Eur. J. Med. Chem. 35 (2000) 511−527

S0223523400001513/FLA

Original article

Spirostanols obtained by cyclization of pseudosaponin derivatives and comparison of anti-platelet agglutination activities of spirostanol glycosides

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Received 28 June 1999; revised 6 December 1999; accepted 9 December 1999

Abstract – Naturally occurring saponins **3** and **4** have a normal type F ring and α-arranged CH_3-21 group. Treatments of pseudosaponin peracetates **18** and **19** derived from **3** and **4**, respectively, with alcoholic KOH, followed by acidification with acetic acid, gave spirostanols **20** and **22** having iso type F rings as major products. Structural analyses of sapogenins and saponins derived from pseudo derivatives **11**, **12**, **18** and **19** were performed by comparisons of their ^{1}H-NMR spectral data and the X-ray analytical data of 3-*O*-*p*-bromobenzoyl sarsasapogenin **7**, 3-*O*-acetyl diosgenin **13** and saponin **20**. The mechanisms of ring-closure reaction of the side chain at C-22 of pseudosapogenins and pseudosaponins were deduced using stereomodels of the spirostanols derived from **11** under various reaction conditions. Inhibitory activities of saponin diglycosides **3**, **4**, **20**, **21** and **25** on human platelet agglutinations induced by ADP and ristocetin were compared. © 2000 Éditions scientifiques et médicales Elsevier SAS

saponin / isospirostanol / pseudosapogenin / pseudosaponin / anti-platelet agglutination

1. Introduction

There are two different structures, normal- (I) and iso-type (II), with respect to the F rings in spirostanols (*figure 1*). So far only the normal-type derivatives have been found in the naturally occurring spirostanols [1]. Marker et al. [2] and Wall et al. [3] reported that iso structures were formed during the acid hydrolysis of the plant steroidal saponins and were in a more stable form in strong acid medium. Furthermore several investigators [2–8] revealed that the isomerization of normal structures to the iso structures proceeded via pseudo type intermediates such as (III) formed during acid hydrolyses. One of the examples of the isomerization is the formation of smilagenin **1** from sarsasapogenin **2**. This procedure was reported by Wall and Walens [8], and Marker and Rhormann [9]; sarsasapogenin **2** was refluxed in alcoholic hydrogen chloride solution to give smilagenin **1**. Sarsasapogenin **2** is the aglycon of timosaponin A-III **3**, which is isolated from rhizomes of *Anemarrhena asphodeloides* BUNGE (Liliaceae) [10–14]. The rhizome has been known to have anti-platelet agglutination activity [10], anti-diabetic activity [15] and anti-diuretic activity [16].

In this paper we report the formation of iso type spirostanols from sarsasapogenin **2**, timosaponin A-III **3** and markogenin diglycoside **4**, the latter of which was isolated together with **3** as a minor constituent from the same plant, in controlled reaction conditions. The structures of the products are established by ^{1}H-NMR and X-ray crystallography. The anti-platelet agglutination activities of saponin diglycosides **3**, **4**, **20**, **21** and **25** are further compared.

2. Chemical results and discussion

In order to obtain smilagenin **1** from timosaponin A-III **3** according to the procedure of Marker et al. [2], **3** was refluxed with 2 N HCl in dioxane/H_2O (1:1) to quantitatively afford a crystalline compound which was acetylated to give acetate **5**. In the ^{1}H-NMR spectra, signals of

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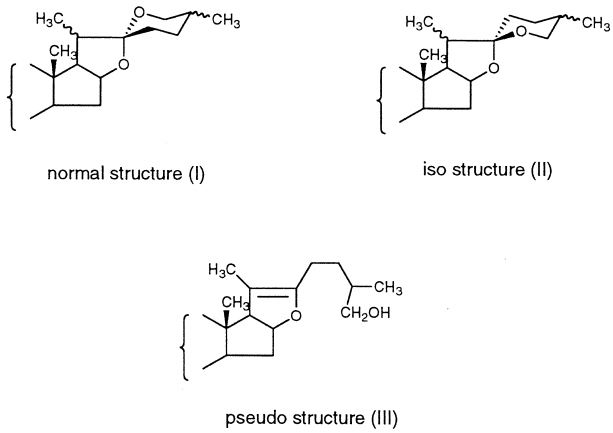

Figure 1. Structures of F rings of spirostanols.

5 were superimposable on the corresponding signals of the aglycon moiety of timosaponin A-III acetate **6** except for the signal of H-3 (see Experimental section). The structure of the hydrolysate was unequivocally confirmed by X-ray diffraction study [17, 18] using single crystals of 3-*O*-*p*-bromobenzoate **7** derived from the hydrolysate (*figure 2*). In the crystal, the F ring of **7** has a normal type structure (*R*-configuration at C-22), and CH_3-21 and CH_3-27 groups arrange in α and β, respectively. These results indicated that the hydrolysis of naturally occurring timosaponin A-III **3** in 2 N HCl caused no isomerization at the 22 position, and consequently gave quantitatively only normal type sapogenin **2**. These results suggested that the crystalline hydrolysate from **3** was sarsasapogenin **2** but not smilagenin **1**, a different result from that of Marker et al. [2]

The preparation of smilagenin **1** from sarsasapogenin **2** was further re-investigated in the same reaction conditions reported by Marker and Rohrmann [9]; sarsasapogenin **2** was refluxed in a solution of 5% HCl in MeOH for 4 days, then post-treated according to the described method to obtain a crystalline residue. However, the residue showed only one peak, whose retention time agreed with that of **2** on high performance liquid chromatography, and also the acetate of the residue gave the same ^{1}H-NMR spectrum as that of **5**. Thus, our results of the treatments of timosaponin A-III **3** and sarsasapogenin **2** with strong mineral acid were different from those reported by Marker and Rohmann.

Next, ring-closure reactions of pseudosapogenin and pseudosaponin derivatives were investigated. Pseudosar-

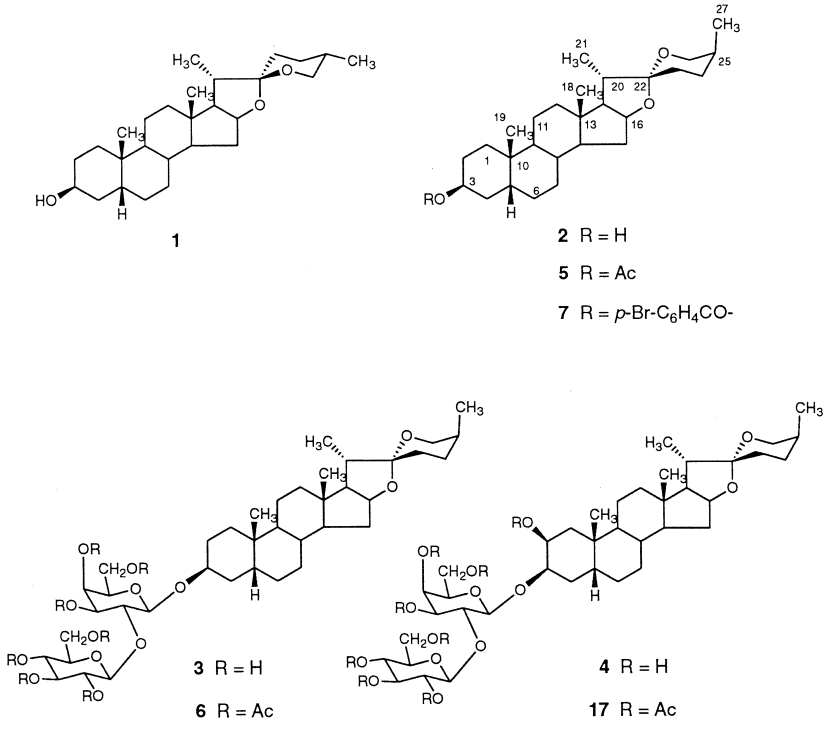

Figure 2. Structures of **1**–**7** and **17**.

sasapogenin **11** and pseudodiosgenin **12** were obtained via the corresponding diacetates **8** and **10** derived from sarsasapogenin **2** and diosgenin **9**, respectively, according to the methods of Scheer et al. [5]. Treatments of **11** and **12** with 5% HCl in MeOH for 12 h at room temperature gave only sarsasapogenin **2** and diosgenin **9**, respectively. The absolute structure of **9** was confirmed by the X-ray analysis using its monoacetate **13** (ORTEP not represented) [17, 18]. In the crystal of **13**, the F ring has a normal type structure (*R*-configuration at C-22) as well as **7**, and CH_3-21 arranged in α (*S*-configuration at C-20), and CH_3-27 groups are equatorial (*R*-configuration at C-25). However, when the ring-closure reaction of **11** was carried out in acetic acid instead of 5% HCl in MeOH, products **2**, **14** and **15** were obtained in the yields of 2.7, 13.2 and 57.4%, respectively (*figure 3*). These products showed the same quasimolecular ion peak $[M + Na]^+$ at *m/z* 439 in the fast-atom-bombardment mass spectra (FABMS). In order to elucidate the structures of the products, ^{1}H-NMR spectra of compounds **2**, **14** and **15** were compared (*figure 4* and *table I*). *Figure 4* showed the spectra of **2**, **14** and **15** in the regions of δ 3.2–4.6, in which the proton signals at positions 3, 16 and 26 were observed. The chemical shifts and coupling constants of H-26a (δ 3.30, d, J = 11.0 Hz) and H-26b (δ 3.95, dd, J = 11.0, 2.8 Hz) on the F ring of **2** were similar to those (δ 3.25, d, J = 11.3 Hz for H-26a; δ 4.03, dd, J = 11.3, 3.1 Hz for H-26b) on the F ring of **14**. From the spectral data and the X-ray evidence, it is suggested that **14** has the same normal type F ring as well as **2**. The difference of the structures between **2** and **14** consequently seemed to be due to that of the configurations of CH_3-21 groups. As the configuration of the CH_3-21 group of **2** was α it was speculated that the CH_3-21 group of **14** had β-configuration. The signals of both protons at the 26 position of **15** closely appeared at δ 3.46–3.52, different from those of **2** and **14**, which indicates that compound **15** has a different F-ring structure, namely iso type structure C or D (*figure 5*), from **2** and **14**. The actual structure of **15** was, however, obscure at this moment, although the structure assignment of **15** was described later.

The ring-closure reaction of pseudodiosgenin **12** in acetic acid gave two products, **9** as a minor product (12.6% yield) and **16** as a major product (68.3% yield). Compounds **9** and **16** showed the same quasimolecular ion peak $[M + Na]^+$ at *m/z* 437 in the FABMS spectra. As it was already confirmed that compound **9** had a normal type F ring and that the CH_3-21 group had α-configuration [19, 20], the ^{1}H-NMR spectrum of **16** was compared with that of **9** to elucidate the structure. In the ^{1}H-NMR spectra (*table I*), the chemical shifts and multiplicities of the H-26a and H-26b of **9** and **16** were similar, which suggests that **9** and **16** have the same normal type F-rings. As the CH_3-21 group of **9** has α-configuration, the CH_3-21 group of **16** is therefore deduced to arrange in β.

Thus, it became apparent that iso type spirostanol **15** was obtained via the pseudosapogenin **11** in acetic acid, though pseudodiosgenin **12** gave only normal type spirostanols **9** and **16**. Our next investigation was focused on the preparations of iso type spirostanol glycosides. Pseudosaponin peracetates **18** and **19** were prepared from saponin acetates **6** and **17**, respectively, in the same manner as pseudosarsasapogenin acetate **8** mentioned above. Treatment of **18** with 5% KOH in EtOH/H_2O (1:1), followed by acidification with acetic acid, gave products **3**, **20** and **21** in the yields of 2.8, 46.5 and 20.1%, respectively (*figure 6*). The products **3**, **20** and **21** showed the same quasimolecular ion peak $[M + Na]^+$ at *m/z* 763 in the FABMS spectra. Compound **3** was identified with authentic timosaponin-AIII by HPLC and ^{1}H- and ^{13}C-NMR spectra. Fortunately compound **20** was obtained as good crystals for the X-ray analysis. From the crystalline structure (*figure 7*), it was confirmed that the F ring of **20** was an iso type structure and the CH_3-21 and CH_3-27 groups of **20** arranged in β and α, respectively. The signals due to the protons at the 26 positions of **20** and **21** appeared at the similar fields at δ 3.6–3.68, suggesting that **21** has the same iso type F ring as **20**. As the CH_3-21 group of **20** was β, it was deduced that compound **21** was an isospirostanol having an α-arranged CH_3-21 group.

