Synthesis and *anti*-human hepatocellular carcinoma activity of new nitric oxide-releasing glycosyl derivatives of oleanolic acid†

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A series of nitric oxide (NO)-releasing glycosyl derivatives (**2–14**) of oleanolic acid were synthesized to improve the aqueous solubility and cytotoxicity of the parent compound **1**. Derivative **3** exhibited better solubility and strong cytotoxicity against human hepatocellular carcinoma (HCC) *in vitro*. Furthermore, **3** displayed low acute toxicity to mice and significantly inhibited the growth of HCC tumors *in vivo*, indicating that **3** may be a promising candidate for the treatment of human HCC.

Introduction

Human hepatocellular carcinoma (HCC) is one of the leading causes for mortality in the world.[1] Currently, there is no effective chemotherapy for HCC. Therefore, development of new therapeutic reagents, particularly injectable drugs, will be of great significance.

High levels of NO usually are toxic to tumor cells.[2] Our previous studies have demonstrated that furoxan-based NO releasing derivatives of oleanolic acid (OA), such as the compound **1** (Chart 1), have strong cytotoxicity against HCC *in vitro* and significantly inhibit the growth of HCC tumors *in vivo*.[3] However, **1** has a very low aqueous solubility (< 1 μg mL^{-1} at pH 6.5–7.8), even with different strategies of formulation, such as microemulsion and nano-liposomes. Hence, it is unfeasible to make **1** injectable for clinical application. Notably, the glycosyl modification of some drugs can improve their aqueous solubilities and cell penetrations, and help in their targeting to specific types of cells, enhancing the selectivity and bioactivity through intra/intercellular carbohydrate-protein interaction.[4] Glycosylated drugs have been successfully marketed worldwide.[5] In this study, we synthesized new glycosyl derivatives (**2–14**) of **1** and examined their cytotoxicities against HCC cells *in vitro*. Furthermore, we evaluated the solubility, NO-releasing ability, *in vivo* acute toxicity, and *anti*-HCC activity of the compound **3**. We found that **3** (galactosyl derivative of **1**) had better aqueous solubility, produced high levels of NO, and displayed strong cytotoxicity selectively against HCC *in vitro* and *in vivo*, but little acute toxicity to mice. We discussed the implication of our novel findings in the future development of *anti*-HCC medicines.

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Results

Chemistry

Ten monosaccharide derivatives (eight hexoses, **2–7**, **13** and **14**, and two pentoses, **8** and **9**) and three disaccharide derivatives (**10–12**) of **1** were synthesized by coupling the corresponding *O*-acetylated glycosyl bromides to the 28-COOH of **1** and subsequent deacetylation (Schemes 1–4). The glycosyl bromides **15a**, **15b**, and **15e–15k** were obtained by the treatment of saccharides with Ac_2O–CH_3COBr–MeOH in one-pot.[6] The compound **15c**

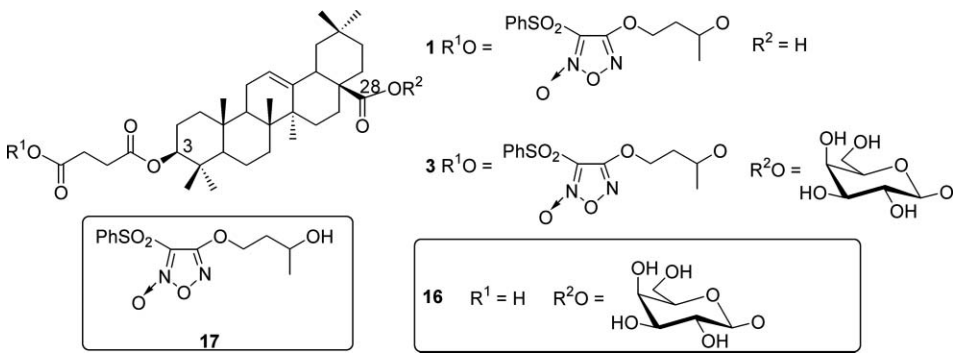
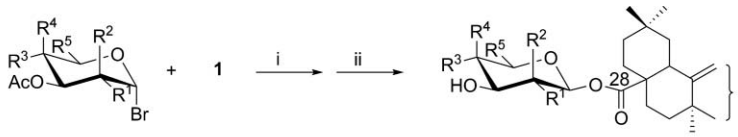


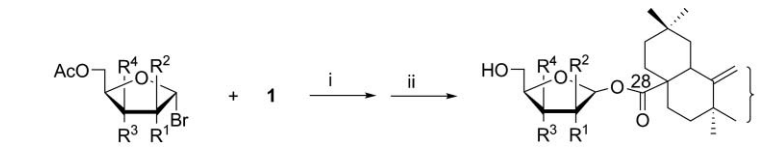
Chart 1 The structures of **1**, **3**, **16** and **17**



15a R^1 = OAc, R^2 = H, R^3 = OAc, R^4 = H, R^5 = CH_2OAc;
15b R^1 = OAc, R^2 = H, R^3 = H, R^4 = OAc, R^5 = CH_2OAc;
15c R^1 = OAc, R^2 = H, R^3 = OAc, R^4 = H, R^5 = COOMe;
15d R^1 = $NHCOCH_3$, R^2 = H, R^3 = OAc, R^4 = H, R^5 = CH_2OAc;
15e R^1 = H, R^2 = H, R^3 = OAc, R^4 = H, R^5 = CH_2OAc;
15f R^1 = H, R^2 = H, R^3 = H, R^4 = OAc, R^5 = CH_2OAc;

2 R^1 = OH, R^2 = H, R^3 = OH, R^4 = H, R^5 = CH_2OH;
3 R^1 = OH, R^2 = H, R^3 = H, R^4 = OH, R^5 = CH_2OH;
4 R^1 = OH, R^2 = H, R^3 = OH, R^4 = H, R^5 = COOH;
5 R^1 = $NHCOCH_3$, R^2 = H, R^3 = OH, R^4 = H, R^5 = CH_2OH;
6 R^1 = H, R^2 = H, R^3 = OH, R^4 = H, R^5 = CH_2OH;
7 R^1 = H, R^2 = H, R^3 = H, R^4 = OH, R^5 = CH_2OH;

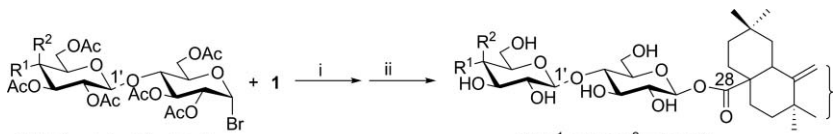
Scheme 1 The synthetic routes of **2–7**. *Regents and conditions*: (i) K_2CO_3, CTAB, H_2O–CH_2Cl_2, r.t., 48 h for **2**, **3**, **5–7**; or K_2CO_3, DMF, r.t., 48 h for **4**; (ii) MeONa, MeOH–CH_2Cl_2, 0–10 °C, 30–60 min.



