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Synthesis and anti-cancer activities of glycosides and glycoconjugates of diterpenoid isosteviol†

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A series of glycosides and glycoconjugates of diterpenoid isosteviol (16-oxo-*ent*-beyeran-19-oic acid) with various monosaccharide residues were synthesized and their cytotoxicity against some human cancer and normal cell lines was assayed. Most of the synthesized compounds demonstrated moderate to significant cytotoxicity against human cancer cell lines M-HeLa and MCF-7. Three lead compounds exhibited selective cytotoxic activities against M-HeLa (IC_{50} = 10.0–15.1 μ M) that were three times better than the cytotoxicity of the anti-cancer drug Tamoxifen (IC_{50} = 28.0 μ M). Moreover, the lead compounds were not cytotoxic with respect to the normal human cell line Chang liver (IC_{50} > 100 μ M), whereas Tamoxifen inhibited the viability of normal human Chang liver cells with an IC_{50} value of 46.0 μ M. It was determined that the cytotoxicity of the lead compounds was due to induction of apoptosis proceeding along the mitochondrial pathway. The cytotoxic activity of the synthesized compounds substantially depended on the nature of the monosaccharide residue and its position, that is, whether the monosaccharide residue was attached directly to the isosteviol skeleton or was moved away from it by means of a polymethylene linker.

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1. Introduction

Cancer is a dreaded disease that ranks as the second-leading cause of death worldwide.¹ Although a variety of methods to overcome and treat cancers have been researched, the number of cancer patients continues to increase each year. Furthermore, an estimated 15.5 million people in the world will become cancer patients by 2030, and 11.5 million of these cases are expected to be fatal.² Cancers have been reported to be caused by the dysregulation of key cellular processes, such as growth signaling, anti-apoptotic signaling, immune response, gene stability, and regulation of the stromal microenvironment.^{3,4} The treatment of cancer has been focused on re-regulating these cellular functions. Up to the present date, numerous clinical trials have investigated potential cures for

cancer *via* radiation, chemotherapy, antibody treatment, and immunotherapy.^{1,2} Radiation and chemotherapy have severe side effects due to their cytotoxicity to normal cells.² Antibody treatment and immunotherapy show a highly specific cancer targeting ability, but they have a limited target range and can be very expensive.⁵ Additionally, many types of cancers tend to relapse and acquire resistance after treatment.^{2,5} Currently, combination therapies involving several drugs or therapies are being used to attempt to overcome the limitations and drawbacks of individual therapies. Furthermore, to reduce the side effects of anti-cancer drugs and to discover more effective drugs, new approaches have been developed to identify novel molecules with anti-cancer activity from new sources.² For this reason, researchers are always looking for inspiration from natural products.¹ Plant species have been used in medical treatment for millennia.⁴ Additionally, plant-derived metabolites have been reported to be useful for a variety of therapeutic purposes and biotechnological applications.⁶ Plant metabolites exhibit a wide range of biological functions, including anti-cancer, analgesic, anti-inflammation, and anti-microbial activities.⁷ Plants have generated about 25% of clinically used drugs.⁸ More than 60% of drugs with anti-cancer activity originated from plants.² As discussed above, the development of new molecules for cancer treatment with fewer side effects and greater efficacy is essential. Plant-derived metabolites are good sources of new anti-cancer drugs with

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reduced cytotoxicity and increased activity.⁹ For example, diterpenoids have been reported to have cytotoxic and antiproliferative properties.^{1,2,10}

In particular, the naturally occurring diterpenoid isosteviol **1** (16-oxo-*ent*-beyeran-19-oic acid) easily obtained by acid hydrolysis of the glycosides of the plant *Stevia rebaudiana* (genus *Stevia*, family *Asteraceae* within the tribe *Eupatorieae*),¹¹ like all natural terpenoids, is a biologically active substance with a wide spectrum of activities. Isosteviol **1** has moderate antihypertensive,¹² antihyperglycemic,¹³ cardioprotective,¹⁴ anti-tumor,¹⁰ anti-tuberculosis,¹⁵ and antimicrobial¹⁶ effects. Chemical modification of isosteviol **1** did not only increase its anti-tuberculosis,^{15,17–19} anti-cancer,^{20,21} and anti-bacterial²² activities but also provided it with antiviral,²³ antimitotic,²⁴ and mitochondriotropic²⁵ effects. It is interesting to note that although glycosylation of biologically active natural compounds that are not glycosides has long been considered as one of the ways to “tune their activity”,²⁶ among more than 200 derivatives of isosteviol **1** (ref. 27 and 28) known in the literature, only a few of its glycosides have been described.^{29–31} In continuation of our studies on the synthesis and biological activity of glycosides and glycoconjugates of isosteviol,^{17–19,30,31} we now

describe the synthesis and anticancer activities of glycosides and glycoconjugates of isosteviol **1** with D-glucopyranose, D-galactopyranose, D-mannopyranose, D-N-acetylglucosamine, and D-glucuronic acid methyl ester residues.

2. Results and discussion

2.1 Chemistry

The synthesis of isosteviol glycosides is shown in Scheme 1. The reaction of isosteviol **1** with bromo derivatives of mono-saccharides **2–6** in the presence of K₂CO₃ and tetrabutylammonium bromide (TBAB) led to glycosides **7–11** with acetylated hydroxyl groups. Then, the acetyl protection of the sugar residues of glycosides **7–9** was removed by sodium methoxide to give glycosides **12–14**. The trichloroethyl-formate protection of the amino group of glycoside **10** was removed by the action of Zn powdered in acetic acid, and then acetyl protective groups were removed by the action of sodium methoxide to give glycoside **16**. By analogy with ref. 32, glycoside **11** was deacetylated by hydrazine hydrate that allowed the introduction of pharmacophoric hydrazide and hydrazone groups in glycoside **17** simultaneously with the

Scheme 1 Synthesis of isosteviol glycosides. Reagents and conditions: (i) K₂CO₃, TBAB, CH₂Cl₂/H₂O = 10 : 1, 20–48 h, 50 °C; (ii) MeONa, MeOH, 2 h, 20 °C; (iii) Zn powdered, AcOH, Ac₂O, 20 °C, 24 h; (iv) NH₂NH₂·H₂O, MeOH, 20 °C.

deprotection of hydroxyl groups. The synthesis of isosteviol glycoconjugates with a carbohydrate residue moved away from the diterpenoid skeleton through the polymethylene linker is shown in Scheme 2. The reaction of isosteviol derivatives 18 and 19 with bromo derivatives of monosaccharides 3–5 in the presence of K_2CO_3 and TBAB led to glycoconjugates 20–25 with acetylated hydroxyl groups. The amine groups of glycoconjugates 24 and 25 were deprotected by Zn powdered in acetic acid to provide glycoconjugates 26 and 27. The deprotection of hydroxyl groups of glycoconjugates 20–23, 26, and 27 carried out according to the standard procedure (sodium methoxide in methanol at room temperature or even at $-40\text{ }^\circ\text{C}$) resulted in the hydrolysis of the glycosidic bond. Deacetylation reactions carried out in methanol in the presence of Na_2CO_3 at room temperature,³³ as well as in a solution of 10% Et_3N in a mixture of methanol–water–hexane,³⁴ also resulted in the hydrolysis of the glycosidic bond. The anomeric protons of glycosides 7, 8, 10, 12, 13, 15, and 16 and glycoconjugates 20, 21, 26, and 27 in the 1H NMR spectra resonated as a doublet at 5.3–5.8 ppm with coupling constants of 7.9–9.7 Hz indicating the formation of a β -anomer. The anomeric protons of glycosides 9 and 14 and glycoconjugates 22 and 23 resonated as doublets at 6.08–6.09 ppm with coupling constants of 1.7–2.0 Hz, which confirmed the α -orientation of the glycosidic bonds.