In order to elucidate the configuration of the CH_3-21 group of **15**, the chemical shifts of the proton at the 20 position of **15** was compared with those of sapogenins **2**, **9**, **14** and **16** and saponins **3**, **20** and **21** in the ^{1}H-NMR spectra (*table I*). The H-20 signals of **14**, **16** and **21** were observed as quintets at δ 2.47–2.70, while those of **2**, **3**, **9** and **20** were shifted to a higher field than δ 2.2. The orientations of the protons at the 20 positions and the oxygen atoms on the F rings of **14**, **16** and **21** are *cis*, whereas those of **2**, **3**, **9** and **20** are *trans*. Thus, when an H-20 signal was observed at lower field than about δ 2.47, the orientation relationship between the proton at the 20 position and the oxygen atom on the F ring in one spirostanol structure was thought to be *cis*. On the other hand, when an H-20 signal shifted at higher field than δ 2.2, the orientation relationship was thought to be *trans*. The H-20 signal of **15** appeared at higher field than δ 2.2 as well as those of **2**, **3**, **9** and **20**, which suggests that the orientation of the H-20 and the oxygen atom on the F-ring of **15** is *trans*. Therefore it was deduced that the structure of **15** was C in *figure 5*, in which the CH_3-21 group arranges in β.

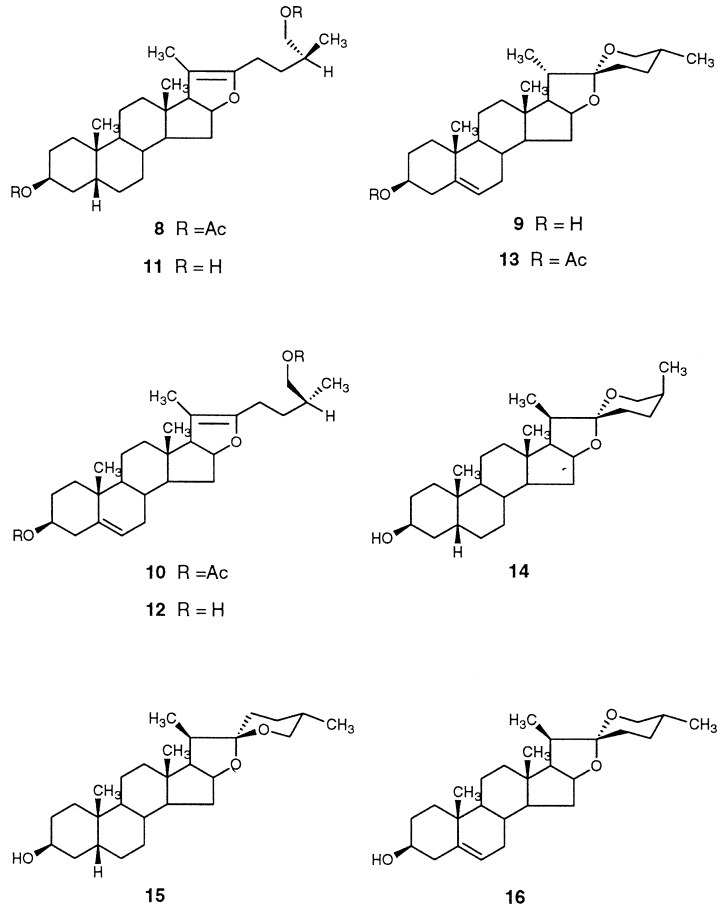

Figure 3. Structures of **8**–**16**.

Treatment of pseudosaponin peracetate **19** with 5% KOH, followed by acidification with acetic acid, four spirostanol diglycosides **4**, **22**, **23** and **24** were obtained in the yields of 3.8, 41.5, 11.3 and 2.0%, respectively. Although X-ray analyses were not performed because they were all obtained as amorphous powders, the structures of the products were analysed by comparison of the ^{1}H-NMR spectra of **4** and **22**–**24** with those of **3**, **20** and **21**. Compounds **4**, **22** and **24** gave similar ^{1}H-NMR spectra to those of **3**, **20** and **21**, respectively, except for the signals due to the 2 positions (*table I* and Experimental section), which suggests that the structures of the F rings and the configurations at the CH_3-21 groups of **4**, **22** and **24** were the same as those of **3**, **20** and **21**, respectively. As compound **4** was one of two obtainable normal type spirostanol diglycosides and both **22** and **24** were iso type spirostanol diglycosides, it was thought that compound **23** was an epimer of **4** with respect to the 20 position. In the ^{1}H-NMR spectrum of **23**, the signals of

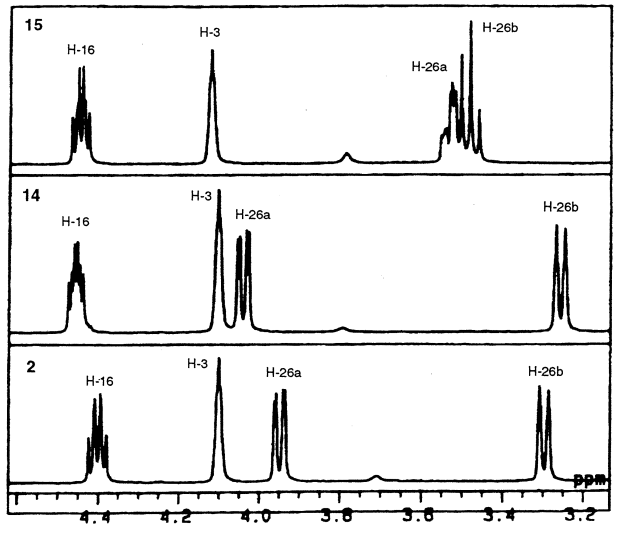

Figure 4. ^{1}H-NMR (500 MHz) spectra (pyridine-d_5) (δ 3.2–4.6) of compounds **2**, **14** and **15**.

Table I. ^{1}H-NMR (pyridine-d_5) signals used for comparisons in structural analyses of spirostanol derivatives, types of F-rings and configurations of CH_3-21 and CH_3-27 groups.

Compound	CH_3-18	CH_3-19	CH_3-21	CH_3-27	H-20	H-26a	H-26b	Type of F-ring	configuration of CH_3-21	configuration of CH_3-27
2	0.76 (s)[a]	0.98 (s)	0.99 (d) (6.7)[b]	1.08 (d) (7.0)	2.2<	3.30 (d) (11.0)	3.95 (dd) (11.0, 2.8)	normal	α	β (axial)
14	0.93 (s)	0.97 (s)	1.17 (d) (7.0)	1.06 (d) (7.3)	2.48 (quin.)[c] (8.5)	3.25 (d) (11.3)	4.03 (dd) (11.3, 3.1)	normal	β	β (axial)
15	1.02 (s)	0.98 (s)	1.11 (d) (7.3)	0.78 (d) (6.4)	2.2<	3.46–3.52*		iso	β	α (equatorial)
9	0.79 (s)	1.03 (s)	0.98 (d) (7.0)	0.79 (d) (6.1)	2.2<	3.37–3.46 (m)		normal	α	α (equatorial)
16	0.97 (s)	1.03 (s)	1.15 (d) (7.9)	0.79 (d) (6.4)	2.47 (quin.)[c] (8.5)	3.48 (m)		normal	β	α (equatorial)
3	0.82 (s)	0.97 (s)	1.08 (d) (7.0)	1.15 (d) (7.0)	2.2<	3.37 (d) (11.0)	4.07 (dd) (11.0, 2.4)	normal	α	β (axial)
20	1.13 (s)	0.99 (s)	1.24 (d) (7.3)	0.71 (d) (5.5)	2.2<	3.60–3.68 (m)		iso	β	α (equatorial)
21	0.96 (s)	0.96 (s)	1.14 (d) (7.9)	0.72 (d) (5.8)	2.70 (quin.)[c] (8.5)	3.60–3.65 (m)		iso	α	α (equatorial)
4	0.80 (s)	0.95 (s)	1.08 (d) (7.3)	1.15 (d) (7.0)	2.2<	3.37 (d) (11.0)	3.83 (dd) (11.0, 3.1)	normal	α	β (axial)
22	1.11 (s)	0.98 (s)	1.22 (d) (7.3)	0.71 (d) (5.5)	2.2<	3.31–3.36 (m)		iso	β	α (equatorial)
23	0.94 (s)	0.96 (s)	1.15 (d) (7.9)	1.08 (d) (7.3)	2.68 (quin.)[c] (8.5)	3.36 (d) (11.0)	3.82 (dd) (11.0, 3.1)	normal	β	β (axial)
24	0.94 (s)	0.96 (s)	1.12 (d) (7.9)	0.71 (d) (5.8)	2.68 (quin.)[c] (8.6)	3.60–3.70 (m)		iso	α	α (equatorial)

[a] Multiplicities were indicated as s (singlet), d (doublet), dd (doublet of doublets), quin. (quintet) and m (multiplet).. [b] Coupling constants (J in Hz) are given in parentheses.. [c] These protons shifted at lower fields in the cases of *cis* relationships between 20-Hs and F-ring oxygens than those of *trans* relationships.. * These proton signals observed closely at δ 3.46 (dd, J = 11.0, 4.6 Hz, H-26a) and δ 3.52 (dd, J = 11.0, 11.0 Hz, H-26b) as shown in *figure 4*.

H-26a and H-26b were observed separately from each other at δ 3.36 (d, J = 11.0 Hz) and 3.82 (dd, J = 11.0 and 3.1 Hz), respectively, as well as **4**. The H-20 signal of **23** was observed at the same position of δ 2.68 as a quintet (J = 8.5 Hz) as that of **24**. From these spectral data it is indicated that compound **23** is a normal type spirostanol diglycoside having β-arranged CH_3-21 group.

The mechanisms of the ring-closure reactions of the side chains at the 22 positions of pseudo derivatives were investigated using pseudosarsasapogenin **11**. As mentioned above, hydrolysis of saponin **3** in 2 N HCl gave only normal type sapogenin **2**. Treatment of pseudosapogenin **11** in 5% HCl in MeOH also quantitatively gave **2**. On the other hand, treatment of **11** with acetic acid gave three products: **2**, **14** and **15**. These results indicated that under strong mineral acid conditions, the normal type sapogenin **2** was the most stable structure, while in a weak acid, such as acetic acid, other cyclized products **14**, which was an epimer of **2**, with respect to C-20 and **15** which was an iso type spirostanol having a β-arranged CH_3-21 group, were obtained as major products accompanied by a minor product **2**. Hereupon, the ring-closure reaction of **11** was performed under control conditions. After 5 min of dissolving **11** in a methanolic hydrochloric acid solution adjusted to pH 4.0, the reaction mixture was poured into 5% KOH solution, then extracted with AcOEt. The extract showed only two peaks due to compounds **14** and **15** as minor and major products, respectively, on the HPLC. From these results together with the fact that **11** was rather stable in neutral and alkaline solutions, the mechanisms of the ring-closure reaction of **11** inferred as follows (*figure 8*): the trigger of the ring-closure reaction of **11** was thought to be protonation at C-20. When the protonation at C-20 occurs from the β-site, the attacking of the proton to C-20 may be greatly sterically hindered by the bulky CH_3-18 group,

