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# Spirostanol glycosides with hemostatic and antimicrobial activities from *Trillium kamtschaticum*

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ABSTRACT

Ten spirostanol glycosides, trillikamtosides A–J, together with eleven known analogues, were isolated from the hemostatic fraction of the 75% aqueous EtOH extract of the whole herbs of *Trillium kamtschaticum*. Their structures were established by extensive spectroscopic data analysis and chemical methods. The aglycones of three of these compounds had unique 3$\beta$,17$\alpha$-dihydroxy-spirostanes featuring a double bond between C-4 and C-5, while two others represent a rare class of spirostanol glycosides which possess a 5(6 → 7) abeo-steroidal aglycone. All the compounds were evaluated for their hemostatic and antimicrobial activities. Three of the spirostanol glycosides exhibited induced-platelet aggregation at a concentration of 300 $\mu$g/mL with maximal induced-platelet aggregation rates of 72%, 71%, and 62% in rabbits, respectively, and their $EC_{50}$ values were 492.7, 203.3, and 109.8 $\mu$M. Five of the spirostanol glycosides showed an anti-*Candida albicans* effect with MIC values of 21.1, 10.6, 8.8, 21.6, and 11.0 $\mu$M, respectively.



## 1. Introduction

The genus *Trillium* (Trilliaceae) consists of approximately 49 species throughout the world, including 9 species in Asia and 40 found in North America (Ohara and Kawano, 2005). However, only three species (*T. kamtschaticum*, *T. tschonoskii*, and *T. govanianum*.) are distributed in China. Some *Trillium* species have been used as folk remedies. The rhizome of *T. erectum* (Beth root), for example, is commonly used as women's herb to aid childbirth and to treat irregular menstrual periods in various native North American Indian tribes (Hayes et al., 2009). The rhizomes of *T. kamtschaticum* and *T. tschonoskii*, called "Toudingyikezhu" in Chinese, have been used as a traditional Chinese medicine usually to treat hypertension, waist and leg pain, as well as traumatic hemorrhage (Jiangsu New Medical College, 1975; Wang et al., 1978). Moreover, some Chinese minorities, namely the Tujia and Miao people, traditionally use *T. tschonoskii* to promote blood flow for regulating menstruation and to treat traumatic hemorrhage (Zhang et al., 2011). In recent pharmacological studies, the crude extract of this species showed anti-inflammatory, analgesic, and procoagulant effects (Yu et al., 2008). Although some phytochemical investigations on *Trillium* plants have been reported (Ono et al., 2003, 2007; Yokosuka and Mimaki, 2008), their hemostatic components are still not clear.

The preliminary pharmacological study herein established that the crude extract of the whole plants of *T. kamtschaticum* exhibited significant induced-platelet aggregation activity at a concentration of 1.5 mg/mL. In an ongoing search to identify bioactive constituents, a macroporous resin column was used to obtain 30%, 70%, and 95% aqueous EtOH eluted fractions. The 70% EtOH fraction showed 76% maximal platelet aggregation rate at a concentration of 1.5 mg/mL, while the other two fractions did not induce platelet aggregation activity obviously. With the aim of searching for bioactive constituents, the 70% EtOH eluent was investigated further, resulting in the isolation of ten new spirostane-type saponins, trillikamtosides A–J (**1**–**10**) (Fig. 1), and 11 known analogues:

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pennogenin 3-*O*-*β*-D-glucopyranoside (**11**) (Ono et al., 2007), pennogenin 3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside (**12**) (Nohara et al., 1975; Ono et al., 2007), floribundasaponin B (**13**) (Mahato et al., 1981), pennogenin 3-*O*-*β*-chacotrioside (**14**) (Nohara et al., 1975), pennogenin 3-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-*α*-L-rhamnopyranosy1-(1 → 4)-*β*-D-glucopyranoside (**15**) (Mimaki et al., 2000), pennogenin 3-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-*α*-L-rhamnopyranosy1-(1 → 4)-[*α*-L-rhamnopyranosy1-(1 → 2)]-*β*-D-glucopyranoside (**16**) (Nohara et al., 1975), polyphyllin V (**17**) (Chen et al., 1981), ophiopogonin B (**18**) (Asano et al., 1993), (25*S*)-27-hydroxypennogenin 3-*O*-*β*-D-glucopyranoside (**19**) (Yokosuka and Mimaki, 2008), (25*S*)-27-hydroxypennogenin-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside (**20**) (Ono et al., 2007), and trikamsteroside A (**21**) (Ono et al., 2007), respectively. All of the purified compounds were evaluated for their hemostatic effects using the induced-platelet aggregation assay. Furthermore, the antimicrobial activities of all the glycosides were tested. Herein, the isolation, structural elucidation, and bioactivities of these isolates are described.

## 2. Results and discussion

Trillikamtoside A (**1**) was obtained as a white amorphous powder. Its molecular formula was deduced as $C_{39}H_{62}O_{14}$ on the basis of its HR-EI-MS ion peak at *m/z* 754.4162 $[M]^+$ (calcd. 754.4140) and its $^{13}C$ NMR data (Table 3), requiring nine degrees of unsaturation. The presence of hydroxy and double bond functionalities was deduced from the IR absorption bands at 3423 and 1636 $cm^{-1}$. The $^1H$ NMR spectrum of **1** exhibited signals for four characteristic steroidal methyls at $\delta_H$ 0.68 (d, $J$ = 4.6 Hz), 1.01 (s), 1.61 (s), and 1.24 (d, $J$ = 6.8 Hz) and one olefinic proton at $\delta_H$ 5.93 (br s), as well as two anomeric protons at $\delta_H$ 5.02 (d, $J$ = 7.8 Hz) and 6.32 (br s). The $^{13}C$ NMR spectrum (Table 3) showed a total of 39 carbon signals, which were ascribed to five methyls, 10 methylenes (including two oxygenated ones), 18 methines (including 13 oxygenated ones), three quaternary carbons (including an oxygenated one at $\delta_C$ 90.4), a trisubstituted double bond, and one ketal carbon at $\delta_C$ 110.3. The double bond and the two monosaccharide units in compound **1** accounted for three degrees of unsaturation, and the remaining six ones of unsaturation required that the aglycone of **1** should be a hexacyclic-ring system. The spectroscopic data of C-16 ($\delta_C$ 90.5) and C-17 ($\delta_C$ 90.4) suggested it was a 17-hydroxylated spirostane-type derivative (Nohara et al., 1975).

Careful analysis of the 1D- and 2D-NMR data for the aglycone of **1** disclosed that it contained the same C—F rings as pennogenin in compounds **11**–**16** (Ono et al., 2007). However, the main differences were observed in A- and B-rings, as well as the presence of an additional oxygenated methine ($\delta_H$ 4.48; $\delta_C$ 74.1). Key $^1H$-$^1H$ COSY correlations (Fig. 2) of $\delta_H$ 5.93 (H-4)/$\delta_H$ 4.43 (H-3) and $\delta_H$ 4.48 (H-6)/$\delta_H$ 1.25 and 2.21 ($H_2$-7) suggested location of a double bond at C-4/C-5 and the oxygenated methine at C-6, respectively. This was also supported by HMBC correlations (Fig. 2) from H-4 to C-2, C-6, and C-10, from H-6 to C-4, C-8, and C-10, from Me-19 to C-1, C-5, and C-9. The *β*-orientation of the OH-6 was deduced from ROESY correlations of $\delta_H$ 5.93 (H-4)/4.48 (H-6), 0.88 (H-9)/1.25(H-7), 4.48 (H-6)/1.25 (H-7). The other parts were determined to be the same as those of pennogenin by 2D NMR experiments. Based on the above evidence, the aglycone of **1** was elucidated as (25*R*)-spirost-4-ene-3*β*,6*β*,17*α*-triol.

Acid hydrolysis of **1** gave D-glucose and L-rhamnose, as determined by HPLC analysis of their L-cysteine methyl esters followed by conversion into *o*-tolyl isothiocyanate derivatives. The large coupling constant ($^3J_{1,2}$ = 7.8 Hz) of the anomeric proton of the D-glucopyranosyl moiety suggested that it was *β*-orientated, while the *α*-orientation for the L-rhamnopyranosyl unit was defined by its chemical shift values of C-3″ ($\delta_C$ 73.1) and C-5″ ($\delta_C$ 69.6) with those of the corresponding carbons of methyl *α*- and *β*-rhamnopyranoside (Kasai et al., 1979). HMBC correlations of $\delta_H$ 5.02 (H-1′) with $\delta_C$ 75.2 (C-3) and of $\delta_H$ 6.32 (H-1″) with $\delta_C$ 78.6 (C-2′) enabled identification of the location of the saccharide chain to C-3, whereas the rhamnopyranosyl was attached to C-2′ of the glucopyranosyl moiety. Therefore, structure **1** was elucidated as (25*R*)-spirost-4-ene-3*β*,6*β*,17*α*-triol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside.

