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Efficient synthesis of α- and β-chacotriosyl glycosides using appropriate donors, and their cytotoxic activity

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Abstract—Natural steroidal glycosides containing α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranose (chacotriose) at the oligosaccharide moiety exhibit anti-cancer and anti-herpes activities. To investigate the structure–activity relationships of the aglycone parts of chacotriosides, we developed a synthesis method for chacotriosyl glycosides having various aglycones. In the process, it was revealed that α-chacotriosyl glycosides could be obtained mainly by using a trichloroacetimidate donor, while β-chacotriosyl glycosides were afforded by using phosphite and phosphate donors. In cytotoxicity tests using the A549 and HepG2 cell lines, naturally occurring β-chacotriosyl diosgenin and cholestanol exhibited higher activities than the corresponding α-chacotriosyl glycosides.

Keywords: Chacotriose; Glycoside; Transglycosylation; Cytotoxicity

1. Introduction

We have studied the steroidal oligosaccharides in many solanaceous plants and have clarified their chemical structures and certain biological activities.[1,2] Our detailed studies on the relationship between the structures and bioactivities of the steroidal glycosides from *Solanum* plants have suggested that spirostanol-type steroidal glycosides containing the chacotriose moiety exhibited potent anti-cancer[3,4] and anti-herpes[5] activities. Therefore, we have developed a facile synthetic method for chacotriosyl donors from D-glucose and L-rhamnose that can be used to transfer the moiety to a variety of aglycones to afford α/β neosaponins, including a natural steroidal glycoside dioscin (β-chacotriosyl diosgenin).[6] In cytotoxicity tests using the PC-12 (lung cancer) and HCT116 (colon cancer) cell lines, dioscin exhibits moderate cytotoxicity [GI_{50}: 2.29 μM (PC-12); 3.07 μM (HCT116)] equivalent to that of cisplatin. On the other hand, the aglycone part (diosgenin), the sugar part (chacotriose), and the α-anomeric glycoside of dioscin exhibit little or no activity. These results obtained by our group indicate that the naturally occurring β-glycosidic linkage in glycosides plays an important role in determining biological activity. Although facile synthetic methods for chacotriosyl derivatives have been demonstrated,[7–14] our method[6] may be useful for directly transferring the chacotriose moiety to various aglycones. However, α-chacotriosyl glycosides with weak biological activities were predominantly afforded in our previous study.[6] Thus, as it is generally known that the reactivity and stereoselectivity of glycosylations are considerably influenced by the properties of the donor, catalyst, and solvent, we describe the facile synthetic route for β-chacotriosyl glycosides predominantly by investigating a variety of chacotriosyl donors. Furthermore, the cytotoxicities of the obtained neosaponins have been determined.

2. Results and discussion

4-Tolyl (2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-(1→4)-[(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)]-3,6-

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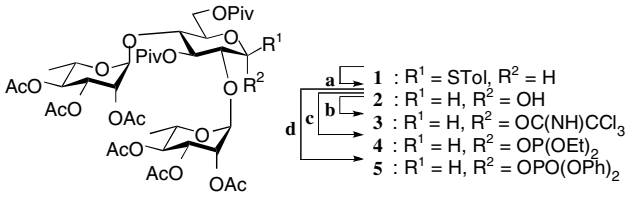
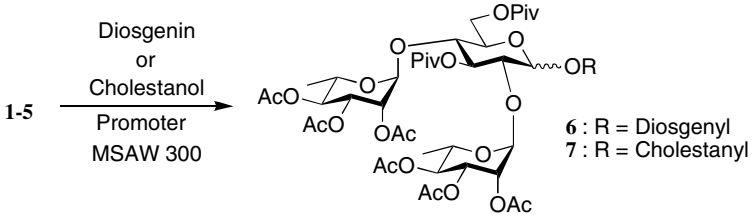
di-*O*-pivaloyl-1-thio-β-D-glucopyranoside (**1**) was prepared from 4-tolyl 3,6-di-*O*-pivaloyl-1-thio-β-D-glucopyranoside[15] and α-L-rhamnopyranosyl trichloroacetimidate according to the method of Cheng et al.[16] The thiotolyl moiety of chacotrioside **1** was deprotected with *N*-iodosuccinimide (NIS) to yield chacotriosyl derivative **2**, and the 1-hydroxy group at the reducing end of **2** (Scheme 1) was converted into various leaving groups for transglycosylation. First, trichloroacetimidate **3**[6] (α-form, 86%) was easily produced from **2** in the presence of trichloroacetonitrile and 1, 8-diazabicyclo[5.4.0]undec-7-ene. Accordingly, diethyl phosphite **4** (α-form, 93%) and diphenyl phosphate **5** (α-form, 86%) were synthesized from **2**, which correspond to diethyl chlorophosphite and diphenyl phosphoryl chloride under triethylamine, respectively.