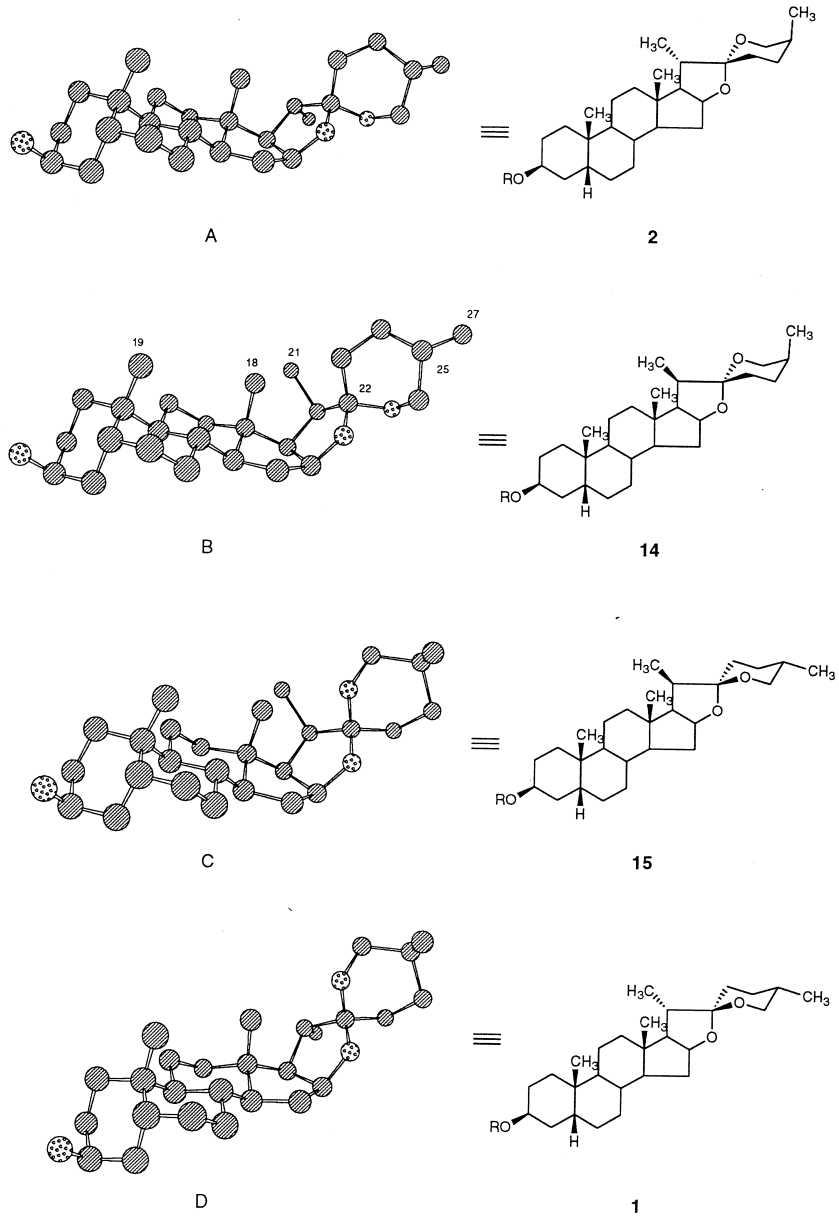

Figure 5. Stereomodels A–D (MM2-based, Chem 3-D plus) of compounds **2**, **14**, **15** and **1**, respectively.

whereas it is thought that the protonation at C-20 from α-site does not suffer from such a steric hindrance. Consequently in the protonation step, pseudostructure **11** seemed to give an intermediate [I], having a β-arranged CH_3-21 group in larger amounts than an intermediate [II] having an α-arranged CH_3-21 group. In the cyclizations of the side chain at the 22 position of the intermediate [I] to form F rings, when the resulting F ring obtained from [I] is a normal type (structure b in *figure 8*), the 1,3-diaxial interactions between the axial CH_3-27 group and a lone pair orbital of the oxygen on the F ring are caused. On the other hand, an equatorial CH_3-27 group on the iso type F ring (structure a) does not suffer from such an interaction. Therefore, it was thought that in the early steps of the ring-closure reaction, **11** was protonated at C-20 from mainly the α-site to give an intermediate [I] whose CH_3-21 group arranged in β, from which iso type spirostanol **15** was obtained in much higher yield than **14**.

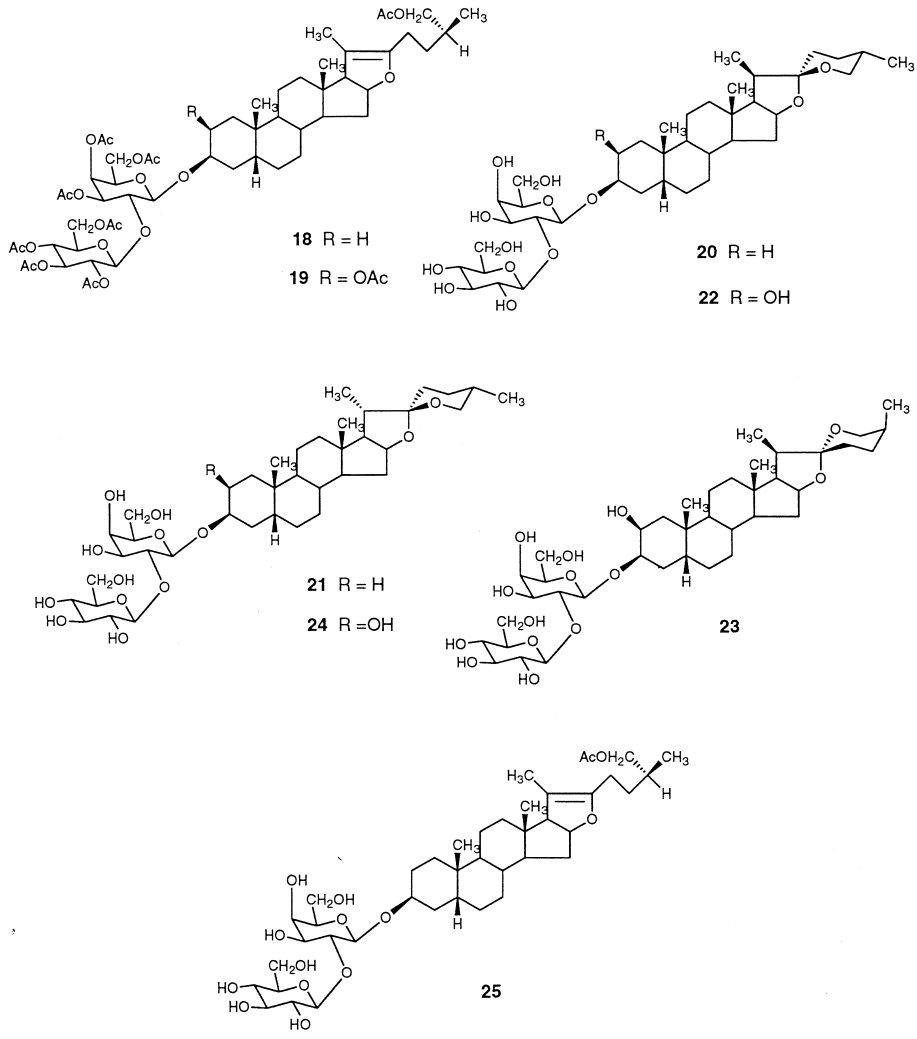

Figure 6. Structures of **18**–**25**.

Another intermediate [II] having an α-arranged CH_3-21 group seems to be produced in small amounts so that **2** was obtained as a minor product in the ring-closure reaction of **11**.

However, treatments of sapogenins **14** and **15** and saponins **20** and **21** as well as that of **3** with strong mineral acid gave only one sapogenin **2**, which suggests that sapogenin **2** has a normal type F ring and an α-arranged CH_3-21 group is the most stable spirostanol structure, and **2** is the thermodynamically favoured product under strong acid condition. This suggestion was revealed by comparisons of the stereomodels A–D which were obtained by molecular dynamic calculations (MM2) (Chem 3-D plus, Chembridge Scientific Company, Inc.) (*figure 5*). In the structures B–D, some steric interactions, among three substituents of CH_3-18, β-CH_3-21 and CH_2-23 in B (**14**), among three substituents of CH_3-18, β-CH_3-21 and the F ring oxygen in C (**15**), and between CH_3-18 and the lone pair orbital on the F-ring oxygen in D (**1**) might have occurred, while such interactions are not considered in structure A (**2**). Therefore compound **2** is thought to be most thermodynamically stable conformer of four spirostanols **1**, **2**, **14** and **15**.

3. Pharmacological results

Anti-platelet agglutination activities of saponins **3**, **4**, **20** and **21** and 26-*O*-acetyl pseudotimosaponin A-III **25** derived from **18** were compared. In this study adenosine-

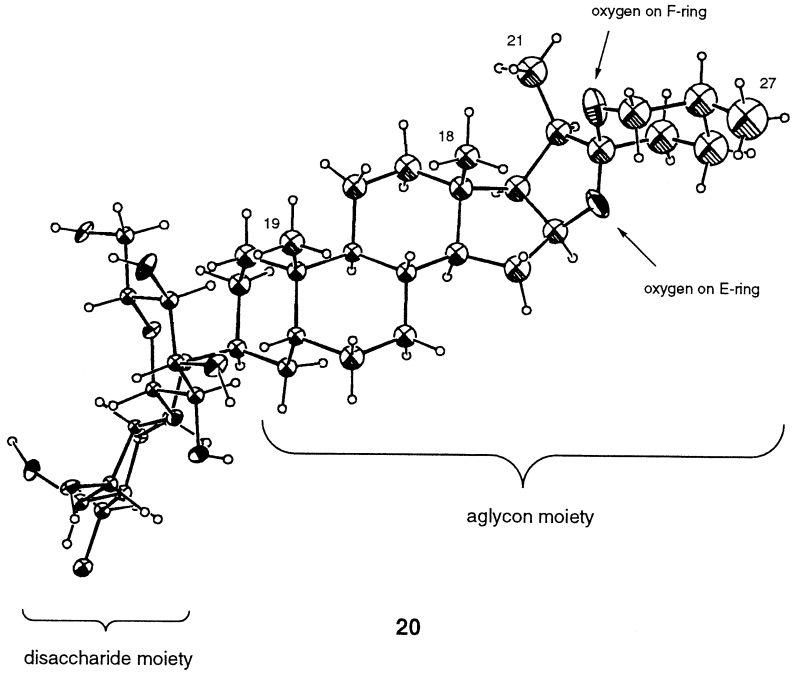

Figure 7. ORTEP representation of **20** at the 20% probability level. Hydrogen atoms are drawn as spheres of arbitrary radius.

5′-diphosphate (ADP) and ristocetin sulfate (ristocetin) were used as agglutination inducers of platelets prepared from healthy adult blood. First, the inhibitory activities of spirostanol diglycosides **3** and **4** were investigated on human platelet agglutinations induced by ADP. The agglutination inhibitions of **3** were 0, 25.4, 79.8 and 100%, and those of **4** were 0, 13.2, 60.0 and 100%, with dose concentrations of 50, 100, 200 and 400 μg/mL, respectively. Thus, it appeared that the inhibitory efficiencies of **3** and **4** on ADP-induced platelet agglutinations were dose-dependent and the results agreed with those reported by Niwa et al. [11].

Next, the inhibitory activities of compounds **20**, **21** and **25** against both ADP- and ristocetin-induced platelet agglutinations were compared with those of **3** (*figure 9*) at the drug concentration of 400 μg/mL that showed 100% inhibition in the assay of **3** on the ADP-induced platelet agglutinations. On the ADP-induced platelet agglutinations, spirostanol diglycosides **3** and **21** possessed potent inhibitory activities, and isospirostnol **20** and pseudosaponin **25** showed 60–70% inhibitory activities. On the contrary, in the inhibitions of the ristocetin-induced platelet agglutinations, **20** and **25** exhibited high activities (100%), although **3** exhibited no activity and **21** poor activity (29%). These results indicate that thermodynamically favoured spirostanols **3**, **4** and **21** have potent activities against the ADP-induced platelet agglutinations. On the other hand, thermodynamically unfavoured saponin **20** and pseudosaponin **25** have potent activity against ristocetin-induced platelet agglutinations. Although these differences of inhibitory activities on both ADP- and ristocetin-induced platelet agglutinations have not yet been elucidated, they might come from the stability of the saponins. Saponins **3** and **21** are stable spirostanols which exhibited potent inhibitory activities on the ADP-induced platelet agglutinations. On the other hand, isospirostanol **20** is thermodynamically unfavoured so that it may produce a pseudosaponin during the assay, and the resulting pseudostructure may appear to be the potent activity against ristocetin-induced platelet agglutinations, because pseudosponin **25** showed potent activity on the agglutinations.

