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Structural revision of periplocosides and periperoxides, natural immunosuppressive agents from the genus *Periploca*

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ABSTRACT

The structures of a series of peroxy function containing pregnane glycosides isolated from *Periploca sepium* and *Periploca forrestii* were revised to be orthoester group bearing ones using 2D NMR spectroscopic techniques, as well as chemical transformations and X-ray crystallographic diffraction analysis. The orthoester function appears to be an essential structural feature for immunosuppressive activity.

1. Introduction

Natural products play an important role in the development of drugs, especially for the treatment of infections and cancer, as well as immunosuppressive compounds (Tietze et al., 2003). Immunosuppressive agents have wide application in organ transplantation and the treatment of autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and Crohn's disease, and almost all the immunosuppressive drugs currently available on the market are derived from a natural source (Mann, 2001). Some traditional Chinese medicines (TCM) have been used for centuries in China to treat various immune system disorders, and represent a valuable resource to find new immunosuppressive agents (Ramgolam et al., 2000).

The root barks of *Periploca sepium* Bge. and the roots of *Periploca forrestii* Schltr. (Asclepiadaceae) have been used as traditional Chinese medicines for the treatment of autoimmune diseases, especially for rheumatoid arthritis (Jiangsu New Medical College, 1998). In our recent research, periplocosides A (**1a**) and E (**2a**) (Fig. 1), the main constituents of *P. sepium*, were found to possess significant activities against the proliferation of T lymphocyte *in vitro* without obvious cytotoxicity (Feng et al., 2008). *In vivo*, periplocoside E (i.p., 10 mg/kg) exhibited potent therapeutical effect in MOG_{35-55} (myelin oligodendrocyte glycoprotein 35–55)-induced experimental autoimmune encephalomyelitis (EAE) by suppressing interleukin 12-dependent CCR5 expression and interferon-γ-dependent CXCR3 expression in T lymphocytes (Zhu et al., 2006a,b), and periplocoside A (p.o., 25 mg/kg) prevented EAE by suppressing IL-17 production and inhibits differentiation of Th17 cells (Zhang et al., 2009). Periplocoside A (i.v., 10 mg/kg) also showed significantly preventive effect on concanavalin A-induced mice hepatitis and has now emerged as a promising natural anti-rheumatoid arthritis agent that shows excellent efficacy in collagen-induced arthritis in mice (Wan et al., 2008).

Previous phytochemical studies on *P. sepium* have been focused on its pregnane glycosides, mainly carried out by two Japanese research groups. Hiroshi Hikino's research group first reported the isolation and structural elucidation of periplosides C (**1**) and A (**2**) (Fig. 2), two pregnane glycosides with an orthoester group (Oshima et al., 1987). Periplocosides A (**1a**), B (**12a**), C (**13a**), D (**3a**), E (**2a**), F (**4a**), J (**5a**), and K (**6a**) (Fig. 3), a series of peroxy function containing pregnane glycosides, were reported by Hideji Itokawa's group from the same plant and a positive color reaction of peroxides was used to support their structural identification (Itokawa et al., 1988a–c). Additionally, periperoxides A (**7a**), B (**8a**), C (**10a**), D (**11a**) and E (**9a**) (Fig. 3), further peroxy group containing pregnane glycosides with immunosuppressive activities isolated from *P. sepium* and *P. forrestii*, were reported in our preceding paper (Feng et al., 2008).

The completely identical ^{1}H NMR and ^{13}C NMR data of periplocoside A (**1a**) and periploside C (**1**) suggested that one of two structures must be incorrect. However, the ambiguous information obtained from NMR spectra and the lack of more direct structural evidence kept the puzzle pending for more than two decades. Our

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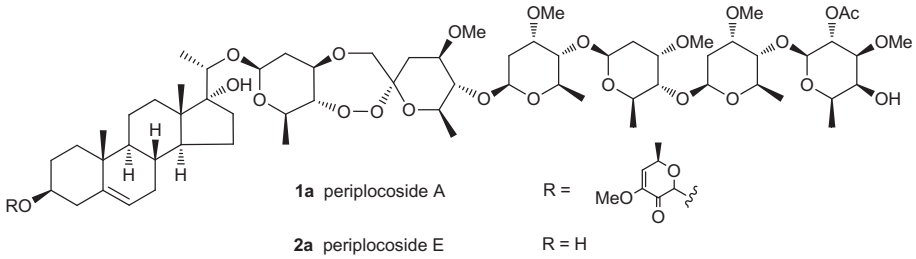


Fig. 1. Structures of periplocosides A (**1a**) and E (**2a**).