Trillikamtoside B (**2**) was isolated as a white amorphous powder with molecular formula $C_{45}H_{72}O_{18}$, as determined by the HR-ESI-MS data exhibiting an $[M + Na]^+$ peak at *m/z* 923.4625 and its $^{13}C$ NMR data (Table 3). By comparing its $^1H$ and $^{13}C$ NMR data (Tables 1 and 3) with those of **1**, compound **2** was deduced to have the same aglycone as **1**. Three monosaccharide moieties were present in structure **2** according to the three anomeric protons at $\delta_H$ 4.91 (d, $J$ = 7.6 Hz, H-1′), 5.87 (br s, H-1‴), and 6.33 (br s, H-1″) and the corresponding anomeric carbons at $\delta_C$ 100.4 (C-1′), 102.8 (C-1‴), and 102.1 (C-1″). The sugars units were determined to be a glucopyranosyl moiety and two rhamnopyranosyls by its $^1H$- and $^{13}C$-NMR, HSQC, HMBC, and ROESY experiments, which was further confirmed by acid hydrolysis and HPLC analysis. The sequence of the trisaccharide chain at C-3 of the aglycone was established from the following HMBC correlations: H-1′ ($\delta_H$ 4.91) of Glc with C-3 ($\delta_C$ 74.6) of the aglycone, H-1″ ($\delta_H$ 6.33) of the inner Rha with C-2′ ($\delta_C$ 77.8) of Glc, and H-1‴ ($\delta_H$ 5.87) of the terminal Rha with C-4′ ($\delta_C$ 78.2) of Glc. Thus, structure **2** was elucidated as (25*R*)-spirost-4-ene-3*β*,6*β*,17*α*-triol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-[*α*-L-rhamnopyranosyl-(1 → 2)]-*β*-D-glucopyranoside.

Trillikamtoside C (**3**) had a molecular formula of $C_{39}H_{60}O_{14}$, based on the HR-ESI-MS data (*m/z* 775.3875 $[M + Na]^+$). Overall, the NMR features of **3** showed a high similarity to those of **1**, except for the replacement of the oxymethine ($\delta_C$ 74.1) at C-6 by an unsaturated ketone group ($\delta_C$ 203.0) in **3**. Its UV absorption at 240 nm supported that **3** possessed an *α*,*β*-unsaturated ketone substructure. This observation was verified by HMBC correlations from H-4 ($\delta_H$ 6.56), $H_2$-7 ($\delta_H$ 2.54, 1.92), and H-8 ($\delta_H$ 1.90) to C-6 ($\delta_C$ 203.0). Consequently, structure **3** was identified as (25*R*)-3*β*,17*α*-dihydroxyspirost-4-ene-6-one-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside.

Trillikamtoside D (**4**) showed a molecular of $C_{39}H_{60}O_{14}$, as evidenced from the HR-ESI-MS data (*m/z* 775.3876 $[M + Na]^+$). Its UV spectrum showed absorption maxima at 255 nm, indicating the presence of a conjugated enal system (Lu et al., 2010), while its IR spectrum with absorptions at 3425 and 1664 $cm^{-1}$ suggested the existence of hydroxy and olefin groups, respectively. The $^1H$ NMR and $^{13}C$ NMR spectroscopic data (Tables 1 and 3) of **4** exhibited signals of an aldehyde ($\delta_H$ 10.3, $\delta_C$ 189.8), a tetrasubstituted double bond ($\delta_C$ 140.4 and 170.3) and other typical resonances of steroidal saponins. Comparison of all signals of **4** with ypsilandroside H (Lu et al., 2010) showed they had the same skeleton of B-nor(7)-6-carboxaldehyde-spirost-5(7)-en. The HMBC correlations (Fig. 2) of $\delta_H$ 0.87 (Me-19) with $\delta_C$ 170.3 (C-5), $\delta_H$ 4.07, 2.53 ($H_2$-4) with $\delta_C$ 140.4 (C-7), and $\delta_H$ 10.3 (H-6) with $\delta_C$ 170.3 (C-5) and 46.8 (C-8) also indicated the basic structure of **4**. Its sugar residues were determined to be the same as those of **1** and **3** based on NMR data, which were confirmed by its acid hydrolysis. The sequence of the disaccharide attached to C-3 was further proved by the observed HMBC correlations of H-1′ ($\delta_H$ 5.12) of the glucopyranosyl moiety with C-3 ($\delta_C$ 78.2) of the aglycone, and H-1″ ($\delta_H$ 6.47) of the rhamnopyranosyl unit with C-2′ ($\delta_C$ 77.7) of the Glc. Thus, compound **4** was characterized as (25*R*)-B-nor(7)-6-carboxaldehyde-spirost-5(7)-en-3*β*-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside.