2.2 Biology

2.2.1 Antimicrobial and anticancer activities. It is widely known that diterpenoid glycosides³⁵ and triterpenoid saponins^{36–39} exhibit strong cytotoxicity against various human cancer cell lines, so one would expect compounds 7–9, 11–17, 20–23, 26 and 27 to be cytotoxic too. The *in vitro* cytotoxic activities of the isosteviol glycosides 7–9 and 11–17 and glycoconjugates 20–23, 26 and 27 were tested on two human cancer cell lines: human cervix epithelioid carcinoma (M-

HeLa) and breast adenocarcinoma pleural fluid (MCF-7), as well as a normal human Chang liver cell line. The results are presented in Table 1. One can see that five compounds, namely, 15, 23, 9, 20, and 22, were one and a half or three times more active against M-HeLa than the anti-cancer drug Tamoxifen. Three compounds, namely, 11, 12, and 21, showed activities that corresponded to the activity of Tamoxifen. Moreover, all the mentioned compounds were completely non-cytotoxic in relation to normal human Chang liver cells. Analysis of the dependence of cytotoxicity on the structure of the studied compounds leads to the following conclusions. First, isosteviol glycosides 7 and 8 with tetra-*O*-acetylated residues of D -glucopyranose (glycoside 7) and D -galactopyranose (glycoside 8) were completely inactive. At the same time, isosteviol glycosides with a tetra-*O*-acetylated D -mannopyranose residue (glycoside 9), a tri-*O*-acetylated D -glucuronic acid methyl ester residue (glycoside 11), and a tri-*O*-acetylated *N*-acetylglucosamine residue (glycoside 15) showed cytotoxicity against M-HeLa and MCF-7 that corresponds to the cytotoxicity of the anticancer drug Tamoxifen (Table 1). Moreover, the cytotoxicity of these glycosides increases (that is, IC_{50} values decrease) when passing from glycoside 11 with a tri-*O*-acetylated residue of D -glucuronic acid methyl ester to glycoside 9 with a tetra-*O*-acetylated D -mannopyranose residue.

Secondly, the removal of acetyl protection of carbohydrate residues in isosteviol glycosides with a tetra-*O*-acetylated D -glucopyranose residue (glycoside 7) and a tetra-*O*-acetylated D -galactopyranose residue (glycoside 8) resulted in the appearance of cytotoxicity against M-HeLa and MCF-7. Unlike glycosides 7 and 8, which did not show any cytotoxicity, the isosteviol glycoside with the D -glucopyranose residue 12 and the isosteviol glycoside with the D -galactopyranose residue 13 were moderately active with IC_{50} values of $32.0 \pm 2.8\text{ }\mu\text{M}$, and $52.0 \pm 4.5\text{ }\mu\text{M}$, respectively. The quite opposite situation is observed for isosteviol glycosides 15 and 16. The isosteviol glycoside 15 with a tri-*O*-acetylated *N*-acetylglucosamine

Scheme 2 Synthesis of isosteviol glycoconjugates with monosaccharides moved from the diterpenoid skeleton. Reagents and conditions: (i) K_2CO_3 , TBAB, CH_2Cl_2/H_2O , 20–48 h, $50\text{ }^\circ\text{C}$; (ii) Zn powdered, $AcOH$, Ac_2O , $20\text{ }^\circ\text{C}$, 24 h.

Table 1 Cytotoxic activity of glycosides and glycoconjugates of isosteviol against human cancer and human normal cell lines (IC₅₀ values in μM with standard error)

Compounds	IC ₅₀ $\pm$ SD ^a (μM)		
	Cancer cell lines		Normal cell line
	M-HeLa ^b	MCF-7 ^c	Chang liver
7	>100	>100	>100
8	>100	>100	>100
9	15.1 $\pm$ 1.2	29.3 $\pm$ 2.5	>100
11	26.0 $\pm$ 1.5	65.0 $\pm$ 4.5	>100
12	32.0 $\pm$ 2.8	>100	>100
13	52.0 $\pm$ 4.5	29.0 $\pm$ 2.6	58.0 $\pm$ 4.4
14	>100	>100	>100
15	18.0 $\pm$ 1.6	36.0 $\pm$ 2.7	86.0 $\pm$ 6.7
16	>100	>100	>100
17	>100	>100	>100
20	10.0 $\pm$ 0.7	32.1 $\pm$ 2.6	>100
21	27.0 $\pm$ 2.1	33.2 $\pm$ 2.4	>100
22	11.0 $\pm$ 0.9	63.2 $\pm$ 4.9	>100
23	18.0 $\pm$ 1.4	31.0 $\pm$ 2.6	>100
26	46 $\pm$ 3.7	>100	51.2 $\pm$ 4.8
27	>100	64.9 $\pm$ 4.9	>100
Tamoxifen	28.0 $\pm$ 2.5	25.0 $\pm$ 2.2	46.0 $\pm$ 3.5

^a IC₅₀: the biological data are generated from three independent experiments. ^b M-HeLa is a human cervix epithelioid carcinoma.

^c MCF-7 is a breast adenocarcinoma (pleural fluid).

residue showed good cytotoxicity against M-HeLa (IC₅₀ = 18.0 $\pm$ 1.6 μM) and moderate cytotoxicity against MCF-7 (IC₅₀ = 36.0 $\pm$ 2.7 μM), whereas the isosteviol glycoside **16** with an *N*-acetylglucosamine residue appeared to be inactive with respect to both the normal human cell line Chang liver, and human cancer cell lines M-HeLa and MCF-7. Thirdly, depending on the nature of the carbohydrate residue, its movement from the diterpenoid (isosteviol) skeleton with the help of a polymethylene linker influenced the cytotoxicity of the compound in different ways. Moving a tetra-*O*-acetylated D -galactopyranose residue away from the isosteviol skeleton to five or nine methylene units, that is, going from glycoside **8** to glycoconjugates **20** and **21**, led to the appearance of cytotoxicity. If glycoside **8** was not active, then glycoconjugate **21** showed cytotoxicity against M-HeLa and MCF-7 (IC₅₀ = 27.0 $\pm$ 2.1 μM and 33.2 $\pm$ 2.4 μM respectively) at the level of the anti-cancer drug Tamoxifen (IC₅₀ = 28 $\pm$ 2.5 μM). Moreover, the cytotoxicity of glycoconjugate **20** against M-HeLa (IC₅₀ = 10.0 $\pm$ 0.7 μM) appeared to be twice as high as the cytotoxicity of the anticancer drug Tamoxifen (IC₅₀ = 28 $\pm$ 2.5 μM). At the same time, moving a tetra-*O*-acetylated D -mannopyranose residue away from the isosteviol skeleton to five or nine methylene units, that is, the transition from glycoside **9** to glycoconjugates **22** and **23** did not lead to a change in cytotoxicity. Thus, glycoside **9** and glycoconjugates **22** and **23** showed almost the same cytotoxicity against M-HeLa (see Table 1). It is interesting to note that moving a three-*O*-acetylated *N*-acetylglucosamine residue away from the isosteviol skeleton to five or nine methylene units, that is, passing from glycoside **15** to glycoconjugates **26** and **27**, led to a dramatic decrease in cytotoxicity against M-HeLa. If glycoside

15 showed good cytotoxicity against M-HeLa with an IC₅₀ value of 18.0 $\pm$ 1.6 μM , which corresponded to the cytotoxicity of Tamoxifen (IC₅₀ = 28 $\pm$ 2.5), then the cytotoxicity of glycoconjugate **26** decreased markedly (IC₅₀ = 46 $\pm$ 3.7), and glycoconjugate **27** was completely inactive. The analysis of Table 1 allows the identification of three lead compounds which showed selective cytotoxicity against M-HeLa that was three times as high as the cytotoxicity of the anti-cancer drug Tamoxifen. These are isosteviol glycoside with the tetra-*O*-acetylated D -mannopyranose residue **9** (IC₅₀ = 15.1 $\pm$ 1.2 μM), isosteviol glycoconjugate with the tetra-*O*-acetylated D -galactopyranose residue **20** (IC₅₀ = 10.0 $\pm$ 0.7 μM), and isosteviol glycoconjugate with the tetra-*O*-acetylated D -mannopyranose residue **22** (IC₅₀ = 11.0 $\pm$ 0.9 μM). It should be emphasized that the lead compounds were not cytotoxic with respect to the normal human cell line Chang liver, whereas the anti-cancer drug Tamoxifen was cytotoxic against both M-HeLa (IC₅₀ = 28.0 $\pm$ 2.5 μM) and MCF-7 (IC₅₀ = 25.0 $\pm$ 2.2 μM), as well as the normal human cell line Chang liver (IC₅₀ = 46.0 $\pm$ 3.5 μM).

