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Oleanane-type triterpene saponins with collagen synthesis-promoting activity from the flowers of *Bellis perennis*

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ABSTRACT

The methanol extract from *Bellis perennis* (Asteraceae) flowers was found to promote collagen synthesis in normal human dermal fibroblasts (NHDFs). Seven oleanane-type triterpene saponins, perennisosides XIII–XIX, and two known saponins, bellissaponins BS5 and BS9, were isolated from the methanol extract. The structures were determined based on chemical and physicochemical data, and confirmed using previously isolated related compounds as references. Among the isolates, including 19 previously reported saponins, perennisosides XVIII, I, II, VII, IX, and XI, asterbatanoside D, bernardioside B_2, and bellissaponins BS5 and BS9 significantly promoted collagen synthesis at 3–30 μM without cytotoxicity.

1. Introduction

Bellis perennis is an Asteraceae plant species commonly known as the daisy, which is widely distributed in Europe and North Africa. The whole flowering plant of *B. perennis* has been used for the treatment of bruises, bleeding, muscular pain, purulent skin diseases, and rheumatism in European folk medicine (Gruenwald et al., 2007). During studies exploring the bioactive constituents of *B. perennis* flowers, isolation and structural elucidation of triterpene saponins, flavonoids, and aromatic and acyclic alcohol glycosides were reported (Karakas et al., 2014; Morikawa et al., 2008, 2010, 2011; Yoshikawa et al., 2008). It was also found that the methanol extract and several saponin constituents inhibited elevation of triglyceride levels in plasma (Yoshikawa et al., 2008) and pancreatic lipase activity (Morikawa et al., 2010), and induced gastric emptying in olive oil-loaded mice (Morikawa et al., 2011). Evaluation of the methanol extract established that it can promote collagen synthesis in normal human dermal fibroblasts (NHDFs). Separation of the active constituents in the extract resulted in isolation of seven new oleanane-type triterpene saponins, called perennisosides XIII–XIX (**1**–**7**). This study deals with the isolation and structural elucidation of these new saponins (**1**–**7**), as well as collagen synthesis activity of its saponin constituents.

2. Results and discussion

2.1. Effects of the methanol extract on collagen synthesis in NHDFs

One of the main causes of skin aging is the reduction of type-I collagen, a primary component of the dermal layer of skin (Meigel et al., 1977). Thus, compounds that maintain type-I collagen levels may be useful to prevent skin aging. (Lee et al., 2007). Collagen is a fibrous extracellular matrix protein. In addition, it is a major component of connective tissue in the human body. Approximately 3–6% of the total tissue protein in the body is collagen, and the functional properties of skin depend on the integrity of collagen in the dermis. Collagen deposition is finely controlled, and is dependent on the physiological status of the body. Changes in the rate of collagen deposition occur during wound healing, development of new bone, and aging. Thus, the control of collagen metabolism may be useful for various therapeutic

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and cosmetic applications (Koya-Miyata et al., 2004). Medicinal foods could be potential resources for these applications because of their low toxicity.

Dried flowers of *B. perennis* were extracted with methanol under conditions of reflux to obtain the methanol extract (25.8% from the dried material). The methanol extract was partitioned using ethyl acetate (EtOAc)–H_2O (1:1, v/v) to yield an EtOAc-soluble fraction (6.7%) and an aqueous phase. The latter was subjected to Diaion HP-20 column chromatography ($H_2O \rightarrow$ MeOH) to yield H_2O- and MeOH-eluted fractions (12.5% and 6.4%, respectively). The methanol extract (1–10 μg/mL) was found to promote collagen synthesis in normal human dermal fibroblasts (NHDFs) (Table 1). Using bioassay-guided fractionation, the MeOH-eluted fraction was found to be the active fraction (% control of collagen content at 10 μg/mL: 147.3 ± 1.9, $p < 0.05$) without cytotoxicity [cell viability (%) in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: 98.8 ± 1.1]. In contrast, the EtOAc-soluble fraction (% control of collagen content at 10 μg/mL: 100.0 ± 4.2; cell viability (%): 102.9 ± 0.8) and the H_2O-eluted fraction (87.8 ± 9.1 and 105.9 ± 0.8, respectively) showed no activity.

2.2. Isolation

In previous studies, 34 saponin constituents were isolated from the active MeOH-eluted fraction including perennisosides I (**8**, 0.0122%), II (**9**, 0.0110%), III (**10**, 0.0026%), VII (**11**, 0.0089%), VIII (**12**, 0.0124%), IX (**13**, 0.0076%), XI (**14**, 0.0132%), and XII (**15**, 0.0111%), perennisaponins B (**16**, 0.0048%), F (**17**, 0.0586%), and K (**18**, 0.0268%), asterbatanoside D (**19**, 0.0063%), bernardioside B_2 (**20**, 0.0128%), bellidioside A (**21**, 0.0575%), bellissaponins BS1 (**22**, 0.0035%) and BS6 (**23**, 0.0233%), and bellisosides D (**24**, 0.0262%), E (**25**, 0.1906%), and F (**26**, 0.0262%) (Morikawa et al., 2008, 2010, 2011; Yoshikawa et al., 2008).

In the present study, several additional constituents in the MeOH-eluted fraction were identified using normal-phase silica gel and reversed-phase ODS column chromatography, followed by HPLC. Seven new oleanane-type triterpene saponins, called perennisosides XIII (**1**, 0.0022%), XIV (**2**, 0.0130%), XV (**3**, 0.0007%), XVI (**4**, 0.0143%), XVII (**5**, 0.0130%), XVIII (**6**, 0.0117%), and XIX (**7**, 0.0005%), and two known saponins, bellissaponins BS5 (**27**, 0.0059%) (Glensk et al., 2001; Wray et al., 1992) and BS9 (**28**, 0.0029%) (Glensk et al., 2001) were isolated as shown in Fig. 1.

2.3. Structures of perennisosides XIII–XIX (*1*–*7*)

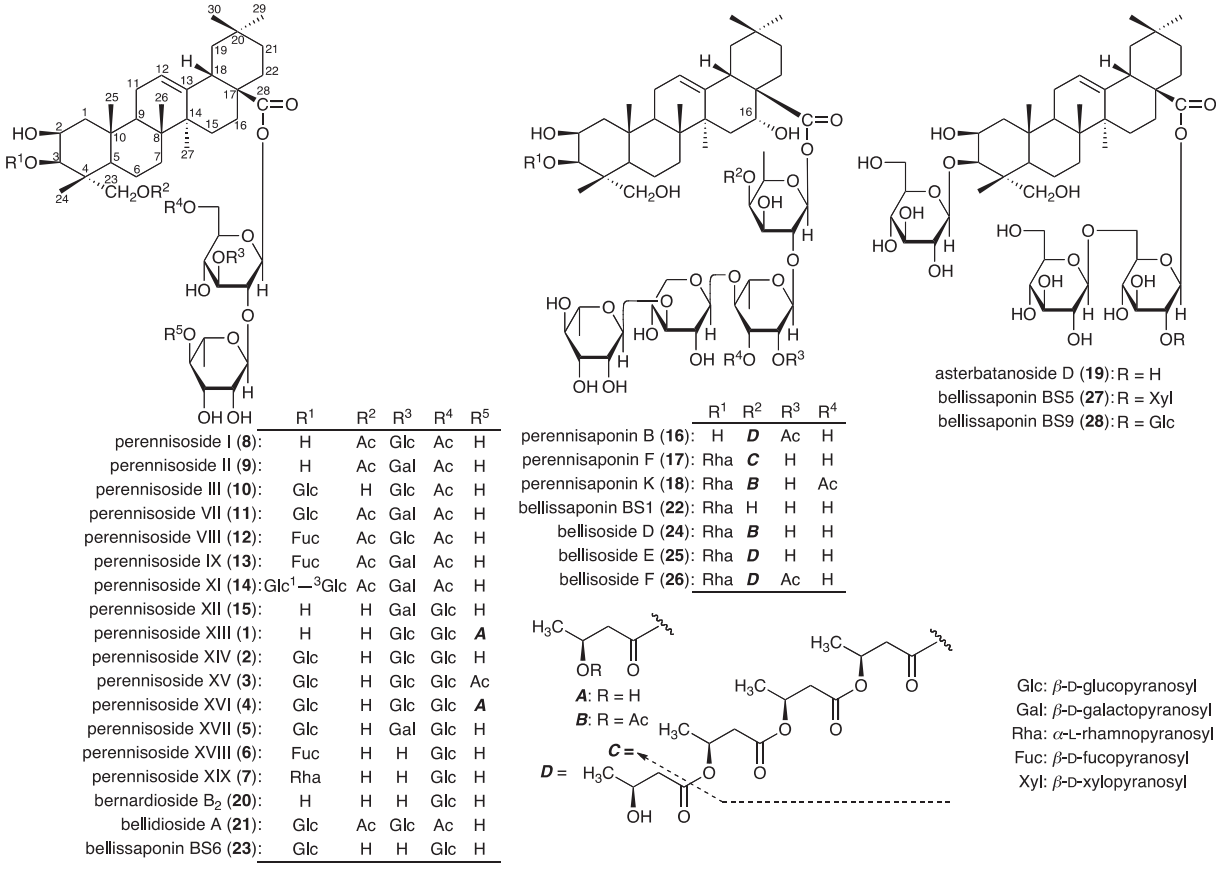
Perennisoside XIII (**1**) was obtained as an amorphous powder with a positive optical rotation ($[\alpha]_D^{27}$ +7.6 in MeOH). Using positive- and negative-ion FABMS, quasimolecular ion peaks were observed at *m*/*z* 1229 $[M+Na]^+$ and 1205 $[M-H]^-$, and HRFABMS analysis established that its molecular formula was $C_{58}H_{94}O_{26}$. Treatment of **1** with 0.5% sodium methoxide (NaOMe)–MeOH produced a des-acyl derivative and methyl (*S*)-(+)-3-hydroxybutyrate (Burk et al., 1995), which was identified by HPLC using an optical rotation detector (Morikawa et al., 2010, 2011; Yoshikawa et al., 2008). The des-acyl derivative was treated successively with 5% aqueous sulfuric acid (H_2SO_4)–1,4-dioxane (1:1, v/v) to liberate bayogenin (Kasai et al., 1988), together with L-rhamnose and D-glucose; these were identified by HPLC using an optical rotation detector (Morikawa et al., 2010, 2011; Yoshikawa et al., 2008). The ^1H (Table 2) and ^{13}C NMR (Table 3) spectra (pyridine-d_5) of **1**, which were assigned with the aid of DEPT, TOCSY, ^1H–^1H COSY, HSQC, HMBC, and phase-sensitive ROESY experiments, showed signals assignable to six methyls [δ 0.85, 0.87, 1.23, 1.34, 1.35, 1.67 (3H each, all s, H_3-29, 30, 26, 27, 24, 25)], a methylene, and two methines bearing an oxygen function [δ 3.66, 4.15 (1H each, both d, J = 10.3 Hz, H_2-23), 4.22 (1H, br s, H-3), 4.55 (1H, br s, H-2)], an olefinic proton [δ 5.49 (1H, t-like, J = *ca.* 3 Hz, H-12)], three glucopyranosyl moieties [δ 4.96 (1H, d, J = 7.6 Hz, Glc-H-1‴″), 5.11 (1H, d, J = 7.9 Hz, Glc-H-1‴′), 6.03 (1H, d, J = 7.6 Hz, Glc-H-1′)], and a rhamnopyranosyl moiety [δ 1.60 (3H, d, J = 6.2 Hz, Rha-H_3-6″), 6.51 (1H, br s, Rha-H-1‴)] with a 3-hydroxybutyryl group {δ 1.54 (3H, d, J = 6.2 Hz, 3HB-H_3-4‴″″), [2.80 (1H, dd, J = 4.8, 13.7 Hz), 2.94 (1H, dd, J = 8.2, 13.7 Hz), 3HB-H_2-2‴″″], 4.69 (1H, m, 3HB-H-3‴″″)}. The ^1H and ^{13}C NMR spectra of **1** resembled that of bernardioside B_2 (**20**) (Schöpke et al., 1996), except for the signals due to an additional β-D-glucopyranosyl moiety (Glc-H-1‴′) and a 3-hydroxybutyryl group. As shown in Fig. S1, the TOCSY and ^1H–^1H COSY experiments on **1** indicated the presence of partial structures written in bold lines. In the HMBC experiment of **1**, long-range correlations were observed between the following protons and carbons: Glc-H-1′ and C-28 (δ_C 176.3); Glc-H-2′ [δ 4.32 (m)] and Rha-C-1 (δ_C 101.1); Rha-H-1‴ and C-2′ (δ_C 75.1); Glc-H-1‴′ and C-3′ (δ_C 88.8); Glc-H-1‴″ and C-6′ (δ_C 69.2); and 3HB-H_2-2‴″″, Rha-H-4‴ and 3HB-C-1‴″″ (δ_C 171.9) (Fig. S1). The 28-*O*-sugar moieties in **1** were also characterized by a phase-sensitive ROESY experiment as shown in Fig. 2. The evidence mentioned above led to deduction of the structure of **1** as bayogenin {28-*O*-[4-*O*-(3*S*)-3-hydroxybutyryl]-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 3)]-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester.