15g R^1 = OAc, R^2 = H, R^3 = H, R^4 = OAc;
15h R^1 = H, R^2 = OAc, R^3 = OAc, R^4 = H;

8 R^1 = OH, R^2 = H, R^3 = H, R^4 = OH;
9 R^1 = H, R^2 = OH, R^3 = OH, R^4 = H;

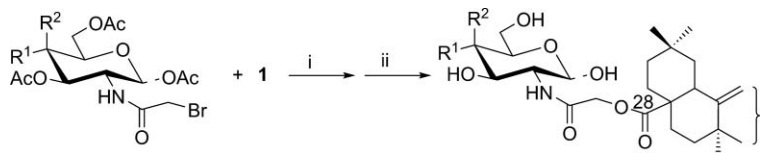
Scheme 2 The synthetic routes of **8** and **9**. *Regents and conditions*: (i) K_2CO_3, CTAB, H_2O–CH_2Cl_2, r.t., 48 h; (ii) MeONa, MeOH–CH_2Cl_2, 0–10 °C, 30–60 min.



15i R^1 = OAc, R^2 = H, 1'α
15j R^1 = OAc, R^2 = H, 1'β
15k R^1 = H, R^2 = OAc, 1' β

10 R^1 = OH, R^2 = H, 1' α
11 R^1 = OH, R^2 = H, 1' β
12 R^1 = H, R^2 = OH, 1' β

Scheme 3 The synthetic routes of **10–12**. *Regents and conditions*: (i) K_2CO_3, CTAB, H_2O–CH_2Cl_2, r.t., 48 h; (ii) MeONa, MeOH–CH_2Cl_2, 0–10 °C, 30–60 min.



15l R^1 = OAc, R^2 = H;
15m R^1 = H, R^2 = OAc.

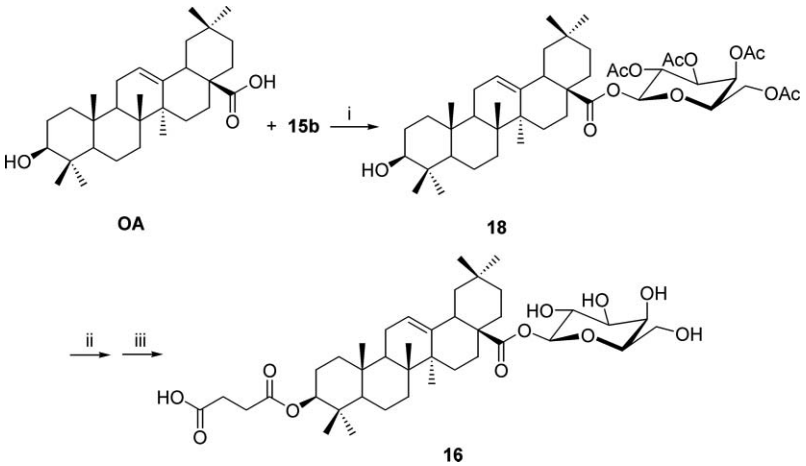
13 R^1 = OH, R^2 = H;
14 R^1 = H, R^2 = OH.

Scheme 4 The synthetic routes of **13** and **14**. *Regents and conditions*: (i) K_2CO_3, DMF, r.t., 8 h; (ii) MeONa, MeOH–CH_2Cl_2, 0–10 °C, 20–40 min.

was achieved through the methylation of D-glucuronic acid with MeONa, and acetylation in Ac_2O catalyzed by perchloric acid and then bromination with 30% hydrobromic acid in acetic acid.[7] The compound **15d** was derived from peracetylated 2-amino-2-deoxy-D-glucose in the presence of 30% hydrobromic acid in acetic acid.[8] The compounds **15l** and **15m** were generated by coupling 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-D-glucose and galactose with bromoacetyl bromide, respectively.[9] The coupling reaction of **1** with *O*-acetylated glycosyl bromides was conducted using K_2CO_3 and CTAB in the mixed solvent of H_2O and CH_2Cl_2 (for **2**, **3**, **5–12**), or in the presence of K_2CO_3 in DMF (for **4**, **13** and **14**). Without purification, the condensation products were directly deacylated using MeONa in anhydrous MeOH and CH_2Cl_2, and neutralized with acidic ion exchange resin to protect the sensitive ester bonds in the target molecule. Notably, LiOH in anhydrous THF was used for the demethylation for the preparation of **4**. OA moiety (**16**) and furoxan moiety (**17**) were obtained *via* condensation of **18** with succinic anhydride and deacylation (Scheme 5), and by the previous procedure,[3] respectively. All of the compounds were subjected to further purification and chemical characterization, and their successful syntheses provided a base for the development of new type of NO-based *anti*-HCC drugs.

In vitro assessment of glycosyl derivatives of 1

The cytotoxicity of synthesized compounds against human HCC SMMC-7721 cells was evaluated by lactate dehydrogenase (LDH) assays[3] using 5-fluorouracil (5-FU) and **1** as positive controls, and their IC_{50} are presented in Fig. 1. The IC_{50} values of **2**, **3**, **9** and **10** (2.7–8.2 μM) were the same order of magnitude as **1**, which was significantly lower than that of 5-FU (43.5 μM).



Scheme 5 The synthetic route of **16**. *Regents and conditions*: (i) K_2CO_3, CTAB, H_2O–CH_2Cl_2, r.t., 48 h; (ii) succinic anhydride, DMAP, anhydrous $CHCl_3$, reflux, 8 h; (iii) MeONa, MeOH–CH_2Cl_2, 0–10 °C, 30 min.

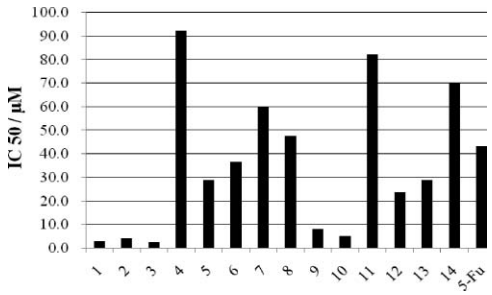


Fig. 1 The IC_{50} of compound **1–14** and 5-FU against human HCC SMMC-7721 cell.