Scheme 1. Reagents and conditions: (a) TMSOTf, CH_2Cl_2, H_2O, 62%; (b) CCl_3CN, DBU, CH_2Cl_2, 86%; (c) $ClP(OEt)_2$, Et_3N, CH_2Cl_2, 93%; (d) $ClPO(OPh)_2$, Et_3N, CH_2Cl_2, 86%.

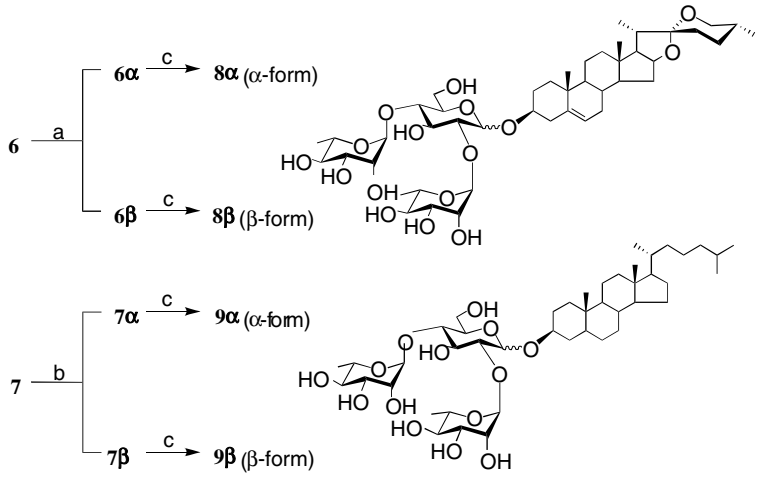
The chacotriosyl donors **1**–**5** that were obtained were transferred to aglycones (diosgenin and cholestanol) using an acid-washed molecular sieve (MSAW 300) and a promoter (Table 1). First, the glycosylation of **1** with diosgenin was carried out in the presence of NIS with boron trifluoride etherate ($BF_3{\cdot}Et_2O$) or trimethylsilyl trifluoromethanesulfonate (TMSOTf) as the promoter in anhydrous dichloromethane (CH_2Cl_2). As a result, the α-form of protected glycoside **6** was predominantly obtained in 48% yield (entry 1, α/β = 71/29). Next, **2** was used with diphenyl sulfoxide and 2,4,6-tri-*tert*-butylpyrimidine (TTBP) as promoters,[17] and then transferred to diosgenin to afford **6** (α/β = 80/20) in 22% yield. Since the yield and β-selectivity of this glycosylation method were not satisfactory, we performed the glycosylation of **3** with diosgenin using $BF_3{\cdot}Et_2O$ as a promoter to afford **6** (α/β = 69/29) in 64% yield. However, because the β-selectivity was not sufficiently high, even though the yield was improved over that using **1** and **2**, glycosylation was attempted in polar solvents (entries 5–7). Glycosylation in all of the polar solvents predominantly afforded β-glycoside, but the total yield was sharply decreased because of the fact that neither **3** nor diosgenin easily dissolve in polar solvents. On the other hand, when **4**[18] was reacted with diosgenin under $BF_3{\cdot}Et_2O$ in CH_2Cl_2, a naturally occurring β-glycoside was mainly afforded (α/β = 26/74) in 48% yield

Table 1. Glycosylation of donors **1**–**5** with diosgenin or cholestanol



Entries	Donor	Acceptor	Promoter	Solvent	α/β	Yield (%)
1	**1**	D	NIS/$BF_3{\cdot}Et_2O$	CH_2Cl_2	71/29	48
2		D	NIS/TMSOTf	CH_2Cl_2	76/24	43
3	**2**	D	Ph_2SO/TTBP/Tf_2O	CH_2Cl_2	80/20	22
4	**3**	D	$BF_3{\cdot}Et_2O$	CH_2Cl_2	69/31	64
5		D	$BF_3{\cdot}Et_2O$	CH_3CN	24/76	9
6		D	$BF_3{\cdot}Et_2O$	CH_3CH_2CN	26/74	14
7		D	$BF_3{\cdot}Et_2O$	CH_3CN/Et_2O	25/75	29
8		C	$BF_3{\cdot}Et_2O$	CH_2Cl_2	81/19	42
9	**4**	D	$BF_3{\cdot}Et_2O$	CH_2Cl_2	26/74	48
10		D	TMSOTf	CH_2Cl_2	24/76	53
11		D	TMSOTf	Et_2O	33/67	34
12		D	TMSOTf	CH_3CN/Et_2O	31/69	31
13		C	$BF_3{\cdot}Et_2O$	CH_2Cl_2	28/72	44
14	**5**	D	TMSOTf	CH_2Cl_2	27/73	43
15		C	TMSOTf	CH_2Cl_2	30/70	46

D: diosgenin, C: cholestanol.