Perennisoside XIV (**2**) was obtained as an amorphous powder with a negative optical rotation ($[\alpha]_D^{23}$ −2.3 in MeOH). In the positive-ion FABMS, a quasimolecular ion peak was observed at *m*/*z* 1305 $[M+Na]^+$, and HRFABMS analysis indicated that the molecular formula was $C_{60}H_{98}O_{29}$. Acid hydrolysis of **2** with 5% aqueous H_2SO_4–1,4-dioxane (1:1, v/v) liberated bayogenin together with L-rhamnose and D-glucose, which were again identified by HPLC using an optical rotation detector. The ^1H (Table 2) and ^{13}C NMR (Table 3) spectra (pyridine-d_5) showed signals assignable to the bayogenin portion [δ 0.84, 0.86, 1.19, 1.22, 1.33, 1.62 (3H each,

Table 1
Effects of methanol extract of *B. perennis* flowers and TGF-β1 on collagen synthesis in NHDFs.

		Collagen content (% of control)			
		0 μg/mL	1 μg/mL	3 μg/mL	10 μg/mL
MeOH extract		100.0 ± 1.7 (100.0 ± 3.5)	115.5 ± 2.8[a] (95.4 ± 1.5)	118.9 ± 5.5[b] (95.8 ± 3.0)	140.2 ± 1.4[b] (89.9 ± 0.9)
	0 ng/mL	1 ng/mL	3 ng/mL	10 ng/mL	30 ng/mL
TGF-β1	100.0 ± 1.8 (100.0 ± 2.4)	196.5 ± 5.7[b] (85.2 ± 1.2)	240.0 ± 5.7[b] (74.1 ± 3.1[c])	255.9 ± 10.1[b] (71.0 ± 2.6[c])	261.0 ± 2.0[b] (70.5 ± 2.0[c])

Each value represents the mean ± S.E.M. (N = 4).
Significantly different from the control.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] Cytotoxic effects were observed, and values in parentheses indicate cell viability (%) in MTT assay.



	R^1	R^2	R^3	R^4	R^5
perennisoside I (**8**):	H	Ac	Glc	Ac	H
perennisoside II (**9**):	H	Ac	Gal	Ac	H
perennisoside III (**10**):	Glc	H	Glc	Ac	H
perennisoside VII (**11**):	Glc	Ac	Gal	Ac	H
perennisoside VIII (**12**):	Fuc	Ac	Glc	Ac	H
perennisoside IX (**13**):	Fuc	Ac	Gal	Ac	H
perennisoside XI (**14**):	Glc^1—^3Glc	Ac	Gal	Ac	H
perennisoside XII (**15**):	H	H	Gal	Glc	H
perennisoside XIII (**1**):	H	H	Glc	Glc	***A***
perennisoside XIV (**2**):	Glc	H	Glc	Glc	H
perennisoside XV (**3**):	Glc	H	Glc	Glc	Ac
perennisoside XVI (**4**):	Glc	H	Glc	Glc	***A***
perennisoside XVII (**5**):	Glc	H	Gal	Glc	H
perennisoside XVIII (**6**):	Fuc	H	H	Glc	H
perennisoside XIX (**7**):	Rha	H	H	Glc	H
bernardioside B_2 (**20**):	H	H	H	Glc	H
bellidioside A (**21**):	Glc	Ac	Glc	Ac	H
bellissaponin BS6 (**23**):	Glc	H	H	Glc	H

	R^1	R^2	R^3	R^4
perennisaponin B (**16**):	H	***D***	Ac	H
perennisaponin F (**17**):	Rha	***C***	H	H
perennisaponin K (**18**):	Rha	***B***	H	Ac
bellissaponin BS1 (**22**):	Rha	H	H	H
bellisoside D (**24**):	Rha	***B***	H	H
bellisoside E (**25**):	Rha	***D***	H	H
bellisoside F (**26**):	Rha	***D***	Ac	H

Fig. 1. Triterpene saponins (**1**–**28**) from *B. perennis* flowers.

all s, H_3-29, 30, 26, 27, 24, 25), 3.60, 4.35 (1H each, both d, J = 11.0 Hz, H_2-23), 4.33, 4.82 (1H each, both br s, H-3, 2), 5.46 (1H, t-like, J = *ca.* 3, H-12)] together with four glucopyranosyl units [δ 4.96 (1H, d, J = 7.0 Hz, Glc-H-1‴″), 5.12 (1H, d, J = 7.6 Hz, Glc-H-1‴), 5.17 (1H, d, J = 7.6 Hz, Glc-H-1′), 6.10 (1H, d, J = 8.2 Hz, Glc-H-1″)] and a rhamnopyranosyl moiety [δ 1.75 (3H, d, J = 6.2 Hz, Rha-H_3-6‴), 6.40 (1H, br s, Rha-H-1‴)]. The ^{1}H and ^{13}C NMR spectroscopic properties were quite similar to those of bellissaponin BS6 (**23**) (Wray et al., 1992), except for the signals due to an additional β-D-glucopyranosyl moiety (Glc-H-1‴′). Finally, the position of the sugar components was determined on the basis of the HMBC experiment, which showed long-range correlations between the following proton and carbon pairs: Glc-H-1′ and C-3 (δ_C 83.0); Glc-H-1″ and C-28 (δ_C 176.4); Rha-H-1‴ and Glc-C-2″ (δ_C 74.9); Glc-H-1‴′ and Glc-C-3″ (δ_C 88.3); and Glc-H-1‴″ and Glc-C-6″ (δ_C 69.4). In a phase-sensitive ROESY experiment of **2**, the ROE correlations in the 28-*O*-sugar moieties observed between the similar proton pairs were as those of **1** (Fig. 2). Thus, the connected position was clarified unambiguously, and the structure of **2** was elucidated to be 3-*O*-β-D-glucopyranosylbayogenin {28-*O*-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 3)]-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester.