2.2.2 Induction of apoptotic effects by the lead compounds. To examine whether the observed cytotoxicity of the lead compounds **9**, **20** and **22** is due to induction of apoptosis, Annexin V-FITC/PI dual staining assay was performed after treatment of M-HeLa cells. As shown in Fig. 1, after 24 h of treatment, significant apoptotic effects were observed for all compounds in M-HeLa cells. Analysis of the apoptotic effects in Fig. 2 shows that after incubation of cells with compounds **20** and **22**, the number of cells in early apoptosis is

Fig. 1 Apoptotic effects of compounds **9**, **20** and **22** on M-HeLa cells. M-HeLa cells were treated with the compounds at indicated concentrations for 24 h. Apoptotic effects were measured by flow cytometry using annexin V-FITC staining protocol. The values are presented as the mean $\pm$ SD (n = 3).

Fig. 2 Representative histograms for the numbers of cells (% of total) in the early and late stages of apoptosis for the control and treatment groups. The values are presented as the mean $\pm$ SD ($n = 3$); (*) $P < 0.01$ compared to control.

much higher in percentage than in late apoptosis. With increasing concentrations of compounds 20 and 22, this effect is enhanced. In the case of compound 9, a different picture is observed. At a concentration of 10 μ M, more cells are in late apoptosis. With an increase in the concentration of compound 9, the numbers of cells in early and late apoptosis are almost the same. It is possible that the differences in the course of apoptotic effects are caused by the structural features of glycoside 9 on the one hand, and of glycoconjugates 20 and 22 on the other. In glycoside 9, a tri-*O*-acetylated β -mannopyranosyl residue was attached directly to the diterpenoid (isosteviol) skeleton, while in glycoconjugates 20 and 22 a monosaccharide residue was moved away from the isosteviol skeleton by five methylene units.

2.2.3 Effects on the mitochondrial membrane potential ($\Delta\psi_m$) by the lead compounds. Next, we examined the possible mechanism of action of the lead compounds 9, 20, 22 on cancer cells. We evaluated their ability to cause a decrease in the mitochondrial membrane potential ($\Delta\psi_m$) in the cells of the M-HeLa culture with the help of flow cytometry methods using JC-10 reagent. In normal cells with a high mitochondrial membrane potential, the dye JC-10 forms aggregates (J-aggregate), near the mitochondrial membranes. When the membrane potential, due to the stimulation of apoptosis, falls, JC-10 is evenly distributed in the cell as a monomer (J-monomer). JC-10 aggregates in normal cells have red fluorescence, while JC-10 monomers are green. The ratio between orange-red and green fluorescence can be used to judge the onset of apoptosis. A reduction in $\Delta\psi_m$ was demonstrated using flow cytometry analysis (Fig. 3). The intensity of the red fluorescence decreased with increasing concentration of the test compound. The results obtained suggest that the mechanism of action of the studied compounds may be associated with the induction of apoptosis, which proceeds along the mitochondrial pathway.

2.2.4 Hemolytic activity of the lead compounds. For the lead compounds 9, 20, 22 the *in vitro* hemolytic effect was investigated both at concentrations close to the IC_{50} values,

Fig. 3 Flow cytometry analysis of M-HeLa cells treated with compounds 9, 20 and 22, along with the quantification of % of cells with red aggregates. The values are presented as mean $\pm$ SD.

Table 2 Hemolytic activity of the lead compounds^a

Compounds	Concentration, μ M	Hemolysis, %
9	10.0 $\pm$ 0.8	0
	50.0 $\pm$ 4.3	0
	100.0 $\pm$ 7.8	0
20	10.0 $\pm$ 0.8	0
	50.0 $\pm$ 4.3	0
	100.0 $\pm$ 7.8	0
22	10.0 $\pm$ 0.8	0
	50.0 $\pm$ 4.3	1.7 $\pm$ 0.1
	100.0 $\pm$ 7.8	5.5 $\pm$ 0.45

^a The biological data are generated from three independent experiments.

and at concentrations exceeding the IC_{50} values five and ten times (Table 2). It can be seen that lead compounds 9 and 20 did not cause the rupturing of human erythrocytes even at concentration of 100 μ M. The lead compound 22 showed very low hemolytic activity only at a concentration of 50 μ M. Even at a concentration of 100 μ M, hemolysis did not exceed 6%.

3. Conclusions

In summary, we synthesized a series of glycosides and glycoconjugates of diterpenoid isosteviol (16-oxo-*ent*-beyeran-19-oic acid) with various monosaccharide residues and assayed their cytotoxicity against some human cancer and normal cell lines. Most of the synthesized compounds demonstrated moderate to significant cytotoxicity against human cancer cell lines M-HeLa and MCF-7. Three lead compounds 9, 20 and 22 exhibited selective cytotoxic activities against M-HeLa ($IC_{50} = 10.0$ – 15.1 μ M) which were three times better

than the cytotoxicity of the anti-cancer drug Tamoxifen ($IC_{50} = 28.0 \mu M$). Moreover, the lead compounds were not cytotoxic with respect to normal human cell line Chang liver ($IC_{50} > 100 \mu M$), whereas Tamoxifen inhibited the viability of normal human cells Chang liver with IC_{50} value of $46.0 \pm 3.5 \mu M$. It was determined that the cytotoxicity of the lead compounds 9, 20 and 22 was due to induction of apoptosis proceeding along the mitochondrial pathway. The cytotoxic activity of the synthesized compounds substantially depended on the nature of a monosaccharide residue and its position, that is, whether a monosaccharide residue was attached directly to the isosteviol skeleton or was moved away from it by means of a polymethylene linker.

4. Experimental section

4.1 Chemistry

The 1H spectra were recorded on a 400 MHz Bruker Advance. ^{13}C NMR spectra were obtained using the above instrument operating at 100.6 MHz. Melting points were obtained on an Electrothermal IA 9000 instrument (Electrothermal, Great Britain). Mass spectra (MALDI) were recorded in positive and negative ion mode on a Bruker Ultraflex III TOF/TOF mass spectrometer for $10^{-3} \text{ mg mL}^{-1}$ solutions in MeOH. Optical rotations were measured using a PerkinElmer-341 automatic digital polarimeter (USA). Flash chromatography was performed on silica gel 60 (40–63 μm , Buchi, Sepacore). Thin-layer chromatography was carried out on plates with silica gel (Sorbfil, Russia). Spots of compounds were visualized by heating the plates (at ca. 150 °C) after immersion in 5% H_2SO_4 and 95% H_2O . All reactions sensitive to air and/or moisture were carried out under an argon atmosphere with anhydrous solvents. Anhydrous solvents were purified and dried (where appropriate) according to standard procedures. D-Galactose, D-mannose, D-glucosamine hydrochloride and 1,2,3,4,6-penta-O-acetyl- β -D-glucopyranose were purchased from Acros (Belgium).

4.1.1 Synthesis of key intermediate 1, 2–6, 11, 17–19. Iso-steviol 1 and its derivatives 18 and 19 were synthesized analogous to the literature.^{18,40} The spectral data of compounds 1, 18, and 19 agreed with those published in the literature.^{31,41} Monosaccharides 2–6 were prepared according to protocols described in ref. 42–45 correspondingly. Their spectral data were in keeping with published ones.^{42–45} Glycosides 11 and 17 were described previously.³²

4.1.2 General procedure for the synthesis of glycosides 7–9, 20–23. Acetylated bromomonosaccharides 2–4 (1.3 mol), K_2CO_3 (2.4 mol), and TBAB (1.3 mol) were added to a stirred solution of isosteviol 1 (1 mol) or its derivatives 18 or 19 (1 mol) in a 10:1 CH_2Cl_2/H_2O mixture (22 mL) and the reaction mixture was refluxed until isosteviol 1 or its derivatives 18 or 19 had completely reacted, as indicated by TLC (30–40 h). After cooling to 25 °C, $CHCl_3$ was added and the mixture was washed with water and brine. The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography

eluted with petroleum ether/EtOAc to afford the corresponding isosteviol glycosides 7–9 and glycoconjugates 20–23.