Scheme 2. Reagents and conditions: (a) ODS column chromatography (90% MeOH); (b) ODS column chromatography (95% MeOH); (c) 3% KOH/MeOH, 65 °C.

(entry 9). Subsequently, to further increase the β-selectivity, we attempted glycosylation using **4** in polar solvents (entries 11 and 12). Unfortunately, the β-selectivity did not increase over that in entry 10. Finally, **5** was transferred to diosgenin, mainly affording a naturally occurring β-glycoside (entry 14), as in the case of **4**. To obtain the other neosaponin, donors **3**–**5** were glycosylated with cholestanol (entries 8, 13, and 15). The $BF_3{\cdot}Et_2O$-promoted glycosylation of **3** with cholestanol predominantly afforded the α-form of protected glycoside **7** in 42% yield (entry 8; α/β = 81/19); on the other hand, the TMSTOf-promoted glycosylation of **5** with cholestanol mainly afforded the β-form of **7** in 44% yield (entry 13; α/β = 28/72). Only by changing the leaving group into a donor could α-glycoside and β-glycoside be easily synthesized separately. The technique of transferring distinct types of chacotriosyl donors to the aglycone after building chacotriose is a useful procedure to construct a library of α- and β-chacotriosyl glycosides having various aglycones.

The α/β anomeric mixtures of protected neoglycosides **6** and **7** thus obtained were separated on an ODS column to afford pure α- and β-protected neoglycosides **6α/β** and **7α/β**. Each protected neoglycoside (**6α/β**, **7α/β**) was deprotected in the usual manner to afford the corresponding neosaponins (**8α/β**, **9α/β**) (Scheme 2).

The cytotoxicities of neosaponins **8α/β** and **9α/β**, diosgenin, cholestanol, and chacotriose[6] toward human lung cancer (A549) and human hepatoblastoma (HepG2) cell lines were determined[19] (Table 2). Dioscin (**8β**) exhibited moderate cytotoxicity against A549, which was the same as that of 5-fluorouracil (5FU). However, compound **8α**, which is an anomeric isomer of dioscin, showed weak activity. Interestingly, the same tendency that the β-chacotriosyl cholestanol **9β** had higher activity than **9α** was observed even though these had weaker activities compared with those of **8α** and **8β**. In addition, **8α** and **8β** exhibited moderate cytotoxicity against HepG2, while **9α** and **9β** did not show activity. Furthermore, only diosgenin, cholestanol, and chacotriose did not exhibit high cytotoxicity against A549 and HepG2. As a consequence, it is suggested that the aglycone moiety and the β-glycosidic linkage of the chacotriosyl glycoside are essential for cytotoxicity. It is thought that our method, in which an oligosaccharide moiety is transferred to various aglycones to obtain the α- and β-linked neosaponins efficiently, is useful to investigate the structure–activity relationships of bioactive glycoconjugate compounds such as natural products.

Table 2. Cytotoxic activity (IC_{50})

	A549	HepG2
5-Fluorouracil	2.4	0.57
8α	12.2	8.1
8β (dioscin)	2.5	4.1
9α	15.1	>50
9β	7.3	>50
Chacotriose	>50	>50
Cholestanol	>50	>50
Diosgenin	>50	>50

3. Experimental

3.1. General methods

Optical rotations were measured using a JASCO DIP-1000 KUY digital polarmeter ($l = 0.5$ cm). ^{1}H NMR and ^{13}C NMR spectra were recorded on JNM-ECX 400 MHz and JEOL α-500 MHz NMR spectrometers. Elucidation of the chemical structures was based on ^{1}H, ^{13}C, COSY, HMQC, and HMBC NMR experiments. The chemical shifts are reported in parts per million (ppm) relative to the residual solvent peaks, and the

coupling constants (*J*) are reported in terms of Hertz. FABMS and HRFABMS spectra were obtained using a glycerol matrix in the positive-ion mode by using a JEOL JMS-DX-303HF spectrometer. HRESIMS analysis was performed using a JMS-T100LC. Column chromatography was carried out using Silica Gel 60 (Kanto Chemical, Co., Inc., 70–230 mesh and 230–400 mesh); Amberlite IR-120B; MB-3 (Organo Co., Ltd); and Chromatorex ODS (Fuji Silysia Chemical Co., Ltd) columns. TLC was performed on precoated Silica Gel 60 F_{254} plates (E. Merck).