The molecular formulae of perennisosides XV (**3**) and XVI (**4**) were determined to be $C_{62}H_{100}O_{30}$ and $C_{64}H_{104}O_{31}$, respectively, by positive- and negative-ion FABMS and HRFABMS analyses. The ^{1}H (Table 2) and ^{13}C NMR (Table 3) spectroscopic properties were quite similar to those of **2**, except for the signals due to an additional acyl moiety {**3**: an acetyl moiety [δ 2.09 (3H, s)]; **4**: a (3*S*)-3-hydroxybutyryl moiety [δ 1.54 (3H, d, J = 6.2 Hz), 2.79 (1H, dd, J = 4.8, 13.7 Hz), 2.91 (1H, dd, J = 8.2, 13.7 Hz), 4.68 (1H, m)]}. Treatment of **3** and **4** with 0.5% sodium methoxide (NaOMe)–MeOH provided the common des-acyl derivative (**2**). The connectivities of the acyl group and oligoglycoside linkage were characterized in HMBC experiments, which showed long-range correlations between the following proton and carbon pairs: Glc-H-1′ [**3**: δ 5.11 (1H, d, J = 7.9 Hz); **4**: δ 5.12 (1H, d, J = 7.6 Hz)] and C-3 (**3**: δ_C 83.2; **4**: δ_C 82.9); Glc-H-1″ [**3**: δ 6.03 (1H, d, J = 7.7 Hz); **4**: δ 6.02 (1H, d, J = 7.6 Hz)] and C-28 (**3**: δ_C 176.4; **4**: δ_C 176.2); Rha-H-1‴ [**3**: δ 6.41 (1H, br s); **4**: δ 6.47 (1H, br s)] and Glc-C-2″ (**3**: δ_C 75.1; **4**: δ_C 75.0); Glc-H-1‴′ [**3**: δ 5.09 (1H, d, J = 7.9 Hz); **4**: δ 5.09 (1H, d, J = 8.2 Hz)] and Glc-C-3″ (**3**: δ_C 88.6; **4**: δ_C 88.7); Glc-H-1‴″ [**3**: δ 4.93 (1H, d, J = 7.7 Hz); **4**: δ 4.94 (1H, d, J = 7.6 Hz)] and Glc-C-6″ (**3**: δ_C 69.4; **4**: δ_C 69.1); and Rha-H-4″ [**3**: δ 5.73 (1H, dd, J = 9.5, 9.6 Hz); **4**: δ 5.84 (1H, dd, J = 9.6, 9.6 Hz)] and the acyl carbonyl carbon (**3**: δ_C 170.7; **4**: δ_C 171.9). On the basis of this evidence, the structures of **3** and **4** were determined to be 3-*O*-β-D-glucopyranosylbayogenin {28-*O*-(4-*O*-acetyl)-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 3)]-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester and 3-*O*-β-D-glucopyranosylbayogenin {28-*O*-[4-*O*-(3*S*)-3-hydroxybutyryl]-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 3)]-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester, respectively.

Perennisoside XVII (**5**) was obtained as an amorphous powder. Its molecular formula, $C_{60}H_{98}O_{29}$, was found to be the same as that of **2** by HRFABMS. Acid hydrolysis of **5** with 5% aqueous H_2SO_4–1,4-dioxane (1:1, v/v) liberated bayogenin together with L-rhamnose, D-glucose, and D-galactose, which were also identified by HPLC using an optical rotation detector. The ^{1}H (Table 2) and ^{13}C NMR (Table 3) spectroscopic properties were quite similar to those of **2**, except for the signals due to a β-D-galactopyranosyl unit [δ5.01 (1H, d, J = 7.7 Hz, Gal-H-1‴′)] instead of the 3″-*O*-β-D-glucopyranosyl moiety in **2**. Connectivity of the β-D-galactopyranosyl moiety

Table 2
^{1}H NMR spectroscopic data (800[a] and 600[b] MHz, pyridine-d_5) of perennisosides XIII–XIX (**1–7**).

Position	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**5**[a]	**6**[b]	**7**[b]
1	1.31 (m)	1.22 (m)	1.28 (m)	1.24 (m)	1.23 (m)	1.32 (m)	1.30 (m)
	2.38 (dd, 3.2, 13.6)	2.28 (dd, 2.4, 14.4)	2.31 (br d, *ca.* 14)	2.30 (br d, *ca.* 13)	2.28 (dd, 2.4, 14.4)	2.35 (br d, *ca.* 14)	2.23 (m)
2	4.52 (br s)	4.77 (br s)	4.80 (br s)	4.82 (br s)	4.78 (br s)	4.77 (br s)	4.69 (br s)
3	4.22 (br s)	4.27 (br s)	4.31 (br s)	4.30 (br s)	4.27 (br s)	4.27 (br s)	4.38 (br s)
5	1.75 (br d, *ca.* 12)	1.73 (m)	1.83 (m)	1.78 (m)	1.73 (br d, *ca.* 10)	1.77 (m)	1.80 (m)
6	1.32 (m)	1.20 (m)	1.30 (m)	1.26 (m)	1.20 (m)	1.23 (m)	1.25 (m)
	1.68 (m)	1.70 (m)	1.82 (m)	1.78 (m)	1.59 (m)	1.80 (m)	1.82 (m)
7	1.78 (m)	1.70 (m)	1.72 (m)	1.77 (m)	1.70 (m)	1.51 (m)	1.60 (m)
	1.90 (dd, 4.0, 12.8)	1.75 (m)	1.83 (m)	1.91 (m)	1.75 (m)	1.72 (m)	1.70 (m)
9	1.82 (dd, 6.4, 11.0)	1.74 (m)	1.79 (m)	1.74 (m)	1.74 (m)	1.77 (m)	1.72 (m)
11	2.06 (m)	2.00 (br dd, *ca.* 5, 14)	2.03 (m)	2.03 (m)	2.00 (m)	2.01 (m)	2.04 (m)
	2.22 (m)	2.17 (m)	2.21 (m)	2.19 (m)	2.16 (ddd, 2.4, 3.2, 14.4)	2.14 (m)	2.16 (m)
12	5.49 (dd, 3.6, 3.7)	5.45 (dd, 3.4, 3.4)	5.48 (t-like, *ca.* 3)	5.47 (t-like, *ca.* 3)	5.45 (dd, 3.4, 3.8)	5.46 (t-like, *ca.* 3)	5.46 (t-like, *ca.* 3)
15	1.54 (m)	1.50 (m)	1.47 (m)	1.58 (m)	1.50 (br dd, *ca.* 3, 14)	1.55 (m)	1.54 (m)
	2.03 (m)	2.03 (m)	2.05 (m)	2.03 (m)	2.01 (m)	2.05 (m)	2.04 (m)
16	1.98 (m)	2.03 (br d, *ca.* 12)	1.98 (m)	1.95 (m)	2.01 (br d, *ca.* 15)	2.05 (m)	2.02 (m)
	2.03 (br dd, *ca.* 3,14)	2.13 (m)	2.19 (m)	2.19 (m)	2.12 (m)	2.19 (m)	2.16 (m)
18	3.15 (dd, 4.0, 13.6)	3.13 (dd, 4.0, 13.6)	3.13 (dd, 4.0, 13.6)	3.12 (dd-like)	3.12 (dd, 4.8, 13.0)	3.13 (br d, *ca.* 15)	3.14 (dd, 4.3, 13.8)
19	1.26 (m)	1.22 (m)	1.25 (m)	1.20 (m)	1.21 (m)	1.23 (m)	1.22 (m)
	1.77 (m)	1.75 (m)	1.78 (m)	1.77 (m)	1.75 (dd, 13.0, 15.2)	1.77 (m)	1.75 (m)
21	1.17 (m)	1.16 (m)	1.18 (m)	1.12 (m)	1.16 (br d, *ca.* 15)	1.15 (m)	1.17 (m)
	1.31 (m)	1.34 (ddd, 4.0, 12.8, 13.6)	1.37 (m)	1.31 (m)	1.34 (ddd, 4.0, 13.6, 14.6)	1.33 (m)	1.36 (m)
22	1.80 (m)	1.85 (ddd, 3.2, 4.0, 13.6)	1.80 (m)	1.80 (m)	1.80 (ddd, 3.2, 4.0, 14.4)	1.82 (m)	1.78 (m)
	1.99 (m)	2.00 (m)	2.00 (m)	1.92 (m)	2.00 (m)	1.96 (m)	2.04 (m)
23	3.66 (d, 10.4)	3.60 (d, 10.4)	3.62 (d, 10.8)	3.62 (d, 11.0)	3.60 (d, 11.0)	3.58 (d, 11.0)	3.57 (d, 10.9)
	4.12 (d, 10.4)	4.29 (d, 10.4)	4.30 (d, 10.8)	4.30 (d, 11.0)	4.28 (d, 11.0)	4.34 (d, 11.0)	3.70 (d, 10.9)
24	1.34 (3H, s)	1.31 (3H, s)	1.31 (3H, s)	1.31 (3H, s)	1.31 (3H, s)	1.29 (3H, s)	1.17 (3H, s)
25	1.66 (3H, s)	1.59 (3H, s)	1.62 (3H, s)	1.61 (3H, s)	1.60 (3H, s)	1.60 (3H, s)	1.60 (3H, s)
26	1.21 (3H, s)	1.18 (3H, s)	1.18 (3H, s)	1.19 (3H, s)	1.19 (3H, s)	1.16 (3H, s)	1.17 (3H, s)
27	1.33 (3H, s)	1.22 (3H, s)	1.31 (3H, s)	1.34 (3H, s)	1.23 (3H, s)	1.23 (3H, s)	1.22 (3H, s)
29	0.86 (3H, s)	0.85 (3H, s)	0.87 (3H, s)	0.86 (3H, s)	0.85 (3H, s)	0.85 (3H, s)	0.85 (3H, s)
30	0.92 (3H, s)	0.92 (3H, s)	0.92 (3H, s)	0.87 (3H, s)	0.92 (3H, s)	0.85 (3H, s)	0.91 (3H, s)
3-*O*-sugar		(Glc)	(Glc)	(Glc)	(Glc)	(Fuc)	(Rha)
1′		5.11 (d, 7.6)	5.11 (d, 7.9)	5.12 (d, 7.6)	5.11 (d, 7.8)	4.99 (d, 7.1)	5.72 (br s)
2′		4.03 (m)	4.02 (m)	4.02 (m)	4.00 (dd, 7.8, 8.8)	4.39 (m)	4.75 (br s)
3′		4.15 (m)	4.17 (m)	4.19 (m)	4.13 (dd, 8.8, 8.8)	4.03 (m)	4.56 (m)
4′		4.17 (m)	4.19 (m)	4.20 (m)	4.14 (dd, 8.8, 8.8)	4.04 (m)	4.32 (m)
5′		3.85 (m)	3.84 (m)	3.83 (m)	3.84 (ddd, 2.4, 5.6, 8.8)	3.80 (q-like)	4.55 (m)
6′		4.30 (m)	4.31 (m)	4.30 (m)	4.32 (dd, 4.8, 12.0)	1.49 (3H, d, 6.1)	1.60 (3H, d, 6.0)
		4.44 (m)	4.43 (m)	4.45 (m)	4.42 (m)		
28-*O*-sugar							
28-*O*-Glc							
1″	6.02 (d, 7.8)	6.07 (d, 7.5)	6.03 (d, 7.7)	6.02 (d, 7.6)	6.03 (d, 7.5)	6.10 (d, 8.0)	6.09 (d, 8.0)
2″	4.30 (dd, 7.8, 8.3)	4.35 (dd, 7.5, 8.8)	4.31 (m)	4.30 (m)	4.28 (dd, 7.5, 8.8)	4.35 (m)	4.34 (m)
3″	4.22 (m)	4.22 (dd, 8.8, 8.8)	4.24 (m)	4.20 (m)	4.19 (dd, 8.8, 9.2)	4.27 (m)	4.24 (m)
4″	4.24 (dd, 8.9, 9.6)	4.28 (m)	4.29 (m)	4.30 (m)	4.20 (dd, 8.8, 9.2)	4.18 (m)	4.16 (m)
5″	4.03 (m)	4.03 (m)	4.00 (m)	4.00 (m)	4.00 (ddd, 2.0, 4.8, 9.2)	4.08 (m)	4.06 (m)
6″	4.15 (dd, 4.8, 12.0)	4.14 (dd, 5.1, 12.0)	4.14 (m)	4.16 (m)	4.13 (dd, 4.8, 12.0)	4.27 (m)	4.24 (m)
	4.54 (dd, 2.0, 12.0)	4.54 (br d, *ca.* 12)	4.53 (m)	4.55 (m)	4.53 (dd, 2.0, 12.0)	4.66 (br d, *ca.* 11)	4.64 (dd, 2.3, 11.5)
2″-*O*-Rha							
1‴	6.43 (br s)	6.32 (br s)	6.41 (br s)	6.47 (br s)	6.29 (br s)	6.55 (br s)	6.46 (br s)
2‴	4.71 (br s)	4.70 (dd, 1.6, 3.2)	4.69 (br s)	4.72 (br s)	4.70 (dd, 1.6, 3.4)	4.77 (br s)	4.79 (br s)
3‴	4.52 (dd, 1.6, 9.6)	4.45 (dd, dd, 3.2, 8.8)	4.50 (dd, 3.3, 9.5)	4.53 (m)	4.42 (dd, 3.4, 8.8)	4.54 (m)	4.55 (m)
4‴	5.81 (dd, 9.6, 9.6)	4.24 (dd, 8.8, 9.6)	5.73 (dd, 9.5, 9.6)	5.84 (dd, 9.6, 9.6)	4.23 (dd, 8.8, 8.8)	4.34 (m)	4.32 (m)
5‴	4.47 (m)	4.41 (m)	4.41 (m)	4.48 (m)	4.39 (m)	4.55 (m)	4.50 (m)
6‴	1.57 (3H, d, 6.4)	1.72 (3H, d, 6.4)	1.46 (3H, d, 6.2)	1.57 (3H, d, 6.0)	1.70 (3H, d, 6.2)	1.77 (3H, d, 6.4)	1.75 (3H, d, 6.3)
3″-*O*-sugar	(Glc)	(Glc)	(Glc)	(Glc)	(Gal)		
1⁗	5.09 (d, 7.8)	5.09 (d, 7.8)	5.09 (d, 7.9)	5.09 (d, 8.2)	4.99 (d, 7.8)		
2⁗	3.98 (dd, 7.8, 8.8)	4.01 (dd, 7.8, 8.8)	3.97 (m)	3.97 (m)	4.42 (dd, 7.8, 9.1)		
3⁗	4.02 (m)	4.13 (m)	4.17 (m)	4.17 (m)	4.09 (dd, 3.4, 9.1)		
4⁗	4.04 (dd, 8.8, 8.8)	4.04 (dd, 9.6, 9.6)	4.02 (m)	4.02 (m)	4.40 (br d, *ca.* 3)		
5⁗	4.13 (m)	4.15 (m)	4.00 (m)	4.00 (m)	4.05 (m)		
6⁗	4.21 (dd, 5.6, 12.0)	4.19 (dd, 5.6, 12.0)	4.19 (m)	4.20 (m)	4.27 (m)		
	4.52 (br d, *ca.* 12)	4.52 (dd, 2.4, 12.0)	4.53 (m)	4.55 (m)	4.45 (m)		
6″-*O*-Glc							
1⁗	4.93 (d, 7.8)	4.93 (d, 7.6)	4.93 (d, 7.7)	4.94 (d, 7.6)	4.92 (d, 7.8)	4.97 (d, 7.3)	4.95 (d, 7.8)
2⁗	3.95 (dd, 7.8, 8.8)	3.95 (dd, 7.6, 8.8)	3.97 (dd, 7.7, 8.8)	3.97 (m)	3.94 (dd, 7.8, 8.8)	3.98 (m)	3.96 (m)
3⁗	4.16 (m)	4.15 (m)	4.17 (m)	4.19 (m)	4.17 (dd, 8.8, 8.8)	4.18 (m)	4.16 (m)