4. Conclusions

It was reported that iso type spirostanol sapogenins were obtained by epimerization at C-22 of the corresponding normal type spirostanol sapogenins and were more stable structures than normal ones in the strong mineral acid solutions [2]. However, in our study, treatments of sarsasapogenin **2** and timosaponin A-III **3** with hot strong mineral acid solutions gave only **2**. Furthermore, treatments of pseudosapogenins **11** and **12** with

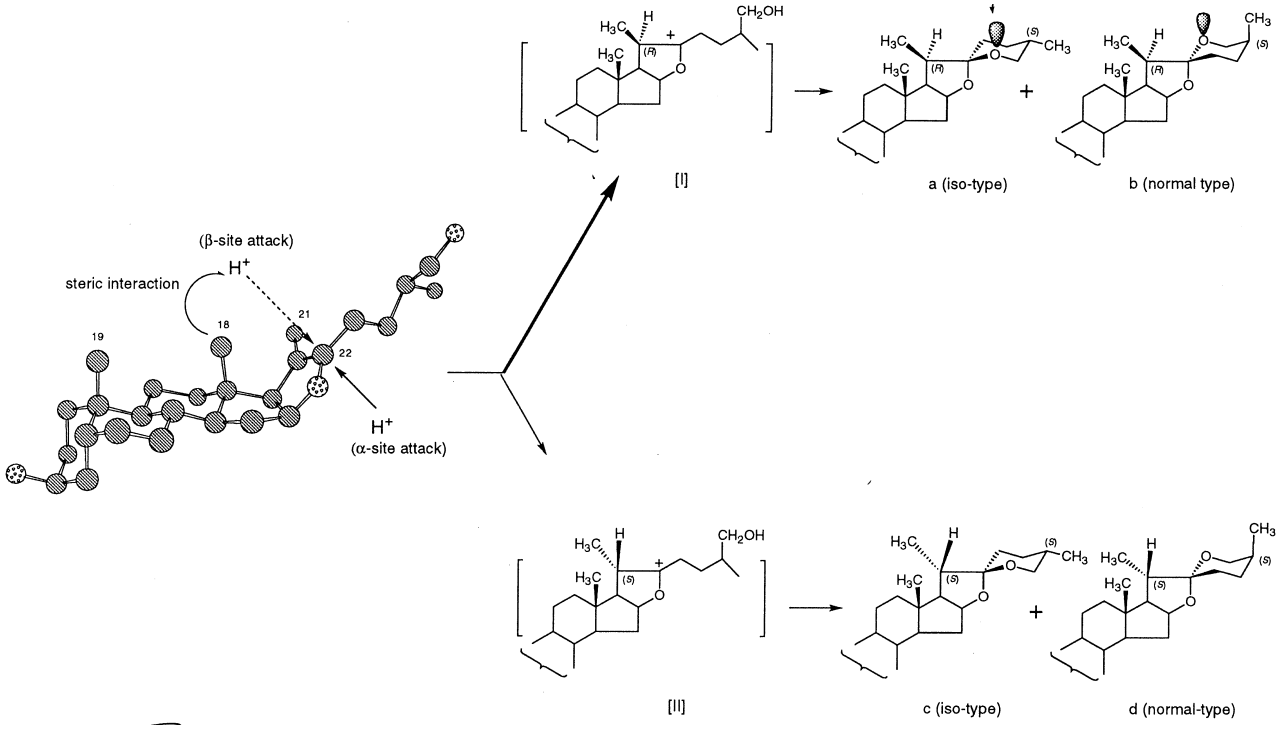

Figure 8. Stereomodel (MM2-based, Chem 3-D plus) of pseudosarsasapogenin **11** and proposed mechanisms for ring-closure reaction of **11**.

strong mineral acid solutions also gave the corresponding normal type spirostanols **2** and **9**, respectively. These results were different from those reported by Marker et al. [2]. On the other hand, treatment of **11** with acetic acid gave iso type spirostanol **15** accompanied by minor normal type spirostanols **2** and **14**, and treatments of pseudosaponin peracetates **18** and **19** with 5% KOH in methanol, followed by acidification with acetic acid produced iso type spirostanol derivatives accompanied by minor normal type ones; **20** and **21** accompanied by **3** from **18**, and **22** and **24** accompanied by **4** and **23** from **19**. It became apparent that the signal assignments of protons at C-26 and C-20 of the spirostanols in the ^{1}H-NMR spectra were a very useful means of determining the conformations of the F rings and the configurations at C-20: when the signals of protons at the 26 position of spirostanols were separately observed at δ 3.25–3.37 (Hs-26a) and 3.81–4.07 (Hs-26b) as shown in *figure 4*, the spirostanols had a normal type F ring. On the other hand, when both protons at the 26 position were closely shifted, the spirostanols had iso type F ring. When the protons at the 20 positions of the spirostanols observed at lower fields than δ 2.47, the orientation relationships between the protons at the 20 positions and the oxygen atoms on the F rings were *cis* as shown in the cases of **14**, **16**, **21**, **23** and **24**. Therefore, the CH_3-21 groups of **14**, **16** and **23** which had normal type F rings arranged in β, and those of **21** and **24** which had iso type F ring arranged in α. On the other hand, when the protons at the 20 position shifted at higher field than δ 2.2, the orientation relationships were *trans*, as shown in the cases of **2**, **3**, **4**, **9**, **15**, **20** and **22**, which describes that the CH_3-21 groups of **2**, **3**, **4** and **9** which had normal type F rings arranged in α, and those of **15**, **20** and **22** which had iso type F rings arranged in β. In addition, the configurations of CH_3-27 groups of spirostanols were deduced from the following results: the signals of β-arranged (axial) CH_3-27 groups were observed at lower fields than δ 1.0, whereas those of α-arranged (equatorial) CH_3-27 groups were at higher fields than δ 0.8.

The mechanisms of the ring closure reactions of the side chains of the pseudo derivatives are explained using **11** as follows (*figure 8*): the attack of a proton at the 20 position of **11** from the β-site exerts a steric hindrance on the CH_3-18 group, while that from the α-site does not. Consequently, intermediate [I] might be produced in much larger amounts than [II]. In the F-ring formation from the intermediate [I], a spirostanol having an iso type

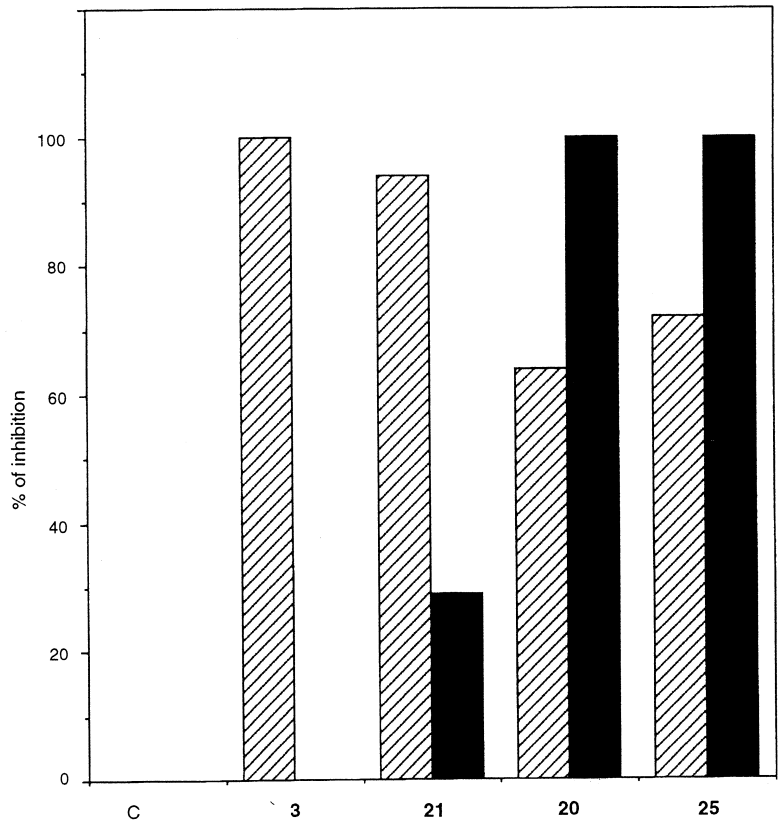

Figure 9. Inhibitory activities on human platelet agglutinations induced by ADP (shaded bars) and ristocetin sulfate (closed bars) at the concentration of 400 μg/mL of compounds **3**, **20**, **21** and **25**. C: control. Materials and assay methods were described in the Experimental section. All data indicate the average of three experiments.

F ring (a in *figure 8*) is obtained as a major product, because in another spirostanol with a normal F ring (b in *figure 8*), a steric interaction between the axial CH_3-27 and a lone pair orbital of oxygen atoms on the F ring is generated, whereas such interaction is not considered in the iso type F ring in structure a. In the four structures A–D in *figure 5*, steric interactions among the CH_3-18, β-CH_3-21 and CH_2-23 in B, among the CH_3-18, β-CH_3-21 and the F-ring oxygen in C, and between the CH_3-18 and the lone pair orbital on the F-ring oxygen in D might occur, while such interactions might not in A. Therefore, it was reasonable that the treatment of **2** with strong mineral acid did not give smilagenin **1** and the hydrolyses of saponin **3**, **20** and **21** in 2 N HCl gave only the most stable sapogenin **2**.

In the ring-closure reaction of pseudodiosgenin **12** in which the configuration at C-25 was *R*, the structures E–H (*figure 10*) were considered for the products. In those structures, the axial CH_3-27 groups of structures F and H which have iso type F rings suffer steric hindrance from the lone pair orbital of the F-ring oxygen. On the other hand, the hindrance is not considered in structures E and G which have a normal type of F ring. Furthermore, an intermediate such as [I] in *figure 8* might be more stable than another intermediate such as [II] in the first step of the reaction. Therefore, the mechanisms for the formations of spirostanols mentioned above also agreed in this case; the ring-closure reaction of **12** in acetic acid gave **16** as a major product and **9** as a minor product.

Although Callow and James [21], Yu et al. [22] and Asano et al. [23] reported that epimerizations at C-25 on normal F rings of spirostanols occurred without any conversion of the F rings in strong mineral acid, however, such epimerizations were not observed in our study.

Inhibitory activities on human platelet agglutinations induced by ADP and restocetin of steroidal saponins were investigated. The inhibitory activities of compounds **20**, **21** and **25** on ADP- and ristocetin-induced platelet agglutinations were compared with those of **3** at the drug

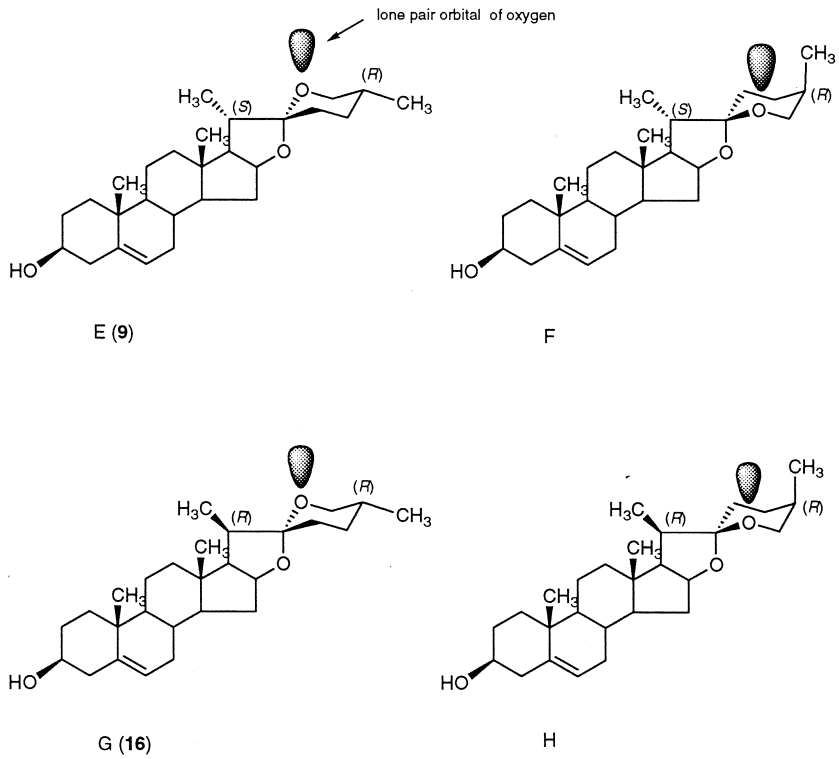

Figure 10. Possible structures E–H formed in the ring-closure reaction of **12**.

concentration of 400 μg/mL (*figure 9*). Stable spirostanols **3** and **21** exhibited potent inhibitory activities, whereas unstable saponin **20** and pseudosaponin **25** showed 60–70% inhibition at the concentration of 400 μg/mL against ADP-induced platelet agglutinations. On the contrary, **25** and **20** showed 100% inhibition on ristocetin-induced platelet agglutinations, although **3** and **21** showed poor inhibitory activities (0% in **3** and 20% in **21**). These results indicate that thermodynamically favoured spirostanols exhibit highly inhibitory activities against ADP-induced platelet agglutination. On the other hand, thermodynamically unfavored saponins showed highly inhibitory activities on ristocetin-induced platelet agglutinations.