3.2. 4-Tolyl (2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-(1→4)-[(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)]-3,6-di-*O*-pivaloyl-1-thio-β-D-glucopyranoside (1)

$BF_3{\cdot}Et_2O$ (2.0 mL, 16.0 mmol) was added to a suspension of 4-tolyl 3,6-di-*O*-pivaloyl-1-thio-β-D-glucopyranoside (2.6 g, 5.8 mmol) and MSAW 300 (4.0 g) in CH_2Cl_2 (10 mL) at −78 °C under nitrogen. After stirring for 1 h, α-L-rhamnopyranosyl trichloroacetimidate (8.6 g, 19.8 mmol) in CH_2Cl_2 (13 mL) was added to the mixture, followed by stirring for 5 h at room temperature. The mixture was diluted with $CHCl_3$ and filtered using Celite. The filtrate was washed with satd aq $NaHCO_3$ and satd aq NaCl, dried over $MgSO_4$, and concentrated under diminished pressure. The residue was purified by silica gel column chromatography (3:1 hexane–EtOAc) to yield **1** (3.4 g, 55%) as a colorless oil: $[\alpha]_D$ +9.2 (*c* 0.13, $CHCl_3$); ^1H NMR (in $CDCl_3$): δ 1.15 (3H, d, *J* 6.1 Hz, Rha H-6), 1.17, 1.19 (each 9H, s, $C(CH_3)_3 \times 2$), 1.23 (3H, d, *J* 6.1 Hz, Rha′ H-6), 1.96 × 2, 2.03, 2.05, 2.11, 2.12 (each 3H, s, $COCH_3 \times 6$), 2.34 (1H, s, $phCH_3$), 4.72 (1H, d, *J* 8.5 Hz, Glc H-1), 4.87 (1H, s, Rha H-1), 4.92 (1H, s, Rha′ H-1); ^{13}C NMR (in $CDCl_3$): δ 99.5, 77.7, 72.0, 77.4, 75.4, 62.7 (Glc C-1–6), 98.0, 69.9, 69.2, 70.5, 67.9, 17.1 (Rha [1→2] C-1–6), 97.3, 69.5, 68.7, 70.2, 66.7, 17.1 (Rha [1→4] C-1–6); HRESIMS: calcd for $C_{47}H_{66}NaO_{21}S$: 1021.3715; found: *m*/*z* 1021.3798 $[M+Na]^+$.

3.3. Preparation of (2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-(1→4)-[(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)]-3,6-di-*O*-pivaloyl-α-D-glucopyranoside (2)[6]

TMSOTf (50 μL, 276 mmol) was added to a suspension of **1** (200 mg, 200 μmol) and NIS (100 mg, 444 μmol) in CH_2Cl_2 (4.0 mL) at −78 °C under nitrogen. After stirring for 10 min, H_2O (1.0 mL, 56 mmol) was added to the mixture, followed by stirring for 3 h at room temperature. Next, $Na_2S_2O_3$ (20 mg, 127 mmol) and $NaHCO_3$ (20 mg, 238 mmol) were added to the mixture, followed by stirring for 1 h. The precipitate was filtered, and the filtrate was concentrated under diminished pressure. The residue was purified by silica gel column chromatography (2:1 hexane–acetone) to yield **2** (111 mg, 62%) as a colorless oil. Physicochemical data were identical to those described in Ref. 6.