Table 2 (*continued*)

Position	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**5**[a]	**6**[b]	**7**[b]
4‴″	4.17 (dd, 8.9, 9.6)	4.17 (m)	4.19 (m)	4.20 (m)	4.15 (dd, 8.8, 8.8)	4.18 (m)	4.16 (m)
5‴″	3.84 (m)	3.88 (ddd, 3.2, 5.6, 9.6)	3.89 (m)	3.88 (m)	3.88 (ddd, 2.4, 5.6, 8.8)	3.86 (m)	3.86 (m)
6‴″	4.32 (dd, 4.8, 12.0)	4.30 (m)	4.31 (m)	4.30 (m)	4.31 (dd, 4.8, 12.0)	4.30 (m)	4.30 (m)
	4.44 (dd, 2.4, 12.0)	4.44 (m)	4.43 (m)	4.45 (m)	4.42 (m)	4.46 (dd, 2.4, 11.8)	4.45 (dd, 2.6, 11.8)
4‴-*O*-acyl	(3HB)		(Ac)	(3HB)			
2‴″	2.78 (dd, 4.8, 14.4)		2.09 (3H, s)	2.79 (dd, 4.8, 13.7)			
	2.91 (dd, 8.0, 14.4)			2.91 (dd, 8.2, 13.7)			
3‴″	4.65 (m)			4.68 (m)			
4‴″	1.52 (3H, d, 6.2)			1.54 (3H, d, 6.2)			

Table 3
^{13}C NMR spectroscopic data (200[a] and 150[b] MHz, pyridine-d_5) of perennisosides XIII–XIX (**1**–**7**).