Trillikamtoside E (**5**) had the molecular formula $C_{45}H_{70}O_{18}$ based

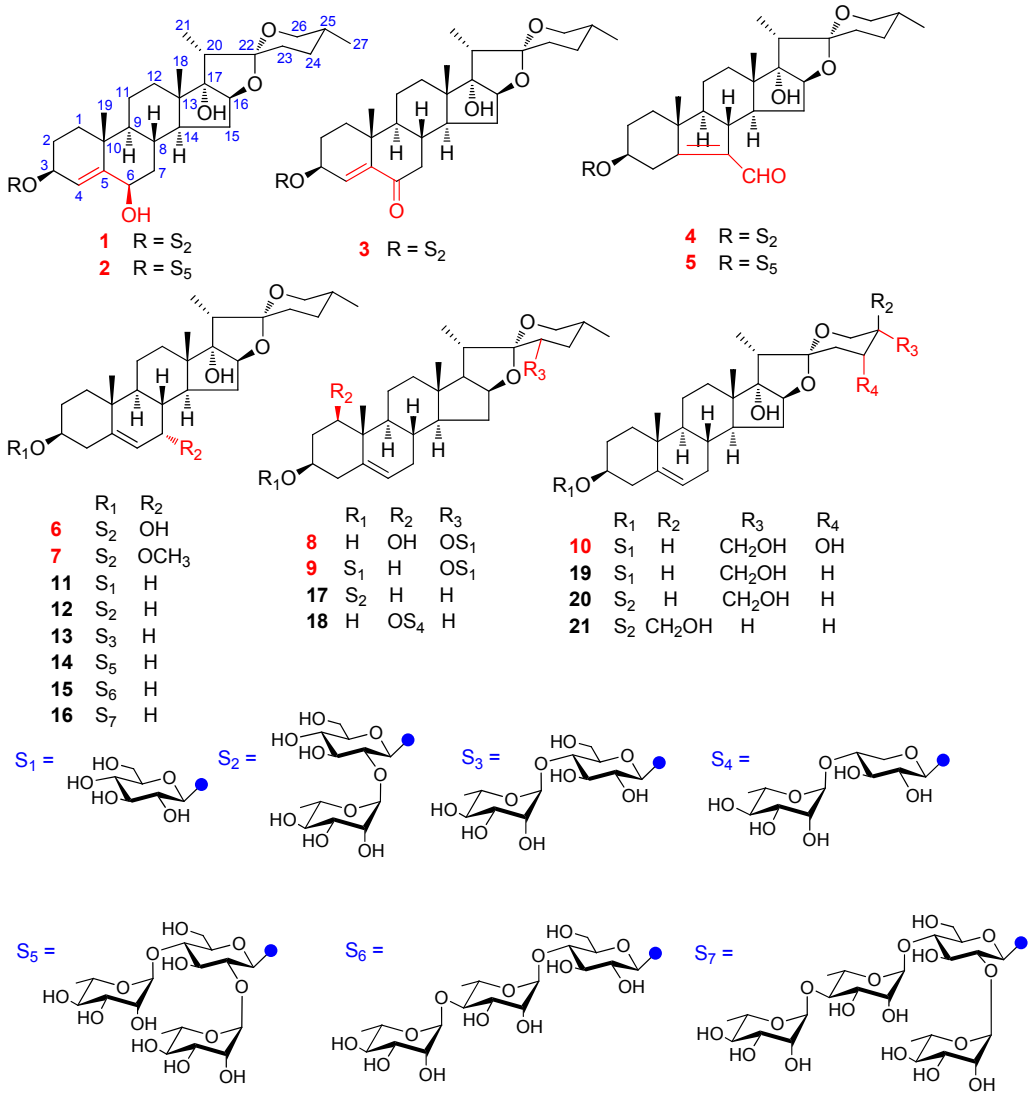


**Fig. 1.** Structures of compounds **1**–**21**.

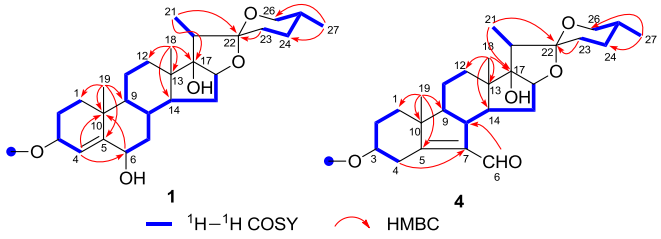


**Fig. 2.** Key HMBC and $^{1}$H-$^{1}$H COSY correlations of the aglycone moieties for **1** and **4**.

on HR-ESI-MS data (*m*/*z* 921.4453 [M + Na]$^{+}$). Moreover, the $^{13}$C-NMR and DEPT spectroscopic data (Table 3) showed compound **5** sharing the same aglycone as **4**. Its $^{1}$H NMR spectrum (Table 1) displayed signals for three anomeric protons ($\delta_H$ 5.02, 5.88, and 6.48) and the $^{13}$C NMR chemical shifts (Table 3) of sugar units of **5** were similar as those of **2**. Thus, compound **5** was determined as (25*R*)-B-nor(7)-6-carboxaldehyde-spirost-5(7)-en-3$\beta$-*O*-$\alpha$-L-rhamnopyranosyl-(1 → 4)-[$\alpha$-L-rhamnopyranosyl-(1 → 2)]-$\beta$-D-glucopyranoside.

Trillikamtoside F (**6**) exhibited a molecular formula of $C_{39}H_{62}O_{14}$ as determined by the positive HR-ESI-MS ion peak at *m*/*z* 777.4042 [M + Na]$^{+}$ (calcd. 777.4037) and the $^{13}$C NMR data (Table 3). Comparison of the NMR spectroscopic data (Tables 2 and 3) of **6** with those of pennogenin 3-*O*-$\alpha$-L-rhamnopyranosyl-(1 → 2)-$\beta$-D-glucopyranoside (**12**) (its $^{13}$C NMR data see Supplementary material S11) indicated that they were very similar, except for the presence of an additional oxymethine ($\delta_H$ 4.05, br s; $\delta_C$ 64.5) and the absence of a methylene in **6**. The oxymethine was placed at C-7 on the basis of the $^{1}$H–$^{1}$H COSY correlation of $\delta_H$ 5.75 (H-6) with $\delta_H$ 4.05 (H-7). The relative configuration of OH-7 was determined to be $\alpha$-oriented by the chemical shift of C-7 ($\delta_C$ 64.5), while the signals for C-7 would be at $\delta_C$ 73.2 for the 7$\beta$-OH isomer (Zhang et al., 2012). The sugar part of **6** was determined to be the same as those of **1**, **3**, and **4** by detailed analysis of their NMR spectroscopic data. Based on the above evidence, structure **6** was defined as (25*R*)-spirost-5-ene-3$\beta$,7$\alpha$,17$\alpha$-triol 3-*O*-$\alpha$-L-rhamnopyranosyl-(1 → 2)-$\beta$-D-glucopyranoside.

Trillikamtoside G (**7**) displayed an ion peak at *m*/*z* 791.4188 [M + Na]$^{+}$ (calcd. for, $C_{40}H_{64}O_{14}Na$, 791.4194) in the HR-ESI-MS, being 14 mass units more than **6**. Its $^{1}$H and $^{13}$C NMR spectroscopic data showed its structure resembling that of **6** with the main difference of an extra methoxy group signals ($\delta_H$ 3.23; $\delta_C$ 56.2). The

**Table 1**
$^1$H NMR spectroscopic data of **1**–**5** in $C_5D_5N$ ($\delta_H$, mult, *J* in Hz).[a]

| Position | **1**[b] | **2**[b] | **3**[c] | **4**[b] | **5**[b] |
|---|---|---|---|---|---|
| 1a | 1.66, o | 1.62, o | 1.53, m | 1.70, m | 1.68, m |
| 1b | 1.17, t (13.4) | 1.15, t (13.5) | 1.15, t (13.4) | 1.10, dd (12.3, 3.4) | 1.10, dd (13.2, 3.4) |
| 2a | 2.03, br s | 1.99, br d (3.6) | 2.09, o | 2.25, brd (12.4) | 2.15, dd (11.8, 3.4) |
| 2b | 1.94, q (11.8) | 1.88, q (11.8) | 1.82, q (11.8) | 1.96, q (11.8) | 1.89, q (11.8) |
| 3 | 4.43, m | 4.43, m | 4.50, m | 4.33, q (8.8) | 4.02, q (8.8) |
| 4a | 5.93, br s | 5.87, br s | 6.56, br s | 4.07, m | 4.05, m |
| 4b | | | | 2.53, td (12.2, 3.5) | 2.49, t (13.0) |
| 6 | 4.48, o | 4.43, o | | 10.3, s | 10.3, s |
| 7a | 2.21, q (13.2) | 2.18, q (13.8) | 2.54, br d (11.8) | | |
| 7b | 1.25, m | 1.20, m | 1.92, q (12.0) | | |
| 8 | 2.43, q (9.0) | 2.40, q (10.8) | 1.90, m | 2.76, td (11.0, 3.8) | 2.75, td (11.0, 3.8) |
| 9 | 0.88, t (9.6) | 0.84, t (9.6) | 1.09, td (11.6, 3.6) | 1.16, td (11.4, 4.0) | 1.15, td (11.4, 4.0) |
| 11a | 1.60, o | 1.57, o | 1.53, m | 1.45, o | 1.44, o |
| 11b | 1.54, o | 1.50, o | 1.33, ddd (3.6, 13.2, 14.6) | 1.45, o | 1.44, o |
| 12a | 2.21, q (13.2) | 2.18, q (13.8) | 2.18, o | 2.18, td (12.6, 5.2) | 2.16, m |
| 12b | 1.55, o | 1.55, o | 1.54, o | 1.53, brd (12.8) | 1.51, brd (12.8) |
| 14 | 2.17, m | 2.13, m | 2.14, m | 2.44, td (12.8, 6.8) | 2.43, td (13.0, 6.0) |
| 15a | 2.31, m | 2.27, m | 2.10, m | 2.89, m | 2.86, m |
| 15b | 1.57, o | 1.55, o | 1.49, q (7.0) | 2.10, td (13.0, 6.0) | 2.10, td (13.0, 6.0) |
| 16 | 4.48, o | 4.44, t (7.2) | 4.42, t (7.2) | 4.50, t (8.3) | 4.48, t (7.2) |
| 17 | | | | | |
| 18 | 1.01, s | 0.98, s | 0.93, s | 1.03, s | 1.05, s |
| 19 | 1.61, s | 1.58, s | 0.93, s | 0.87, s | 0.86, s |
| 20 | 2.28, q (7.2) | 2.25, q (7.2) | 2.25, q (7.2) | 2.30, q (7.2) | 2.28, q (7.0) |
| 21 | 1.24, d (6.8) | 1.20, d (6.8) | 1.21, d (6.8) | 1.25, d (7.2) | 1.24, d (7.2) |
| 23a | 1.71, o | 1.67, o | 1.70, o | 1.73, o | 1.73, o |
| 23b | 1.65, o | 1.59, o | 1.65, o | 1.73, o | 1.73, o |
| 24a | 1.55, o | 1.54, o | 1.55, o | 1.58, o | 1.58, o |
| 24b | 1.55, o | 1.54, o | 1.55, o | 1.58, o | 1.58, o |
| 25 | 1.55, o | 1.55, o | 1.55, o | 1.62, o | 1.58, o |
| 26a | 3.50, o | 3.48, o | 3.48, o | 3.52, o | 3.50, o |
| 26b | 3.50, o | 3.48, o | 3.48, o | 3.52, o | 3.50, o |
| 27 | 0.68, d (4.6) | 0.65, d (5.4) | 0.65, d (5.4) | 0.72, d (6.0) | 0.70, d (6.0) |
| **3-*O*-Glc** | | | | | |
| 1′ | 5.02, d (7.8) | 4.91, d (7.6) | 4.95, d (7.8) | 5.12, d (7.3) | 5.02, d (7.2) |
| 2′ | 4.26, t (8.3) | 4.23, o | 4.18, o | 4.34, o | 4.37, o |
| 3′ | 4.33, q (8.0) | 4.18, q (8.0) | 4.24, q (9.0) | 4.32, dd (12.8, 8.0) | 4.22, o |
| 4′ | 4.18, t (9.0) | 4.38, o | 4.09, t (9.0) | 4.20, t (9.1) | 4.42, o |
| 5′ | 3.97, br s | 3.71, br d (9.6) | 3.91, o | 3.96, br d (9.4) | 3.67, br d (9.4) |
| 6′a | 4.59, o | 4.26, m | 4.54, m | 4.58, br d (11.8) | 4.25, m |
| 6′′b | 4.41, m | 4.10, br d (11.8) | 4.32, m | 4.39, m | 4.10, m |
| **2′-*O*-Rha** | | | | | |
| 1′′ | 6.32, br s | 6.33, br s | 6.25, br s | 6.47, br s | 6.48, br s |
| 2′′ | 4.82, br s | 4.84, br s | 4.78, br s | 4.81, br s | 4.84, br s |
| 3′′ | 4.56, o | 4.54, o | 4.62, dd (9.6,3.0) | 4.66, dd (9.0, 3.0) | 4.63, dd (9.2, 3.0) |
| 4′′ | 4.33, m | 4.34, o | 4.25, o | 4.37, o | 4.38, o |
| 5′′ | 4.93, t (6.3) | 4.86, o | 4.71, o | 5.03, o | 4.97, o |
| 6′′ | 1.72, d (4.8) | 1.67, d (6.0) | 1.68, d (6.0) | 1.77, d (6.0) | 1.75, d (6.5) |
| **4′-*O*-Rha** | | | | | |
| 1′′′ | | 5.87, br s | | | 5.88, br s |
| 2′′′ | | 4.70, br s | | | 4.70, br s |
| 3′′′ | | 4.54, o | | | 4.57, dd (9.2, 3.0) |
| 4′′′ | | 4.34, o | | | 4.38, o |
| 5′′′ | | 4.96, t (7.6) | | | 4.98, o |
| 6′′′ | | 1.62, d (6.6) | | | 1.65, d (6.6) |

