.65 kcal mol^{-1}, which confirmed that the conjugate of a hydrophilic GalNAc moiety with a hydrophobic molecule

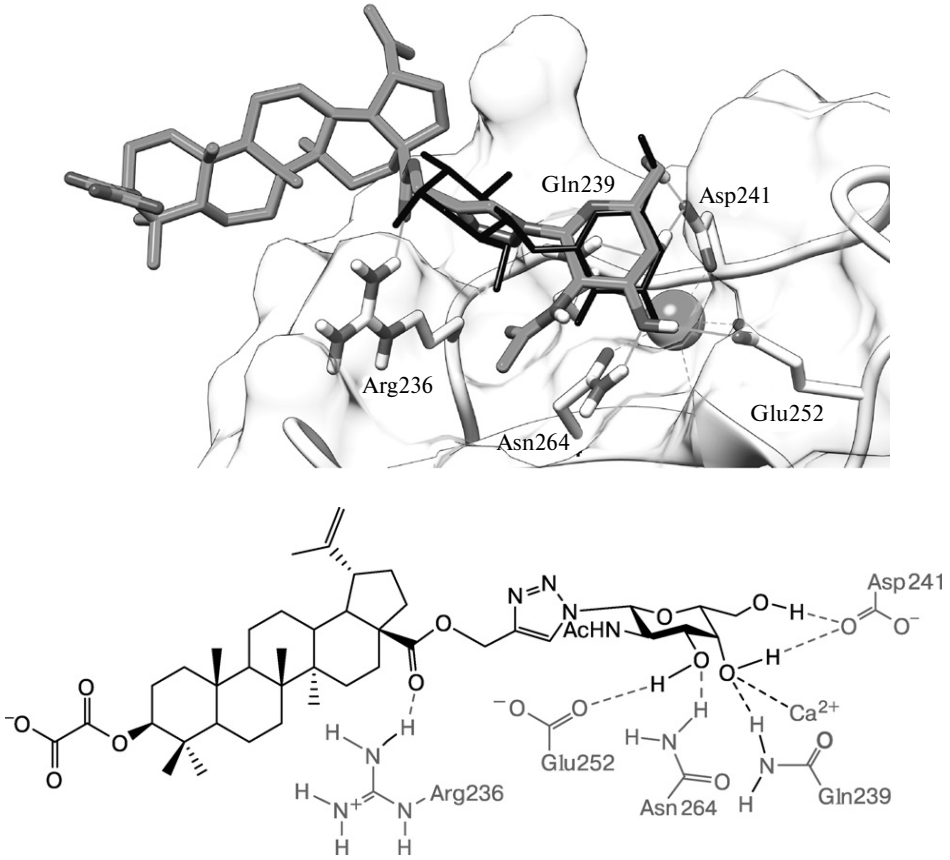


Fig. 1. Binding position of conjugate **5** on the surface of ASGPR as predicted by molecular docking.
Note. Fig. 1 is available in full color on the web page of the journal (https://link.springer.com/journal/volumesAndIssues/11172).

of betulinic acid has a high affinity to ASGPR. It is likely that, in this case, we succeeded in finding the required amphiphilicity balance in the structure of conjugate **5**, which is supplemented by a unique geometry of the polyfunctional carbon skeleton of the natural triterpenoid. Pooled data from the SPR spectra and *in silico* molecular simulation for compound **5** were found to be comparable with those for the earlier synthesized promising branched-structure ASGPR conjugates based on tris(hydroxylmethyl)-aminomethane.[29] Due to its high cytotoxicity, compound **5** can be used in further development of the targeted therapeutic agent against HCC.

Thus, we proposed an original and simple approach to the synthesis of the new betulinic acid conjugate with *N*-acetyl-D-galactosamine, which consists in sequential esterification of the starting triterpenoid and conjugation with the azido derivative of the amino monosaccharide. The reaction between propargyl betulinate and oxalyl chloride followed by acid hydrolysis, which provides insertion of the hemioxalate moiety into the structure of betulinic acid, is a key step in the developed scheme. The low-molecular-weight conjugate possesses *in vitro* activity against hepatocarcinoma cell lines, selectivity of cytotoxic action, and excellent binding to the asialoglycoprotein receptor. The affinity to ASGPR was established based on the analysis of data from SPR spectroscopy and confirmed by *in silico* molecular docking calculations.