5. Experimental protocols

5.1. General procedures

Timosaponin A-III was isolated from rhizomes of *Anemarrhena asphodeloides* BUNGE (Liliaceae) according to the method by Saito et al. [24]. Diosgenin, adenosine-5′-diphosphate and ristocetin sulfate were purchased from SIGMA Chemical Co., USA. Other chemicals and solvents were of reagent grade and were obtained from commercial sources. Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. The thin-layer chromatography (TLC) utilized Kieselgel 60 F_{254} (E. Merck), and spots were detected by spraying the plates with 1:9 $Ce(SO_4)_2$/10% H_2SO_4 reagent, followed by heating at 100 °C for l0 min. Column chromatography was carried out on a Wakogel C-200, and the eluates were monitored by TLC. An SSC-6300 HPLC instrument (Senshu Scientific Co. Ltd.) was employed for analytical HPLC using a DOCOSIL (10 × 250 mm; flow rate, 1.0 mL/min, column temp, 40 °C) column, and was further equipped with an SSC autoinjector 6310 and an SSC fraction collector 6320 for preparative HPLC using a DOCOSIL (10 × 250 mm; flow rate, 1.0 mL/min, column temp, 40 °C) preparative column. ^{1}H- and ^{13}C-NMR at 500 and 125 MHz, respectively, as well as ^{1}H-^{1}H and ^{1}H-^{13}C COSY, DEPT and HMBC spectra, were obtained with a JEOL JNM-A500 FT NMR spectrometer. Tetramethylsilane was used as an internal standard, and chemical shifts are given in ppm. Multiplicities of ^{1}H-NMR signals are indicated as s

(singlet), d (doublet), dd (doublet of doublets), quin. (quintet) and m (multiplet). Fast-atom-bombardment mass spectra (FABMS) were recorded on a JEOL JMS-DX 300 mass spectrometer.

5.1.1. Chemistry

5.1.1.1. Acid hydrolysis of **3**

After refluxing a solution of **3** (3.5 g, 4.72 mmol) in 2 N HCl in dioxane/H_2O (1:1, 20 mL) for 24 h, the mixture was diluted with distilled H_2O (50 mL), extracted with AcOEt (50 mL × 3). The combined extracts were washed with H_2O, dried over Na_2SO_4, then filtered. The filtrate was evaporated to give a residue. The residue was subjected to column chromatography (a gradient of 0–5% MeOH in CH_2Cl_2) to give sapogenin **2** (1.6 g, 81.3%) as fine needles: m.p. more than 300 °C after recrystallization from acetone/H_2O (lit. [11] 317–322 °C); FABMS: *m/z* 439 $[M + Na]^+$; ^{1}H-NMR (d_5-pyridine) δ: 4.10 (1H, br. s, H-3), 4.40 (1H, ddd, *J* = 7.0, 7.0, 3.7 Hz, H-16). Other assignable signals in the ^{1}H-NMR spectrum were listed in *table I*; ^{13}C-NMR spectral data was listed in *table II*.

5.1.1.2. Acetylations of **2** *and* **3**

Acetylations of **2** and **3** were performed in Ac_2O/pyridine (1:1). After-treatments of the reaction mixtures in the usual way quantitatively gave acetates **5** and **6**, respectively. FABMS of **5**: *m/z* 481 $[M + Na]^+$. FABMS of **6**: *m/z* 1057 $[M + Na]^+$. The ^{1}H-NMR spectra of **5** ($CDCl_3$) (only assignable signals were listed) δ 5.06 (1H, br. s, H-3), 4.41 (1H, ddd, *J* = 7.6, 7.6, 6.4 Hz, H-16), 3.95 (1H, dd, *J* = 11.0, 2.8 Hz, H-26a), 3.30 (1H, d, *J* = 11.0 Hz, H-26b), 2.04 (3H, s, Ac), 1.08 (3H, d, *J* = 7.0 Hz, CH_3-27), 0.99 (3H, d, *J* = 6.9 Hz, CH_3-21), 0.98 (3H, s, CH_3-19) and 0.76 (3H, s, CH_3-18): The ^{1}H-NMR spectra of **6** ($CDCl_3$) (only assignable signals were listed) δ 5.31 (1H, d, *J* = 3.4 Hz, H-4′), 5.14 (1H, dd, *J* = 9.5, 9.5 Hz, H-3″), 5.05 (1H, dd, *J* = 9.5, 9.5 Hz, H-4″), 4.96 (1H, dd, *J* = 10.1, 7.9 Hz, H-2′), 4.89 (1H, dd, *J* = 9.5, 8.2 Hz, H-2″), 4.78 (1H, d, *J* = 8.2 Hz, H-1″), 4.33 (1H, d, *J* = 7.9 Hz, H-1′), 4.41 (1H, ddd, *J* = 7.6, 7.6, 6.4 Hz, H-16), 4.33 (1H, dd, *J* = 12.5, 4.6 Hz, H-6″a), 4.14 (1H, dd, *J* = 11.3, 6.7 Hz, H-6′a), 4.06–4.10 (2H, H-6′b and 6″b), 4.00 (1H, br. s, H-3), 3.96 (1H, dd, *J* = 11.0, 2.7 Hz, H-26a), 3.88 (1H, dd, *J* = 10.1, 7.9 Hz, H-2′), 3.83 (1H, dd, *J* = 7.2, 7.2 Hz, H-5′), 3.71 (1H, ddd, *J* = 9.8, 4.6, 2.8 Hz, H-5″), 3.30 (1H, d, J = 11.3 Hz, H-26b) and 2.14, 2.07, 2.06, 2.02, 2.01, 2.00 and 1.99 (each 3H, s, Ac).

5.1.1.3. Sarsasapogenin p-bromobenzoate **7**

To a solution of **2** (1.0 g, 2.4 mmol) in pyridine (5 mL), *p*-bromobenzoyl chloride (1.0 g, 4.56 mmol) was added and allowed to stand overnight. The reaction mixture was poured into ice water (100 mL) and extracted with CH_2Cl_2 (50 mL × 3). The combined extracts were successively washed with 5% HCl, $NaHCO_3$-saturated water, and water, then dried over anhydrous Na_2SO_4, and filtered. The filtrate was evaporated to obtain a residue that was subjected to column chromatography (a gradient of 0–10% AcOEt in benzene) to give compound **7** (1.2 g, 83.4%): m.p. 207–209 °C (after recrystallization from ether/*n*-hexane); FABMS: *m/z* 621 $[M + Na]^+$ and 623 $[M + Na + 2]^+$ (1:1 as the peak-height ratios); ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 7.90 and 7.57 (each 2H, AB quartet, aromatic protons), 5.32 (1H, br. s, 3-H), 4.42 (1H, ddd, *J* = 7.6, 7.6, 6.4 Hz, H-16), 3.96 (1H, dd, *J* = 11.2, 2.6 Hz, H-26a), 3.30 (1H, d, *J* = 11.2 Hz, H-26b), 1.08 (3H, d, *J* = 6.9 Hz, 27-CH_3), 1.03 (3H, s, 19- CH_3), 1.00 (3H, d, *J* = 6.6 Hz, 21- CH_3), 0.77 (3H, s, 18- CH_3); ^{13}C-NMR ($CDCl_3$) δ: 165.2 (CO), 131.7 (C-2′ and 6′), 131.0 (C-3′ and 5′), 130.0 (C-4′), 127.7 (C-1′), 109.7 (C-22), 80.7 (C-16), 71.8 (C-3), 65.1

Table II. ^{13}C-NMR spectral data of compounds **2**, **9** and **14**–**16**[a].

	2	**14**	**15**	**9**	**16**
C-1	29.9[b]	29.9	29.9	37.2	37.2
C-2	27.7	27.8	27.9	31.6	31.5
C-3	67.0	67.0	67.1	71.7	71.6
C-4	33.5	33.5	34.0	42.2	42.2
C-5	36.4	36.5	36.6	140.8	140.8
C-6	26.5	26.5	26.6	121.4	121.3
C-7	26.5	26.2	26.5	32.0	31.7
C-8	35.2	35.0	34.5	31.4	30.5
C-9	39.8	39.7	39.9	50.0	49.9
C-10	35.2	35.1	35.3	36.6	36.5
C-11	20.8	20.4	20.6	20.8	20.4
C-12	40.2	40.2	41.1	39.7	39.7
C-13	40.6	42.2	42.0	40.2	41.7
C-14	56.4	57.5	57.0	56.5	57.5
C-15	31.7	32.0	33.6	31.8	32.1
C-16	81.0	81.1	83.2	80.8	81.0
C-17	62.0	60.8	60.1	62.0	60.4
C-18	16.4	16.2	15.9	16.2	16.1
C-19	23.8	23.9	23.9	19.4	19.4
C-20	42.0	46.6	45.9	41.6	46.4
C-21	14.3	11.5	9.9	14.5	11.3
C-22	109.7	108.8	106.8	109.3	108.3
C-23	25.7	25.9	29.1	31.3	30.6
C-24	25.9	25.7	34.9	28.7	28.5
C-25	27.0	27.4	30.0	30.2	31.2
C-26	65.1	66.8	67.5	66.8	68.1
C-27	16.0	16.5	17.1	17.1	17.1

[a] Spectra were obtained in $CDCl_3$.. [b] Chemical shifts are in ppm from internal $(CH_3)_4Si$. Signal assignments were based on DEPT, ^{1}H-^{1}H, ^{1}H-^{13}C COSY and ^{1}H-^{13}C-long-range COSY spectral data.

(C-26), 62.1 (C-17), 56.4 (C-14), 42.1 (C-20), 40.7 (C-13), 40.2 (C-12), 40.0 (C-9), 37.7 (C-5), 35.3 (C-8), 35.1 (C-10), 31.8 (C-15), 31.1 (C-1), 30.7 (C-4), 27.1 (C-25), 26.5 (C-6), 26.5 (C-7), 25.9 (C-24), 25.8 (C-23), 25.1 (C-2), 24.1 (C-19), 20.9 (C-11), 16.5 (C-18), 16.1 (C-27) and 14.4 (C-21); Anal. calcd. for $C_{34}H_{47}O_4Br$: C, 68.10; H, 7.90. Found: C, 67.75; H, 7.69.

5.1.1.4. Pseudosarsasapogenin diacetate **8**

A solution of **2** (5.0 g, 12.0 mmol) in acetic anhydride (50 mL) was heated in a bomb tube at 200 °C for 12 h. The reaction mixture was co-evaporated with toluene (50 mL × 5) to give a residue that was subjected to column chromatography (a gradient of 0–10% AcOEt in benzene), followed by application of preparative HPLC (1:9 H_2O/acetone), to give compound **8** as white foam (3.8 g, 63.2%): FABMS: *m/z* 523 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.06 (1H, br. s, H-3), 4.72 (ddd, *J* = 10.4, 7.9, 5.8 Hz, H-16), 3.94 (1H, dd, *J* = 10.7, 6.7 Hz, H-26a), 3.87 (1H, dd, *J* = 10.7, 5.8 Hz, H-26b), 2.47 (1H, d, *J* = 10.4 Hz, H-17), 2.05 (3H, s, Ac), 2.04 (3H, s, Ac), 1.58 (3H, s, CH_3-21), 0.98 (3H, s, CH_3-19), 0.94 (3H, d, *J* = 6.7 Hz, CH_3-27) and 0.66 (3H, s, CH_3-18); ^{13}C-NMR ($CDCl_3$) δ 171.1 (CO), 170.5 (CO), 151.3 (C-22), 103.7 (C-20), 84.2 (C-16), 70.5 (C-3), 69.1 (C-26), 64.2 (C-17), 54.8 (C-14), 43.6 (C-13), 39.8 (C-9), 39.8 (C-12), 37.2 (C-5), 35.0 (C-8), 34.9 (C-10), 33.9 (C-15), 32.0 (C-25), 30.6 (C-24), 30.6 (C-1), 30.5 (C-4), 26.4 (C-7), 26.3 (C-6), 24.9 (C-2), 23.7 (C-19), 23.1 (C-11), 21.4 ($COCH_3$), 20.9 (C-23), 20.8 ($COCH_3$), 16.5 (C-27), 14.0 (C-18) and 11.5 (C-21); Anal. calcd. for $C_{31}H_{48}O_5$: C, 71.36; H, 9.66. Found: C, 71.17; H, 9.68.