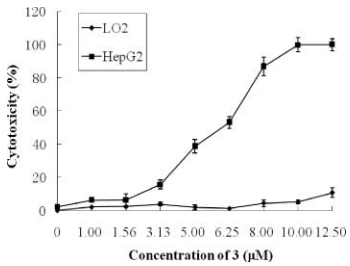


Fig. 2 Cytotoxicity of 3 against HepG2 and LO2 cells. HepG2 and LO2 cells were cultured in medium in the presence of indicated concentrations of **3** for 24 h. The cytotoxicity of **3** was determined by MTT assay. Data are means ± SEM of cytotoxicity (%) from three independent experiments.

In comparison with **1**, **3** displayed similar and stronger cytotoxicity against human HepG2, SMMC-7721 and BEL-7402, respectively, determined by the MTT assay (Table 1).[10] Further analysis revealed that treatment with increased doses of **3**, similar to treatment with vehicle, had no significant effect on the survival of non-HCC LO2 cells while the same treatment induced majority of HepG2 cell death (Fig. 2). Therefore, **3** had strong cytotoxicity selectively to human HCC cells *in vitro*.

Given that **3** contained both OA (**16**) and furoxan (**17**) moieties (Chart 1), we further characterized the cytotoxicity of **16** and **17** against HCC cells, simultaneously. While **16** had little cytotoxicity against HCC in our experimental conditions, both **17** and **3** displayed strong cytotoxicity against HepG2 cells (Fig. 3a), suggesting that NO produced by furoxan and **3** was responsible for their cytotoxicity. Indeed, treatment with 20 μg ml^{-1} haemoglobin, a known NO quencher,[11] abolished and significantly reduced the *anti*-HCC activities of **17** and **3** (Fig. 3b). Importantly, treatment with **3** induced high levels of NO production in HepG2 cells, which was 2- to 5-fold higher than that in LO2 cells (Fig. 2c). Collectively, our data indicate that the furoxan moiety in 3 promotes high levels of NO production, which are responsible for its strong cytotoxicity selectively against HCC cells *in vitro*.

Table 1 Effects of compound **1** and **3** on proliferation in human HCC[a]

HCC	Compd.	Inhibitory rates 10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	IC_{50}/μM
HepG2	**1**	98.0%	85.2%	62.1%	5.3%	1.37
	3	99.5%	95.8%	15.7%	1.2%	2.13
SMMC-7721	**1**	98.5%	24.2%	10.2%	8.7%	4.78
	3	99.6%	98.4%	11.6%	7.9%	1.18
BEL -7402	**1**	92.9%	18.0%	5.5%	4.9%	14.5
	3	99.7%	82.6%	7.8%	1.1%	2.96

[a] Data are expressed as mean (%) of three independent experiments.

The solubility of **1** and **3** in aqueous and nonaqueous solutions was determined, as previously reported.[12] We found that 16.2 mg mL^{-1} of **3** was soluble in 1,2-propylene glycol and 0.826 mg mL^{-1} in 5% macrogol 15 hydroxystearate (solutol HS-15) aqueous solution, which were obviously higher than 2.23 mg mL^{-1} and 0.158 mg mL^{-1} of **1** in corresponding solutions, respectively. Furthermore, the solubility of **3** in 20% solutol HS15, transcutol P, 2% tween 80 or labrasol reached at 16.5, 4.6, 2.2 or 1.5 mg mL^{-1}, respectively. Accordingly, the injectable solution of **3** (5 mg mL^{-1} in 5% 1,2-propylene glycol, 5% solutol HS15, 5% anhydrous ethanol in H_2O) was successfully generated for the evaluation of its biological activity *in vivo*.

In vivo anti-HCC activity of 3

To evaluate the safety of **3**, groups of mice were injected intravenously with a single dose of **3** at 150.0, 120.0, 96.0, 76.8, 61.4 mg kg^{-1} or vehicle control, respectively. The survival of mice

Table 2 The acute toxicity of **3** in mice

Dose/mg kg^{-1}	Number of mice	Number of dead mice						Total death	Survival (%) on day 14
		1h	4h	1d	2d	3d	4–14d		
150.0	10	0	10	—	—	—	—	10	0
120.0	10	0	4	5	0	0	0	9	10
96.0	10	0	1	2	2	1	0	6	40
76.8	10	0	0	0	1	0	0	1	90
61.4	10	0	0	0	0	0	0	0	100
vehicle	10	0	0	0	0	0	0	0	100

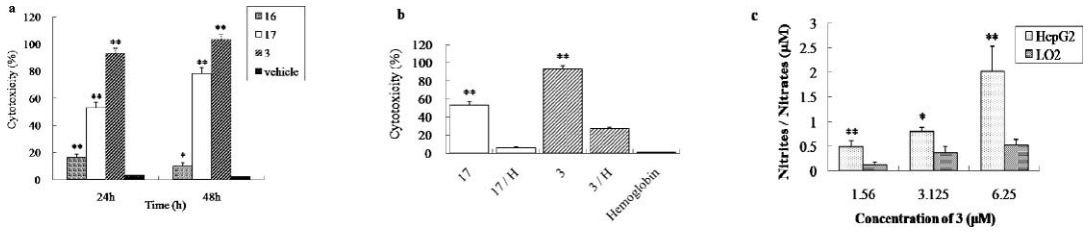


Fig. 3 Determination of relevant components for cytotoxicity. (a) Cytotoxicity of different components. HepG2 cells were treated with 12.5 μM of **3**, **16** and **17** and the viability of the cells was determined by MTT assays. (b) Effect of haemoglobin on the cytotoxicity of **3** and **17**. HepG2 cells were pretreated with, or without, 20 μM of haemoglobin for 1 h and then exposed to 12.5 μM of **3** or **17**. The viability of the cells was determined by MTT assays 24 h later. (c) The levels of nitrites/nitrates. HepG2 and LO2 cells were treated with different doses of **3** for 6 h and the intracellular levels of nitrites/nitrates were determined. Data shown are mean±SEM of individual groups from three separated experiments. *P <0.05, **P <0.01 *vs.* vehicle control (a), haemoglobin treated (b), or LO2 (c), respectively.

was monitored up to 14 days after injection. Treatment with **3** at a high dose (120 or 150 mg kg^{-1}) killed almost all mice (Table 2), and the LD_{50} value of **3** was 94.1 mg kg^{-1} for this strain of mice. In contrast, injection with **3** at 61.4 mg kg^{-1}, like with vehicle alone, did not cause any abnormality in the mice throughout the observation period. Therefore, injection with **3** at or below this dose is supposed to be safe for mice.