[a] s, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, multiplet; o, overlapped.
[b] Recorded at 600 MHz.
[c] Recorded at 400 MHz.

methoxy functionality was assigned to be at C-7 by the HMBC correlations of $\delta_H$ 3.23 with $\delta_C$ 73.6 (C-7). The ROESY correlation between 7-OMe ($\delta_H$ 3.23)/H-14 ($\delta_H$ 2.83) indicated that the methoxy moiety was $\alpha$-oriented. Consequently, structure **7** was elucidated to be (25*R*)-spirost-5-ene-3$\beta$,17$\alpha$-diol-7$\alpha$-methoxy-3-*O*-$\alpha$-L-rhamnopyranosyl-(1 → 2)-$\beta$-D-glucopyranoside.

Trillikamtoside H (**8**) was assigned the molecular formula $C_{33}H_{52}O_{10}$ based on the HR-ESI-MS analysis (*m/z* 631.3461 $[M + Na]^+$). Comparison of the NMR data of **8** with those of aculeoside B (Mimaki et al., 1998) showed that it had the same overall structure, except for the absence of a sugar moiety at C-1. This was supported by the upfield chemical shift of C-1 ($\delta_C$ 85.1 ppm → 78.1 ppm) and the long-range correlation of the anomeric proton signal at $\delta_H$ 5.00 (H-1′) with the carbon resonance at $\delta_C$ 76.4 (C-23) in the HMBC spectrum. ROESY correlations suggested the same relative configuration as aculeoside B. Accordingly, structure **8** was confirmed as (23*S*,25*R*)-spirost-5-ene-1$\beta$,3$\beta$,23-triol-23-*O*-$\beta$-D-glucopyranoside.

Trillikamtoside I (**9**) showed a molecular of $C_{39}H_{62}O_{14}$ by the HR-ESI-MS (*m/z* 777.4045 $[M + Na]^+$) and its $^{13}$C NMR data (Table 3). Its $^1$H and $^{13}$C NMR spectroscopic data (Tables 2 and 3) verified that it was similar to compound **8** with a glucosylated C-23. The $^1$H and

$^{13}$C NMR spectroscopic data of the aglycone of **9** (Tables 2 and 3) indicated that the basic skeleton of **9** was similar to diosgenin in compound **17** (its $^{13}$C NMR data see Supplementary material S12), except for presence of an oxygenated methine ($\delta_H$ 4.06; $\delta_C$ 76.6) and the absence of a methylene in F-ring. The oxymethine was assigned to be C-23 on the grounds of the $^1$H-$^1$H COSY of H-23 ($\delta_H$ 4.06) with $H_2$-24 ($\delta_H$ 1.91 and 2.41), of $H_2$-24 with H-25 ($\delta_H$ 1.81) and the HMBC correlations from H-20 ($\delta_H$ 3.24) to C-23 ($\delta_C$ 76.6). The 23*S* configuration was deduced from ROESY correlations of H-23 ($\delta_H$ 4.06)/H-20 ($\delta_H$ 3.24) and H-23/H-25 ($\delta_H$ 1.81). Furthermore, two glucopyranosyl moieties were identified by extensive 1D and 2D NMR analysis, and hydrolysis. The HMBC correlation of $\delta_H$ 5.04 (H-1′) of 3-Glc with $\delta_C$78.1 (C-3) of the aglycone, and $\delta_H$ 5.00 (H-1″) of 23-Glc with $\delta_C$ 76.6 (C-23) of the aglycone, indicated that C-3 and C-23 were glycosylated, and **9** was a bisdesmoside. Thus, structure **9** was defined as (23*S*,25*R*)-23-*O*-*β*-D-glucopyranosyl-spirost-5-ene-3*β*,23-diol-3-*O*-*β*-D-glucopyranoside.

Trillikamtoside J (**10**) displayed a molecular of $C_{33}H_{52}O_{11}$ as determined by HR-ESI-MS (*m/z* 647.3397 $[M + Na]^+$). Its $^{13}$C NMR data (Table 3) suggested that its aglycone was a 17*α*,27-dihydroxy-$\Delta^5$-spirostane (Yokosuka and Mimaki, 2008) with an additional oxymethine ($\delta_C$ 66.3). The $^1$H-$^1$H COSY correlations of H-24 ($\delta_H$ 4.55)/H-25 ($\delta_H$ 2.25) and an HMBC correlation from $H_a$-27 ($\delta_H$ 4.38) to $\delta_C$ 66.3 (C-24) verified the presence of a 24-OH group. Its 24*R* and 25*S* configurations were determined by ROESY correlations of H-23ax ($\delta_H$ 2.11)/H-20 ($\delta_H$ 2.39), H-23ax/H-21 ($\delta_H$ 1.28), H-24 ($\delta_H$ 4.55)/H-23ax, and H-25 ($\delta_H$ 2.25)/H-23ax. Furthermore, detailed analysis of 1D and 2D NMR spectra showed that the sugar moiety of **10** was a glucopyranosyl moiety. Based on this evidence, structure **10** was established as (24*R*,25*S*)- spirost-5-ene-3*β*,17*α*,24,27-tetraol-3-*O*-*β*-D-glucopyranoside.

Considering the fact that steroidal glycosides show antimicrobial (Qin et al., 2012, 2013) and induced rabbit platelet aggregation (Sun et al., 2014) properties, all of the compounds were evaluated for hemostatic and antimicrobial effects against one fungal (*Candida albicans*) and three bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*) (Table 4). Pennogenin-type saponins **12**, **14**, and **16** showed maximal induced-platelet aggregation rates (MPAR) of 72%, 71%, and 62% with $EC_{50}$ values of 492.7 ± 33.3, 203.3 ± 29.9, and 109.8 ± 45.4 *μ*M, respectively. This finding suggested that the hydroxy group at C-17 in pennogenin glycosides was essential towards the hemostatic effect, whereas the introduction of different functional groups in the A, B, or F-ring of pennogenin glycosides could make the hemostatic effect weak or disappear. In addition, the type of monosaccharide, the length of sugar moiety, and the linkage of sugar sequence attached to penogenin also had an effect on hemostatic activity. Moreover, **12**, **13**, **14**, **17**, and **18** exhibited significant antifungal effects against *C. albicans* with MIC values of 21.1, 10.6, 8.8, 21.6, and 11.0 *μ*M, respectively, compared to positive control fluconazole (MIC 52.3 *μ*M). None of the compounds showed antibacterial effects against the selected bacteria as compared to vancomycin and amikacin.