3.4. (2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-(1→4)-[2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1→2)]-3,6-di-*O*-pivaloyl-α-D-glucopyranosyl diethyl phosphite (4)

Diethyl chlorophosphite (20 μL, 138 μmol) was added to a stirred solution of trisaccharide **2** (55 mg, 55 μmol) and Et_3N (25 μL, 181 μmol) in CH_2Cl_2 (1.5 mL) at 0 °C under nitrogen. After stirring for 30 min, the reaction was quenched with MeOH, followed by stirring at room temperature for 30 min. The mixture was concentrated under diminished pressure, and the residue was purified by silica gel column chromatography (2:1 hexane–EtOAc) to yield **4** (52 mg, 93%) as a colorless oil: ^1H NMR (in $CDCl_3$): δ 1.20, 1.23 (each 9H, s, $C(CH_3)_3 \times 2$), 1.31 (3H, t, *J* 7.3 Hz, OCH_2CH_3), 1.33 (3H, t, *J* 7.0 Hz, OCH_2CH_3), 1.94, 1.98, 2.02, 2.04, 2.11, 2.12 (each 3H, s, $COCH_3 \times 6$), 4.76 (1H, s, Rha H-1), 4.89 (1H, s, Rha′ H-1), 5.20 (1H, dd, *J* 3.7 Hz, Glc H-1), 5.55 (1H, dd, *J* 9.1, 9.2 Hz, Glc H-3).

3.5. (2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-(1→4)-[2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1→2)]-3,6-di-*O*-pivaloyl-α-D-glucopyranosyl diphenyl phosphate (5)

Diphenyl phosphoryl chloride (40 mL, 193 μmol) was added to a stirred solution of trisaccharide **2** (20 mg, 20 μmol), DMAP (3 mg, 25 μmol), and Et_3N (30 μL, 217 μmol) in CH_2Cl_2 (1.2 mL) at 0 °C under nitrogen. After stirring for 1 h, the reaction was quenched with MeOH, followed by stirring at room temperature for 30 min. The mixture was concentrated under diminished pressure, and the residue was purified by silica gel column chromatography (3:2 hexane–EtOAc) to yield **5** (19 mg, 86%) as a colorless oil: ^1H NMR (in $CDCl_3$): δ 1.19, 1.22 (each 9H, s, $C(CH_3)_3 \times 2$), 1.87, 1.93, 1.98, 2.05, 2.11 × 2 (each 3H, s, $COCH_3 \times 2$), 4.79 (1H, s, Rha H-1), 4.83 (1H, s, Rha′ H-1), 5.52 (1H, dd, *J* 9.1, 9.8 Hz, Glc H-3), 6.00 (1H, d, *J* 3.7 Hz, Glc H-1).

3.6. General procedure for the glycosylation of (2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-(1→4)-[2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl(1→2)]-3,6-di-*O*-pivaloyl-D-glucopyranosyl diosgenin (6α, 6β)

3.6.1. Procedure A using 4-tolyl thio-β-chacotorioside. 4-Tolyl thio-β-chacotorioside **1** (50 mg, 50 μmol), diosgenin (60 mg, 144 μmol), NIS (25 mg, 111 μmol), and MSAW 300 (200 mg) were suspended in CH_2Cl_2 (2.0 mL) at room temperature. After stirring for 30 min, TMSTOf (1 M solution in Et_2O, 10 μL) was added to the mixture dropwise. The mixture was stirred for 30 min at room temperature, diluted with $CHCl_3$,

washed with satd aq $NaHCO_3$ and satd aq NaCl, and concentrated under diminished pressure. The obtained residue was purified by column chromatography (2:1 hexane–EtOAc) to yield a mixture of **6α** and **6β** as a colorless oil. The mixture was separated by ODS column chromatography (9:1 MeOH–H_2O) to yield **6α** (22 mg, 34%) and **6β** (9 mg, 14%), both as white solids. Physicochemical data were identical to those described in Ref. 6.

3.6.2. Procedure B using 1-hydroxy-α-chacotorioside. Trifluoromethanesulfonic anhydride (15 μL, 9 μmol) was added to a solution of **2** (22 mg, 22 μmol), diphenyl sulfoxide (40 mg, 198 μmol), and TTBP (50 mg, 202 mmol) in CH_2Cl_2 at −40 °C. After stirring for 1.5 h, diosgenin (35 mg, 84 μmol) was added to the mixture at −40 °C. The reaction mixture was stirred for 1 h before the addition of excess Et_3N. The mixture was diluted with CH_2Cl_2, washed with satd aq $NaHCO_3$ and satd aq NaCl, and concentrated under diminished pressure. The residue was purified by silica gel column chromatography (2:1 hexane–EtOAc) to yield a mixture of **6α** and **6β** as a colorless oil. The mixture was separated by ODS column chromatography (9:1 MeOH–H_2O) to yield **6α** (5 mg, 18%) and **6β** (1 mg, 4%), both as white solids. Physicochemical data were identical to the products of Section 3.6.1.