To evaluate the *in vivo* activity of **3**, groups of Balb/c nude mice were inoculated subcutaneously with SMMC7721 cells. After the establishment of solid tumor, the mice were randomized and treated with 12.5 or 25 mg kg^{-1} of **3**, positive control 5-FU (25 mg kg^{-1}) or vehicle alone, respectively. Treatment with vehicle alone did not change the growth of tumors *in vivo* because the growth of HCC tumors in the vehicle-treated mice was dynamically similar to that in untreated controls (data not shown). In contrast, treatment with **3** at 12.5 mg kg^{-1} significantly inhibited the growth of HCC tumor ($p < 0.01$ *vs.* controls). Treatment with 25 mg kg^{-1} of **3** enhanced the inhibitory effect on the growth of HCC tumors ($p < 0.01$ *vs.* treatment with 12.5 mg kg^{-1}), and its inhibitory effect was similar to that of 5-FU treatment (Fig. 4). Importantly, the tumor weights in the mice treated with **3** at 12.5 or 25 mg kg^{-1} was 0.48 ± 0.33 g or 0.31 ± 0.13 g, which were significantly lower than that from the vehicle-treated controls (0.87 ± 0.31 g, $p <$ 0.01). Notably, the mean body weight in the mice treated with **3** was heavier than that of the mice treated with 5-FU at the end of this experiment. Together, our data demonstrate that the NO-releasing glycosyl derivative of OA is a potent inhibitor of the growth of solid HCC tumors and has a better safety *in vivo*.

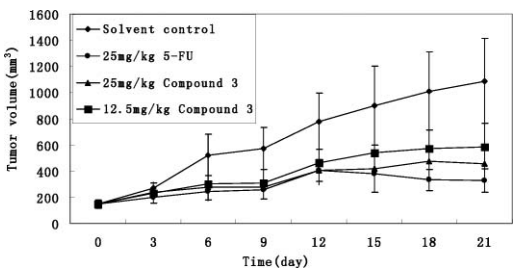


Fig. 4 The effect of **3** on the growth of HCC tumors *in vivo*. Groups of Balb/c nude mice were inoculated with SMMC7721 cells. After establishment of solid tumor, the mice were treated with, or without, indicated reagent and the growth of tumors were measured. Data shown are mean ±SEM of tumor volumes for each group of mice ($n = 10$ per group). The kinetics of HCC tumor growth in untreated controls were similar to that solvent controls (data not shown).

Discussion and conclusions

Analysis of structure and activity relationship (SAR) revealed that the monosaccharide derivatives had stronger cytotoxicity than that of disaccharide derivatives except for the maltosyl compound. Among ten monosaccharide derivatives, the glucosyl (**2**) and galactosyl (**3**) derivatives displayed the strongest cytotoxicity against HCC cells. Notably, high levels of glucose transporter proteins and asialoglycoprotein receptors (ASGPR) are expressed in human HCC cells[4,13] and they may interact with those glucosyl and galactosyl derivatives by recognization and endocytosis,[10] leading to the efficient entry of those compounds into HCC cells. However, the precise mechanisms underlying the potent cytotoxicity of those compounds on HCC cells remain to be further investigated. Indeed, **3** showed stronger cytotoxicity against different types of HCC cells (HepG2, SMMC-7721 and BEL-7402), but did not affect the survival of non-HCC human liver cells *in vitro*. The selective cytotoxicity is likely mediated by high levels of NO produced in HCC cells. Evidently, the furoxan moiety (**17**) and **3**, but not the OA moiety (**16**), displayed potent *anti*-HCC

activity, which was neutralized by NO quencher haemoglobin. Furthermore, the intracellular levels of NO produced by **3** in HCC cells were about 2- to 5-fold higher than that in non-HCC LO2 cells. The differential levels of NO produced by **3** between HCC and non-HCC liver cells may be crucial for its selective *anti*-HCC activity.

Interestingly, the *anti*-HCC activity of **3** was more potent than that of **17**. The higher *anti*-HCC activity may result from the more efficient entry of **3** into the cells, the synergistic effect of NO with OA, and/or higher stability of **3**. As expected, **3** had a better aqueous solubility and could be prepared for intravenous injection. Importantly, intravenous injection with **3** had low acute toxicity in mice. Treatment with **3** at lower doses significantly inhibited the growth of HCC tumors in nude mice and its inhibitory effects were dose-dependent. The selective *anti*-HCC activity *in vitro*, lower acute toxicity in mice and highly inhibitory effect on the growth of HCC tumors *in vivo* of **3** make it a promising candidate for preclinical study. Our findings indicate that NO-releasing glycosyl derivatives of OA may be suitable for preparing injectable medicine for intervention of human HCC.

Experimental section

General

The compound **1** was prepared as described previously.[3] Melting points were determined using a capillary apparatus (RDCSY-I). All of the synthesized compounds were purified by column chromatography (CC) on silica gel 60 (200-300 mesh) or thin-layer chromatography (TLC) on silica gel 60 F254 plates (250 μm; Qingdao Ocean Chemical Company, China). Subsequently, they were routinely analyzed by IR (Shimadzu FTIR-8400*S*), ^{1}H-NMR (Bruker ACF-300Q, 500 MHz), and MS (Hewlett-Packard 1100 LC/MSD spectrometer). The purity of the compounds tested, except for **16** that was analyzed by elemental analysis (Elementar Vario EL III instrument), was characterized by the HPLC analysis (LC-10A HPLC system consisting of LC-10ATvp pumps and SPD-10Avp UV detector) and high resolution mass spectrometry (Agilent technologies LC/MSD TOF). Individual compounds with a purity of >95% were used for subsequent experiments (see the ESI†).

General procedure for the preparation of 2, 3, 5–12

Compound **1** (852 mg, 1 mmol) was dissolved in the mixture of 15 mL CH_2Cl_2 and 20 mL H_2O in the presence of K_2CO_3 (207 mg, 1.5 mmol) and CTAB (91 mg, 0.25 mmol). The mixture was vigorously stirred and reacted with the corresponding *O*-acetylated glycosyl bromides (1.2 mmol) in several fractions at RT for 48 h. The organic layer was harvested and the remaining organic solvents in the aqueous layer were extracted with CH_2Cl_2 (10 mL×3), followed by drying over sodium sulfate and then evaporating. The resulting oil-like materials were dissolved in the mixture of 1 : 1 anhydrous CH_2Cl_2 and MeOH on ice and its pH was adjusted to 9.0 with 0.1 M MeONa/MeOH. The deacetylation was monitored by TLC (1 : 10 v/v MeOH–CH_2Cl_2) and its pH was then adjusted to 7.0 with acidic ion exchange resin 001 × 7 (732). After filtration, the filtrate was evaporated *in vacuo* and the resulting residue was purified by column chromatography (E to MeOH–CH_2Cl_2 1 : 10 v/v) to give the title compounds (20–36%).