Position	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**5**[a]	**6**[b]	**7**[b]	Position	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**5**[a]	**6**[b]	**7**[b]
1	45.2	44.2	44.2	44.2	44.2	44.3	44.8	28-*O*-sugar							
2	71.7	70.5	70.5	70.5	70.5	70.6	71.0	28-*O*-Glc							
3	73.4	83.3	83.2	82.9	83.3	83.0	81.6	1″	94.4	94.4	94.3	94.1	94.4	94.8	94.8
4	42.6	42.8	42.8	42.7	42.8	42.7	43.0	2″	75.26	75.1	75.1	75.0	75.3	75.1	75.8
5	48.5	48.0	47.9	47.7	48.0	47.8	47.5	3″	88.8	88.2	88.6	88.7	88.3	79.5	78.3
6	18.5	18.0	18.3	18.1	18.2	18.1	18.2	4″	69.1	69.1	69.1	68.8	69.1	71.1	71.4
7	33.5	33.1	33.3	33.4	33.1	33.1	33.0	5″	77.4	77.2	77.4	77.3	77.3	77.7	77.7
8	40.2	40.2	40.2	40.3	40.2	40.1	40.3	6″	69.4	69.6	69.4	69.1	69.5	69.4	69.6
9	48.7	48.7	48.6	48.5	48.7	48.6	48.7	2″-*O*-Rha							
10	37.3	37.1	37.1	36.9	37.1	37.0	37.2	1‴	101.1	101.3	100.9	101.0	101.3	101.4	101.5
11	24.1	24.1	24.1	24.0	24.1	24.0	24.1	2‴	72.3	72.3	72.2	72.2	72.3	72.2	72.2
12	123.1	122.9	122.8	122.9	122.9	122.8	122.8	3‴	70.3	72.6	70.1	70.1	72.58	72.5	72.5
13	144.1	144.2	143.9	144.0	144.2	144.3	144.3	4‴	75.4	73.8	75.0	74.9	73.8	73.8	73.9
14	42.5	42.6	42.5	42.5	42.6	42.5	42.5	5‴	67.7	70.1	67.6	67.6	70.07	69.7	70.3
15	29.0	28.7	28.9	28.9	28.7	28.7	28.7	6‴	18.2	18.7	18.1	18.1	18.8	18.7	18.7
16	23.3	23.4	23.2	23.1	23.4	23.4	23.5	3″-*O*-sugar	(Glc)	(Glc)	(Glc)	(Glc)	(Gal)		
17	47.3	47.3	47.3	47.2	47.3	47.2	47.2	1‴″	104.1	104.0	104.1	104.0	104.6		
18	42.0	42.1	42.0	41.8	42.1	42.0	42.0	2‴″	75.15	75.5	75.5	75.2	72.60		
19	46.6	46.5	46.5	46.3	46.5	46.4	46.5	3‴″	78.56	78.33	78.3	78.2	77.4		
20	30.8	30.7	30.7	30.6	30.7	30.7	30.7	4‴″	71.7	71.7	71.7	71.5	70.11		
21	34.2	34.2	34.2	34.0	34.2	34.0	34.1	5‴″	78.64	78.64	78.6	78.5	75.1		
22	32.3	32.3	32.3	32.2	32.3	32.3	32.4	6‴″	62.5	62.5	62.5	62.3	62.1		
23	68.0	65.9	65.6	65.3	65.9	65.5	65.2	6″-*O*-Glc							
24	14.5	15.0	15.1	15.1	15.0	15.0	14.8	1⁗″	105.5	105.4	105.4	105.4	105.4	105.3	105.3
25	17.5	17.5	17.4	17.3	17.5	17.4	17.6	2⁗″	75.32	75.3	75.3	75.2	75.4	75.3	75.2
26	17.7	17.6	17.7	17.6	17.6	17.5	17.6	3⁗″	78.30	78.25	78.2	78.1	78.34	78.3	78.3
27	26.1	26.0	26.0	26.0	26.0	25.9	26.0	4⁗″	71.7	71.67	71.6	71.3	71.72	71.4	71.6
28	176.3	176.5	176.4	176.2	176.5	176.5	176.6	5⁗″	78.34	78.5	78.6	78.4	78.2	78.3	78.3
29	33.1	33.1	33.1	33.1	33.1	33.1	33.1	6⁗″	62.8	62.8	62.7	62.5	62.8	62.5	62.7
30	23.9	23.9	23.8	23.7	23.9	23.8	23.9	4‴-*O*-acyl	(3HB)		(Ac)	(3HB)			
3-*O*-sugar		(Glc)	(Glc)	(Glc)	(Glc)	(Fuc)	(Rha)	1⁗″	172.0		170.7	171.9			
1′		105.6	105.6	105.7	105.6	106.1	104.1	2⁗″	45.6		21.1	45.5			
2′		75.5	75.5	75.4	75.5	72.5	72.6	3⁗″	64.9			64.8			
3′		78.29	78.3	78.2	78.6	75.3	72.8	4⁗″	24.2			24.2			
4′		71.7	71.7	71.5	71.67	72.6	74.0								
5′		78.59	78.6	78.4	78.27	71.4	69.8								
6′		62.8	62.7	62.5	62.8	17.3	18.6								

was characterized on the basis of the HMBC spectrum, in which a long-range correlation was observed between Gal-H-1⁗″ and Glc-C-3″ (δ_C 88.0). As was expected, ROE correlations by the phase-sensitive ROESY experiment of **5** were observed between the following proton pairs in the 3″-*O*-β-D-galactopyranosyl part in **5** {Gal-H-4⁗″ [δ 4.40 (1H, br d, J = *ca.* 3 Hz)] and Gal-H-3⁗″ [δ 4.09 (1H, dd, J = 3.4, 9.1 Hz)]/Gal-H-5⁗″ [δ 4.05 (1H, m)]} (Fig. 2). Consequently, the structure of **5** was determined to be 3-*O*-β-D-glucopyranosylbayogenin {28-*O*-α-L-rhamnopyranosyl(1 → 2)-[β-D-galactopyranosyl (1 → 3)]-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester.

Perennisosides XVIII (**6**) and XIX (**7**) were also obtained as amorphous powders with negative optical rotations (**6**: $[\alpha]_D^{26}$ −3.8; **7**; $[\alpha]_D^{24}$ −7.1 both in MeOH). In the positive- and negative-ion FABMS, common quasimolecular ion peaks were observed at *m*/*z* 1127 $[M+Na]^+$ and 1103 $[M-H]^-$, and HRFABMS analysis established that the molecular formula was $C_{54}H_{88}O_{23}$. The acid hydrolysis of **6** and **7** with 5% aqueous H_2SO_4–1,4-dioxane (1:1, v/v) liberated bayogenin together with L-rhamnose (for **6** and **7**), D-fucose (for **6**), and D-glucose (for **6** and **7**), which were identified by HPLC using an optical rotation detector. The ^{1}H (Table 2) and ^{13}C NMR (Table 3) spectra (pyridine-d_5) of **6** showed signals assignable to six methyls [δ 0.85, 0.85, 1.16, 1.23, 1.29, 1.60 (3H each, all s, H_3-29, 30, 26, 27, 24, 25)], a methylene and two methines bearing an oxygen function [δ 3.58, 4.34 (1H each, both d, J = 11.0 Hz H_2-23), 4.27 (1H, br s, H-3), 4.77 (1H, br s, H-2)], an olefinic proton [δ 5.46 (1H, t-like, J = *ca.* 3 Hz, H-12)], a β-D-fucopyranosyl [δ 4.99 (1H, d, J = 7.1 Hz, Fuc-H-1′)], two β-D-glucopyranosyl [δ 4.97 (1H, d, J = 7.3 Hz, Glc-H-1⁗″), 6.10 (1H, d, J = 8.0 Hz, Glc-H-1″)], and a

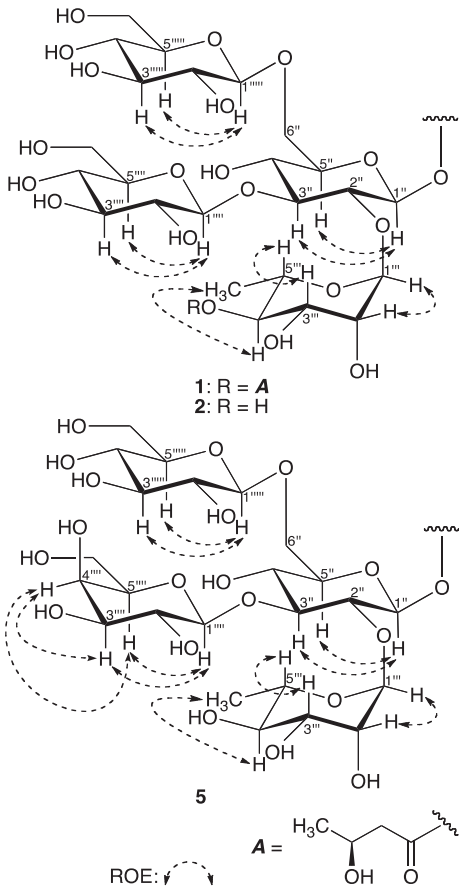


Fig. 2. Important ROE correlations of the 28-*O*-sugar moieties in **1**, **2**, and **5**.

α-L-rhamnopyranosyl moiety [δ 1.77 (3H, d, J = 6.4 Hz, Rha-H$_3$-6‴), 6.55 (1H, br s, Rha-H-1‴)]. The ^{1}H and ^{13}C NMR spectroscopic properties of **6** were quite similar to those of bellissaponin BS6 (**23**) (Wray et al., 1992), except for signals due to the 3-*O*-glycosyl moiety. In the HMBC experiment of **6**, long-range correlations were observed between the following proton and carbon pairs: Fuc-H-1′ and C-3 (δ_C 83.0); Glc-H-1″ and C-28 (δ_C 176.5); Rha-H-1‴ and Glc-C-2″ (δ_C 75.1); and Glc-H-1⁗ and Glc-C-6″ (δ_C 69.4). Thus the connected position was clarified unambiguously, and the structure of **6** was elucidated to be 3-*O*-β-D-fucopyranosylbayogenin {28-*O*-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester. On the other hand, the 3-*O*-α-L-rhamnopyranosyl analog of **6**, perennisoside XIX (**7**), was also determined by various 2D NMR measurements. Consequently, the structure of **7** was elucidated to be 3-*O*-α-L-rhamnopyranosylbayogenin {28-*O*-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester.

2.4. Effects on collagen synthesis in NHDFs

In previous studies, ursane-type triterpene 28-*O*-oligoglycosyl esters, asiaticoside and madecassoside, were isolated from *Centella asiatica*, a herb used in Sri Lankan and Indian Ayurvedic traditional medicine (Matsuda et al., 2001a,b). These triterpene saponins were reported to be promoters of human type-I collagen synthesis (Bonte et al., 1994; Lee et al., 2007; Liu et al., 2008). The structures of asiaticoside and madecassoside resembled saponin constituents from *B. perennis* flowers; thus, similar activities were expected. As shown in Table 4, perennisosides XVIII (**6**,% control of collagen content: 113.1 ± 1.1 at 30 μM), I (**8**, 120.6 ± 3.4 at 3 μM), II (**9**, 131.7 ± 3.1 at 30 μM), VII (**11**, 132.0 ± 5.0 at 10 μM), IX (**13**, 126.1 ± 6.0 at 10 μM), and XI (**14**, 124.8 ± 4.6 at 10 μM), as well as asterbatanoside D (**19**, 121.7 ± 2.8 at 10 μM), bernardioside B$_2$ (**20**, 115.4 ± 1.9 at 30 μM), and bellissaponins BS5 (**27**, 127.0 ± 2.7 at 10 μM) and BS9 (**28**, 127.6 ± 2.9 at 30 μM), significantly promoted collagen synthesis without cytotoxicity. These activities were more potent than those of asiaticoside (138.1 ± 2.3 at 100 μM) and madecassoside (113.5 ± 1.9 at 100 μM).