5.1.1.5. Pseudodiosgenin diacetate **10**

The general procedure was employed with diosgenin **9** (5.0 g, 12.1 mmol) in acetic anhydride (50 mL) to give a residue that was subjected to column chromatography (a gradient of 0–10% AcOEt in benzene), followed by application of preparative HPLC (1:9 H_2O/acetone), to give compound **10** as a white foam (3.4 g, 56.3%): FABMS: *m/z* 521 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.38 (1H, d, *J* = 4.3 Hz, H-6), 4.74 (1H, ddd, *J* = 10.2, 7.9, 5.6 Hz, H-16), 4.60 (1H, m, H-3), 3.95 (1H, dd, *J* = 10.9, 5.9 Hz, H-26a), 3.87 (1H, dd, *J* = 10.9, 6.6 Hz, H-26b), 2.47 (1H, d, *J* = 10.2 Hz, H-17), 2.05 (3H, s, Ac), 2.03 (3H, s, Ac), 1.59 (3H, s, CH_3-21), 1.04 (3H, s, CH_3-19), 0.94 (3H, d, *J* = 6.7 Hz, CH_3-27) and 0.69 (3H, s, CH_3-18); ^{13}C-NMR ($CDCl_3$) δ 171.2 (CO), 170.5 (CO), 151.4 (C-22), 139.7 (C-5), 122.3 (C-6), 103.8 (C-20), 84.3 (C-16), 73.8 (C-3), 69.1 (C-26), 64.2 (C-17), 54.9 (C-14), 50.0 (C-9), 43.2 (C-13), 39.5 (C-1), 38.1 (C-4), 37.0 (C-12), 36.7 (C-10), 34.1 (C-15), 32.1 (C-7), 32.1 (C-8), 31.2 (C-25), 30.8 (C-24), 27.7 (C-2), 23.2 (C-11), 21.4 ($COCH_3$), 21.0 ($COCH_3$), 21.0 (C-23), 19.3 (C-19), 16.7 (C-27), 13.9 (C-18) and 11.6 (C-21); Anal. calcd. for $C_{31}H_{46}O_5$: C, 74.66; H, 9.30. Found: C, 74.49; H, 9.33.

5.1.1.6. Pseudosarsasapogenin **11**

A solution of **8** (3.0 g, 6.0 mmol) in 5% methanolic potassium hydroxide (20 mL) was allowed to stand overnight at room temperature. The solution was diluted with ice water (100 mL) and the mixture was extracted with CH_2Cl_2 (100 mL × 2). The combined extracts were washed with water, then dried over anhydrous Na_2SO_4, and filtered. The filtrate was evaporated to obtain a residue that was subjected to column chromatography (a gradient of 0–5% AcOEt in benzene) to give compound **11** (2.1 g, 84.1%, m.p. 169–170 °C after recrystallization from ether) (lit. [2] m.p. 171–173 °C): FABMS: *m/z* 439 $[M + Na]^+$; ^{1}H-NMR (d_5-pyridine) (only assignable signals were listed) δ 4.87 (1H, ddd, *J* = 10.1, 7.9, 5.8 Hz, H-16), 4.38 (1H, br. s, H-3), 3.77 (1H, dd, *J* = 10.4, 5.8 Hz, H-26a), 3.69 (1H, dd, *J* = 10.4, 6.4 Hz, H-26b), 2.54 (1H, d, *J* = 10.1 Hz, H-17), 1.67 (3H, s, CH_3-21), 1.11 (3H, d, *J* = 6.7 Hz, CH_3-27), 1.03 (3H, s, CH_3-19) and 0.75 (3H, s, CH_3-18); ^{13}C-NMR (pyridine-d_5) δ 152.6 (C-22), 103.5 (C-20), 84.6 (C-16), 67.5 (C-26), 66.1 (C-3), 64.7 (C-17), 54.9 (C-14), 43.9 (C-13), 40.2 (C-12), 40.0 (C-9), 37.0 (C-5), 36.1 (C-25), 35.6 (C-8), 35.3 (C-10), 34.5 (C-15), 34.4 (C-4), 31.3 (C-1), 30.6 (C-24), 28.6 (C-7), 27.2 (C-6), 27.0 (C-2), 24.3 (C-19), 23.8 (C-11), 21.4 (C-23), 17.1 (C-27), 14.5 (C-18) and 11.9 (C-21); Anal. calcd. for $C_{27}H_{44}O_3$: C, 77.84; H, 10.64. Found: C, 77.49; H, 10.28.

5.1.1.7. Pseudodiosgenin **12**

Compound **10** (2.0 g, 4.0 mmol) was deacetylated in the same manner as **8** to obtain **12** (1.3 g, 78.5%): FABMS of **12**: *m/z* 437 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ: 5.34 (1H, d, *J* = 5.2 Hz, H-6), 4.73 (1H, ddd, *J* = 10.1, 7.9, 5.8 Hz, H-16), 3.50 (1H, m, H-3), 3.49 (1H, dd, *J* = 10.6, 5.5 Hz, H-26a), 3.43 (1H, dd, *J* = 10.6, 6.0 Hz, H-26b), 2.47 (1H, d, *J* = 10.1 Hz, H-17), 1.59 (3H, s, CH_3-21), 1.02 (3H, s, CH_3-19), 0.93 (3H, d, *J* = 6.7 Hz, CH_3-27) and 0.69 (3H, s, CH_3-18); ^{13}C-NMR ($CDCl_3$) δ: 151.5 (C-22), 140.8 (C-5), 121.2 (C-6), 103.7 (C-20), 84.2 (C-16), 71.5 (C-3), 67.7 (C-26), 64.1 (C-17), 55.0 (C-14), 50.0 (C-9), 43.2 (C-13), 42.2 (C-4), 39.4 (C-1), 37.2 (C-12), 36.5 (C-10), 35.3 (C-25), 34.0 (C-15), 32.1 (C-7), 31.5 (C-24), 31.2 (C-8), 30.4 (C-2), 23.1 (C-11), 20.9 (C-23), 19.4 (C-19),

16.5 (C-27), 13.9 (C-18) and 11.6 (C-21); Anal. calcd. for $C_{27}H_{42}O_3$: C, 78.21; H, 10.21. Found: C, 78.01; H, 10.25.

5.1.1.8. Ring-closure reactions of **11** *and* **12** *in 5% HCl in methanol*

Solutions of **11** and **12** in 5% HCl in methanol were allowed to stand for 12 h at room temperature. These reactions quantitatively gave **2** and **9**, respectively. Compounds **2** and **9** were identified as sarsasapogenin and diosgenin, respectively, with HPLC and ^{1}H-NMR spectra.

5.1.1.9. Ring-closure reaction of **11** *in acetic acid*

Compound **11** (1.0 g, 2.4 mmol) was dissolved in acetic acid (5 mL) and the mixture was allowed to stand at room temperature for 12 h. The reaction mixture was poured into ice water (50 mL) and extracted with CH_2Cl_2 (30 mL × 3). The combined extracts were successively washed with water, $NaHCO_3$-saturated water and water, then dried over anhydrous $MgSO_4$, and filtered. The filtrate was evaporated to give a residue that was subjected to preparative HPLC (2:8 H_2O/MeOH) to give compounds **2**, **14** and **15** in 2.7, 13.2 and 57.4% yields, respectively. Compounds **2**, **14** and **15** showed the same quasimolecular ion peak at *m/z* 439 $[M + Na]^+$.

Signals of CH_3-18, CH_3-19, CH_3-21, CH_3-27, H-20, H-26a and H-26b in the ^{1}H-NMR spectra ($CDCl_3$) of **14** and **15** were listed in *table I*. Other assignable signals of **14** and **15** were as follows; δ 4.45 (1H, ddd, *J* = 7.0, 7.0, 3.7 Hz, H-16) and 4.10 (1H, br. s, H-3) for **14**, and δ 4.42 (1H, ddd, *J* = 7.6, 7.6, 5.5 Hz, H-16) and 4.10 (1H, br. s, H-3) for **15**. ^{13}C-NMR ($CDCl_3$) spectra of **14** and **15** were listed in *table II*. Anal. calcd. for $C_{27}H_{44}O_3$ of **14**: C, 77.84; H, 10.64. Found: C, 78.53; H, 10.35. Anal. calcd. for $C_{27}H_{44}O_3$ of **15**: C, 77.84; H, 10.64. Found: C, 78.67; H, 10.50.

5.1.1.10. Ring-closure reaction of **12** *in acetic acid*

Treatment of compound **12** (1.0 g, 2.4 mmol) with acetic acid gave a residue that was subjected to preparative HPLC (2:8 H_2O/acetone) to give compounds **9** (white powder, 126 mg, 12.6%) and **16** (white powder, 683 mg, 68.3%). Compound **9** was identified as diosgenin with HPLC and ^{1}H-NMR spectrum. FABMS of **16**: *m/z* 437 $[M + Na]^+$. Signals of CH_3-18, CH_3-19, CH_3-21, CH_3-27, H-20, H-26a and H-26b in the ^{1}H-NMR spectra ($CDCl_3$) of **16** were listed in *table I*. Other assignable signals of **16** were as follows: δ 5.34 (1H, d, *J* = 5.27 Hz, H-6), 4.44 (1H, ddd, *J* = 7.0, 7.0, 4.0 Hz, H-16) and 3.50 (1H, m, H-3); Anal. calcd. for $C_{27}H_{42}O_3$: C, 78.21; H, 10.21. Found: C, 78.10; H, 10.24. ^{13}C-NMR ($CDCl_3$) spectra of **9** and **16** were listed in *table II*.