Procedure for the preparation of 4

Compound **1** (852 mg, 1 mmol) and K_2CO_3 (207 mg, 1.5 mmol) were dissolved in DMF 10 mL and then reacted with 1.2 mmol methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)-uronate in several fractions at RT for 48 h. Subsequently, the mixture was poured into H_2O (150 mL) and the resulting rough compound was extracted with CH_2Cl_2 (50 mL×3). The collected CH_2Cl_2 layers were washed, dried and concentrated to obtain oil-like materials, followed by deacetylation as described above. The generated residue was dissolved in anhydrous THF 10 mL in the presence of 200 mg LiOH for 24 h and reacted with MeOH (10 mL), followed by adjusting the pH up to 7.0 with acidic ion exchange resin 001 × 7 (732). After filtration and evaporation, the resulting residue was purified by column chromatography (E to MeOH–CH_2Cl_2 1 : 8 v/v) to give the title compounds (15%).

General procedure for the preparation of 13 and 14

Compound **1** (852 mg, 1 mmol) and K_2CO_3 (207 mg, 1.5 mmol) were dissolved in DMF 10 mL and reacted with 1.2 mmol corresponding *O*-acetylated glycosyl bromides in several fractions at RT for 48 h. Subsequently, the mixture was poured into H_2O (150 mL) and the resulting rough compounds were extracted with CH_2Cl_2 (50 mL×3). The collected CH_2Cl_2 layers were washed sequentially with 1 M HCl (80 mL×3), water and saturated NaCl solution, dried over sodium sulfate, and concentrated *in vacuo* to obtain oil-like materials, which were then deacetylated using the procedures described above to give the title compounds (19–30%).

3-[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl] oxy-12-en-28-glucosyl oleanolate (2). The title compound was obtained in 21% yield as white solid. m.p. 101–103 °C. ESI-MS: 1032 $[M+NH_4]^+$, 1049 $[M+Cl]^-$; IR (KBr): 3439, 2947, 2879, 1732, 1617, 1553, 1366, 1170 cm^{-1}; ^{1}H NMR (500 MHz, DMSO), δ (ppm): 0.75 (s, 3H, CH_3), 0.82 (s, 6H, 2×CH_3), 0.85 (s, 3H, CH_3), 0.89 (s, 6H, 2×CH_3), 1.23 (s, 3H, CH_3), 2.67 (s, 4H, 2×$COCH_2$), 2.77~2.88 (m, 1H, C_{18}–H), 3.65 (m, 1H, H_3), 3.78 (m, 1H, H_5), 4.09 (m, 1H, H_4), 4.18 (brs, 1H, 3α-H), 4.21 (t, 2H, OCH_2, J = 6 Hz), 4.43 (m, 1H, H_2), 4.76 (d, 1H, J = 4.5 Hz, $H_{6'}$), 4.83 (d, 1H, J = 6.0 Hz, H_6), 4.99~5.13 (m, 1H, OCH), 5.26 (brs, 1H, C_{12}–H), 5.49 (d, 1H, J = 8 Hz, H_1), 7.61~7.64 (m, 2H, ArH), 7.73~7.76 (m, 1H, ArH), 8.06 (d, 2H, ArH, J = 8.2 Hz). HRMS: calculated 1037.4651, found 1037.4655, PPM error 0.3584.

3-[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl] oxy-12-en-28-galactosyl oleanolate (3). The title compound was obtained in 28% yield as white solid. m.p. 121–124 °C. ESI-MS:1032 $[M+NH_4]^+$, 1049 $[M+Cl]^-$; IR (KBr): 3437, 2947, 1733, 1612, 1552, 1367, 1175 cm^{-1}; ^{1}H NMR (500 MHz, DMSO), δ (ppm): 0.67 (s, 3H, CH_3), 0.74 (s, 6H, 2×CH_3), 0.83 (s, 3H, CH_3), 0.87 (s, 6H, 2×CH_3), 1.1 (s, 3H, CH_3), 2.53 (s, 4H, 2×$COCH_2$), 2.77~2.83 (m, 1H, C_{18}–H), 3.51 (m, 1H, H_3), 3.68 (m, 1H, H_5), 4.28 (m, 1H, H_4), 4.41 (brs, 1H, 3α-H), 4.45 (t, 2H, OCH_2, J = 6 Hz), 4.51 (m, 1H, H_2), 4.71 (d, 1H, J = 4.5 Hz, $H_{6'}$), 4.93 (d, 1H, J = 6.0 Hz, H_6), 4.99~5.03 (m, 1H, OCH), 5.16 (brs, 1H, C_{12}–H), 5.21 (d, 1H, J = 8 Hz, H_1),

7.72~7.75 (m, 2H, ArH), 7.88~7.91 (m, 1H, ArH), 8.01 (d, 2H, ArH, $J = 8$ Hz)° HRMS: calculated 1037.4651, found 1037.4662, PPM error 1.0331.

3-[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl] oxy-12-en-28-glucuronyl oleanolate (4). The title compound was obtained in 15% yield as white syrup. ESI-MS 1027 $[M - H]^-$; IR (KBr): 3429, 2922, 2851, 1733, 1618, 1553, 1367, 1171 cm^{-1}; ^1H NMR (500 MHz, DMSO), δ (ppm): 0.68 (s, 3H, CH_3), 0.73 (s, 6H, 2×CH_3), 0.83 (s, 3H, CH_3), 0.90 (s, 6H, 2×CH_3), 1.11 (s, 3H, CH_3), 2.54 (s, 4H, 2×$COCH_2$), 2.67–2.93 (m, 1H, C_{18}–H), 3.06–3.10 (m, 2H, H_3, H_4), 4.28 (m, 1H, 3α-H), 4.39–4.43 (m, 2H, OCH_2), 4.91–5.12 (m, 2H, OCH, H_2), 5.12 (d, 2H, $J = 5.7$ Hz), 5.16 (brs, 1H, C_{12}–H), 5.22 (d, 1H, $J = 8$ Hz, H_1), 7.71~7.77 (m, 2H, ArH), 7.87~7.92 (m, 1H, ArH), 8.01 (d, 2H, ArH, $J = 8$ Hz). HRMS: calculated 1028.1884, found 1028.1870, PPM error −1.2621.