Experimental

^{1}H and ^{13}C NMR were recorded on a Bruker DPX-300 instrument (300 and 75.47 MHz, respectively). High-resolution mass spectra (ESI-HRMS) were obtained on a Thermo Scientific Orbitrap Elite spectrometer. IR spectra were recorded on a Nicolet iS5 FT-IR spectrometer (Thermo Scientific, USA) equipped with an iD7 ATR accessory with a ZnSe crystal (7800—550 cm^{-1}). Melting points were determined on an OptiMelt automated melting point system. Reversed-phase chromatography was carried out on an Interchim Puriflash 4250 chromatograph. Specific rotation (expressed in deg mL g^{-1} dm^{-1}) was determined on an A. Krupps Optronic P-800 automated polarimeter. TLC analysis was performed on Merck TLC Silicagel 60 F254 plates in a CH_2Cl_2—MeOH system. The spots of compounds were developed using 10% H_2SO_4 followed by heating at 100—120 °C for 2—3 min. Commercially available reagents (Sigma-Aldrich) were used as received. Compounds **2** and **4** were synthesized according to the published procedures.[19,22]

(Prop-2-yn-1-yl) 3β-*O*-carboxycarbonyl-lup-20(29)-en-28-oate (3). Oxalyl chloride (0.4 mL, 4 mmol, 4 equiv.) was added to a solution of compound **2** (0.5 g, 1 mmol) in 10 mL CH_2Cl_2.

The reaction mixture was stirred for 24 h at ~20 °C. The solvent was removed under reduced pressure on a rotary evaporator. The residue was dissolved in 1,4-dioxane (1 mL) and poured with stirring in a 0.1% solution of HCl in H_2O (50 mL). The precipitate formed was filtered off, washed with water until pH 7, and dried in air. The product was isolated by reversed-phase chromatography using H_2O—MeCN (1 : 1) as the eluent to obtain a white powder (0.28 g, 48%). R_f 0.27 (CH_2Cl_2—MeOH, 5 : 1). M.p. 242—243 °C. $[\alpha]_D^{25}$ +55 (*c* 0.4, CH_2Cl_2). IR, ν/cm^{-1}: 1173, 1686, 1743, 1774, 3302. ^{1}H NMR ($CDCl_3$), δ: 0.86 (s, 3 H, CH_3); 0.88 (s, 3 H, CH_3); 0.90 (s, 3 H, CH_3); 0.93 (s, 3 H, CH_3); 0.97 (s, 3 H, CH_3); 1.69 (s, 3 H, H(30)); 2.27—0.98 (m, 25 H, CH, CH_2); 2.56 (t, 1 H, H(33), J = 2.4 Hz); 3.00 (m, 1 H, H(19)); 4.61 (s, 1 H, C(29)$\underline{H}_b$); 4.65 (m, 1 H, H(3)); 4.73 (s, 1 H, C(29)$\underline{H}_a$); 4.85 (m, 2 H, H(31)). ^{13}C NMR ($CDCl_3$), δ: 14.65, 16.03, 16.16, 16.38, 18.10, 19.35, 20.85, 23.34, 25.37, 27.92, 29.67, 30.56, 32.13, 34.16, 37.05, 37.09, 38.07, 38.26, 38.40, 40.66, 42.41, 46.94, 49.22, 50.33, 53.94, 55.30, 56.43, 76.05 (C(33)), 76.35 (C(32)), 85.08 (C(3)), 109.79 (C(29)), 150.31 (C(20)), 157.05 (C(35)), 157.33 (C(34)), 183.11 (C(28)). MS (ESI), found: *m/z* 565.3530 $[M - H]^-$. $C_{35}H_{50}O_6$. Calculated: 565.3535.

{[1-(2-Acetamido-2-deoxy-β-D-galactopyranosyl)-1*H*-1,2,3-triazol-4-yl]methyl} 3β-*O*-carboxycarbonyl-lup-20(29)-en-28-oate (5). Compound **3** (0.55 g, 1 mmol) was dissolved in DMF (25 mL) and azide **4** (0.3 g, 1.2 mmol, 1.2 equiv.), anhydrous $CuSO_4$ (0.2 g, 1.2 mmol, 1.2 equiv.), and NaAsc (0.28 g, 1.4 mmol, 1.4 equiv.) were added. The reaction mixture was stirred for 24 h in the argon atmosphere at ~20 °C, concentrated on a rotary evaporator, and twice re-evaporated with toluene. The product was isolated by reversed-phase chromatography using H_2O—CH_3CN (1 : 1) as the eluent to obtain a white powder (3.85 g, 81%). R_f 0.30 (CH_2Cl_2—MeOH, 5 : 1). M.p. 198—199 °C. $[\alpha]_D^{25}$ +15.4 (*c* 0.3, MeOH). IR, ν/cm^{-1}: 1201, 1550, 1644, 1693, 1739, 2938. ^{1}H NMR (DMSO-d_6), δ: 0.81 (s, 6 H, 2 CH_3); 0.86 (s, 3 H, CH_3); 0.87 (s, 3 H, CH_3); 0.94 (s, 3 H, CH_3); 1.63 (s, 3 H, H(30)); 1.64 (s, 3 H, NHC(O)C$\underline{H}_3$); 2.22—0.97 (m, 28 H, CH, CH_2); 2.96—2.91 (m, 1 H, H(19)); 3.56—3.47 (m, 2 H); 3.71—3.65 (m, 2 H); 3.77 (d, 1 H, J = 3.0 Hz); 4.44—4.36 (m, 1 H); 4.55—4.51 (m, 1 H, H(3)); 4.49 (s, 2 H); 4.56 (s, 1 H, C(29)$\underline{H}_b$); 4.69 (s, 1 H, C(29)$\underline{H}_a$); 5.61 (d, 1 H, H(1′), J = 10.0 Hz); 7.78 (d, 1 H, N$\underline{H}$C(O)CH_3, J = 9.3 Hz); 7.97 (s, 1 H, H(33)). ^{13}C NMR (DMSO-d_6), δ: 14.82, 16.13, 16.32, 16.77, 18.42, 19.39, 20.90, 23.22, 25.51, 27.94, 28.55, 29.66, 30.54, 32.14, 36.78, 37.07, 37.18, 38.03, 38.95, 40.70, 42.45, 42.49, 47.07, 48.97, 50.05, 51.25, 55.43, 55.87, 60.91, 68.17, 71.66, 77.24, 78.92, 82.97, 86.94, 110.15 (C(29)), 121.57 (C(33)), 148.26 (C(32)), 150.77 (C(20)), 167.76 (C(35)), 169.84 (C(34)), 177.72 (NH$\underline{C}$(O)CH_3), 177.75 (C(28)). MS (ESI), found: *m/z* 847.4262 $[M + Cl]^-$. $C_{43}H_{64}ClN_4O_{11}$. Calculated: 847.4266.