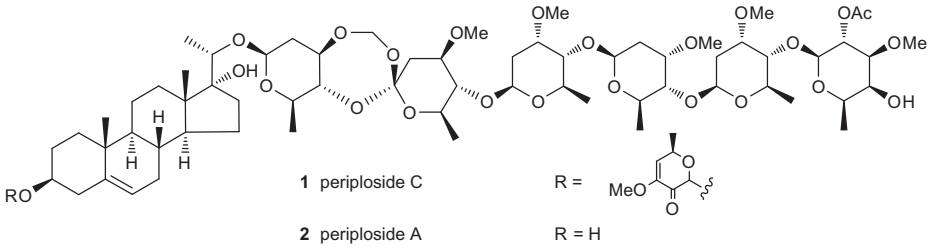


Fig. 2. Structures of periplosides C (**1**) and A (**2**).

extensive investigation using 2D NMR spectroscopic techniques, as well as chemical transformations and X-ray crystallographic diffraction analysis, established that the peroxy function in the sugar chain of these pregnane glycosides should be revised to an orthoester group.

2. Results and discussion

In the course of the reexamination of the HMBC spectrum of periplocoside A (**1a**), a $^{4}J_{CH}$ ^{1}H-^{13}C long-range correlation between H-4 of the canarose and C-2′ of the heptulose, and a $^{2}J_{CH}$ ^{1}H–^{13}C coupling between H-1′ and C-2′ of the heptulose, were found at the same time. In the case of periploside C (**1**), the aforementioned signals could be more reasonably interpreted as $^{3}J_{CH}$ ^{1}H–^{13}C long-range correlations between H-4 of the canarose and C-1′ of the oleandrose, and between the two gem-protons of the dioxymethylene and C-1′ of the oleandrose in **1** (Fig. 4). The two proton resonances at δ 4.74 and 5.14 (each 1H, d, J = 7.6 Hz) in the ^{1}H NMR spectrum should be more reasonably assigned for the two gem-protons of dioxymethylene group in periploside C (**1**) than H-1′ of the heptulose in periplocoside A (**1a**). Structure **1** was therefore thought to be more reliable.

Chemical transformations were then carried out for more evidence (Scheme 1). Partial acidic hydrolysis of **1** was undertaken with 0.001 N H_2SO_4–MeOH according to H. Itokawa's report to give compounds **14**, **15**, and **16**; however, such subacidity seemed impossible to break the C–C bond between C-1′ and C-2′ of the heptulose as in structure **1a** (Itokawa et al., 1988b). In contrast, treatment of **1** with 1 N aqueous HCl in THF led to compound **17** as well as **14** and **16**. Basic hydrolysis of **1** with NaOH–MeOH afforded **4** as the main product. Additionally, the reduction of **4** with diisobutylaluminum hydride (DIBAL-H) in dry toluene (0 °C, Ar atmosphere) yielded **18**. All the chemical transformations mentioned above belong to the reaction type of orthoesters, and are in accordance with the fact that orthoesters are highly vulnerable under acidic conditions and are relatively stable in neutral or basic aqueous solution (Huang et al., 2007).