3.6.3. Procedure C using chacotriosyl trichloroacetimidate. Chacotriosyl imidate **3** (48 mg, 64 μmol), diosgenin (400 mg, 0.97 mmol), and MSAW 300 (800 mg) were suspended in CH_2Cl_2 (8.0 mL) at 20 °C. After stirring for 30 min, $BF_3{\cdot}Et_2O$ (10% solution in CH_2Cl_2, 2.0 μmol) was added dropwise to the mixture. The mixture was stirred for 2 h at room temperature, diluted with $CHCl_3$, and filtered using Celite. The filtrate was washed with satd aq $NaHCO_3$ and satd aq NaCl, dried over $MgSO_4$, and concentrated under diminished pressure. The residue was purified by column chromatography (2:1 hexane–EtOAc) to yield a mixture of **6α** and **6β** as a colorless oil. The mixture was separated by ODS column chromatography (9:1 MeOH–H_2O) to yield **6α** (26 mg, 44%) and **6β** (12 mg, 20%), both as white solids. Physicochemical data were identical to the products of Section 3.6.1.

3.6.4. Procedure D using chacotriosyl diethyl phosphite. Chacotriosyl diethyl phosphite **4** (25 mg, 25 μmol), diosgenin (15 mg, 36 μmol), and MSAW 300 (140 mg) were suspended in CH_2Cl_2 (1.5 mL) at room temperature. After stirring for 30 min, TMSTOf (1 M solution in Et_2O, 20 μL) was added to the mixture in a dropwise fashion. After stirring for 1.5 h at room temperature, the reaction was quenched with MeOH, followed by stirring at room temperature for 30 min. The residue was concentrated under diminished pressure and then purified by column chromatography (2:1 hexane–EtOAc) to yield a mixture of **6α** and **6β** as a colorless oil. The mixture was separated by ODS column chromatography (9:1 MeOH–H_2O) to yield **6α** (4 mg, 12%) and **6β** (13 mg, 41%), both as white solids. Physicochemical data were identical to the products of Section 3.6.1.

3.6.5. Procedure E using chacotriosyl diphenyl phosphate. Chacotriosyl diphenyl phosphate **5** (30 mg, 27 μmol), diosgenin (20 mg, 48 μmol), and MSAW 300 (150 mg) were suspended in CH_2Cl_2 (1.5 mL) at room temperature. After stirring for 30 min, TMSTOf (1 M solution in Et_2O, 20 μL) was added to the mixture in a dropwise fashion. After stirring for 2 h at room temperature, the reaction was quenched with MeOH, followed by stirring at room temperature for 30 min. The residue was concentrated under diminished pressure and then purified by column chromatography (2:1 hexane–EtOAc) to yield a mixture of **6α** and **6β** as a colorless oil. The mixture was separated by ODS column chromatography (9:1 MeOH–H_2O) to yield **6α** (4 mg, 11%) and **6β** (11 mg, 32%), both as white solids. Physicochemical data were identical to the products of Section 3.6.1.

3.7. General procedure for the glycosylation of (2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-(1→4)-[2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl(1→2)]-3,6-di-*O*-pivaloyl-D-glucopyranosyl cholestanol (7α, 7β)

3.7.1. Procedure A using chacotriosyl trichloroacetimidate. Glycosylation of **3** (40 mg, 39 μmol) with cholestanol (60 mg, 178 μmol) was accomplished as described in Section 3.6.3, followed by purification by column chromatography (3:1 hexane–EtOAc) to yield a mixture of **7α** and **7β** as a colorless oil. The mixture was separated by ODS column chromatography (19:1 MeOH–H_2O) to afford **7α** (17 mg, 34%) and **7β** (4 mg, 8%), both as white solids. Data for α-glycoside (**7α**): $[\alpha]_D^{15}$ −22.1 (*c* 0.21, $CHCl_3$); ^1H NMR (in $CDCl_3$): δ 0.66 (3H, s, H-19), 0.86 (3H, d, *J* 6.1 Hz, H-27), 0.87 (3H, s, H-18), 0.89 (3H, d, *J* 6.1 Hz, H-21), 1.16 (3H, d, *J* 6.7 Hz, Rha H-6), 1.21 (3H, d, *J* 6.1 Hz, Rha′ H-6), 1.20, 1.23 (each 9H, s, $C(CH_3)_3 \times 2$), 1.95, 1.98, 2.02, 2.03, 2.11, 2.12 (each 3H, s, $COCH_3 \times 6$), 4.72 (1H, s, Rha H-1), 4.88 (1H, s, Rha′ H-1), 5.03 (1H, d, *J* 3.1 Hz, Glc H-1); ^{13}C NMR (in $CDCl_3$): δ 37.1, 27.9, 78.8, 29.7, 45.2, 29.3, 31.9, 35.6, 54.5, 35.6, 21.3, 28.3, 39.0, 56.4, 24.2, 28.8, 56.6, 12.1, 12.4, 35.8, 20.6, 36.2, 23.9, 39.6, 29.3, 22.8, 22.6 (C-1–27), 97.6, 80.0, 71.3, 77.5, 71.2, 62.6 (Glc C-1–6), 99.9, 69.6, 68.8, 71.0, 67.9, 17.6 (Rha [1→2] C-1–6), 96.3, 68.9, 68.0, 70.3, 66.9, 17.2 (Rha′ [1→4] C-1–6); HRESIMS: calcd for $C_{67}H_{106}O_{22}Na$: 1286.7151; found: *m*/*z* 1286.6833 $[M+Na]^+$. Data for β-glycoside (**7β**): $[\alpha]_D^{15}$ −52.1 (*c*