5.1.1.11. Pseudotimosaponin A-III octaacetate **18**

The general procedure was employed with timosaponin A-III heptaacetate **6** (10.0 g, 9.7 mmol) which was heated in acetic anhydride (50 mL) to give a residue that was subjected to column chromatography (a gradient of 0–10% AcOEt in benzene), followed by application of preparative HPLC (1:9 H_2O/acetone), to give compound **18** (white foam, 5.8 g, 55.8%). FABMS: *m/z* 1099 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) (protons on the aglycon) δ 4.72 (1H, ddd, *J* = 10.1, 7.9, 5.8 Hz, H-16), 4.00 (1H, br. s, H-3), 3.94 (1H, dd, *J* = 11.0, 6.1 Hz, H-26a), 3.88 (1H, d, *J* = 11.0 Hz, H-26b), 2.47 (1H, d, *J* = 10.1 Hz, H-17), 1.58 (3H, s, CH_3-21), 1.00 (3H, s, CH_3-19), 0.93 (3H, d, *J* = 7.0 Hz, CH_3-27) and 0.66 (3H, s, CH_3-18); (protons on galactopyranose) δ 5.30 (1H, d, *J* = 3.4 Hz, H-4′), 4.96 (1H, dd, *J* = 10.1, 3.4 Hz, H-3′), 4.45 (1H, d, *J* = 7.6 Hz, H-1′), 4.13 (1H, dd, *J* = 11.3, 6.4 Hz, H-6′a), 4.06–4.10 (H-6′b,), 3.86 (1H, dd, *J* = 10.1, 7.6 Hz, H-2′) and 3.83 (1H, dd, *J* = 6.4, 6.4 Hz, H-5′); (protons on glucopyranose) δ 5.14 (1H, dd, *J* = 9.5, 9.5 Hz, H-3″), 5.05 (1H, dd, *J* = 9.5, 7.3 Hz, H-4″), 4.83 (1H, dd, *J* = 9.5, 8.2 Hz, H-2″), 4.78 (1H, d, *J* = 8.2 Hz, H-1″), 4.33 (1H, dd, *J* = 12.5, 4.6 Hz, H-6″), 4.06–4.10 (6″a and 6″b) and 3.70 (1H, ddd, *J* = 7.3, 4.9, 2.8 Hz, H-5″), and 2.14, 2.07, 2.06, 2.05, 2.03, 2.01, 2.00 and 2.00 (each 3H, Ac); ^{13}C-NMR ($CDCl_3$) δ 171.1, 170.5, 170.3, 170.2, 170.1, 169.8, 169.2 and 169.1 (each CO), 151.3 (C-22), 103.7 (C-20), 100.4 (C-1″), 100.1 (C-1′), 84.3 (C-16), 75.6 (C-3), 74.9 (C-2′), 73.0 (C-3′), 72.8 (C-3″), 71.8 (C-2″), 71.8 (C-5″), 70.3 (C-5′), 69.1 (C-26), 68.4 (C-4″), 67.3 (C-4′), 64.3 (C-17), 62.2 (C-6″), 61.1 (C-6′), 54.8 (C-14), 43.6 (C-13), 40.0 (C-9), 39.9 (C-12), 36.2 (C-5), 35.0 (C-8), 34.9 (C-10), 33.9 (C-15), 32.0 (C-25), 30.6 (C-24), 30.0 (C-1), 30.0 (C-4), 26.6 (C-7), 26.4 (C-6), 26.2 (C-2), 23.8 (C-19), 23.1 (C-23), 20.9 (C-11), 20.9 (CO*C*H$_3$), 20.7 (CO*C*H$_3$), 20.6 (CO*C*H$_3$), 20.6 (CO*C*H$_3$), 20.5 (CO*C*H$_3$), 20.5 (CO*C*H$_3$), 20.4 (CO*C*H$_3$), 20.4 (CO*C*H$_3$), 16.5 (C-27), 14.0 (C-18) and 11.5 (C-21); Anal. calcd. for $C_{55}H_{80}O_{21}$: C, 61.32; H, 7.49. Found: C, 61.05; H, 7.56.

5.1.1.12. Pseudomarcogenin diglycoside nonaacetate **19**

The general procedure was employed with compound **17** (1.0 g, 0.91 mmol) which was heated in acetic anhydride (10 mL) to give a residue that was subjected to column chromatography (a gradient of 0–10% AcOEt in benzene), followed by application of preparative HPLC (1:9 H_2O/acetone), to give compound **19** (white foam, 640 mg, 61.7%): FABMS: *m/z* 1157 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) (protons on the aglycon) δ 4.73 (2H, H-3 and H-16), 4.10 (1H, m, H-2), 3.94 (1H, dd, *J* = 10.7, 6.4 Hz, H-26a), 3.88 (1H,

dd, *J* = 10.7, 6.1 Hz, H-26b), 2.47 (1H, d, *J* = 10.1 Hz, H-17), 1.57 (3H, s, CH_3-21), 1.02 (3H, s, CH_3-19), 0.94 (3H, d, *J* = 6.7 Hz, CH_3-27) and 0.65 (3H, s, CH_3-18); (protons on galactopyranose) δ 5.29 (1H, d, *J* = 3.7 Hz, H-4′), 4.93 (1H, dd, *J* = 10.1, 3.7 Hz, H-3′), 4.32 (1H, d, *J* = 7.6 Hz, H-1′), 3.98 (1H, dd, *J* = 10.1, 7.6 Hz, H-2′), 3.82 (1H, dd, *J* = 7.3, 7.3 Hz, H-5′) and 4.05–4.15 (H-6′a and H-6′b); (protons on glucopyranose) δ 5.13 (1H, dd, *J* = 9.8, 9.5 Hz, H-3″), 4.98 (1H, dd, *J* = 9.8, 9.8 Hz, H-4″), 4.83 (1H, dd, *J* = 9.5, 8.2 Hz, H-2″), 4.73 (1H, d, *J* = 8.2 Hz, H-1″) and 4.05–4.15 (H-6″a and H-6″b), 3.73 (1H, ddd, J = 9.8, 5.2, 3.7 Hz, H-5″) and 2.15, 2.09, 2.08, 2.06, 2.05, 2.04, 2.01, 1.99 and 1.98 (each 3H, Ac); ^{13}C-NMR ($CDCl_3$) δ 171.1, 170.5, 170.5, 170.3, 170.1, 170.1, 169.8, 169.3 and 169.2 (each CO), 151.3 (C-22), 103.7 (C-20), 100.8 (C-1′), 100.4 (C-1″), 84.2 (C-16), 77.0 (C-2), 74.0 (C-2′), 73.7 (C-3′), 72.7 (C-3″), 71.7 (C-5″), 71.4 (C-2″), 70.5 (C-5′), 70.0 (C-3), 69.1 (C-26), 68.7 (C-4″), 67.3 (C-4′), 64.2 (C-17), 62.5 (C-6″), 61.2 (C-6′), 54.6 (C-14), 45.3 (C-13), 41.0 (C-9), 39.6 (C-12), 36.7 (C-10), 35.1 (C-8), 35.0 (C-5), 33.9 (C-15), 30.6 (C-1), 30.6 (C-24), 30.1 (C-4), 26.6 (C-6), 25.6 (C-7), 23.5 (C-19), 23.1 (C-23), 21.0 (C-11), 21.2 (C-25), 20.9–20.3 ($COCH_3 \times 9$), 16.6 (C-27), 14.0 (C-18) and 11.5 (C-21); Anal. calcd. for $C_{57}H_{82}O_{23}$: C, 60.31; H, 7.28. Found: C, 60.01; H, 7.30.

5.1.1.13. Ring-closure reaction of **18**

A solution of **18** (5 g, 4.64 mmol) in 5% KOH in EtOH/H_2O (1:1, 20 mL) was allowed to stand overnight at room temperature. The reaction mixture was acidified with acetic acid (pH 4.0), then neutralized with pyridine. The mixture was evaporated to give a residue that was subjected to column chromatography ($CHCl_3$/MeOH/H_2O, 65:35:10, lower layer), followed by application of preparative HPLC (3:7 H_2O/MeOH), to give compound **3** (fine needles, m.p. more than 300 °C after recrystallization from acetone/ethanol, 95 mg, 2.8%), **20** (white needles, m.p. more than 300 °C after recrystallization from acetone/ethanol, 1.6 g, 46.5%), and **21** (white foam, 690 mg, 20.1%). Compound **3** was identified as timosaponin A-III by HPLC and ^{1}H-NMR spectrum. FABMS of **20** ***m/z*** 763 $[M + Na]^+$; signals of CH_3-18, CH_3-19, CH_3-21, CH_3-27, H-20, H-26a and H-26b in the ^{1}H-NMR spectra (pyridine-d_5) were listed in *table I*. Other assignable signals were as follows: δ 5.25 (1H, d, *J* = 7.6 Hz, H-1″), 4.90 (1H, d, *J* = 7.6 Hz, H-1′), 4.64 (1H, dd, *J* = 9.5, 7.6 Hz, H-2′), 4.54 (1H, d, *J* = 3.1 Hz, H-4′), 4.53 (1H, ddd, J = 7.3, 7.3, 5.5 Hz, H-16), 4.35–4.55 (4H, H-6′a, 6′b, 6″a and 6″b), 4.32 (1H, br. s, H-3), 4.29 (1H, dd, *J* = 9.5, 9.2 Hz, H-4″), 4.26 (1H, dd, *J* = 9.5, 3.1 Hz, H-3′), 4.17 (1H, dd, *J* = 9.2, 8.9 Hz, H-3″), 4.06 (1H, dd, *J* = 8.9, 7.6 Hz, H-2″),.4.01 (1H, dd, *J* = 6.4, 6.1 Hz, H-5′) and 3.85 (1H, ddd, *J* = 9.5, 6.1, 3.4 Hz, H-5″); ^{13}C-NMR spectrum of **20** was listed in *table III*; Anal. calcd. for $C_{39}H_{64}O_{13}.H_2O$: C, 61.72; H, 8.77. Found; C, 61.66; H, 8.81. FABMS of **21** *m/z* 763 $[M + Na]^+$; signals of CH_3-18, CH_3-19, CH_3-21, CH_3-27, H-20, H-26a and H-26b in the ^{1}H-NMR spectra (pyridine-d_5) were listed in *table I*; other assignable signals were as follows: δ 5.23 (1H, d, *J* = 7.6 Hz, H-1″), 4.89 (1H, d, *J* = 7.6 Hz, H-1′), 4.62 (1H, dd, *J* = 9.5, 7.6 Hz, H-2′), 4.61 (1H, ddd, *J* = 7.3, 7.3, 3.4 Hz, H-16), 4.58 (1H, d, *J* = 4.0 Hz, H-4′), 4.53 (1H, m, H-16), 4.31 (1H, br. s, H-3), 4.35–4.47 (4H, H-6′a, 6′b, 6″a and 6″b), 4.27 (1H, dd, *J* = 9.5, 9.2 Hz, H-4″), 4.24 (1H, dd, *J* = 9.5, 4.0 Hz, H-3′), 4.14 (1H, dd, *J* = 9.2, 8.9 Hz, H-3″), 4.03 (1H, dd, *J* = 8.9, 7.6 Hz, H-2″), 4.00 (1H, dd, J = 6.1, 6.1 Hz, H-5′) and 3.81 (1H, ddd, *J* = 9.5, 5.5, 4.0 Hz, H-5″); ^{13}C-NMR spectrum of **21** was listed in *table III*; Anal. calcd. for $C_{39}H_{64}O_{13}.H_2O$: C, 61.72; H, 8.77. Found: C, 61.33; H, 8.65.

5.1.1.14. Acid hydrolysis of **20** *and* **21**

Solutions of **20** and **21** in 2 N HCl in dioxane/H_2O were heated at 80 °C for 16 h. After-treatment of both solutions in the same manner as hydrolysis of **3**, **2** was obtained quantitatively.

5.1.1.15. Ring-closure reaction of **19**

A solution of **19** (0.6 g, 0.53 mmol) in 5% KOH in EtOH/H_2O (1:1, 5 mL) was allowed to stand overnight at room temperature. After-treatment of the solution in the same manner as the ring-closure reaction of **18** gave compounds **4** (15 mg, 3.8%), **22** (166 mg, 41.5%), **23** (45 mg, 11.3%) and **24** (8 mg, 2.0%). Compound **4** was identified with marcogenin diglycoside by comparison of HPLC and ^{1}H-NMR spectrum. FABMS of **22** *m/z* 779 $[M + Na]^+$; signals of CH_3-18, CH_3-19, CH_3-21, CH_3-27, H-20, H-26a and H-26b in the ^{1}H-NMR specrum (pyridine-d_5) were listed in *table I*. Other assignable signals were as follows: δ 5.27 (1H, d, *J* = 7.6 Hz, H-1″), 4.99 (1H, d, *J* = 7.6 Hz, H-1′), 4.71 (1H, dd, *J* = 8.9, 7.6 Hz, H-2′), 4.51 (1H, d, *J* = 3.1 Hz, H-4′), 4.53–4.43 (5H, H-16, 6′a, 6′b, 6″a and 6″b), 4.38 (1H, br. s, H-3), 4.27 (2H, H-3′ and 4″), 4.19 (1H, dd, *J* = 8.9, 8.9 Hz, H-3″), 4.11 (1H, dd, *J* = 8.9, 7.6 Hz, H-2″), 4.09 (1H, m, H-5′) and 3.85 (1H, m, H-5″); ^{13}C-NMR spectrum were listed in *table III*; Anal. calcd. for $C_{39}H_{64}O_{14}.H_2O$: C, 60.45; H, 8.58. Found: C, 60.19; H, 8.47. FABMS of **23** *m/z* 779 $[M + Na]^+$; signals of CH_3-18, CH_3-19, CH_3-21, CH_3-27, H-20, H-26a and H-26b in the ^{1}H-NMR specrum (pyridine-d_5) were listed in *table I*; other assignable signals of **23** were as follows; δ 5.29 (1H, d, *J* = 7.6 Hz, H-1″), 5.03 (1H, d, *J* = 7.6 Hz, H-1′), 4.73 (1H, dd, *J* =

Table III. ^{13}C-NMR spectral data of compounds **3**, **4** and **20**–**24**[a].