2.5. Concluding remarks

In conclusion, seven new oleanane-type triterpene saponins, perennisosides XIII–XIX (**1**–**7**), were isolated the methanol extract of *B. perennis* flowers, and promoted collagen synthesis in NHDFs. Among the isolates, which included 19 previously reported saponins (**8**–**26**), 10 saponin constituents (**6**, **8**, **9**, **11**, **13**, **14**, **19**, **20**, **27**, and **28**) significantly promoted collagen synthesis at concentrations of 10–30 μM, without causing cytotoxicity. These data demonstrated that the methanol extract and several saponin constituents from *B. perennis* flowers could promote the synthesis of type-I collagen. The detailed mechanism of action should be studied further.

3. Experimental

3.1. General experimental procedures

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l = 5 cm); IR spectra, Shimadzu FTIR-8100 spectrometer; ^{1}H NMR spectra, JEOL JNM-ECA800 (800 MHz), JNM-ECA600 (600 MHz), and JNM-ECS400 (400 MHz) spectrometers; ^{13}C NMR spectra, JEOL JNM-ECA800 (200 MHz), JNM-ECA600 (150 MHz), and JNM-ECS400 (100 MHz) spectrometers with tetramethylsilane as an internal standard; FABMS and HRFABMS, JEOL JMS-SX 102A mass spectrometer; HPLC detector, Shimadzu RID-6A refractive index and SPD-10A UV–VIS detectors, and Shodex OR-2 optical rotation detector; HPLC column, Cosmosil 5C$_{18}$-MS-II and HILIC (Nacalai Tesque, Inc., Kyoto, Japan) and Wakopak Navi C-30–5 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) 4.6 mm i.d. × 250 mm and 20 mm i.d. × 250 mm for analytical and preparative purposes, respectively, YMC-Pack ODS-AQ (YMC Co., Ltd., Kyoto, Japan) and Kaseisorb LC NH$_2$-60-5 (Tokyo Kasei Co., Ltd., Tokyo, Japan), 4.6 mm i.d. × 250 mm for identification of acyl and sugar moieties, respectively.

The following experimental conditions were used for column chromatography (CC): highly porous synthetic resin, Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan); normal-phase silica gel CC, silica gel 60 N (Kanto Chemical Co., Ltd., Tokyo, Japan; 63–210 mesh, spherical, neutral); reversed-phase ODS CC, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., Aichi, Japan; 100–200 mesh); TLC, pre-coated TLC plates with silica gel 60F$_{254}$ (Merck, Darmstadt, Germany, 0.25 mm, normal-phase) and silica gel RP-18 WF$_{254S}$ (Merck, Darmstadt, Germany, 0.25 mm, reversed-phase); reversed-phase HPTLC, pre-coated TLC plates with silica gel RP-18 WF$_{254S}$ (Merck, Darmstadt, Germany, 0.25 mm); detection was carried out by spraying 1% Ce(SO$_4$)$_2$–10% aqueous H$_2$SO$_4$, followed by heating.

3.2. Plant material

The flowers of *B. perennis*, which were cultivated in Albania, were purchased from Tochimoto Tenkaido Co., Ltd., Osaka, Japan in November 2006 as described previously (Morikawa et al., 2008). A voucher specimen (lot. No. 20860501K) of this plant material is on file in our laboratory.

Table 4
Effects of saponin constituents from *B. perennis* flowers on collagen synthesis in NHDFs.

	Collagen content (% of control)			
	0 μM	3 μM	10 μM	30 μM
Perennisoside XIII (**1**)	100.0 ± 5.3	104.5 ± 6.5	99.8 ± 0.7	112.4 ± 4.1
	(100.0 ± 3.3)	(89.0 ± 2.2)	(94.8 ± 3.7)	(107.0 ± 0.8)
Perennisoside XIV (**2**)	100.0 ± 4.5	98.5 ± 2.2	103.4 ± 3.7	109.0 ± 2.5
	(100.0 ± 2.6)	(99.3 ± 1.1)	(98.2 ± 0.5)	(74.9 ± 2.1[c])
Perennisoside XV (**3**)	100.0 ± 2.6	98.8 ± 2.5	97.1 ± 1.0	
	(100.0 ± 3.3)	(88.5 ± 1.4)	(65.4 ± 2.7[c])	
Perennisoside XVI (**4**)	100.0 ± 2.1	99.4 ± 1.2	100.1 ± 5.7	101.8 ± 8.0
	(100.0 ± 0.8)	(97.7 ± 1.3)	(105.6 ± 6.1)	(100.3 ± 3.6)
Perennisoside XVII (**5**)	100.0 ± 3.0	110.5 ± 8.5	90.4 ± 9.3	85.9 ± 3.5
	(100.0 ± 5.0)	(90.0 ± 3.4)	(103.1 ± 12.8)	(122.3 ± 12.8)
Perennisoside XVIII (**6**)	100.0 ± 1.3	98.0 ± 2.9	101.9 ± 1.5	113.1 ± 1.1[b]
	(100.0 ± 2.7)	(100.5 ± 1.4)	(104.3 ± 1.3)	(102.1 ± 2.0)
PerennisosideXIX (**7**)	100.0 ± 1.3	100.7 ± 0.9	111.3 ± 8.9	79.4 ± 6.5
	(100.0 ± 0.4)	(99.4 ± 0.4)	(97.7 ± 1.0)	(104.3 ± 1.7)
Perennisoside I (**8**)	100.0 ± 7.6	120.6 ± 3.4[b]	175.1 ± 11.7[b]	135.8 ± 12.4[b]
	(100.0 ± 0.9)	(105.3 ± 1.6)	(101.7 ± 1.7)	(99.9 ± 2.0)
Perennisoside II (**9**)	100.0 ± 2.1	103.1 ± 5.6	116.1 ± 7.2	131.7 ± 3.1[b]
	(100.0 ± 3.7)	(106.7 ± 1.5)	(112.6 ± 2.0)	(116.4 ± 1.0)
PerennisosideIII (**10**)	100.0 ± 2.6	109.5 ± 4.1	113.1 ± 3.3	106.0 ± 5.7
	(100.0 ± 2.7)	(102.8 ± 1.3)	(99.8 ± 0.1)	(103.1 ± 2.0)
Perennisoside VII (**11**)	100.0 ± 6.1	109.2 ± 8.8	132.0 ± 5.0[b]	
	(100.0 ± 0.7)	(89.5 ± 0.4)	(76.2 ± 0.4[c])	(73.1 ± 0.8[c])
Perennisoside VIII (**12**)	100.0 ± 9.1	86.9 ± 3.0	79.5 ± 4.0	90.5 ± 3.7
	(100.0 ± 0.6)	(99.6 ± 1.7)	(108.5 ± 1.9)	(120.0 ± 1.2[b])
Perennisoside IX (**13**)	100.0 ± 1.2	126.1 ± 6.0[b]	133.9 ± 1.5[b]	143.8 ± 7.1[b]
	(100.0 ± 1.2)	(102.7 ± 1.3)	(105.3 ± 2.1)	(111.1 ± 2.9[a])
Perennisoside XI (**14**)	100.0 ± 3.2	124.8 ± 4.6[b]	120.3 ± 2.9[b]	120.0 ± 1.4[b]
	(100.0 ± 0.8)	(111.1 ± 4.0)	(112.3 ± 2.2)	(97.9 ± 2.3)
Perennisoside XII (**15**)	100.0 ± 1.4	97.8 ± 2.3	96.7 ± 4.6	89.5 ± 14.3
	(100.0 ± 2.5)	(95.5 ± 2.8)	(117.0 ± 9.0)	(125.6 ± 8.5[b])
Perennisaponin B (**16**)	100.0 ± 5.1	114.7 ± 2.6		
	(100.0 ± 1.2)	(113.5 ± 2.6)	(32.0 ± 9.4[c])	(1.0 ± 16.3[c])
Perennisaponin F (**17**)	100.0 ± 1.0	109.4 ± 6.0		
	(100.0 ± 1.9)	(78.1 ± 3.6[c])	(2.7 ± 17.7[c])	
Perennisaponin K (**18**)	100.0 ± 3.7	102.8 ± 2.8		
	(100.0 ± 5.4)	(115.8 ± 12.1)	(25.8 ± 10.8[c])	(3.2 ± 14.1[c])
Asterbatanoside D (**19**)	100.0 ± 3.9	113.1 ± 4.1	121.7 ± 2.8[b]	94.7 ± 4.5
	(100.0 ± 0.9)	(105.6 ± 3.0)	(109.9 ± 1.9)	(133.6 ± 2.6[b])
Bernardioside B_2 (**20**)	100.0 ± 2.6	106.7 ± 0.8	112.6 ± 1.2	115.4 ± 1.9[b]
	(100.0 ± 0.9)	(120.3 ± 2.8[b])	(121.1 ± 7.9[b])	(124.8 ± 4.1[b])
Bellidioside A (**21**)	100.0 ± 3.9	105.4 ± 4.5	113.3 ± 3.3	108.3 ± 1.4
	(100.0 ± 2.7)	(103.6 ± 1.3)	(92.8 ± 1.7)	(91.3 ± 0.7)
Bellissaponin BS1 (**22**)	100.0 ± 5.3	90.9 ± 1.5	92.1 ± 2.2	
	(100.0 ± 2.4)	(101.6 ± 1.0)	(106.0 ± 3.1)	(39.6 ± 5.4[c])
Bellissaponin BS6 (**23**)	100.0 ± 5.8	107.6 ± 4.7	98.2 ± 3.3	112.0 ± 7.3
	(100.0 ± 1.6)	(99.0 ± 2.1)	(96.8 ± 1.9)	(79.5 ± 1.5[c])
Bellisoside D (**24**)	100.0 ± 4.3	84.9 ± 3.2		
	(100.0 ± 1.5)	(105.8 ± 4.7)	(62.2 ± 14.2[c])	(3.5 ± 2.6[c])
Bellisoside E (**25**)	100.0 ± 2.4	107.9 ± 8.1		
	(100.0 ± 3.3)	(105.1 ± 3.6)	(26.2 ± 15.7[c])	(1.7 ± 10.1[c])
Bellisoside F (**26**)	100.0 ± 2.6	55.3 ± 5.6		
	(100.0 ± 1.2)	(107.3 ± 4.5)	(1.9 ± 5.9[c])	
Bellissaponin BS5 (**27**)	100.0 ± 4.9	108.3 ± 6.1	127.0 ± 2.7[b]	125.4 ± 4.3[b]
	(100.0 ± 5.3)	(148.5 ± 1.5[b])	(157.7 ± 2.7[b])	(159.2 ± 1.4[b])
Bellissaponin BS9 (**28**)	100.0 ± 4.4	110.0 ± 4.0	121.7 ± 5.4[b]	127.6 ± 2.9[b]
	(100.0 ± 1.1)	(102.3 ± 1.4)	(102.4 ± 1.3)	(95.9 ± 1.3)
	0 μM	10 μM	30 μM	100 μM
Asiaticoside	100.0 ± 3.1	105.5 ± 2.7	109.4 ± 4.1	138.1 ± 2.3[b]
	(100.0 ± 0.9)	(99.8 ± 0.1)	(102.3 ± 0.9)	(94.7 ± 0.8)
Madecassoside	100.0 ± 2.0	104.8 ± 1.4	102.7 ± 1.3	113.5 ± 1.9[b]
	(100.0 ± 4.2)	(97.1 ± 1.1)	(94.1 ± 2.1)	(91.9 ± 0.6)