4.1.2.1. 2,3,4,6-Tetra-O-acetyl- β -D-glucopyranosyl-16-oxo-ent-beyeran-19-oate (7). Pure white crystals; yield: 0.63 g (60%); mp: 180 °C; $[\alpha]_D^{20} = -44.0$ (1; CH_2Cl_2). Eluent: petroleum ether/EtOAc = 3:1. 1H NMR (400 MHz, $CDCl_3$): δ 0.69 (s, 3H, H-20), 0.96 (s, 3H, H-17), 1.19 (s, 3H, H-18), 0.85–1.89 (m, 18H, ent-beyerane), 2.00 (s, 3H, CH_3CO), 2.02 (s, 6H, 2 CH_3CO), 2.05 (s, 3H, CH_3CO), 2.15 (d, 1H, $J = 13.5$ Hz, H-3 eq.), 2.54 (dd, 1H, $J = 18.6, 3.5$ Hz, H-15 α), 3.77–3.81 (m, 1H, H-5'), 4.06 (dd, 1H, $J = 12.3, 1.9$ Hz, H-6'a), 4.27 (dd, 1H, $J = 12.3, 5.0$ Hz, H-6'b), 5.10 (t, 1H, $J = 9.6$ Hz, H-4'), 5.18 (t, 1H, $J = 8.7$ Hz, H-2'), 5.23 (t, 1H, $J = 9.2$ Hz, H-3'), 5.71 (d, 1H, $J = 8.1$ Hz, H-1'). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 13.67 (C-20), 18.98 (C-2), 19.96 (C-17), 20.46 (C-11), 20.68 (2 CH_3CO), 20.80 (CH_3CO), 20.85 (CH_3CO), 21.63 (C-6), 28.98 (C-18), 37.38 (C-3), 37.95 (C-10), 38.25 (C-12), 39.52 (C-13), 39.78 (C-1), 41.58 (C-7), 44.23 (C-4), 48.64 (C-8), 48.79 (C-15), 54.44 (C-14), 54.90 (C-9), 57.20 (C-5), 61.81 (C-6'), 68.31 (C-4'), 70.35 (C-2'), 72.67 (C-3'), 73.21 (C-5'), 91.35 (C-1'), 169.19 (CH_3CO), 169.52 (CH_3CO), 170.19 (CH_3CO), 170.61 (CH_3CO), 175.58 (C-19), 222.11 (C-16). MALDI-MS: m/z : 671.42 $[M + Na]^+$, 687.42 $[M + K]^+$. Anal., %: C 63.09; H 7.52. $C_{34}H_{48}O_{12}$. Calcd., %: C 62.95; H 7.46. M 648.74.

4.1.2.2. 2,3,4,6-Tetra-O-acetyl- β -D-galactopyranosyl-16-oxo-ent-beyeran-19-oate (8). White foam; yield: 0.55 g (45%); $[\alpha]_D^{20} = -43.0$ (1; CH_2Cl_2). Eluent: petroleum ether/EtOAc = 3:1. 1H NMR (400 MHz, $CDCl_3$): δ 0.69 (s, 3H, H-20), 0.97 (s, 3H, H-17), 1.19 (s, 3H, H-18), 0.81–1.91 (m, 18H, ent-beyerane), 1.98 (s, 3H, CH_3CO), 2.01 (s, 3H, CH_3CO), 2.03 (s, 3H, CH_3CO), 2.17 (s, 4H, CH_3CO , H-3 eq.), 2.56 (dd, 1H, $J = 18.6, 3.2$ Hz, H-15 α), 4.01 (t, 1H, $J = 6.7$ Hz, H-5'), 4.06–4.16 (m, 2H, H-6'a, H-6'b), 5.07 (dd, 1H, $J = 10.3, 3.5$ Hz, H-3'), 5.34 (t, 1H, $J = 9.4$ Hz, H-2'), 5.41 (d, 1H, $J = 2.1$ Hz, H-4'), 5.68 (d, 1H, $J = 8.3$ Hz, H-1'). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 13.69 (C-20), 19.04 (C-2), 19.96 (C-17), 20.45 (C-11), 20.62 (CH_3CO), 20.73 (CH_3CO), 20.80 (CH_3CO), 20.96 (CH_3CO), 21.59 (C-6), 29.06 (C-18), 37.39 (C-3), 37.91 (C-10), 38.25 (C-12), 39.52 (C-13), 39.78 (C-1), 41.58 (C-7), 44.23 (C-4), 48.65 (C-8), 48.80 (C-15), 54.46 (C-14), 54.90 (C-9), 57.25 (C-5), 60.93 (C-6'), 66.92 (C-4'), 67.89 (C-2'), 71.24 (C-3'), 71.66 (C-5'), 91.79 (C-1'), 169.35 (CH_3CO), 170.03 (CH_3CO), 170.33 (CH_3CO), 170.39 (CH_3CO), 175.64 (C-19), 222.19 (C-16). MALDI-MS: m/z : 671.45 $[M + Na]^+$. Anal., %: C 63.03; H 7.48. $C_{34}H_{48}O_{12}$. Calcd., %: C 62.95; H 7.46. M 648.74.

4.1.2.3. 2,3,4,6-Tetra-O-acetyl- α -D-mannopyranosyl-16-oxo-ent-beyeran-19-oate (9). White foam; yield: 0.1 g (8%); $[\alpha]_D^{20} = -32.7$ (0.5; CH_2Cl_2). Eluent: petroleum ether/EtOAc = 3:1. 1H NMR (400 MHz, $CDCl_3$): δ 0.76 (s, 3H, H-20), 0.98 (s, 3H, H-17), 1.28 (s, 3H, H-18), 0.80–1.99 (m, 18H, ent-beyerane), 2.00 (s, 3H, CH_3CO), 2.07 (s, 3H, CH_3CO), 2.09 (s, 3H, CH_3CO), 2.17 (s, 3H, CH_3CO), 2.21 (d, 1H, $J = 12.7$ Hz, H-3 eq.), 2.63 (dd, 1H, $J = 18.6, 3.6$ Hz, H-15 α), 4.00–4.06 (m, 1H, H-5'), 4.09 (dd, 1H, $J = 12.3, 2.6$ Hz, H-6'a), 4.28 (dd, 1H, $J = 12.2, 5.7$ Hz, H-6'b), 5.22–5.36 (m, 3H, H-3', H-2', H-4'), 6.08 (d, 1H, $J = 2.0$ Hz, H-1'). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 13.63 (C-20), 18.79 (C-2), 19.79 (C-17), 20.34 (C-11), 20.52 (CH_3CO), 20.53

(CH_3CO), 20.64 (CH_3CO), 20.70 (CH_3CO), 21.81 (C-6), 28.90 (C-18), 37.20 (C-3), 37.88 (C-10), 38.11 (C-12), 39.43 (C-13), 39.67 (C-1), 41.44 (C-7), 44.31 (C-4), 48.27 (C-8), 48.66 (C-15), 54.20 (C-14), 54.63 (C-9), 56.87 (C-5), 62.50 (C-6'), 65.50 (C-4'), 67.89 (C-5'), 71.24 (C-3'), 71.62 (C-2'), 90.79 (C-1'), 169.47 (CH_3CO), 169.59 (CH_3CO), 169.92 (CH_3CO), 170.45 (CH_3CO), 174.37 (C-19), 221.83 (C-16). MALDI-MS: m/z : 671.55 [$\text{M} + \text{Na}$] $^+$. Anal., %: C 63.13; H 7.58. $\text{C}_{34}\text{H}_{48}\text{O}_{12}$. Calcd., %: C 62.95; H 7.46. M 648.74.