0.22, $CHCl_3$); ^1H NMR (in $CDCl_3$): δ 0.66 (3H, s, H-19), 0.86 (3H, d, J 6.7 Hz, H-27), 0.89 (3H, d, J 6.7 Hz, H-21), 1.08 (3H, s, H-18), 1.15 (3H, d, J 6.1 Hz, Rha H-6), 1.17 (9H, s, $C(CH_3)_3$), 1.19 (3H, d, J 6.1 Hz, Rha′ H-6), 1.21 (each 9H, s, $C(CH_3)_3$), 1.95, 1.98, 2.02, 2.04, 2.10, 2.12 (each 3H, s, $COCH_3 \times 6$), 4.60 (1H, d, J 7.4 Hz, Glc H-1), 4.82 (1H, s, Rha H-1), 4.88 (1H, s, Rha′ H-1); ^{13}C NMR (in $CDCl_3$): δ 37.1, 27.1, 79.2, 29.1, 45.1, 29.4, 31.9, 35.6, 54.5, 35.5, 21.3, 28.0, 39.1, 56.4, 24.1, 28.6, 56.4, 12.3, 12.4, 35.9, 20.6, 36.1, 23.9, 39.6, 29.3, 22.7, 22.5 (C-1–27), 97.5, 80.2, 71.1, 77.5, 71.1, 62.6 (Glc C-1–6), 99.8, 69.5, 68.8, 71.2, 67.9, 17.8 (Rha [1→2] C-1–6), 96.6, 68.8, 68.0, 70.3, 66.8, 17.5 (Rha′ [1→4] C-1–6); HRESIMS: calcd for $C_{67}H_{106}O_{22}Na$: 1286.7151; found: m/z 1286.6945 $[M+Na]^+$.

3.7.2. Procedure B using chacotriosyl diethyl phosphite. Glycosylation of **4** (52 mg, 51 μmol) with cholestanol (80 mg, 206 μmol) was accomplished as described in Section 3.6.4, followed by purification by column chromatography (3:1 hexane–EtOAc) to yield a mixture of **7α** and **7β** as a colorless oil. The mixture was separated by ODS column chromatography (19:1 MeOH–H_2O) to afford **7α** (8 mg, 12%) and **7β** (21 mg, 32%), both as white solids. Physicochemical data were identical to the products of Section 3.7.1.

3.7.3. Procedure E using chacotriosyl diphenyl phosphate. Glycosylation of **4** (19 mg, 17 μmol) with cholestanol (30 mg, 77 μmol) was accomplished as described in Section 3.6.5, followed by purification by column chromatography (3:1 hexane–EtOAc) to yield a mixture of **7α** and **7β** as a colorless oil. The mixture was separated by ODS column chromatography (19:1 MeOH–H_2O) to afford **7α** (3 mg, 14%) and **7β** (7 mg, 32%), both as white solids. Physicochemical data were identical to the products of Section 3.7.1.