## 3. Conclusion

The present phytochemical investigation of the bioactive part of *T. kamtschaticum* led to isolation of 21 spirostane-type steroidal glycosides. Among them, 17 compounds were elucidated as pennogenin glycosides and their derivatives, and the remaining four were diosgenin glycosides and their derivatives. The aglycones of **1**–**3** have a unique 3*β*,17*α*-dihydroxy-spirostane skeleton containing a double bond between C-4 and C-5, while **4** and **5** possess a rare 5(6 → 7) abeo-steroidal aglycone containing a 6-5-6-5-5-6 fused ring spirostane with an *α*,*β*-unsaturated aldehyde group in B ring. This is the first report of pennogenin glycosides being responsible for the hemostatic effect of title species, which also supports its traditional use as a hemostatic agent.

## 4. Experimental

### 4.1. General experimental procedures

Optical rotations were measured on a SEPA-3000 automatic digital polarimeter. EI-MS and HR-EI-MS were recorded on Waters AutoSpec Premier P776. ESI-MS spectra were obtained on a Bruker HTC/Esquire spectrometer. HR-ESI-MS spectra were acquired on an API QStar Pulsar instrument. IR spectra were measured on a Bio-Rad FTS-135 spectrometer with KBr pellets, whereas UV spectra were obtained using a Shimadzu UV-2401PC spectrophotometer. NMR spectra were run on Bruker DRX-500 and Avance III 600 instruments with TMS as internal standard. Column chromatography (CC) was performed over silica gel (200–300 mesh, 10–40 *μ*m, Qingdao Marine Chemical Co., China), RP-18 (40–63 *μ*m, Merck), and Sephadex LH-20 (GE Healthcare, Sweden). TLC was performed on $HSGF_{254}$ (0.2 mm, Qingdao Marine Chemical Co., China) or RP-18 $F_{254}$ (0.25 mm, Merck). Fractions were monitored by TLC and spots were visualized by heating silica gel plates after spraying with 10% $H_2SO_4$ in EtOH. Semi-preparative HPLC was run on an Agilent 1100 liquid chromatograph with a diode array detector (DAD) setting of 203 and 254 nm, and using a ZORBAX SB-$C_{18}$ (5 *μ*m) column (250 × 9.4 mm i.d.). Analytical HPLC analysis was carried out on an Agilent 1100 liquid chromatograph with diode array detector (DAD) setting at 203 nm and 254 nm, ZORBAX SB-$C_{18}$ (5 *μ*m) column (250 × 4.6 mm i.d.). Turbidometric measurements of platelet aggregation of the samples were performed in a Chrono-log Model 700 Aggregometer (Chrono-log Corporation, Havertown, PA, USA).

### 4.2. Plant material

Whole plants of *T. kamtschaticum* were collected in July 2012 from Panshi city, Jilin Province, China, and identified by Dr. Yun-Heng Ji of the Kunming Institute of Botany. A voucher specimen (No. HY0016) is deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, CAS.

### 4.3. Extraction and isolation

Air-dried and powdered plant material (10.0 kg) was extracted with EtOH/$H_2O$ (75:25, v/v, 3 × 20 L), with solvent extraction carried out under conditions of reflux for a total of 6 h (3 × 2 h). The combined extracts were concentrated under reduced pressure, then extracted with *n*-BuOH (20 L × 3). The evaporated combined *n*-BuOH extracts (1.2 kg) were subjected to D101 macroporous resin CC eluted successively with an EtOH:$H_2O$ gradient system (0:100; 30:70; 70:30; 95:5, v/v). The EtOH:$H_2O$ (70:30, v/v) fraction (270.2 g) was passed through an MCI gel column and subsequently through a silica gel column (200–300 mesh) with gradients of $CHCl_3$:MeOH:$H_2O$ (10:1:0; 8:2:0.2; 7:3:0.5; and 2:1:0.5, v/v) to give four fractions (Fr. 1– Fr. 4). Fr. 2 (97.8 g) was further subjected to MPLC (RP-18; MeOH:$H_2O$, 50:50 → 90:10, v/v) to give six subfractions (Fr. 2.1–Fr. 2.6). Compounds **12** (10.0 mg) and **17** (23.5 mg) were obtained from Fr.2.2 (15.5 g) after repeated silica gel CC ($CHCl_3$:MeOH, 20:1 → 10:1, v/v). Fr. 2.3 (19.8 g) and Fr. 2.4 (10.3 g) were subjected to Sephadex LH-20 CC (MeOH:$CHCl_3$, 1:1, v/v) followed by semi-preparative HPLC to yield **11** (2.0 mg), **13** (13.5 mg), **15** (7.6 mg), and **18** (20.9 mg). Fr. 3 (86.0 g) was applied to MPLC (RP-18; MeOH:$H_2O$, 60:40 → 100:0, v/v) separately to give five subfractions (Fr. 3.1–Fr. 3.5). Likewise, Fr. 3.1 (33.3 g) was subjected

**Table 2**
$^1$H NMR spectroscopic data of **6**–**10** in $C_5D_5N$ ($\delta_H$, mult, *J* in Hz).[a]

| Position | **6**[b] | **7**[b] | **8**[c] | **9**[c] | **10**[b] |
|---|---|---|---|---|---|
| 1a | 1.70, m | 1.69, m | 3.70, d (10.4) | 1.65, m | 1.69, m |
| 1b | 0.89, td (13.8, 3.0) | 0.96, o | | 0.92, dd (13.6, 3.0) | 0.92, o |
| 2a | 2.02, m | 2.06, br d (13.8) | 2.58, br d (11.4) | 2.12, br d (11.6) | 2.12, brd (11.2) |
| 2b | 1.87, q (11.8) | 1.87, q (11.8) | 2.22, q (11.8) | 1.71, o | 1.72, m |
| 3 | 3.83, m | 3.91, m | 3.94, m | 3.94, m | 3.88, m |
| 4a | 2.83, br d (8.0) | 2.89, dd (13.6, 4.6) | 2.65, q (12.0) | 2.69, br d (12.2) | 2.69, dd (12.8, 3.0) |
| 4b | 2.83, br d (8.0) | 2.85, m | 2.65, q (12.0) | 2.42, br d (9.6) | 2.46, dd (12.8, 3.0) |
| 6 | 5.75, d (5.4) | 5.77, d (5.4) | 5.53, d (4.8) | 5.25, d (5.4) | 5.27, d (4.8) |
| 7a | 4.05, br s | 3.30, m | 1.86, o | 1.75, o | 1.87, o |
| 7b | | | 1.53, o | 1.75, o | 1.50, o |
| 8 | 1.72, o | 1.69, o | 1.53, o | 1.47, o | 1.50, o |
| 9 | 1.64, o | 1.55, o | 1.37, td (11.0, 4.0) | 0.87, o | 0.92, o |
| 11a | 1.61, o | 1.55, o | 2.87, br d (4.4) | 1.38, o | 1.57, o |
| 11b | 1.61, o | 1.55, o | 1.75, o | 1.38, o | 1.46, o |
| 12a | 2.24, td (12.8, 4.6) | 2.16, td (13.4, 4.6) | 1.82, o | 1.72, o | 1.50, o |
| 12b | 1.55, o | 1.49, m | 1.28, o | 1.12, o | 1.50, o |
| 14 | 3.04, td (12.8, 5.8) | 2.83, m | 2.02, m | 1.06, o | 2.07, o |
| 15a | 2.72, q (12.8) | 2.35, q (12.8) | 1.53, o | 1.98, o | 2.16, o |
| 15b | 1.56, o | 1.58, o | 1.53, o | 1.47, o | 1.48, o |
| 16 | 4.55, t (7.2) | 4.53, t (7.2) | 4.60, t (7.2) | 4.61, o | 4.49, t (6.6) |
| 17 | | | 1.89, m | 1.91, m | |
| 18 | 1.02, s | 0.97, s | 1.22, s | 1.13, s | 0.95, s |
| 19 | 1.09, s | 1.04, s | 1.27, s | 0.83, s | 0.91, s |
| 20 | 2.32, q (7.2) | 2.29, q (7.0) | 3.26, t (7.2) | 3.24, t (6.8) | 2.39, q (7.2) |
| 21 | 1.25, d (7.2) | 1.23, d (7.0) | 1.19, d (6.6) | 1.24, d (7.0) | 1.28, d (7.2) |
| 23a | 1.70, o | 1.72, o | 4.06, m | 4.06, m | 2.42, m |
| 23b | 1.70, o | 1.72, o | 4.06, m | 4.06, m | 2.11, o |
| 24a | 1.72, o | 1.55, o | 2.42, o | 2.41, o | 4.55, dd (11.8, 2.4) |
| 24b | | | 1.91, m | 1.91, m | |
| 25 | 1.56, o | 1.55, o | 1.78, o | 1.81, o | 2.25, m |
| 26a | 3.47, o | 3.54, o | 3.48, o | 3.49, o | 4.18, dd (11.2, 4.8) |
| 26b | 3.47, o | 3.54, o | 3.48, o | 3.49, o | 4.06, o |
| 27a | 0.64, d (6.0) | 0.67, d (5.0) | 0.57, d (5.0) | 0.57, d (9.6) | 4.38, o |
| 27b | | | | | 4.03, o |
| **7-OMe** | | 3.23, s | | | |
| **3-*O*-Glc** | | | | | |
| 1′ | 4.97, d (7.2) | 5.01, d (7.2) | | 5.04, d (7.6) | 5.00, d (5.2) |
| 2′ | 4.27, o | 4.28, o | | 4.09, o | 4.05, o |
| 3′ | 4.28, o | 4.29, o | | 4.32, o | 4.28, o |
| 4′ | 4.16, t (9.6) | 4.20, t (9.6) | | 4.29, o | 4.27, o |
| 5′ | 3.89, m | 3.85, m | | 4.01, o | 3.97, m |
| 6′a | 4.52, t (6.8) | 4.46, dd (12.0, 2.0) | | 4.56, o | 4.54, dd (11.8, 2.4) |
| 6″b | 4.37, o | 4.36, o | | 4.41, o | 4.37, o |
| **2′-*O*-Rha** | | | **23-*O*-Glc** | **23-*O*-Glc** | |
| 1″ | 6.38, br s | 6.41, br s | 5.00, d (7.6) | 5.00, d (7.6) | |
| 2″ | 4.82, br s | 4.83, br s | 4.02, m | 4.04, o | |
| 3″ | 4.62, m | 4.66, dd (9.2, 3.2) | 4.24, o | 4.28, o | |
| 4″ | 4.36, t (9.2) | 4.37, t (9.4) | 4.25, o | 4.28, o | |
| 5″ | 4.99, o | 5.00, o | 4.00, m | 4.01, o | |
| 6″a | 1.70, d (6.0) | 1.77, d (6.0) | 4.53, br d (10.4) | 4.56, o | |
| 6″b | | | 4.40, m | 4.41, o | |