4.1.2.4. (2,3,4,6-Tetra-O-acetyl- β -D-galactopyranosyloxopentyl)-16-oxo-ent-beyeran-19-oate (20). White foam; yield: 0.31 g (49%); $[\alpha]_{\text{D}}^{20} = -38.8$ (0.8; CH_2Cl_2). Eluent: petroleum ether/EtOAc = 5:1. ^1H NMR (400 MHz, CDCl_3): δ 0.70 (s, 3H, H-20), 0.98 (s, 3H, H-17), 1.19 (s, 3H, H-18), 0.81–1.94 (m, 24H, ent-beyerane, $(\text{CH}_2)_3$ – spacer), 1.99 (s, 3H, CH_3CO), 2.03 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO), 2.16 (s, 4H, CH_3CO , H-3 eq.), 2.38 (td, 2H, $J = 7.4$, 3.0 Hz, H-2''), 2.62 (dd, 1H, $J = 18.6$, 3.7 Hz, H-15 α), 3.93–4.18 (m, 5H, H-5', H-6'', H-6'a, H-6'b), 5.09 (dd, 1H, $J = 10.4$, 3.4 Hz, H-3'), 5.33 (dd, 1H, $J = 10.4$, 8.3 Hz, H-2'), 5.43 (d, 1H, $J = 3.0$ Hz, H-4'), 5.72 (d, 1H, $J = 8.3$ Hz, H-1'). ^{13}C NMR (100.6 MHz, CDCl_3): δ 13.53 (C-20), 19.10 (C-2), 19.96 (C-17), 20.46 (C-11), 20.62 (CH_3CO), 20.71 (CH_3CO), 20.73 (CH_3CO), 20.96 (CH_3CO), 21.85 (C-6), 24.21, 25.69, 28.28 (C-2''–C-4''), 29.09 (C-18), 33.96 (C-5''), 37.44 (C-3), 38.08 (C-10), 38.15 (C-12), 39.58 (C-13), 39.98 (C-1), 41.66 (C-7), 43.96 (C-4), 48.55 (C-8), 48.81 (C-15), 54.43 (C-14), 54.86 (C-9), 57.22 (C-5), 61.08 (C-6'), 63.94 (C-6''), 66.94 (C-4'), 68.05 (C-2'), 70.92 (C-3'), 71.85 (C-5'), 92.23 (C-1'), 169.38 (CH_3CO), 170.01 (CH_3CO), 170.20 (CH_3CO), 170.39 (CH_3CO), 171.49 (C-1''), 177.38 (C-19), 222.40 (C-16). MALDI-MS: m/z : 785.5 [$\text{M} + \text{Na}$] $^+$, 801.5 [$\text{M} + \text{K}$] $^+$. Anal., %: C 62.91; H 7.69. $\text{C}_{40}\text{H}_{58}\text{O}_{14}$. Calcd., %: C 62.98; H 7.66. M 762.88.

4.1.2.5. (2,3,4,6-Tetra-O-acetyl- β -D-galactopyranosyloxononyl)-16-oxo-ent-beyeran-19-oate (21). White foam; yield: 0.22 g (39%); $[\alpha]_{\text{D}}^{20} = -46.2$ (1; CH_2Cl_2). Eluent: petroleum ether/EtOAc = 6:1. ^1H NMR (400 MHz, CDCl_3): δ 0.70 (s, 3H, H-20), 0.97 (s, 3H, H-17), 1.18 (s, 3H, H-18), 0.86–1.92 (m, 32H, ent-beyerane, $(\text{CH}_2)_7$ – spacer), 1.99 (s, 3H, CH_3CO), 2.02 (s, 3H, CH_3CO), 2.03 (s, 3H, CH_3CO), 2.16 (s, 4H, CH_3CO , H-3 eq.), 2.33–2.98 (m, 2H, H-2''), 2.62 (dd, 1H, $J = 18.5$, 3.7 Hz, H-15 α), 3.94–4.17 (m, 5H, H-5', H-10'', H-6'a, H-6'b), 5.08 (dd, 1H, $J = 10.4$, 3.4 Hz, H-3'), 5.31–5.34 (m, 1H, H-2'), 5.42 (d, 1H, $J = 3.3$ Hz, H-4'), 5.70 (d, 1H, $J = 8.3$ Hz, H-1'). ^{13}C NMR (100.6 MHz, CDCl_3): δ : 13.56 (C-20), 19.14 (C-2), 19.99 (C-17), 20.49 (C-11), 20.65 (CH_3CO), 20.74 ($3\text{CH}_3\text{CO}$), 21.89 (C-6), 24.66, 26.27, 28.63 (C-6''–C-8''), 29.06 (C-18), 29.11, 29.24, 29.27, 29.43 (C-2''–C-5''), 34.17 (C-9''), 37.49 (C-3), 38.14 (C-10), 38.20 (C-12), 39.63 (C-13), 40.05 (C-1), 41.73 (C-7), 43.99 (C-4), 48.60 (C-8), 48.85 (C-15), 54.49 (C-14), 54.90 (C-9), 57.28 (C-5), 61.12 (C-6'), 64.41 (C-10''), 66.98 (C-4'), 68.06 (C-2'), 71.01 (C-3'), 71.86 (C-5'), 92.21 (C-1'), 169.38 (CH_3CO), 170.05 (CH_3CO), 170.23 (CH_3CO), 170.41 (CH_3CO), 171.88 (C-1''), 177.48 (C-19), 222.44 (C-16). MALDI-MS: m/z : 841.5 [$\text{M} + \text{Na}$] $^+$. Anal., %: C 64.58; H 8.08. $\text{C}_{44}\text{H}_{66}\text{O}_{14}$. Calcd., %: C 64.53; H 8.12. M 818.99.

4.1.2.6. (2,3,4,6-Tetra-O-acetyl- α -D-mannopyranosyloxopentyl)-16-oxo-ent-beyeran-19-oate (22). Colorless oil; yield 0.08 g (20%); $[\alpha]_{\text{D}}^{20} = -43.5$ (0.6; CH_2Cl_2). Eluent: petroleum ether/EtOAc = 5:1. ^1H NMR (400 MHz, CDCl_3): δ 0.70 (s, 3H, H-20), 0.96 (s, 3H, H-17), 1.18 (s, 3H, H-18), 0.85–1.93 (m, 24H, ent-beyerane, $(\text{CH}_2)_3$ – spacer), 1.99 (s, 3H, CH_3CO), 2.05 (s, 3H, CH_3CO), 2.08 (s, 3H, CH_3CO), 2.17 (s, 4H, CH_3CO , H-3 eq.), 2.42 (t, 2H, $J = 7.4$ Hz, H-2''), 2.62 (dd, 1H, $J = 18.6$, 3.6 Hz, H-15 α), 3.96–4.13 (m, 4H, H-5', H-6'', H-6'a), 4.28 (dd, 1H, $J = 12.3$, 4.9 Hz, H-6'b), 5.23–5.26 (m, 1H, H-3'), 5.31–5.35 (m, 2H, H-2', H-4'), 6.09 (d, 1H, $J = 1.8$ Hz, H-1'). ^{13}C NMR (100.6 MHz, CDCl_3): δ 13.42 (C-20), 18.98 (C-2), 19.85 (C-17), 20.33 (C-11), 20.60 (CH_3CO), 20.69 ($2\text{CH}_3\text{CO}$), 20.75 (CH_3CO), 21.74 (C-6), 24.21, 25.65, 28.20 (C-2''–C-4''), 28.98 (C-18), 33.82 (C-5''), 37.33 (C-3), 37.95 (C-10), 38.03 (C-12), 39.46 (C-13), 39.85 (C-1), 41.52 (C-7), 43.84 (C-4), 48.43 (C-8), 48.68 (C-15), 54.30 (C-14), 54.74 (C-9), 57.11 (C-5), 62.13 (C-6'), 63.85 (C-6''), 65.57 (C-4'), 68.39 (C-5'), 68.79 (C-3'), 70.70 (C-2'), 90.57 (C-1'), 169.49 (CH_3CO), 169.68 (CH_3CO), 169.92 (CH_3CO), 170.47 (CH_3CO), 170.54 (C-1''), 177.27 (C-19), 222.29 (C-16). MALDI-MS: m/z : 785.3 [$\text{M} + \text{Na}$] $^+$, 801.3 [$\text{M} + \text{K}$] $^+$. Anal., %: C 63.04; H 7.71. $\text{C}_{40}\text{H}_{58}\text{O}_{14}$. Calcd., %: C 62.98; H 7.66. M 762.88.