Cytotoxicity study of synthesized compounds. *Culturing of cell lines*. Cells were cultured in a DMEM-F12 medium which contained 10% of bovine serum albumin, GlutaMAX (2 mmol L^{-1} glutamine), and penicillin—streptomycin mixture (50 U mL^{-1} of penicillin and 0.05 mg mL^{-1} of streptomycin) (Gibco, USA) at 37 °C in the 5% CO_2 atmosphere. Cells lines were controlled for the absence of mycoplasm.

Determination of cytotoxicity. Huh7 and HepG2 cells ($4 \cdot 10^3$ per well) and PC3 cells ($2.5 \cdot 10^3$ per well) in the medium (140 μL) were placed in a 96-well plate and incubated for 16 h. The studied compounds were dissolved in DMSO until a concentration of 20 mmol L^{-1} and diluted with the culture medium. The resulting solutions (11 μL each) were added to cells until final concentrations from 100 μmol L^{-1} to 46 nmol L^{-1} in wells (eight dilutions, three-fold dilution; the final concentration of DMSO in each well did not exceed 0.5%). Solutions were incubated under standard conditions for 72 h. The cell viability was estimated by the MTT test.[25] The negative control was cells cultured in the incubation medium with equivalent content of DMSO. The reference sample was doxorubicin. Data were processed using GraphPad Prism Software to calculate IC_{50}.

Surface plasmon resonance spectroscopy. The K_D value was determined *in vitro* by SPR spectroscopy on a Biacore X100 instrument (Biacore AB, Sweden) using a CM5 carrier chip, which consisted of a gold plate coated with carboxymethylated dextran. The chip surface included two flow-through cells. The ASGPR enzyme from rabbit liver (Generic Assays, Germany, >95% purity) was immobilized onto the surface of one cell using a pH 7.4 buffer mixture of 150 mmol L^{-1} NaCl, 50 mmol L^{-1} $CaCl_2$, and 50 mmol L^{-1} Tris. The studied compounds were dissolved in DMSO and diluted with a pH 7.4 buffer solution (150 mmol L^{-1} NaCl, 50 mmol L^{-1} $CaCl_2$, and 50 mmol L^{-1} Tris), so that the portion of organic solvent at main studied concentrations was <1 wt.%. The reference sample was *N*-acetyl-D-galactosamine. The negative control was glucose. The analysis was performed using samples in a wide concentration range from 10^{-2} to 10^{-7} mol L^{-1}. The new betulinic acid conjugate **5** was used in experiments at concentrations from 10^{-5} to $5 \cdot 10^{-11}$ mol L^{-1}.

The studied compound was fed at a flow rate of 20 μL min^{-1} for 180 sec. Then, the dissociation of the complex was studied for 60 sec. The K_D value (thermodynamic) was determined using the Langmuir adsorption isotherm model (1 : 1 Langmuir association). The carrier chip was regenerated with 20 mmol L^{-1} EDTA (20 μL). The obtained data were processed using the BIAevaluation 3.0 program.

Molecular docking. The ASGPR model was prepared using Protein Preparation Wizard in the Maestro software (Schrodinger Inc.) based on the crystal structure of 5JPV.[30] The obtained receptor model was validated by redocking of the native ligand from the corresponding crystal structure. The energy lattice was plotted in a cubic box with dimensions of 36×36×36 Å.

The molecular docking was carried out in the Glide module of the Maestro software (Schrodinger Inc.). The obtained data were visualized using the UCSF Chimera program package.[31]

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