Each value represents the mean ± S.E.M. ($N = 4$). Significantly different from the control.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] Cytotoxic effects were observed, and values in parentheses indicate cell viability (%) in MTT assay.

3.3. Extraction and isolation

The dried flowers of *B. perennis* (3.0 kg) were extracted three times with MeOH under conditions of reflux for 3 h. Evaporation of the solvent under reduced pressure provided the MeOH extract (775.0 g, 25.8%). The bulk of the MeOH extract (720.0 g) was partitioned between an EtOAc–H2O (1:1) mixture, and removal of the solvents *in vacuo* yielded an EtOAc-soluble fraction (187.1 g, 6.7%) and an aqueous phase (532.0 g). The aqueous phase was subjected to Diaion HP-20 CC (4.0 kg, H2O → MeOH, twice) to obtain H2O-eluted (350.0 g, 12.5%) and MeOH-eluted (180.0 g, 6.4%) fractions, as described previously (Morikawa et al., 2008).

The MeOH-eluted fraction (140.0 g) was subjected to normal-phase silica gel CC [3.0 kg, $CHCl_3$–MeOH–H_2O (20:3:1 → 10:3:1 → 7:3:1, v/v/v, lower layer → 6:4:1) → MeOH] to obtain eight fractions [Fr. 1 (0.85 g), Fr. 2 (5.67 g), Fr. 3 (2.41 g), Fr. 4 (1.24 g), Fr. 5 (7.73 g), Fr. 6 (96.05 g), Fr. 7 (10.11 g), and Fr. 8 (16.09 g)]. Fraction 7 (10.11 g) was subjected to reversed-phase silica gel CC [300 g, MeOH–H_2O (20:80 → 30:70 → 40:60 → 50:50 → 70:30, v/v) → MeOH] to afford nine fractions [Fr. 7–1 (796.8 mg), Fr. 7–2 (2520.6 mg), Fr. 7–3 (641.1 mg), Fr. 7–4 (713.4 mg), Fr. 7–5 (1910.7 mg), Fr. 7–6 (3098.7 mg), Fr. 7–7 (257.8 mg), Fr. 7–8 (286.5 mg), and Fr. 7–9 (361.2 mg)] as reported previously (Morikawa et al., 2008). Fraction 7–5 (1910.7 mg) was separated by HPLC [Cosmosil 5C_{18}-MS-II, CH_3CN–MeOH–H_2O (26:16:58, v/v/v)] to afford 10 fractions {Fr. 7–5–1 (174.1 mg), Fr. 7–5–2 (150.2 mg), Fr. 7–5–3 [=perennisoside XVIII (**6**, 272.3 mg, 0.0117%)], Fr. 7–5–4 (228.7 mg), Fr. 7–5–5 (135.4 mg), Fr. 7–5–6 (87.1 mg), Fr. 7–5–7 (70.7 mg), Fr. 7–5–8 [=bellissaponin BS5 (**27**, 136.4 mg, 0.0059%) (Glensk et al., 2001; Wray et al., 1992)], Fr. 7–5–9 [=bellissaponin BS9 (**28**, 68.6 mg, 0.0029%) (Glensk et al., 2001)], and Fr. 7–5–10 (153.9 mg)}. Fraction 7–5–4 (228.7 mg) was further purified by HPLC [Cosmosil HILIC, CH_3CN–H_2O (90:10, v/v)] to furnish perennisoside XIX (**7**, 10.9 mg, 0.0005%). Fraction 7–5–5 (135.4 mg) was further purified by HPLC [Cosmosil 5C_{18}-MS-II, CH_3CN–MeOH–H_2O (25:16:59, v/v/v)] to furnish perennisoside XIII (**1**, 51.1 mg, 0.0022%). Fraction 8 (16.09 g) was subjected to reversed-phase silica gel CC [300 g, MeOH–H_2O (20:80 → 30:70 → 40:60 → 50:50 → 70:30, v/v) → MeOH] to afford nine fractions [Fr. 8–1 (3977.2 mg), Fr. 8–2 (759.6 mg), Fr. 8–3 (774.2 mg), Fr. 8–4 (5033.2 mg), Fr. 8–5 (427.2 mg), Fr. 8–6 (946.7 mg), Fr. 8–7 (2280.8 mg), Fr. 8–8 (2189.0 mg), and Fr. 8–9 (710.1 mg)] as reported previously (Morikawa et al., 2008). Fraction 8–7 (960.0 mg) was separated by HPLC [Cosmosil 5C_{18}-MS-II, CH_3CN–MeOH–H_2O (22:16:62, v/v)] to afford nine fractions {Fr. 8–7–1 (38.6 mg), Fr. 8–7–2 (43.6 mg), Fr. 8–7–3 (52.7 mg), Fr. 8–7–4 (39.4 mg), Fr. 8–7–5 (57.6 mg), Fr. 8–7–6 [=perennisoside XVI (**4**, 133.1 mg, 0.0135%)], Fr. 8–7–7 (126.6 mg), Fr. 8–7–8 (119.9 mg), and Fr. 8–7–9 (46.7 mg)}. Fraction 8–7–7 (126.2 mg) was further purified by HPLC [Cosmosil HILIC, CH_3CN–H_2O (80:20, v/v)] to furnish perennisosides XV (**3**, 7.3 mg, 0.0007%) and XVI (**4**, 8.4 mg, 0.0008%). Fraction 8–8 (506.5 mg) was further purified by HPLC [Cosmosil 5C_{18}-MS-II, CH_3CN–MeOH–1% aqueous AcOH (20:16:64, v/v)] to furnish perennisoside XVII (**5**, 70.4 mg, 0.0130%) and XIV (**2**, 19.4 mg, 0.0036%).

*3.3.1. Perennisoside XIII (**1** = bayogenin {28-O-[4-O-(3S)-3-hydroxybutyryl]-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 3)]-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester)*

Amorphous powder, $[\alpha]_D^{27}$ +7.6 (*c* 3.11, MeOH); IR (KBr) ν_{max} cm^{-1}: 3440, 1736, 1655, 1260, 1075; For ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 1229 $[M+Na]^+$; Negative-ion FABMS *m/z*: 1205 $[M-H]^-$, 1119 $[M-C_4H_7O_2]^-$, 957 $[M-C_{10}H_{17}O_7]^-$, 811 $[M-C_{16}H_{27}O_{11}]^-$, 649 $[M-C_{22}H_{37}O_{16}]^-$, 487 $[M-C_{28}H_{47}O_{21}]^-$; HRFABMS *m/z*: 1229.5924 $[M+Na]^+$ (calcd for $C_{58}H_{94}O_{26}Na$, 1229.5931).

*3.3.2. Perennisoside XIV (**2** = 3-O-β-D-glucopyranosylbayogenin {28-O-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 3)]-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester)*

Amorphous powder, $[\alpha]_D^{23}$ −2.3 (*c* 1.30, MeOH); IR (KBr) ν_{max} cm^{-1}: 3440, 1736, 1656, 1260, 1075; For ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 1305 $[M+Na]^+$; Negative-ion FABMS *m/z*: 1281 $[M-H]^-$, 1119 $[M-C_6H_{11}O_5]^-$, 957 $[M-C_{12}H_{21}O_{10}]^-$, 811 $[M-C_{18}H_{31}O_{14}]^-$, 649 $[M-C_{24}H_{41}O_{19}]^-$, 487 $[M-C_{30}H_{51}O_{24}]^-$; HRFABMS *m/z*: 1305.6088 $[M+Na]^+$ (calcd for $C_{60}H_{98}O_{29}Na$, 1305.6091).

*3.3.3. Perennisoside XV (**3** = 3-O-β-D-glucopyranosylbayogenin {28-O-(4-O-acetyl)-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 3)]-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester)*

Amorphous powder, $[\alpha]_D^{25}$ −2.5 (*c* 0.56, MeOH); IR (KBr) ν_{max} cm^{-1}: 3445, 1736, 1655, 1260, 1076; For ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 1347 $[M+Na]^+$; Negative-ion FABMS *m/z*: 1323 $[M-H]^-$, 1161 $[M-C_6H_{11}O_5]^-$, 999 $[M-C_{12}H_{21}O_{10}]^-$, 649 $[M-C_{26}H_{43}O_{22}]^-$, 487 $[M-C_{32}H_{53}O_{27}]^-$; HRFABMS *m/z*: 1347.6190 $[M+Na]^+$ (calcd for $C_{62}H_{100}O_{30}Na$, 1347.6197).