4.1.2.7. (2,3,4,6-Tetra-O-acetyl- α -D-mannopyranosyloxononyl)-16-oxo-ent-beyeran-19-oate (23). Colorless oil; yield: 0.06 g (13%); $[\alpha]_{\text{D}}^{20} = -47.6$ (0.5; CH_2Cl_2). Eluent: petroleum ether/EtOAc = 4:1. ^1H NMR (400 MHz, CDCl_3): δ 0.70 (s, 3H, H-20), 0.96 (s, 3H, H-17), 1.18 (s, 3H, H-18), 0.79–1.94 (m, 32H, ent-beyerane, $(\text{CH}_2)_7$ – spacer), 1.99 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO), 2.08 (s, 3H, CH_3CO), 2.17 (s, 4H, CH_3CO , H-3 eq.), 2.40 (t, 2H, $J = 7.5$ Hz, H-2''), 2.62 (dd, 1H, $J = 18.4$, 3.6 Hz, H-15 α), 3.95–4.11 (m, 4H, H-5', H-10'', H-6'a), 4.27 (dd, 1H, $J = 12.3$, 5.0 Hz, H-6'b), 5.25 (s, 1H, H-3'), 5.31–5.36 (m, 2H, H-2', H-4'), 6.09 (d, 1H, $J = 1.7$ Hz, H-1'). ^{13}C NMR (100.6 MHz, CDCl_3): δ 13.39 (C-20), 18.97 (C-2), 19.83 (C-17), 20.33 (C-11), 20.59 (CH_3CO), 20.62, 20.66, 20.72 ($3\text{CH}_3\text{CO}$), 21.73 (C-6), 24.61, 26.12, 28.47 (C-6''–C-8''), 28.95 (C-4'', C-5''), 29.11, 29.12 (C-2''–C-3''), 29.28 (C-18), 34.00 (C-9''), 37.32 (C-3), 37.97 (C-10), 38.03 (C-12), 39.46 (C-13), 39.88 (C-1), 41.56 (C-7), 43.83 (C-4), 48.43 (C-8), 48.68 (C-15), 54.31 (C-14), 54.73 (C-9), 57.11 (C-5), 62.13 (C-6'), 64.27 (C-10''), 65.60 (C-4'), 68.40 (C-5'), 68.81 (C-3'), 70.62 (C-2'), 90.44 (C-1'), 169.48 (CH_3CO), 169.67 (CH_3CO), 169.89 (CH_3CO), 170.54 (CH_3CO), 170.81 (C-1''), 177.33 (C-19), 222.32 (C-16). MALDI-MS: m/z : 841.9 [$\text{M} + \text{Na}$] $^+$. Anal., %: C 64.62; H 8.03. $\text{C}_{44}\text{H}_{66}\text{O}_{14}$. Calcd., %: C 64.53; H 8.12. M 818.99.

4.1.3 General procedure for the synthesis of glycosides 15, 26 and 27. Acetylated bromomonosaccharide 5 (1.3 mol), K_2CO_3 (2.4 mol), and TBAB (1.3 mol) were added to a stirred solution of isosteviol 1 (1 mol) or its derivatives 18 or 19 (1 mol) in a 10:1 $\text{CH}_2\text{Cl}_2/\text{H}_2\text{O}$ mixture (22 mL) and the reaction mixture was refluxed until isosteviol 1 or its derivatives 18 or 19 had completely reacted, as indicated by TLC (30–40 h). After cooling to 25 °C, CHCl_3 was added and the mixture washed with water and brine. The organic layer was dried over MgSO_4 , filtered, and concentrated under reduced

pressure, and the residue was used without further purification in the next step. A suspension of compounds **10**, **24**, or **25** (1 mol) and Zn powdered (19 mol) in AcOH (10 mL) was stirred for 10 min at room temperature; then Ac₂O (1 mL) was added, and the mixture was stirred for 24 h at room temperature. The suspension was filtered, washed with CH₂Cl₂ and concentrated. The residue was diluted with CH₂Cl₂ and washed with water. The organic layer was separated, dried over MgSO₄, and concentrated. Chromatography of the residue gave compounds **15**, **26** or **27**.

4.1.3.1. (3,4,6-Tri-O-acetyl-2-deoxy-2-acetamido-β-D-glucopyranosyl-16-oxo-ent-beyeran-19-oate (15). Colorless oil; yield: 0.17 g (37%), $[\alpha]_D^{20} = -25.5$ (1.7; CHCl₃). Eluent: petroleum ether/EtOAc = 1:2. ¹H NMR (400 MHz, CDCl₃): δ 0.69 (s, 3H, H-20), 0.97 (s, 3H, H-17), 1.18 (s, 3H, H-18), 0.73–1.93 (m, 18H, *ent*-beyerane), 2.02 (s, 3H, CH₃CO), 2.03 (s, 3H, CH₃CO), 2.04 (s, 3H, CH₃CO), 2.06 (s, 3H, CH₃CO), 2.20 (d, 1H, *J* = 13.5 Hz, H-3 eq.), 2.57 (dd, 1H, *J* = 18.8, 3.6 Hz, H-15α), 3.72–3.80 (m, 1H, H-5'), 4.04–4.15 (m, 1H, H-6'a), 4.26 (dd, 1H, *J* = 12.3, 5.2 Hz, H-6'b), 4.32–4.41 (m, 1H, H-2'), 5.04–5.15 (m, 2H, H-3', H-4'), 5.46 (d, 1H, *J* = 9.8 Hz, NHC(O)CH₃), 5.64 (d, 1H, *J* = 8.8 Hz, H-1'). ¹³C NMR (100.6 MHz, CDCl₃): δ 13.66 (C-20), 19.03 (C-2), 19.97 (C-17), 20.46 (C-11), 20.71 (CH₃CO), 20.75 (CH₃CO), 20.83 (CH₃CO), 21.56 (C-6), 23.42 (CH₃CONH), 29.05 (C-18), 37.41 (C-3), 37.77 (C-10), 38.26 (C-12), 39.54 (C-13), 39.76 (C-1), 41.59 (C-7), 44.24 (C-4), 48.69 (C-8), 48.81 (C-15), 52.79 (C-2'), 54.44 (C-14), 54.87 (C-9), 57.28 (C-5), 62.00 (C-6'), 68.25 (C-4'), 72.86 (C-3'), 73.15 (C-5'), 92.16 (C-1'), 169.39 (CH₃CO), 169.86 (CH₃CO), 170.70 (CH₃CO), 171.37 (CH₃CO), 176.17 (C-19), 222.37 (C-16). MALDI-MS: *m/z*: 671.3 [M + Na + H]⁺. Anal., %: C 63.50; H 7.59; N 2.19. C₃₄H₄₉NO₁₁. Calcd., %: C 63.04; H 7.62; N 2.16. M 647.75.

4.1.3.2. (3,4,6-Tri-O-acetyl-2-deoxy-2-acetamido-β-D-glucopyranosyloxopentyl)-16-oxo-ent-beyeran-19-oate (26). Colorless oil; yield: 0.25 g (95%), $[\alpha]_D^{20} = -29.7$ (2.9; CHCl₃). ¹H NMR (400 MHz, CDCl₃): δ 0.69 (s, 3H, H-20), 0.96 (s, 3H, H-17), 1.17 (s, 3H, H-18), 0.77–1.96 (m, 24H, *ent*-beyerane, (CH₂)₃ – spacer), 1.89 (s, 3H, CH₃CO), 2.02 (s, 3H, CH₃CO), 2.03 (s, 3H, CH₃CO), 2.07 (s, 3H, CH₃CO), 2.15 (d, 1H, *J* = 13.9 Hz, H-3 eq.), 2.30–2.42 (m, 2H, H-2''), 2.60 (dd, 1H, *J* = 18.6, 3.3 Hz, H-15α), 3.77–3.83 (m, 1H, H-5'), 3.94–4.04 (m, 2H, H-6''), 4.07–4.13 (m, 1H, H-6'a), 4.19–4.29 (m, 2H, H-2', H-6'b), 5.05–5.21 (m, 2H, H-3', H-4'), 5.72 (d, 1H, *J* = 8.8 Hz, H-1'), 5.87 (d, 1H, *J* = 9.4 Hz, NHC(O)CH₃). ¹³C NMR (100.6 MHz, CDCl₃): δ 13.54 (C-20), 19.08 (C-2), 19.96 (C-17), 20.47 (C-11), 20.68 (CH₃CO), 20.74 (CH₃CO), 20.82 (CH₃CO), 21.87 (C-6), 23.24 (CH₃CONH), 24.13, 25.69, 28.25 (C-2''–C-4''), 29.07 (C-18), 33.97 (C-5''), 37.43 (C-3), 38.10 (C-10), 38.15 (C-12), 39.60 (C-13), 39.98 (C-1), 41.64 (C-7), 43.97 (C-4), 48.55 (C-8), 48.86 (C-15), 53.25 (C-2'), 54.41 (C-14), 54.81 (C-9), 57.19 (C-5), 61.81 (C-6'), 63.94 (C-6''), 68.02 (C-4'), 72.73 (C-3'), 72.99 (C-5'), 92.61 (C-1'), 169.38 (CH₃CO), 170.18 (CH₃CO), 170.75 (CH₃CO), 171.20 (C-1'), 172.04 (CH₃CONH), 177.49 (C-19), 222.74 (C-16). MALDI-MS: *m/z*: 784.7 [M + Na]⁺, 800.7 [M + K]⁺. Anal., %: C 62.99; H 7.77; N 1.85. C₄₀H₅₉NO₁₃. Calcd., %: C 63.06; H 7.81; N 1.84. M 761.9.