[a] s, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, multiplet; o, overlapped.
[b] Recorded at 600 MHz.
[c] Recorded at 400 MHz.

to silica get CC ($CHCl_3$:MeOH:$H_2O$, 10:1:0 → 7:3:0.5, v/v) and followed by semi-preparative HPLC to afford **3** (13.5 mg), **9** (13.4 mg), **10** (1.2 mg), **19** (44.0 mg), **20** (81.3 mg), and **21** (20.0 mg), respectively. Fr. 3.2 (19.5 g) and Fr. 3.4 (10.4 g) were crystallized to afford **14** (12.8 mg) and **16** (20.0 mg), respectively. The mother liquor of Fr. 3.2 was then purified by HPLC ($CH_3CN$:$H_2O$, 50:50 → 60:40, v/v) to obtain **1** (12.6 mg), **4** (13.6 mg), **6** (4.2 mg), and **7** (3.3 mg). Similarly, the mother liquor of Fr. 3.4 was further purified by semi-preparative HPLC ($CH_3CN$:$H_2O$, 50:50 → 55:45, v/v) to yield **2** (6.5 mg), **5** (3.4 mg), and **8** (24.7 mg).

#### 4.3.1. Trillikamtoside A (*1*)

White amorphous powder; $\alpha_D^{18}$ −107 (*c* 0.10, MeOH); IR (KBr) $\nu_{max}$ 3423, 2952, 1636, 1053, 978, 918, 898, 870 cm$^{-1}$ (intensity: 898 cm$^{-1}$ > 918 cm$^{-1}$); for $^1$H (pyridine-$d_5$, 600 MHz) and $^{13}$C NMR (pyridine-$d_5$, 150 MHz) spectroscopic data, see Tables 1 and 3; EIMS *m*/*z* 754 $[M]^+$; HREIMS *m*/*z* 754.4162 $[M]^+$ (calcd. for $C_{39}H_{62}O_{14}$, 754.4140).

#### 4.3.2. Trillikamtoside B (*2*)

White amorphous powder; $\alpha_D^{18}$ −89 (*c* 0.16, MeOH); IR (KBr) $\nu_{max}$ 3425, 2932, 1634, 1054, 978, 917, 898, 872 cm$^{-1}$ (intensity: 898 cm$^{-1}$ > 917 cm$^{-1}$); for $^1$H (pyridine-$d_5$, 600 MHz) and $^{13}$C NMR (pyridine-$d_5$, 150 MHz) spectroscopic data, see Tables 1 and 3; positive ESIMS *m*/*z* 923 $[M + Na]^+$; positive HRESIMS *m*/*z* 923.4625 $[M + Na]^+$ (calcd. for $C_{45}H_{72}O_{18}Na$, 923.4616).

#### 4.3.3. Trillikamtoside C (*3*)

White amorphous powder; $\alpha_D^{18}$ −86 (*c* 0.18, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 240 (3.7) nm; IR (KBr) $\nu_{max}$ 3441, 2951, 1680, 1632, 1054,