*3.3.4. Perennisoside XVI (**4** = 3-O-β-D-glucopyranosylbayogenin {28-O-[4-O-(3S)-3-hydroxybutyryl]-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 3)]-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester)*

Amorphous powder, $[\alpha]_D^{27}$ +1.5 (*c* 3.58, MeOH); IR (KBr) ν_{max} cm^{-1}: 3440, 1736, 1655, 1260, 1076; For ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 1391 $[M+Na]^+$; Negative-ion FABMS *m/z*: 1367 $[M-H]^-$, 1281 $[M-C_4H_7O_2]^-$, 1119 $[M-C_{10}H_{17}O_7]^-$, 811 $[M-C_{22}H_{37}O_{16}]^-$, 649 $[M-C_{28}H_{47}O_{21}]^-$, 487 $[M-C_{34}H_{57}O_{26}]^-$; HRFABMS *m/z*: 1391.6454 $[M+Na]^+$ (calcd for $C_{64}H_{104}O_{31}Na$, 1391.6459).

*3.3.5. Perennisoside XVII (**5** = 3-O-β-D-glucopyranosylbayogenin {28-O-α-L-rhamnopyranosyl(1 → 2)-[β-D-galactopyranosyl(1 → 3)]-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester)*

Amorphous powder, $[\alpha]_D^{27}$ −1.1 (*c* 3.27, MeOH); IR (KBr) ν_{max} cm^{-1}: 3440, 1736, 1655, 1230, 1075; For ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 1305 $[M+Na]^+$; Negative-ion FABMS *m/z*: 1281 $[M-H]^-$, 1119 $[M-C_6H_{11}O_5]^-$, 957 $[M-C_{12}H_{21}O_{10}]^-$, 811 $[M-C_{18}H_{31}O_{14}]^-$, 649 $[M-C_{24}H_{41}O_{19}]^-$, 487 $[M-C_{30}H_{51}O_{24}]^-$; HRFABMS *m/z*: 1305.6097 $[M+Na]^+$ (calcd for $C_{60}H_{98}O_{29}Na$, 1305.6091).

*3.3.6. Perennisoside XVIII (**6** = 3-O-β-D-fucopyranosylbayogenin {28-O-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester)*

Amorphous powder, $[\alpha]_D^{26}$ −3.8 (*c* 3.39, MeOH); IR (KBr) ν_{max} cm^{-1}: 3440, 1736, 1655, 1260, 1063; For ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 1127 $[M+Na]^+$; Negative-ion FABMS *m/z*: 1103 $[M-H]^-$, 957 $[M-C_6H_{11}O_4]^-$, 941 $[M-C_6H_{11}O_5]^-$, 633 $[M-C_{18}H_{31}O_{14}]^-$, 487 $[M-C_{24}H_{41}O_{18}]^-$; HRFABMS *m/z*: 1127.5619 $[M+Na]^+$ (calcd for $C_{54}H_{88}O_{23}Na$, 1127.5614).

*3.3.7. Perennisoside XIX (**7** = 3-O-α-L-rhamnopyranosylbayogenin {28-O-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester)*

Amorphous powder, $[\alpha]_D^{24}$ −7.1 (*c* 0.57, MeOH); IR (KBr) ν_{max} cm^{-1}: 3451, 1741, 1655, 1084, 1061; For ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 1127 $[M+Na]^+$; Negative-ion FABMS *m/z*: 1103 $[M-H]^-$, 649 $[M-C_{18}H_{33}O_{13}]^-$, 633 $[M-C_{18}H_{33}O_{14}]^-$; HRFABMS *m/z*: 1127.5619 $[M+Na]^+$ (calcd for $C_{54}H_{88}O_{23}Na$, 1127.5614).

*3.4. Solvolysis and acid hydrolysis of perennisosides XIII (**1**)*

A solution of **1** (2.0 mg) in 0.5% NaOMe–MeOH (1.0 mL) was stirred at room temperature for 3 h. The reaction mixture was neutralized with Dowex HCR-W2 (H^+ form) and the resin was removed by filtration. Evaporation of the solvent under reduced pressure gave a residue that was subjected to HPLC analysis [column: YMC-Pack ODS-AQ, 250 × 4.6 mm i.d.; mobile phase: MeOH–H_2O (20:80, v/v); detection: optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan)]; flow rate: 0.7 mL/min] to identify methyl (*S*)-(+)-3-hydroxybutrate [t_R 9.2 min (positive)], which

was identified by a commercially obtained sample {$[\alpha]_D^{20}$ +41.3 (c = 0.32, $CHCl_3$)} (Burk et al., 1995). The deacylated product was dissolved in 5% aqueous H_2SO_4–1,4-dioxane (1:1, v/v, 1 mL) and heated under conditions of reflux for 1 h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH^- form), and the resin removed by filtration. Upon removal of the solvent under reduced pressure, the residue was partitioned in EtOAc–H_2O (1:1, v/v), with removal of the solvent *in vacuo* from the EtOAc-soluble fraction and an aqueous phase, respectively. The EtOAc-soluble fraction was purified by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–H_2O (80:20, v/v)] to furnish bayogenin (0.6 mg, 74.1%) (Kasai et al., 1988). In turn, the aqueous layer was subjected to HPLC analysis under the following conditions: HPLC column, Kaseisorb LC NH_2-60-5, 4.6 mm i.d. × 250 mm (Tokyo Kasei Co., Ltd., Tokyo, Japan); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan); mobile phase, CH_3CN–H_2O (85:15, v/v); flow rate 0.5 mL/min]. Identification of L-rhamnose [t_R: 12.0 min (negative optical rotation)] and D-glucose [t_R: 20.7 min (positive optical rotation)] in the aqueous layer was carried out by comparison of its retention time and optical rotation with those of authentic samples.

3.5. Deacylation of perennisosides XV (**3**) and XVI (**4**)

Solutions of **3** (1.8 mg) and **4** (4.0 mg) in 0.5% NaOMe–MeOH (1.0 mL) were stirred at room temperature for 3 h. Through the same work-up and identification procedure used for **1**, a common desacyl derivative **2** (1.5 mg, 88.5% from **3**; 3.1 mg, 82.5% from **4**) was purified by HPLC [Cosmosil 5C_{18}-MS-II, CH_3CN–MeOH–H_2O (20:16:64, v/v/v)]. Furthermore, methyl (*S*)-(+)-3-hydroxybutrate was identified from a part of reaction mixture of **4** by the HPLC analysis mentioned above using an optical rotation detector.

3.6. Acid hydrolysis of perennisosides XIV (**2**), XVII (**5**), XVIII (**6**), and XIX (**7**)

Solutions of **2** and **5**–**7** (each 2.0 mg) were heated in 5% aqueous H_2SO_4–1,4-dioxane (1:1, v/v, 1 mL) under conditions of reflux for 1 h. Through the same procedure, bayogenin (0.5 mg, 65.7% from **2**; 0.6 mg, 78.8% from **5**; 0.6 mg, 67.9% from **6**; 0.7 mg, 79.1% from **7**) was purified from each EtOAc-soluble fraction by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–H_2O (80:20, v/v)], and the sugar components L-rhamnose from **2** and **5**–**7**, D-fucose [15.5 min (positive optical rotation)] from **6**, D-glucose from **2** and **5**–**7**, and D-galactose [22.2 min (positive optical rotation)] from **5** were identified in the aqueous layer under the HPLC conditions mentioned above.

3.7. Bioassays

3.7.1. Reagents

Dulbeco's minimum essential medium (DMEM, DL: glucose 1000 mg/L) was from Sigma–Aldrich Chemical (St. Louis, MO, USA); fetal bovine serum (FBS) was from Life Technologies (Rockville, MD, USA), and other chemicals were from Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan). The 96-well microplates were purchased from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan).

3.7.2. Cell culture

NHDFs, derived from human fetal foreskin fibroblasts, were purchased from Kurabo Industries Ltd. (Osaka, Japan). The cells were cultured in Kurabo's modified medium 106S supplemented with 2% FBS and human skin fibroblast growth factor (Kurabo Industries Ltd.) at 37 °C in a humidified atmosphere containing 5% CO_2.

3.7.3. Effect on collagen synthesis in NHDFs

Collagen synthesis was evaluated as described previously, with modifications (Koya-Miyata et al., 2004). Briefly, a cell suspension of 2.5×10^4 NHDFs in 100 μL DMEM containing FBS (10%), penicillin G (100 U/mL), and streptomycin (100 μg/mL) was inoculated in a 96-well microplate and pre-incubated for 24 h at 37 °C under a 5% CO_2 atmosphere. The medium was exchanged with 100 μL of fresh medium [FBS (−)] with or without the test sample added to the medium. After incubation for 48 h, type-I collagen content in the supernatant was determined using a commercial kit [Procollagen Type I C-peptide (PIP) EIA Kit (Takara Bio Inc., Shiga, Japan)] (Lee et al., 2007). Data were expressed as the percent of control collagen content.

In addition, the cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Thus, the medium was exchanged with 100 μL of fresh medium, and 10 μL of MTT {5 mg/mL in phosphate-buffered saline [PBS (−)]} solution was added. After incubation for 4 h, the medium was removed, and *i*-PrOH (100 μL containing 0.04 M HCl) was added to dissolve the formazan produced in the cells. The optical density (O.D.) of the formazan solution was measured by a microplate reader at 570 nm (reference: 655 nm). Each test compound was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration, 0.5%). Transforming growth factor (TGF)-β1 (from human recombinant), asiaticoside (Matsuda et al., 2001a,b), and madecassoside (Matsuda et al., 2001a,b) were used as reference compounds.

3.8. Statistics

Values are expressed as the mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical analyses. Probability (p) values less than 0.05 were considered significant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.05.011.

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