A single crystal of intermediate **4** was obtained via slow evaporation of a petroleum ether–acetone (1:1) solution and subjected to X-ray crystallographic diffraction analysis. The result obtained unambiguously showed the existence of an orthoester rather than a peroxy functional group in its sugar chain (Fig. 5). Therefore, the previously proposed structure of periplocoside F (**4a**) was revised to **4** and named periploside F. Accordingly, the incorrect structures of periplocosides A (**1a**), B (**12a**), C (**13a**), D (**3a**), E (**2a**), J (**5a**), K (**6a**), and periperoxides A (**7a**), B (**8a**), C (**10a**), D (**11a**), E (**9a**) previously characterized were revised, and with the names periplosides C (**1**), M (**12**), N (**13**), D (**3**), A (**2**), J (**5**), K (**6**), E (**7**), G (**8**), I (**10**), L (**11**), and H (**9**) assigned, respectively, using **4** as a model compound (Fig. 6).

The remaining doubt laid in the positive color reaction for peroxides of these pregnane glycosides. In our repeated TLC examinations, however, all of these compounds showed negative results compared with that of 30% hydrogen peroxide, which gave a purple spot as soon as sprayed with the color developing agent. Noticeably, each compound, as well as the color agent that employed as the blank control, turned purple when standing exposed to the air for a few minutes after they were sprayed with the color agent. Since no positive control and blank control were mentioned in H. Itokawa's reports, the observation might be explained by the aerial oxidation of *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride, the color developing agent used in the detection of peroxides (Itokawa et al., 1988a–c).

As reported in our previous paper, orthoester possessing pregnane glycosides showed significant activity against the proliferation of T lymphocyte *in vitro* with IC_{50} values in the range of 0.29–1.97 μM without obvious cytotoxicity, whereas those pregnane glycosides without orthoester group in their structures exhibit no obvious activity at up to 10 μM (Feng et al., 2008). To further clarify the importance of the orthoester function in these compounds to the immunosuppressive activity, the inhibitory effects of T cell proliferation of **4** and **14–18** were evaluated. As a result, the IC_{50} of compound **4** was found to be 1.13 μM, and compounds **14–18** exhibited no obvious activity at up to 10 μM. More specifically, removal of the unique spiro orthoester group removes the activity, suggesting that the orthoester function in the sugar chain of these pregnane glycosides is critical for immunosuppressive activity. To the best of our knowledge, the orthoester has been widely discovered as a structure subunit in natural products of plant origin, and some of the orthoester containing compounds have shown important biological activities and have been widely