**Table 3**
$^{13}$C NMR spectroscopic data of **1**–**10** in $C_5D_5N$.

| Position | **1**[a] | **2**[a] | **3**[b] | **4**[a] | **5**[a] | **6**[a] | **7**[a] | **8**[b] | **9**[b] | **10**[a] |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 37.8 | 37.1 | 34.9 | 36.8 | 36.1 | 37.1 | 36.9 | 78.1 | 37.4 | 37.4 |
| 2 | 27.8 | 27.2 | 26.3 | 30.3 | 29.7 | 29.9 | 29.9 | 43.6 | 30.3 | 30.1 |
| 3 | 75.2 | 74.6 | 74.5 | 78.2 | 77.8 | 77.4 | 77.5 | 68.1 | 78.1 | 78.0 |
| 4 | 125.8 | 125.0 | 130.7 | 31.2 | 30.6 | 38.8 | 39.0 | 44.0 | 39.3 | 39.2 |
| 5 | 148.7 | 148.2 | 147.2 | 170.3 | 169.5 | 143.6 | 146.0 | 140.2 | 140.9 | 140.7 |
| 6 | 74.1 | 73.4 | 203.0 | 189.8 | 189.3 | 125.9 | 121.2 | 124.5 | 121.8 | 121.6 |
| 7 | 40.8 | 40.1 | 46.6 | 140.4 | 139.8 | 64.5 | 73.6 | 32.3 | 32.3 | 31.9 |
| 8 | 31.6 | 31.0 | 34.8 | 46.8 | 46.2 | 38.5 | 37.7 | 32.8 | 31.5 | 32.2 |
| 9 | 55.3 | 54.7 | 51.1 | 60.6 | 60.0 | 42.3 | 42.9 | 51.3 | 50.2 | 50.5 |
| 10 | 37.9 | 37.1 | 38.5 | 47.0 | 46.3 | 37.8 | 37.9 | 43.6 | 37.1 | 36.9 |
| 11 | 21.4 | 20.8 | 20.5 | 20.8 | 20.2 | 20.6 | 20.6 | 24.2 | 21.1 | 20.8 |
| 12 | 32.5 | 32.1 | 31.8 | 33.0 | 32.4 | 31.9 | 32.0 | 40.8 | 40.2 | 32.2 |
| 13 | 45.5 | 45.3 | 45.5 | 48.4 | 47.8 | 44.8 | 44.8 | 41.0 | 41.0 | 45.1 |
| 14 | 53.1 | 52.4 | 52.9 | 50.9 | 50.3 | 46.1 | 45.5 | 57.3 | 56.6 | 52.3 |
| 15 | 32.2 | 31.5 | 31.3 | 34.9 | 34.3 | 32.0 | 31.8 | 32.3 | 32.1 | 31.6 |
| 16 | 90.5 | 89.8 | 90.0 | 90.4 | 89.7 | 90.2 | 90.0 | 81.4 | 81.4 | 90.2 |
| 17 | 90.4 | 89.9 | 90.0 | 89.5 | 89.0 | 90.2 | 90.1 | 62.4 | 62.2 | 90.0 |
| 18 | 17.8 | 17.2 | 17.3 | 17.8 | 17.4 | 17.0 | 16.7 | 17.4 | 17.1 | 17.0 |
| 19 | 22.0 | 21.3 | 19.7 | 16.0 | 15.3 | 18.2 | 18.2 | 14.8 | 19.3 | 19.3 |
| 20 | 45.3 | 44.7 | 44.8 | 45.1 | 44.5 | 44.8 | 44.8 | 35.8 | 35.7 | 44.9 |
| 21 | 10.3 | 9.7 | 9.8 | 10.5 | 9.8 | 9.8 | 9.7 | 14.9 | 14.8 | 9.7 |
| 22 | 110.3 | 109.6 | 109.9 | 110.1 | 109.5 | 109.7 | 109.7 | 110.8 | 110.7 | 112.0 |
| 23 | 32.5 | 31.9 | 32.1 | 32.6 | 32.0 | 31.9 | 31.8 | 76.4 | 76.6 | 42.0 |
| 24 | 29.3 | 28.6 | 28.8 | 29.3 | 28.7 | 28.7 | 28.7 | 37.3 | 37.2 | 66.3 |
| 25 | 30.9 | 30.3 | 30.5 | 31.0 | 30.3 | 30.3 | 30.3 | 31.5 | 31.6 | 47.6 |
| 26 | 67.1 | 66.5 | 66.7 | 67.1 | 66.5 | 66.5 | 66.5 | 65.7 | 65.7 | 62.2 |
| 27 | 17.9 | 17.2 | 17.2 | 17.8 | 17.2 | 17.2 | 17.2 | 16.8 | 16.7 | 61.4 |
| 7-OMe | | | | | | | 56.2 | | | |
| **3-*O*-Glc** | | | | | | | | | | |
| 1′ | 101.5 | 100.4 | 101.2 | 101.3 | 100.7 | 100.2 | 100.4 | | 102.6 | 102.5 |
| 2′ | 78.6 | 77.8 | 78.6 | 77.7 | 77.0 | 77.5 | 77.7 | | 75.4 | 75.3 |
| 3′ | 80.2 | 77.9 | 79.5 | 80.1 | 77.8 | 79.5 | 79.5 | | 78.7 | 78.5 |
| 4′ | 72.1 | 78.2 | 71.8 | 72.2 | 78.3 | 71.7 | 71.5 | | 71.7 | 71.6 |
| 5′ | 79.0 | 77.0 | 78.6 | 79.0 | 76.9 | 78.3 | 78.1 | | 78.6 | 78.4 |
| 6′ | 63.1 | 61.1 | 62.5 | 63.1 | 61.2 | 62.5 | 62.3 | | 62.8 | 62.7 |
| **2′-*O*-Rha** | | | | | | | | **23-*O*-Glc** | **23-*O*-Glc** | |
| 1″ | 102.7 | 102.1 | 102.5 | 102.3 | 101.6 | 101.9 | 101.9 | 106.3 | 106.3 | |
| 2″ | 73.2 | 72.4 | 72.7 | 73.0 | 72.4 | 72.7 | 72.7 | 75.4 | 75.4 | |
| 3″ | 73.1 | 72.5 | 73.0 | 73.4 | 72.7 | 72.5 | 72.5 | 78.9 | 78.7 | |
| 4″ | 75.0 | 74.3 | 74.5 | 74.6 | 74.0 | 74.0 | 74.0 | 71.6 | 71.7 | |
| 5″ | 69.6 | 69.1 | 69.9 | 69.9 | 69.3 | 69.4 | 69.4 | 78.5 | 78.6 | |
| 6″ | 19.0 | 18.3 | 18.6 | 18.0 | 18.5 | 18.6 | 18.6 | 62.7 | 62.8 | |
| **4′-*O*-Rha** | | | | | | | | | | |
| 1‴ | | 102.8 | | | 102.8 | | | | | |
| 2‴ | | 72.5 | | | 72.5 | | | | | |
| 3‴ | | 72.6 | | | 72.6 | | | | | |
| 4‴ | | 73.8 | | | 73.8 | | | | | |
| 5‴ | | 70.1 | | | 70.3 | | | | | |
| 6‴ | | 18.4 | | | 18.4 | | | | | |

[a] Recorded at 150 MHz.
[b] Recorded at 100 MHz.

**Table 4**
Hemostatic and antimicrobial effects of **12**–**14** and **16**–**18**.[a]

| Compounds | Induced-platelet aggregation effect ($EC_{50}$, $\mu$M) | Anti-*Candida albicans* activity (MIC, $\mu$M) |
|---|---|---|
| **12** | 492.7 ± 33.3 | 21.1 |
| **13** | – | 10.6 |
| **14** | 203.3 ± 29.9 | 8.8 |
| **16** | 109.8 ± 45.4 | – |
| **17** | – | 21.6 |
| **18** | – | 11.0 |
| Adenosine diphosphate[b] | 53.3 ± 6.5 | – |
| Fluconazole[b] | – | 52.3 |

[a] Other compounds were inactive in antimicrobial assay (MIC > 100 $\mu$M).
[b] Positive controls.

978, 916, 900, 865 $cm^{-1}$ (intensity: 900 $cm^{-1}$ > 916 $cm^{-1}$); for $^1$H (pyridine-$d_5$, 400 MHz) and $^{13}$C NMR (pyridine-$d_5$, 100 MHz) spectroscopic data, see Tables 1 and 3; positive ESIMS *m*/*z* 775 $[M + Na]^+$; positive HRESIMS *m*/*z* 775.3875 $[M + Na]^+$ (calcd. for $C_{39}H_{60}O_{14}Na$, 775.3881).

#### 4.3.4. Trillikamtoside D (**4**)

White amorphous powder; $\alpha_D^{18}$ −53.2 (*c* 0.14, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 255 (3.7), 202 (3.6) nm; IR (KBr) $\nu_{max}$ 3424, 2930, 1664, 1383, 980, 918, 896, 867 $cm^{-1}$ (intensity: 896 $cm^{-1}$ > 918 $cm^{-1}$); for $^1H$ (pyridine-$d_5$, 600 MHz) and $^{13}C$ NMR (pyridine-$d_5$, 150 MHz) spectroscopic data, see Tables 1 and 3; positive ESIMS *m/z* 775 $[M + Na]^+$; positive HRESIMS *m/z* 775.3876 $[M + Na]^+$ (calcd. for $C_{39}H_{60}O_{14}Na$, 775.3881).

#### 4.3.5. Trillikamtoside E (**5**)

White amorphous powder; $\alpha_D^{18}$ −53.7 (*c* 0.08, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 255 (3.5), 202(3.4) nm; IR (KBr) $\nu_{max}$ 3441, 2931, 1638, 1383, 1055, 979, 917, 895, 866 $cm^{-1}$ (intensity: 895 $cm^{-1}$ > 918 $cm^{-1}$); for $^1H$ (pyridine-$d_5$, 600 MHz) and $^{13}C$ NMR (pyridine-$d_5$, 150 MHz) data, see Tables 1 and 3; positive ESIMS *m/z* 921 $[M + Na]^+$; positive HRESIMS *m/z* 921.4453 $[M + Na]^+$ (calcd. for $C_{45}H_{70}O_{18}Na$, 921.4460).

#### 4.3.6. Trillikamtoside F (**6**)

White amorphous powder; $\alpha_D^{18}$ −88 (*c* 0.15, MeOH); IR (KBr) $\nu_{max}$ 3441, 2931, 1631, 1054, 1053, 979, 953, 917, 896, 866 $cm^{-1}$ (intensity: 896 $cm^{-1}$ > 917 $cm^{-1}$); for $^1H$ (pyridine-$d_5$, 600 MHz) and $^{13}C$ NMR (pyridine-$d_5$, 150 MHz) spectroscopic data, see Tables 2 and 3; positive ESIMS *m/z* 777 $[M + Na]^+$; positive HRESIMS *m/z* 777.4042 $[M + Na]^+$ (calcd. for $C_{39}H_{62}O_{14}Na$, 777.4037).