3-[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl] oxy-12-en-28-(2-acetylamino-2-deoxy-glucosyl) oleanolate (5). The title compound was obtained in 36% yield as white solid. m.p. 142–146 °C. ESI-MS 1078 $[M+Na]^+$; IR (KBr): 3424, 2948, 2879, 1734, 1618, 1552, 1370, 1170 cm^{-1}; ^1H NMR (500 MHz, DMSO), δ (ppm): 0.73 (s, 3H, CH_3), 0.77 (s, 6H, 2×CH_3), 0.89 (s, 3H, CH_3), 0.91 (s, 6H, 2×CH_3), 1.21 (s, 3H, CH_3), 2.59 (s, 4H, 2×$COCH_2$), 2.73~2.99 (m, 1H, C_{18}–H), 3.17 (m, 3H, $COCH_3$), 3.40~3.51 (brs, 1H, NH), 3.64~3.67 (m, 2H, H_3, H_5), 4.32~4.36 (brs, 1H, 3α-H), 4.40–4.47 (m, 4H, OCH_2, H_2, H_4), 4.99 (d, 1H, $J = 5.5$ Hz, H_6), 5.03–5.05 (m, 2H, OCH, $H_{6'}$), 5.20 (brs, 1H, C_{12}–H), 5.26 (d, 1H, $J = 8.9$ Hz, H_1), 7.75–7.78 (m, 2H, ArH), 7.91–7.94 (m, 1H, ArH), 8.02–8.04 (m, 2H, ArH). HRMS: calculated 1056.5097, found 1056.5084, PPM error −1.2613.

3-[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl] oxy-12-en-28-(2-deoxy-glucosyl) oleanolate (6). The title compound was obtained in 27% yield as white syrup. ESI-MS: 997 $[M - H]^-$; IR (KBr): 3427, 2945, 2879, 1733, 1617, 1553, 1367, 1170 cm^{-1}; ^1H NMR (500 MHz, DMSO), δ (ppm): 0.57 (s, 3H, CH_3), 0.69 (s, 6H, 2×CH_3), 0.77 (s, 3H, CH_3), 0.81 (s, 6H, 2×CH_3), 0.98 (s, 3H, CH_3), 2.57 (s, 4H, 2×$COCH_2$), 2.87~2.93 (m, 1H, C_{18}–H), 4.43~3.51 (m, 1H, H_4), 3.62–3.79 (3 m, 3H, H_5, H_6, $H_{6'}$), 3.86~4.01 (m, 1H, H_3), 4.42 (brs, 1H, 3α-H), 4.43 (t, 2H, OCH_2, $J = 6$ Hz), 4.68~4.98 (m, 1H, OCH_2), 5.18 (brs, 1H, C_{12}–H), 5.24 (dd, 1H, H_1), 7.73~7.74 (m, 2H, ArH), 7.88~7.95 (m, 1H, ArH), 7.99~8.07(m, 2H, ArH). HRMS: calculated 1021.4702, found 1021.4696, PPM error −0.6006.

3-[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl] oxy-12-en-28-(2-deoxy-galatosyl) oleanolate (7). The title compound was obtained in 21% yield as white syrup. ESI-MS: 1016 $[M+NH_4]^+$; IR (KBr): 3431, 2946, 1732, 1618, 1553, 1367, 1170 cm^{-1}; ^1H NMR (500 MHz, DMSO), δ (ppm): 0.61 (s, 3H, CH_3), 0.71 (s, 6H, 2×CH_3), 0.82 (s, 3H, CH_3), 0.85 (s, 6H, 2×CH_3), 1.2 (s, 3H, CH_3), 2.58 (s, 4H, 2×$COCH_2$), 2.67~2.81 (m, 1H, C_{18}–H), 4.53~3.61 (m, 1H, H_4), 3.65~3.82 (m, 3H, H_5, H_6, $H_{6'}$), 3.90~4.0 (m, 1H, H_3), 4.41 (brs, 1H, 3α-H), 4.45 (t, 2H, OCH_2, $J = 6$ Hz), 4.78~5.00 (m, 1H, OCH_2), 5.14 (brs, 1H, C_{12}–H), 5.21 (dd, 1H, H_1), 7.72~7.74 (m, 2H, ArH), 7.88~7.91 (m, 1H, ArH), 7.99~8.01 (m, 2H, ArH). HRMS: calculated 1021.4702, found 1021.4712, PPM error 0.9657.

3-[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl] oxy-12-en-28-xylosyl oleanolate (8). The title compound was obtained in 21% yield as white solid. m.p. 103–106 °C. ESI-MS: 1007 $[M+Na]^+$, 1019 $[M+Cl]^-$; IR (KBr): 3424, 2942, 1733, 1618, 1553, 1367, 1170 cm^{-1}; ^1H NMR (500 MHz, DMSO), δ (ppm): 0.65 (s, 3H, CH_3), 0.74 (s, 6H, 2×CH_3), 0.85 (s, 3H, CH_3), 0.91 (s, 6H, 2×CH_3), 1.18 (s, 3H, CH_3), 2.54 (s, 4H, 2×$COCH_2$), 2.62~2.73 (m, 1H, C_{18}–H), 3.68~3.78 (m, 2H, H_3, H_4), 4.28~4.34 (m, 1H, 3α-H), 4.48~4.55 (m, 2H, OCH_2), 4.67~4.81 (m, 2H, H_5, $H_{5'}$), 4.91~5.00 (m, 1H, OCH_2), 5.12~5.22 (m, 1H, H_2), 5.28 (brs, 1H, C_{12}–H), 5.32 (d, 1H, $J = 5$ Hz, H_1), 7.71~7.76 (m, 2H, ArH), 7.89~7.90 (m, 1H, ArH), 7.99~8.02 (m, 2H, ArH). HRMS: calculated 1007.4545 found 1007.4553, PPM error 0.7311.