4.1.3.3. (3,4,6-Tri-O-acetyl-2-deoxy-2-acetamido-β-D-glucopyranosyloxononyl)-16-oxo-ent-beyeran-19-oate (27). Colorless foam; yield: 0.16 g (20%); $[\alpha]_D^{20} = -20.9$ (0.7; CH₃OH). ¹H NMR (400 MHz, CDCl₃): δ 0.70 (s, 3H, H-20), 0.97 (s, 3H, H-17), 1.18 (s, 3H, H-18), 0.84–1.87 (m, 32H, *ent*-beyerane, (CH₂)₇ – spacer), 1.90 (s, 3H, CH₃CO), 2.02 (s, 3H, CH₃CO), 2.03 (s, 3H, CH₃CO), 2.07 (s, 3H, CH₃CO), 2.17 (d, 1H, *J* = 13.0 Hz, H-3 eq.), 2.35 (t, 2H, *J* = 7.5 Hz, H-2''), 2.62 (dd, 1H, *J* = 18.6, 3.7 Hz, H-15α), 3.75–3.82 (m, 1H, H-5'), 3.94–4.06 (m, 2H, H-10''), 4.11 (dd, 1H, *J* = 12.4, 2.2 Hz, H-6'a), 4.22–4.34 (m, 2H, H-2', H-6'b), 5.10–5.17 (m, 2H, H-3', H-4'), 5.69 (d, 1H, *J* = 8.8 Hz, H-1'), 5.73 (d, 1H, *J* = 9.6 Hz, NHC(O)CH₃). ¹³C NMR (100.6 MHz, CDCl₃): δ 13.57 (C-20), 19.15 (C-2), 20.01 (C-17), 20.47 (C-11), 20.71 (CH₃CO), 20.76 (CH₃CO), 20.84 (CH₃CO), 21.91 (C-6), 23.31 (CH₃CONH), 24.66 (C-6''), 26.26 (C-7''), 28.58 (C-8''), 29.13, 29.18, 29.22, 29.39 (C-2''–C-5''), 29.01 (C-18), 34.19 (C-9''), 37.52 (C-3), 38.15 (C-10), 38.21 (C-12), 40.06 (C-13), 39.65 (C-1), 41.73 (C-7), 44.01 (C-4), 48.62 (C-8), 48.90 (C-15), 53.21 (C-2'), 54.48 (C-14), 54.89 (C-9), 57.28 (C-5), 61.84 (C-6'), 64.41 (C-10''), 67.98 (C-4'), 72.86 (C-3'), 73.13 (C-5'), 92.64 (C-1'), 169.35 (CH₃CO), 170.04 (CH₃CO), 170.77 (CH₃CO), 171.24 (C-1''), 172.45 (CH₃CONH), 177.54 (C-19), 222.71 (C-16). MALDI-MS: *m/z*: 840.8 [M + Na]⁺, 856.7 [M + K]⁺. Anal., %: C 64.54; H 8.29; N 1.75. C₄₄H₆₇NO₁₃. Calcd., %: C 64.61; H 8.26; N 1.71. M 818.

4.1.4 General procedure for the preparation of compounds 12–14 and 16. The corresponding compounds **7–9** and **15** were dissolved in anhydrous MeOH at room temperature and the pH was adjusted to 9.0 using 0.1 N MeONa/MeOH. The deacetylation procedure was monitored by TLC and upon its completion the pH adjusted to 7.0 with acidic ion-exchange resin Amberlyst 15. After filtration, the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography eluted with petroleum ether/EtOAc to afford the corresponding glycosides **12–14**, and **16**.

4.1.4.1. β-D-Glucopyranosyl-16-oxo-ent-beyeran-19-oate (12). White foam; yield: 0.36 g (92%), $[\alpha]_D^{20} = -30.5$ (0.5; CH₂Cl₂). Eluent: CH₂Cl₂/MeOH = 4:1. ¹H NMR (400 MHz, CDCl₃): δ 0.77 (s, 3H, H-20), 0.96 (s, 3H, H-17), 1.24 (s, 3H, H-18), 0.82–1.91 (m, 18H, *ent*-beyerane), 2.19 (d, 1H, *J* = 13.9 Hz, H-3 eq.), 2.67 (dd, 1H, *J* = 18.3, 3.4 Hz, H-15α), 3.46–3.90 (m, 6H, H-5', H-6'a, H-6'b, H-3', H-2', H-4'), 5.49 (d, 1H, *J* = 8.0 Hz, H-1'). ¹³C NMR (100.6 MHz, CD₃OD): δ 14.12 (C-20), 19.96 (C-2), 20.21 (C-17), 21.47 (C-11), 22.74 (C-6), 29.06 (C-18), 38.51 (C-3), 38.94 (C-10), 39.32 (C-12), 40.77 (C-13), 40.92 (C-1), 42.52 (C-7), 45.06 (C-4), 49.47 (C-8), 49.85 (C-15), 55.17 (C-14), 55.83 (C-9), 58.63 (C-5), 62.42 (C-6'), 71.13 (C-4'), 74.05 (C-2'), 78.67 (C-3'), 78.71 (C-5'), 95.62 (C-1'), 178.07 (C-19), 225.27 (C-16). MALDI-MS: *m/z*: 502.9 [M + Na]⁺. Anal., %: C 64.90; H 8.48. C₂₆H₄₀O₈. Calcd., %: C 64.98; H 8.39. M 480.59.

4.1.4.2. β-D-Galactopyranosyl-16-oxo-ent-beyeran-19-oate (13). White powder; yield: 0.09 g (60%); mp: 153 °C, $[\alpha]_D^{20} = -37.2$ (1; MeOH). ¹H NMR (400 MHz, CDCl₃ + CD₃OD): δ 0.72 (s, 3H, H-20), 0.90 (s, 3H, H-17), 1.18 (s, 3H, H-18), 0.79–1.85 (m, 18H, *ent*-beyerane), 2.13 (d, 1H, *J* = 13.5 Hz, H-3 eq.), 2.58 (dd, 1H, *J* = 18.6, 3.5 Hz, H-15α), 3.43–3.88 (m, 6H, H-5', H-6'

a, H-6'b, H-3', H-2', H-4'), 5.29 (d, 1H, $J = 8.2$ Hz, H-1'). ^{13}C NMR (100.6 MHz, CD_3OD): δ 14.14 (C-20), 19.99 (C-2), 20.19 (C-17), 21.48 (C-11), 22.77 (C-6), 29.10 (C-18), 38.52 (C-3), 38.95 (C-10), 39.33 (C-12), 40.78 (C-13), 40.95 (C-1), 42.55 (C-7), 45.09 (C-4), 48.99 (C-8), 49.86 (C-15), 55.19 (C-14), 55.85 (C-9), 58.66 (C-5), 62.07 (C-6'), 70.10 (C-4'), 71.33 (C-2'), 75.48 (C-3'), 77.38 (C-5'), 96.13 (C-1'), 178.15 (C-19), 225.25 (C-16). MALDI-MS: m/z : 503.2 $[\text{M} + \text{Na}]^+$. Anal., %: C 64.95; H 8.45. $\text{C}_{26}\text{H}_{40}\text{O}_8$. Calcd., %: C 64.98; H 8.39. M 480.59.