#### 4.3.7. Trillikamtoside G (**7**)

White amorphous powder; $\alpha_D^{18}$ −88 (*c* 0.16, MeOH); IR (KBr) $\nu_{max}$ 3431, 2903, 1631, 1381, 980, 918, 894, 865 $cm^{-1}$ (intensity: 894 $cm^{-1}$ > 918 $cm^{-1}$); for $^1H$ (pyridine-$d_5$, 600 MHz) and $^{13}C$ NMR (pyridine-$d_5$, 150 MHz) spectroscopic data, see Tables 2 and 3; positive ESIMS *m/z* 791 $[M + Na]^+$; positive HRESIMS *m/z* 791.4188 $[M + Na]^+$ (calcd. for $C_{40}H_{64}O_{14}Na$, 791.4194).

#### 4.3.8. Trillikamtoside H (**8**)

White amorphous powder; $\alpha_D^{18}$ −45.7 (*c* 0.10, MeOH); IR (KBr) $\nu_{max}$ 3425, 2903, 1632, 1072, 963, 920, 900, 870 $cm^{-1}$; for $^1H$ (pyridine-$d_5$, 400 MHz) and $^{13}C$ NMR (pyridine-$d_5$, 100 MHz) spectroscopic data, see Tables 2 and 3; positive ESIMS *m/z* 631 $[M + Na]^+$; positive HRESIMS *m/z* 631.3461 $[M + Na]^+$ (calcd. for $C_{33}H_{52}O_{10}Na$, 631.3458).

#### 4.3.9. Trillikamtoside I (**9**)

White amorphous powder; $\alpha_D^{18}$ −38 (*c* 0.08, MeOH); IR (KBr) $\nu_{max}$ 3425, 2953, 1632, 1382, 963, 924, 902, 873 $cm^{-1}$; for $^1H$ (pyridine-$d_5$, 400 MHz) and $^{13}C$ NMR (pyridine-$d_5$, 100 MHz) spectroscopic data, see Tables 2 and 3; positive ESIMS *m/z* 777 $[M + Na]^+$; positive HRESIMS *m/z* 777.4045 $[M + Na]^+$ (calcd. for $C_{39}H_{62}O_{14}Na$, 777.4037).

#### 4.3.10. Trillikamtoside J (**10**)

White amorphous powder; $\alpha_D^{18}$ −70.3 (*c* 0.18, MeOH); IR (KBr) $\nu_{max}$ 3427, 2930, 1632, 1380, 978, 903 $cm^{-1}$; for $^1H$ (pyridine-$d_5$, 600 MHz) and $^{13}C$ NMR (pyridine-$d_5$, 150 MHz) spectroscopic data, see Tables 2 and 3; positive ESIMS *m/z* 647 $[M + Na]^+$; positive HRESIMS *m/z* 647.3397 $[M + Na]^+$ (calcd. for $C_{33}H_{52}O_{11}Na$, 647.3407).

### 4.4. Determination of absolute configuration of sugars by HPLC

The absolute configurations of the sugar moieties were determined by the method described in the literature (Born, 1962; Born and Cross, 1963). Standard monosaccharides, L-rhamnose (5 mg) and D-glucose (5 mg), were incubated with L-cysteine methyl ester hydrochloride (5 mg) (Aldrich, Japan) in pyridine (5 mL) respectively. After heating at 60 °C for 1 h, *o*-tolyl isothiocyanate (10 μL) (Tokyo Chemical Industry Co., Ltd., Japan) was next added to each and the solutions were kept at 60 °C for 1 h. The products of D-glucose and L-rhamnose were separated by silica gel CC (200–300 mesh) eluted with $CHCl_3$ and $CHCl_3$:MeOH (100:1, v/v) to remove the low polar by-products independently, while $CHCl_3$:MeOH (100:1, v/v) and $CHCl_3$:MeOH (30:1, v/v) were employed to afford the target products methyl 2-(polyhydroxyalkyl)-3-(*o*-tolylthiocarbamoyl)-thiazolidine-4(*R*)-carboxylates. The products were further confirmed by their peaks at *m/z* 469 $[M + Na]^+$ and 455 $[M + Na]^+$ in the ESI-MS. Analytical HPLC was performed on a ZORBAX SB-$C_{18}$ column (250 × 4.6 mm i.d., 5 μm, Agilent, U.S.A) at 35 °C with gradient elution of $CH_3CN$:$H_2O$ (20:80 → 50:50, v/v) for 30 min at a flow rate of 1 mL/min. Peaks were detected by UV detection at 254 nm, with peaks of standard monosaccharide derivatives recorded at $t_R$ 13.83 (D-Glc), $t_R$ 17.74 (L-Rha).

Compounds **1**–**10** (1 mg, each) were individually treated with 6 M $CF_3COOH$ (1,4-dioxane/$H_2O$ 1:1, 1 mL) under conditions of refluxing on a water bath for 2 h at 90 °C. After cooling, each reaction mixture was extracted with $CHCl_3$ (3 × 5 mL). Next, each aqueous layer was evaporated to dryness using rotary evaporation. Each dried residue was dissolved in pyridine (1 mL) mixed with L-cysteine methyl ester hydrochloride (1 mg) (Aldrich, Japan) and heated at 60 °C for 1 h. Then, *o*-tolyl isothiocyanate (5 μL) (Tokyo Chemical Industry Co., Ltd., Japan) was added to each mixture, this being heated at 60 °C for 1 h. Each reaction mixture was directly analyzed by reversed phase HPLC following the above procedure. Under these conditions, the absolute configurations of the sugars for ten new saponins were identified as D-glucose ($t_R$ = 13.90 min) and L-rhamnose ($t_R$ = 17.66 min), respectively.

### 4.5. Platelet aggregation assays

Turbidometric measurements of platelet aggregation of the samples were performed in a Chrono-log Model 700 Aggregometer (Chrono-log Corporation, Havertown, PA, USA) according to Born's method (Born, 1962; Born and Cross, 1963). Platelet aggregation studies were completed within 3 h of preparation of PRP. Immediately after preparation of PRP, 250 μL was incubated in each of the test tubes at 37 °C for 5 min and then 2.5 μL of compounds (300 μg/mL) were individually added. The changes in absorbance as a result of platelet aggregation were recorded. The extent of aggregation was estimated by the percentage of maximum increase in light transmittance, with the buffer representing 100% transmittance. ADP (Adenosine diphosphate) was used as a positive control with a 53.3 ± 6.5% maximal platelet aggregation rate at a concentration of 10 μM. 1% DMSO was used as a blank control with a 3.0% maximal platelet aggregation. Data counting and analysis was done on SPSS 16.0, with experimental results expressed as mean ± standard error.

### 4.6. Antimicrobial assays

Four bacterial strains: *Candida albicans* (ATCCY0109), *Staphylococcus aureus* (ATCC25913), *Escherichia coli* (ATCC25922), and *Pseudomonas aeruginosa* (ATCC27853) (Chinese Food and Drug Inspection Institute) were cultivated in ordinary AGAR medium (*C. albicans* was cultivated in Solid Sabouraud Medium) at 37 °C for 24 h and then transplanted *in vitro*. The final concentration of the suspension was adjusted to $10^6$ CFU/mL by direct microscopic counts. The MIC (minimal inhibitory concentration) of the samples against the bacterial and fungal strains was determined according to America Clinical and Laboratory Standards Institute, using the micro liquid dilution method. The MIC is defined as the minimum concentration of the compounds at which the microorganisms do

not demonstrate visible growth. In order to determine MIC values of the test compounds, ten two-fold serial dilutions were performed for each compound. Culture media (100 μL) were placed in a 96-well micro-titer plate in 1–12 holes. Test compounds were dissolved in DMSO (100 μL) and then added to the second hole of each row containing already placed culture media (100 μL). After blending, a 100 μL of the mixture from second hole was moved to third hole, and so on for times until the 11th hole. A 100 μL mixture taken from 11th hole was not put in 12th hole, instead, it was discarded. Finally, bacteria or fungi suspension of 100 μL was placed in 2–12 holes. In this way, every first hole of the row contained only culture media as sterility check while every 12th hole of the row contained only bacterial/fungal liquid as negative control. The plate was incubated under anaerobic condition at 37 °C for 24 h. After incubation, the wells were examined for growth of microorganisms and the MIC values were determined. Each experiment was repeated three times. MIC > 100 μM were considered to be inactive.

Fluconazole and vancomycin were used as positive controls, with MIC values of 52.3 and 0.7 μM against *C. albicans* and *Staphylococcus aureus*, respectively, while amikacin was used as the positive control with MIC values of 3.4 and 1.7 μM against *E. coli* and *P. aeruginosa*, respectively.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2016.09.006.

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