3-[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl] oxy-12-en-28-arabinosyl oleanolate (9). The title compound was obtained in 21% yield as white solid. m.p. 117–120 °C. ESI-MS: 983 $[M - H]^-$, 1019 $[M+Cl]^-$; IR (KBr): 3444, 2947, 1733, 1617, 1552, 1367, 1170 cm^{-1}; ^1H NMR (500 MHz, DMSO), δ (ppm): 0.68 (s, 3H, CH_3), 0.79 (s, 6H, 2×CH_3), 0.81 (s, 3H, CH_3), 0.89 (s, 6H, 2×CH_3), 1.08 (s, 3H, CH_3), 2.52 (s, 4H, 2×$COCH_2$), 2.67~2.73 (m, 1H, C_{18}–H), 3.68~3.70 (m, 2H, H_3, H_4), 4.29~4.30 (m, 1H, 3α-H), 4.41~4.44 (m, 2H, OCH_2), 4.57~4.71 (m, 2H, H_5, $H_{5'}$), 4.98~5.02 (m, 1H, H_2), 4.99~5.08 (m, 1H, OCH_2), 5.21 (brs, 1H, C_{12}–H), 5.33 (d, 1H, $J = 5$ Hz, H_1), 7.72~7.76 (m, 2H, ArH), 7.88~7.89 (m, 1H, ArH), 7.99~8.01 (m, 2H, ArH). HRMS: calculated 1007.4545 found 1007.4552, PPM error 0.6318.

3-[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl] oxy-12-en-28-maltosyl oleanolate (10). The title compound was obtained in 29% yield as white syrup. ESI-MS: 1194 $[M+NH_4^+]^+$, 1199 $[M+Na]^+$; IR (KBr): 3423, 2963, 1733, 1618, 1553, 1368 cm^{-1};^1H NMR (500 MHz, DMSO), δ (ppm): 0.69 (s, 3H, CH_3), 0.71 (s, 6H, 2×CH_3), 0.81 (s, 3H, CH_3), 0.85 (s, 6H, 2×CH_3), 1.1 (s, 3H, CH_3), 2.53 (s, 4H, 2×$COCH_2$), 2.71~2.83 (m, 1H, C_{18}–H), 3.41~3.43 (m, 1H, H_4), 3.61~3.67 (m, 2H, $H_{5'}$, $H_{2'}$), 3.71~3.78 (m, 1H, H_2), 4.13~4.32 (m, 2H, H_5, $H_{4'}$), 4.38~4.46 (m, 2H, H_3, $H_{3'}$), 4.54~4.61 (m,1H, $H_{6b'}$), 4.67~4.83 (m, 1H, H_{6b}), 5.00~5.03 (m, 2H, H_{6a}, $H_{6a'}$), 5.09~5.21 (m, 4H, OCH, 3α-H, OCH_2), 5.23~5.31 (m, 2H, C_{12}–H, $H_{1'}$), 5.62 (d, 1H, $J = 8$ Hz, H_1), 7.72~7.75 (m, 2H, ArH), 7.88~7.91 (m, 1H, ArH), 7.98~8.00 (m, 2H, ArH). HRMS: calculated 1199.5179 found 1199.5173, PPM error −0.5433.

3-[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl] oxy-12-en-28-cellobiosyl oleanolate (11). The title compound was obtained in 23% yield as white solid. m.p. 126–129 °C. ESI-MS: 1175 $[M - H]^-$; IR (KBr): 3422, 2931, 1734, 1617, 1553, 1367, 1170 cm^{-1}; ^1H NMR (500 MHz, DMSO), δ (ppm): 0.65 (s, 3H, CH_3), 0.78 (s, 6H, 2×CH_3), 0.89 (s, 3H, CH_3), 0.95 (s, 6H, 2×CH_3), 1.13 (s, 3H, CH_3), 2.53 (s, 4H, 2×$COCH_2$), 2.76~2.83 (m, 1H, C_{18}–H), 3.42~3.43 (m, 1H, H_4), 3.62~3.67 (m, 2H, $H_{5'}$, $H_{2'}$), 3.74~3.78 (m, 1H, H_2), 4.23~4.33 (m, 2H, H_5, $H_{4'}$), 4.38~4.46 (m, 2H, H_3, $H_{3'}$), 4.50~4.61 (m,1H, $H_{6b'}$), 4.67~4.77 (m, 1H, H6b), 5.00~5.03 (m, 2H, H_{6a}, $H_{6a'}$), 5.09~5.18 (m, 4H, OCH, 3α-H, OCH_2), 5.27~5.31 (m, 2H, C_{12}–H, $H_{1'}$), 5.61 (d, 1H, $J = 8$ Hz, H_1), 7.72~7.75 (m, 2H,

ArH), 7.88~7.91 (m, 1H, ArH), 7.98~8.00 (m, 2H, ArH). HRMS: calculated 1199.5179 found 1199.5171, PPM error –0.7100.

3-[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl] oxy-12-en-28-lactosyl oleanolate (12). The title compound was obtained in 23% yield as white solid. m.p. 150–153 °C. ESI-MS: 1175 $[M - H]^-$; IR (KBr): 3428, 2945, 2879, 1732, 1614, 1553, 1368, 1170 cm^{-1};^1H NMR (500 MHz, DMSO), δ (ppm): 0.65 (s, 3H, CH_3), 0.78 (s, 6H, 2×CH_3), 0.89 (s, 3H, CH_3), 0.95 s, 6H, 2×CH_3), 1.13 (s, 3H, CH_3), 2.53(s, 4H, 2×$COCH_2$), 2.76~2.83 (m, 1H, C_{18}–H), 3.39~3.43 (m, 1H, H_4), 3.59~3.67 (m, 2H, $H_{5'}$, $H_{2'}$), 3.74~3.80 (m, 1H, H_2), 4.13~4.33 (m, 2H, H_5, $H_{4'}$), 4.36~4.46 (m, 2H, H_3, $H_{3'}$), 4.50~4.67 (m, 1H, $H_{6b'}$), 4.67~4.77 (m, 1H, H6b), 4.89~5.02 (m, 2H, H_{6a}, $H_{6a'}$), 5.09~5.16 (m, 4H, OCH, 3α-H, OCH_2), 5.27~5.32 (m, 2H, C_{12}–H, $H_{1'}$), 5.54 (d, 1H, J = 8 Hz, H_1), 7.72~7.76 (m, 2H, ArH), 7.88~7.91 (m, 1H, ArH), 7.98~8.01 (m, 2H, ArH). HRMS: calculated 1199.5179 found 1199.5178, PPM error –0.1264.