4.1.4.3. α -D-Mannopyranosyl-16-oxo-ent-beyeran-19-oate (14). White powder; yield: 0.05 g (90%); mp: 212 °C, $[\alpha]_{\text{D}}^{20} = -31.5$ (0.7; MeOH). ^1H NMR (400 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$): δ 0.75 (s, 3H, H-20), 0.94 (s, 3H, H-17), 1.21 (s, 3H, H-18), 1.03–1.94 (m, 18H, ent-beyerane), 2.16 (d, 1H, $J = 14.2$ Hz, H-3 eq.), 2.60 (dd, 1H, $J = 18.8, 3.7$ Hz, H-15 α), 3.57–3.83 (m, 6H, H-5', H-6'a, H-6'b, H-3', H-2', H-4'), 6.02 (d, 1H, $J = 1.5$ Hz, H-1'). ^{13}C NMR (100.6 MHz, $\text{CD}_3\text{OD} + \text{CDCl}_3$): δ 13.97 (C-20), 19.57 (C-2), 20.01 (C-17), 20.97 (C-11), 22.46 (C-6), 29.19 (C-18), 37.93 (C-3), 38.36 (C-10), 38.76 (C-12), 40.21 (C-13), 40.31 (C-1), 41.89 (C-7), 44.92 (C-4), 49.99 (C-8), 49.46 (C-15), 54.71 (C-14), 55.18 (C-9), 57.58 (C-5), 62.07 (C-6'), 67.24 (C-4'), 70.38 (C-2'), 72.13 (C-3'), 78.29 (C-5'), 95.05 (C-1'), 176.53 (C-19), 224.80 (C-16). MALDI-MS: m/z : 503.5 $[\text{M} + \text{Na}]^+$. Anal., %: C 64.72; H 8.35. $\text{C}_{26}\text{H}_{40}\text{O}_8$. Calcd., %: C 64.98; H 8.39. M 480.59.

4.1.4.4. 2-Deoxy-2-acetamido- β -D-glucopyranosyl-16-oxo-ent-beyeran-19-oate (16). White powder; yield: 0.07 g (78%); mp: 154–155 °C, $[\alpha]_{\text{D}}^{20} = -44.5$ (0.45; MeOH). ^1H NMR (400 MHz, CD_3OD): δ 0.76 (s, 3H, H-20), 0.94 (s, 3H, H-17), 1.20 (s, 3H, H-18), 0.80–2.07 (m, 18H, ent-beyerane), 1.98 (s, 3H, CH_3CONH), 2.17 (d, 1H, $J = 13.2$ Hz, H-3 eq.), 2.58 (dd, 1H, $J = 18.8, 3.6$ Hz, H-15 α), 3.33–3.51 (m, 3H, H-3', H-4', H-5'), 3.70 (dd, 1H, $J = 12.1, 4.9$ Hz, H-6'a), 3.83 (dd, 1H, $J = 12.0, 2.2$ Hz, H-6'b), 3.89 (t, 1H, $J = 9.1$ Hz, H-5'), 5.60 (d, 1H, $J = 8.9$ Hz, H-1'). ^{13}C NMR (100.6 MHz, CD_3OD): δ 14.25 (C-20), 20.00 (C-2), 20.15 (C-17), 21.43 (C-11), 22.76 (C-6), 29.27 (C-18), 38.43 (C-3), 39.00 (C-10), 39.23 (C-12), 40.65 (C-13), 40.82 (C-1), 42.40 (C-7), 45.15 (C-4), 49.78 (C-8), 49.82 (C-15), 55.15 (C-14), 55.86 (C-9), 55.92 (C-2'), 58.25 (C-5), 62.45 (C-6'), 71.57 (C-4'), 76.35 (C-3'), 78.75 (C-5'), 93.84 (C-1'), 177.75 (C-19), 225.00 (C-16). ESI-MS: m/z : 544.4 $[\text{M} + \text{Na}]^+$. Anal., %: C 64.29; H 8.40; N 2.65. $\text{C}_{28}\text{H}_{43}\text{NO}_8$. Calcd., %: C 64.47; H 8.31; N 2.69. M 521.64.

4.2 Biology

4.2.1 Cytotoxicity of the test compounds on cancer and normal human cell lines. Cytotoxic effects of compounds 7–9, 11–17, 20–23, 26, and 27 on human cancer and normal cell lines were estimated by means of the multifunctional Cytell Cell Imaging System (GE Health Care Life Science, Sweden) using the Cell Viability BioApp which precisely counts the number of cells and evaluates their viability from fluorescence intensity data. Two fluorescent dyes that selectively penetrate the cell membranes and fluoresce at different wavelengths were used in the experiments. Low-molecular-weight 4',6-

diamidin-2-phenylindol dye (DAPI) is able to penetrate intact membranes of living cells and color nuclei in blue. High-molecular propidium iodide dye penetrates only dead cells with damaged membranes, staining them in yellow. As a result, living cells are painted in blue and dead cells are painted in yellow. DAPI and propidium iodide were purchased from Sigma. The M-HeLa clone 11 human, epithelioid cervical carcinoma, a strain of HeLa, and a clone of M-HeLa, a human breast adenocarcinoma cell line (MCF-7) and the Chang liver cell line (human liver cells) from the N. F. Gamaleya Research Center of Epidemiology and Microbiology were used in the experiments. The cells were cultured in standard Eagle's nutrient medium manufactured at the Chumakov Institute of Poliomyelitis and Virus Encephalitis (PanEco Company) and supplemented with 10% fetal calf serum and 1% nonessential amino acids. The cells were plated into a 96-well plate (Eppendorf) at a concentration of 100 000 cells per mL, 150 μL of medium per well, and cultured in a CO_2 incubator at 37 °C. Twenty four hours after seeding the cells into the wells, the nutrient medium was taken and the test compound was added at a predetermined dilution, 150 μL to each well. The dilutions of the compounds were prepared immediately in nutrient media; 5% DMSO that does not induce the inhibition of cells at this concentration was added for better solubility. Cells were incubated with the test substances for 24 hours. Then the medium with the test compounds was removed, and the cells were washed with phosphate buffered saline PBS (pH –6.8) and stained with DAPI and propidium iodide for 40 minutes. From stock solutions of DAPI and propidium iodide (1 mg mL^{-1}), 1% and 0.07% were added per well, respectively. The cytometric results were analyzed using the Cell Viability BioApp and obtained using protocols in the form of percentage of live and dead cells. The experiments were repeated three times. Intact cells cultured in parallel with experimental cells were used as a control.

4.2.2 Flow cytometry assay

4.2.2.1. Cell culture. M-HeLa cells at 1×10^6 cells per well in a final volume of 2 mL were seeded into six-well plates. After 24 h of incubation, various concentrations of compounds 9, 20 and 22 were added to wells for various periods of time.

4.2.2.2. Cell apoptosis analysis. The cells were harvested at 2000 rpm for 5 min and then washed twice with ice-cold PBS, followed by resuspension in binding buffer. Next, the samples were incubated with 5 μL of Annexin V-FITC and 5 μL of propidium iodide for 15 min at room temperature in the dark. Finally, the cells were analyzed by flow cytometry (Guava easyCyte, MERCK, USA). The experiments were repeated three times.

4.2.2.3. Mitochondrial membrane potential. The cells were harvested at 2000 rpm for 5 min and then washed twice with ice-cold PBS, followed by resuspension in JC-10 (10 $\mu\text{g mL}^{-1}$) and incubation at 37 °C for 10 min. After the cells were rinsed three times and suspended in PBS, the JC-10 fluorescence was observed by flow cytometry.

4.2.2.4. Statistical analysis. The cytometric results were analyzed by the Cytell Cell Imaging Multifunctional System

using the Cell Viability BioApp and Apoptosis BioApp application. The data in the table and graphs are given as the mean $\pm$ standard error.

Conflicts of interest

There are no conflicts to declare.

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