3.8. Cholestanol-3β-yl α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-α-D-glucopyranoside (9α)

Compound **7α** (41 mg, 32 μmol) was dissolved in 3% KOH/MeOH (5.0 mL) and heated at 65 °C. After stirring for 20 h, the mixture was purified by column chromatography (MCI gel, CHP20P; water, 250 mL; MeOH, 150 mL) and then concentrated under diminished pressure to afford **9α** (27 mg, 98%) as a white solid: $[\alpha]_D^{15}$ +35.1 (c 0.16, $CHCl_3$); ^1H NMR (in $CDCl_3$): δ 0.65 (6H, s, H-18, 19), 0.90 (3H, d, J 6.7 Hz, H-26, 27), 0.96 (3H, d, J 6.7 Hz, H-21), 1.67 (3H, d, J 6.1 Hz, Rha H-6), 1.72 (3H, d, J 6.1 Hz, Rha′ H-6), 5.52 (1H, s, Glc H-1), 5.85 (1H, s, Rha H-1), 5.97 (1H, s, Rha′ H-1); ^{13}C NMR (in C_5D_5N): δ 37.6, 30.2, 78.7, 39.0, 45.3, 29.4, 32.3, 32.1, 50.5, 37.0, 21.3, 28.5, 42.5, 56.9, 24.6, 40.0, 56.4, 12.0, 19.5, 36.1, 18.9, 36.5, 24.2, 39.8, 20.8, 23.0, 22.7 (C-1–27), 100.3, 78.2, 76.9, 78.0, 77.8, 61.3 (Glc C-1–6), 102.2, 72.6, 72.7, 74.2, 69.5, 18.7 (Rha [1→2] C-1–6), 102.9, 72.6, 72.9, 73.9, 70.4, 18.5 (Rha [1→4] C-1–6); HRESIMS: calcd for $C_{45}H_{78}O_{14}Na$: 865.5289; found: m/z 865.5183 $[M+Na]^+$.

3.9. Cholestanol-3β-yl α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (9β)

Compound **7β** (18 mg, 14 μmol) was dissolved in 3% KOH/MeOH (3.0 mL) and heated at 65 °C. After stirring for 20 h, the mixture was purified by column chromatography (MCI gel, CHP20P; water, 100 mL; MeOH, 80 mL) and then concentrated under diminished pressure to afford **9a** (11 mg, 92%) as a white solid: $[\alpha]_D^{15}$ −46.3 (c 0.23, $CHCl_3$); ^1H NMR (in $CDCl_3$) δ: 0.65 (3H, s, H-18), 0.90 (3H, d, J 6.7 Hz, H-26), 0.91 (3H, d, J 6.7 Hz, H-27), 0.98 (3H, d, J 6.7 Hz, H-21), 1.09 (3H, s, H-19), 1.63 (3H, d, J 6.1 Hz, Rha H-6), 1.78 (3H, d, J 6.1 Hz, Rha′ H-6), 4.37 (1H, d, J 7.3 Hz, Glc H-1), 5.87 (1H, s, Rha H-1), 6.41 (1H, s, Rha′ H-1); ^{13}C NMR (in C_5D_5N): δ 37.6, 29.9, 79.8, 39.1, 45.3, 29.3, 32.1, 32.0, 50.4, 37.0, 21.3, 28.5, 42.5, 56.9, 24.6, 40.0, 56.4, 12.0, 19.5, 36.1, 18.9, 36.5, 24.2, 39.8, 20.7, 23.0, 22.6 (C-1–27), 97.2, 78.1, 76.8, 78.0, 77.9, 61.3 (Glc C-1–6), 102.1, 72.6, 72.7, 74.2, 69.5, 18.6 (Rha [1→2] C-1–6), 102.9, 72.6, 72.9, 73.9, 70.4, 18.5 (Rha [1→4] C-1–6); HRESIMS: calcd for $C_{45}H_{78}O_{14}Na$: 865.5289; found: m/z 865.5112 $[M+Na]^+$.

3.10. Cancer cell growth inhibitory bioassay

The in vitro inhibitory effects of compounds **8α**–**9β** on the proliferation of human lung carcinoma (A549) and hepatoblastoma (HepG2) cell lines were measured with the MTT method.[20] Human tumor cells were maintained as adherent cell cultures in the MEM medium supplemented with 1 mM sodium pyruvate and 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator containing 5% CO_2. The cells were counted, transferred into 96-well microtiter plates at a density of 5×10^3 cells per well in 100 μL of culture medium, and then incubated with 10% FBS for 24 h prior to the addition of the test compounds. The test compounds were dissolved in DMSO (100 μL each) and diluted with the medium (without FBS) to the desired concentration. To microtiter wells emptied of their original media, the test compounds were then added, followed by incubation for 24 h. At the final concentration of 0.5%, DMSO exhibited no interference with the growth of the cell lines. A MTT solution (10 μL/well) was added to cells, which were then incubated for 2 h at 37 °C. The absorbances of the resulting solutions were measured at 450 nm on an automated microplate reader (Labsystems Multiskan MS). Each experiment was repeated twice in triplicate.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.02.012.

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