3-[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl] oxy-12-en-28-[2-*N*-(2-deoxy-glucose)-2-oxo]-ethyl oleanolate (13). The title compound was obtained in 19% yield as white syrup. ESI-MS: 1071 $[M - H]^-$; IR (KBr): 3424, 2947, 1733, 1619, 1551, 1366, 1170 cm^{-1}; ^1H NMR (500 MHz, DMSO), δ (ppm): 0.67 (s, 3H, CH_3), 0.77 (s, 6H, 2×CH_3), 0.83 (s, 3H, CH_3), 0.89 (s, 6H, 2×CH_3), 1.2 (s, 3H, CH_3), 2.58 (s, 4H, 2×$COCH_2$), 2.79~2.83 (m, 1H, C_{18}–H), 3.52~3.59 (m, 2H, H_5, H_3), 3.72~3.74 (m, 1H, H_4), 3.81 (m, 1H, H_2), 4.28 (m, 1H, H_4), 4.44~4.45 (m, 4H, 3α-H, OCH_2, H_6, $H_{6'}$), 4.93~4.94 (m, 1H, $OCH_)$, 5.00 (brs, 1H, C_{12}–H), 5.21 (m, 1H, H_1), 7.72~7.76 (m, 2H, ArH), 7.88~7.91 (m, 1H, ArH), 8.00 (d, 2H, ArH, J = 7.8 Hz). HRMS: calculated 1094.4865 found 1094.4868, PPM error 0.1901.

3-[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl] oxy-12-en-28-[2-*N*-(2-deoxy-galactose)-2-oxo]-ethyl oleanolate (14). The title compound was obtained in 30% yield as white syrup. ESI-MS: 1071 $[M - H]^-$; IR (KBr): 3426, 2946, 1732, 1618, 1552, 1367, 1170 cm^{-1}; ^1H NMR (500 MHz, DMSO), δ (ppm): 0.68 (s, 3H, CH_3), 0.78 (s, 6H, 2×CH_3), 0.84 (s, 3H, CH_3), 0.89 (s, 6H, 2×CH_3), 1.1 (s, 3H, CH_3), 2.58 (s, 4H, 2×$COCH_2$), 2.57~2.83 (m, 1H, C_{18}–H), 3.53~3.59 (m, 2H, H_5, H_3), 3.72~3.74 (m, 1H, H_4), 3.92 (m, 1H, H_2), 4.34 (m, 1H, H_4), 4.34~4.45 (m, 4H, 3α-H, OCH_2, H_6, $H_{6'}$), 4.80~4.94 (m, 1H, OCH), 5.10 (brs, 1H, C_{12}–H), 5.21 (d, 1H, H_1, J = 1.5 Hz), 7.72~7.76 (m, 2H, ArH), 7.88~7.91 (m, 1H, ArH), 8.00 (d, 2H, ArH, J = 7.8 Hz). HRMS: calculated 1094.4865 found 1094.4881, PPM error 1.3779.

3-(3-Carboxy-1-oxopropoxy)-12-en-28-galactosyl oleanolate (16). The title compound was obtained from the condensation of succinyl oleanolic acid with 2, 3, 4, 6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide and then deacetylation in 45% yield as white solid. m.p. 121–123 °C. ESI-MS: 717 $[M - H]^-$; ^1H NMR (500 MHz, DMSO), δ (ppm): 0.69 (s, 3H, CH_3), 0.80 (s, 6H, 2×CH_3), 0.88–0.89 (s, 9H, 3CH_3), 1.02 (s, 3H, CH_3), 2.51 (s, 4H, 2×$COCH_2$), 3.51 (m, 1H, H_3), 3.68 (m, 1H, H_5), 4.38 (m, 1H, H_4), 4.41 (brs, 1H, 3α-H), 4.51 (m, 1H, H_2), 4.71 (d, 1H, J = 5.2 Hz, $H_{6'}$), 4.93 (d, 1H, J = 5.8 Hz, H_6), 5.16 (brs, 1H, C_{12}–H), 5.21 (d, 1H, J = 8 Hz, H_1). Anal. calcd for $C_{40}H_{62}O_{11}·2H_2O$: C, 63.64 H, 8.81; found C, 63.94 H, 8.93.

Cytotoxicity assay *in vitro*

HepG2 and non-HCC liver LO2 cells at 10^4 cells/well were in triplicate cultured in 10% FBS DMEM in 96-well flat-bottom microplates overnight. The cells were treated with, or without, different concentrations of each compound for various periods. During the last 4 h incubation, 30 μL tetrazolium dye (MTT) solution (5 mg ml^{-1}) was added to each well. The resulting MTT-formazan crystals were dissolved in 150 μL DMSO, and the absorbance was spectrophotometrically measured at 570 nm on an ELISA plate reader. The cell viability was expressed as the optical density ratio of the treatment to vehicle control. Additional experiments were performed by pre-treatment of HepG2 cells with, or without, 20 μM haemoglobin for 1 h and exposure to individual compounds tested.

Solubility Studies

The solubility of **1** and **3** was tested, as described previously.[10] Briefly, an excess amount of **1** or **3** powder was introduced in triplicate into 5 ml of each medium (5% propylene glycol, 5% macrogol 15 hydroxystearate (HS-15), 20% solutol HS15, 20% transcutol P, 2% tween 80 or 2% labrasol in water) in a small flask with a glass stopper and shaken at 37.0 °C in dark for 3 days. Aliquots (about 2 ml) of the solution were collected, centrifuged at 10,000 g for 5 min and suitably diluted. The concentrations of each compound were determined by HPLC.

Nitrate/nitrite measurement *in vitro*

NO levels were determined by using a nitrate/nitrite colorimetric assay kit (Beyotime Institute of Biotechnology), according to the manufacturers' instructions. The cells (5×10^6/well) were treated in triplicate with different concentrations of the compound for 6 h and lyzed. After microfuge ultrafiltration, the contents of nitrate/nitrite in the lysates were measured. Individual values were obtained by subtracting the background (diluent treated wells) and calculated according to the standard curve.

Acute toxicity assay

Seven-week-old male and female KM mice were from Shanghai SLAC and housed individually in a specific pathogen free facility. Groups of mice (n = 10 per group) were injected intravenously with a single dose of 150.0, 120.0, 96.0, 76.8, 61.4 mg kg^{-1} or vehicle control, respectively. The mouse death was monitored daily and recorded up to 14 days after treatment. The experimental protocols were approved by the Animal Research Protection Committee of our campus.

Tumor growth inhibition *in vivo*

Female Balb/c nude mice at 5–6 weeks old from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, were inoculated subcutaneously with 10^6 SMMC7721 cells. After the formation of solid tumor with a volume of about 100~300 mm^3, the tumor-bearing mice were randomized and treated intravenously with, or without, 12.5, 25 mg kg^{-1} of 3, 25 mg kg^{-1} of 5-FU or vehicle alone (0.4 ml) three times per week for 21 days, respectively. Their body weights were measured and the growth of tumors was closely monitored, as described previously.[3]

At the end of the experiment, the mice were sacrificed and their tumors were dissected and measured.

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