	3	**20**	**21**	**4**	**22**	**23**	**24**
C-1	30.9[b]	30.9	31.0	40.5	40.5	40.6	40.3
C-2	27.0	27.0	26.9	67.2	67.2	67.2	67.2
C-3	75.4	75.6	75.5	81.7	81.7	81.8	81.8
C-4	30.9	30.8	30.9	31.8	31.8	31.8	31.9
C-5	36.8	36.9	36.9	36.5	36.6	36.6	36.6
C-6	26.8	26.6	26.5	26.7	26.7	26.5	26.5
C-7	26.8	26.8	26.8	26.3	26.3	26.3	26.3
C-8	35.5	34.7	35.1	35.5	34.7	35.2	35.2
C-9	40.2	40.2	40.1	41.3	41.4	41.3	41.3
C-10	35.2	35.2	35.1	37.0	37.0	37.0	37.0
C-11	21.1	20.8	20.7	21.3	21.0	20.9	20.9
C-12	40.3	41.2	40.0	40.2	41.1	40.3	40.6
C-13	40.9	42.2	42.3	40.8	42.1	42.3	42.3
C-14	56.4	56.9	57.4	56.3	56.7	57.3	57.3
C-15	35.2	34.2	32.3	32.1	34.2	32.3	32.3
C-16	81.3	83.3	81.4	81.3	83.3	81.4	81.4
C-17	62.9	60.3	60.0	62.9	60.3	61.0	61.0
C-18	16.6	16.2	16.4	16.5	16.2	16.4	16.4
C-19	24.0	24.0	24.0	23.8	23.9	23.9	23.9
C-20	40.3	46.1	46.6	42.4	46.1	47.0	46.7
C-21	14.9	10.2	11.5	14.9	10.2	11.6	11.5
C-22	109.7	106.8	106.4	109.6	106.8	109.0	108.4
C-23	26.4	35.2	30.9	26.2	35.2	26.2*	31.0*
C-24	26.2	29.5	29.9	26.2	29.5	25.9*	29.0*
C-25	27.5	30.2	30.9	27.5	30.2	27.8	30.9
C-26	65.1	67.7	68.1	65.1	67.7	66.6	68.1
C-27	16.3	17.3	17.4	16.3	17.3	16.6	17.4
C-1′	102.5	102.5	102.5	103.3	103.3	103.4	103.5
C-2′	81.7	81.8	81.7	81.9	82.0	82.1	82.1
C-3′	75.1	75.1	75.1	76.9	76.9	77.0	77.0
C-4′	69.8	69.7	69.7	69.7	69.7	69.7	69.7
C-5′	76.5	76.5	76.5	76.9	76.9	77.0	77.0
C-6′	62.1	62.1	62.1	61.9	61.9	62.0	62.0
C-1″	106.0	106.0	106.0	106.1	106.2	106.2	106.2
C-2″	76.8	76.8	76.8	75.1	75.1	75.1	75.2
C-3″	77.9	77.9	77.9	78.5	78.5	78.5	78.6
C-4″	71.6	71.6	71.6	71.6	71.6	71.7	71.7
C-5″	78.3	78.3	78.3	77.9	77.9	78.0	78.0
C-6″	62.7	62.7	62.7	62.7	62.7	62.8	62.8

[a] Spectra were obtained in pyridine-d_5.. [b] Chemical shifts are in ppm from internal $(CH_3)_4Si$. Signal assignments were based on DEPT, ^{1}H-^{1}H and ^{1}H-^{13}C COSY and ^{1}H-^{13}C long-range COSY spectral data.. * These values may be interchangeable in each column.

8.0, 7.6 Hz, H-2′), 4.58 (1H, dd, *J* = 10.7, 7.3 Hz, H-6″a), 4.53 (1H, d, *J* = 3.1 Hz, H-4′), 4.50–4.39 (4H, H-16, 6′a, 6′b and 6″b), 4.39 (1H, br. s, H-3), 4.31 (1H, dd, *J* = 9.9, 9.5 Hz, H-4″), 4.29 (1H, dd, *J* = 8.0, 3.1 Hz, H-3′), 4.21 (1H, dd, *J* = 9.9, 8.2 Hz, H-3″), 4.11 (2H, H-5′ and 2″) and 3.87 (1H, ddd, *J* = 9.5, 7.3, 4.3 Hz, H-5″); ^{13}C-NMR spectrum were listed in *table III*; Anal. calcd. for $C_{39}H_{64}O_{14}.H_2O$: C, 60.45; H, 8.58. Found: C, 60.33; H, 8.61. FABMS of **24** *m/z* 779 $[M + Na]^+$; signals of CH_3-18, CH_3-19, CH_3-21, CH_3-27, H-20, H-26a and H-26b in the ^{1}H-NMR specrum (pyridine-d_5) were listed in *table I*; other assignable signals of **24** were as follows: δ 5.29 (1H, d, *J* = 7.6 Hz, H-1″), 5.00 (1H, d, *J* = 7.6 Hz, H-1′), 4.74 (1H, dd, *J* = 8.0, 7.6 Hz, H-2′), 4.60 (1H, dd, *J* = 10.1, 7.6 Hz, H-6″a), 4.58 (1H, d, *J* = 4.0 Hz, H-4′), 4.53–4.42 (4H, H-16, 6′a, 6′b and 6″b), 4.39 (1H, br. s, H-3), 4.32 (1H, dd, *J* = 9.5, 9.2 Hz, H-4″), 4.30 (1H, dd, *J* = 7.9, 4.0 Hz, H-3′), 4.21 (1H, dd, *J* = 9.2, 8.9 Hz, H-3″), 4.12 (2H, H-5′ and 2″) and 3.87 (1H, ddd, *J* = 9.5, 7.6, 4.3 Hz, H-5″); ^{13}C-NMR spectrum were listed in *table III*; Anal. calcd. for $C_{39}H_{64}O_{14}.H_2O$: C, 60.45; H, 8.58. Found: C, 60.22; H, 8.46.

5.1.1.16. 26-O-Acetyl pseudotimosaponin A-III **25**

A solution of **18** (1 g, 0.93 mmol) in 5% NH_3 in MeOH (20 mL) was allowed to stand overnight at room temperature. The mixture was sucked for 10 min and evaporated to give a residue that was subjected to column chromatography (0–10% AcOEt in benzene) to give **25** (510 mg, 70.2%) as white powder: FABMS *m/z* 805 $[M + Na]^+$; ^{1}H-NMR (pyridine-d_5) (only assignable signals were listed) δ 5.24 (1H, d, *J* = 7.6 Hz, H-1″), 4.90 (1H, d, *J* = 7.6 Hz, H-1′), 4.86 (2H, H-16, overlapped with H-1′), 4.62 (1H, dd, *J* = 9.2, 8.2 Hz, H-2′), 4.54 (1H, br.s, H-4′), 4.35–4.50 (5H, m, H-26a, 6′a, 6′b, 6″a and 6″b), 4.33 (1H, br. s, H-3), 4.20–4.30 (2H, m, H-3′ and 4″), 4.16 (1H, dd, *J* = 9.2, 8.9 Hz, H-3″), 4.00–4.10 (3H, m, H-26b, 5′ and 2″), 3.81 (1H, m, H-5″), 2.50 (1H, d, *J* = 7.0 Hz, H-17), 2.01 (3H, s, Ac), 1.64 (3H, s, CH_3-21), 0.98 (3H, s, CH_3-19), 0.95 (3H, d, *J* = 6.4 Hz, CH_3-27) and 0.72 (3H, s, CH_3-18); ^{13}C-NMR (pyridine-d_5) δ 170.8 (CO), 151.9 (C-22), 107.5 (C-1″), 103.9 (C-20), 102.5 (C-1′), 84.6 (C-16), 81.7 (C-2′), 78.3 (C-5″), 77.9 (C-3″), 76.8 (C-2″), 76.5 (C-5′), 75.4 (C-3), 75.1 (C-3′), 71.6 (C-4″), 69.7 (C-4′), 69.1 (C-26), 64.6 (C-17), 62.7 (C-6″), 62.1 (C-6′), 54.7 (C-14), 43.8 (C-13), 40.1 (C-9), 40.1 (C-12), 36.9 (C-5), 35.2 (C-8), 35.2 (C-10), 34.4 (C-15), 32.3 (C-25), 31.1 (C-1), 30.9 (C-4), 30.9 (C-7), 26.9 (C-2), 26.8 (C-6), 26.8 (C-24), 24.0 (C-19), 23.4 (C-11), 21.3 (C-23), 20.8 ($COCH_3$), 16.7 (C-27), 14.4 (C-18) and 11.8 (C-21); Anal. calcd. for $C_{41}H_{66}O_{14}.H_2O$: C, 61.48; H, 8.56. Found: C, 61.19; H, 8.67.

5.1.1.17. X-ray diffraction

The measurements of **7**, **13** and **20** were made on a Bruker SMART diffractometer with graphite monochromated Mo Kα radiation (λ = 0.71073 Å), a Mac Science MXC 3KHF diffractometer with graphite monochromated Mo Kα radiation (λ = 0.71073 Å) and a Rigaku

AFC5 diffractometer with graphite monochromated Cu Kα radiation (λ = 1.5418 Å), respectively.

5.1.1.18. Crystal data for **7**

$C_{34}H_{47}BrO_4$, M = 599.63, prismatic crystal (colourless, 0.63 × 0.15 × 0.15 mm), monoclinic, space group $P2_1$, a = 10.339 (1), b = 6.450 (1), c = 23.533 (2) Å, β = 101.697 (2)°, V = 3725.0 (6) Å^3, Z = 2, D_X = 1.296 g/cm^3, final R = 0.042, $_wR$ = 0.075, GOF = 0.833.

5.1.1.19. Crystal data for **13**

$C_{29}H_{44}O_4$, M = 456.64, prismatic crystal (colourless, 0.25 × 0.20 × 0.15 mm), monoclinic, space group $P2_1$, a = 14.553 (2), b = 6.211 (1), c = 14.795 (2) Å, β = 100.036 (7)°, V = 1316.8 (3) Å^3, Z = 2, D_X = 1.150 g/cm^3, final R = 0.049, $_wR$ = 0.050, GOF = 0.563.

5.1.1.20. Crystal data for **20**

$C_{39}H_{64}O_{13}$, M = 730.93, prismatic crystal (colourless, 0.45 × 0.30 × 0.20 mm), orthoronbic, space group $P2_12_12_1$, a = 11.579 (2), b = 43.263 (3), c = 7.967 (2) Å, V = 3990 (3) Å^3, Z = 4, D_X = 1.233 g/cm^3, final R = 0.109, $_wR$ = 0.092, GOF = 5.678.

5.1.2. Pharmacology

5.1.2.1. Materials

Platelet rich plasma (PRP) was prepared with freshly collected blood from healthy adults, and its concentration was adjusted to 3.0 × 10^5 cells/μL by dilution with platelet poor plasma (PPP) [25, 26]. The PRP was used within 3 h of preparation. Spirostanol diglycosides were dissolved in DMSO at a concentration of 40 mg/mL as stock solutions.

5.1.2.2. Assay of platelet agglutination activities

The platelet agglutination was monitored with an aggregometer using PPP as a reference [27]. Two hundred and ninety-four microlitres of PRP in a glass cell apparatus were pre-incubated with 3 μL of samples for 5 min at 37 °C in a glass chamber. Final concentration of each samples was adjusted to 400, 200, 100 and 50 μg/mL. After 5 min of pre-incubation, assays were started by addition of agglutination inducers; 3 μL of 1 mM ADP or 100 mg/mL ristocetin, and the change of light transmittance induced by platelet agglutination was monitored with an Platelet Aggregometer (Chrono-Log Co. Tokyo). Agglutination activity and effect of each sample on it were evaluated by agglutination intensity. The agglutination activity with DMSO instead of a specimen was considered as 100% agglutination. Suppression rate of agglutination was calculated by the following equation:

suppression efficiency of agglutination = 100 × (agglutination intensity with DMSO – agglutination intensity with sample)/agglutination intensity with DMSO.

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