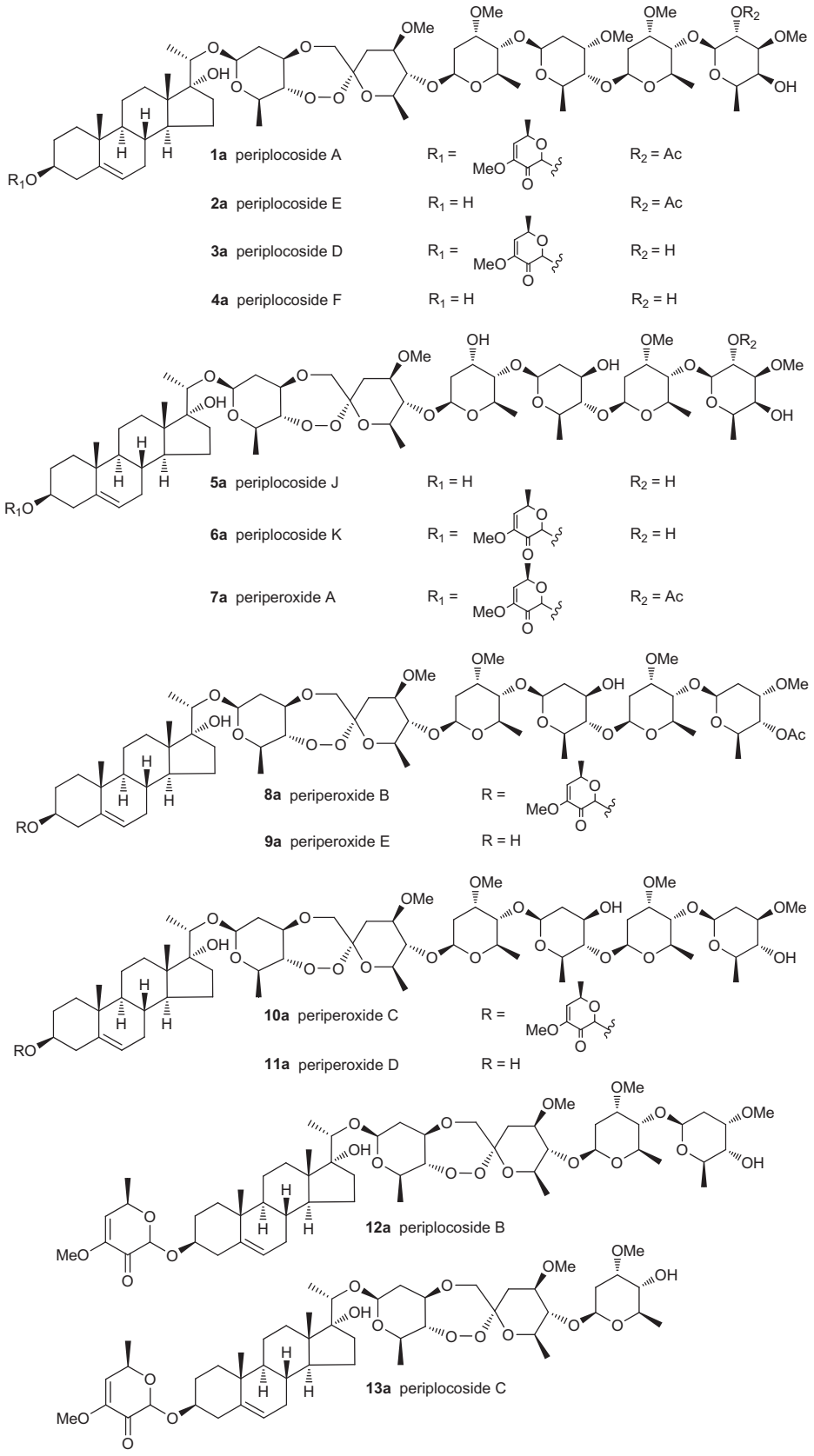


Fig. 3. Structures of peroxy function containing pregnane glycosides from *Periploca sepium* and *P. forrestii*.

used as pharmacological tools in the study of biological processes or as drug leads. Tremendous efforts aimed at the structure-activity relationship among the biologically active orthoester containing natural compounds established that the orthoester group

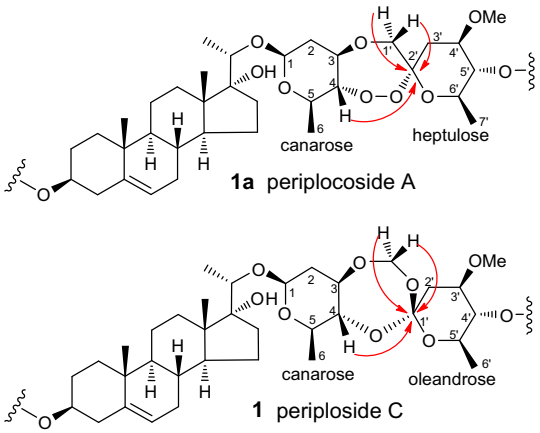


Fig. 4. Important HMBC ($^{1}H \rightarrow {}^{13}C$) correlations for **1a** and **1**.

may function as an essential pharmacophore or serve as a stereochemical constraining element to maintain the desired conformation for the biological activities (Liao et al., 2009).

3. Concluding remarks

In summary, the structures of a series of peroxy function containing immunosuppressive pregnane glycosides isolated from *P. sepium* and *P. forrestii* were revised to be orthoester group bearing ones using 2D NMR techniques as well as chemical transformations and X-ray crystallographic diffraction analysis. The study of structure-activity relationship indicated that the unique spiro orthoester group in the sugar chain of these pregnane glycosides is critical for the immunosuppressive activity. Although these orthoester containing pregnane glycosides have complicated structures compared with small molecular drugs, they have shown therapeutic effects against rheumatoid arthritis, multiple sclerosis, and autoimmune hepatitis in animal tests, and could represent a new type of natural therapeutic agent against autoimmune diseases.

4. Experimental

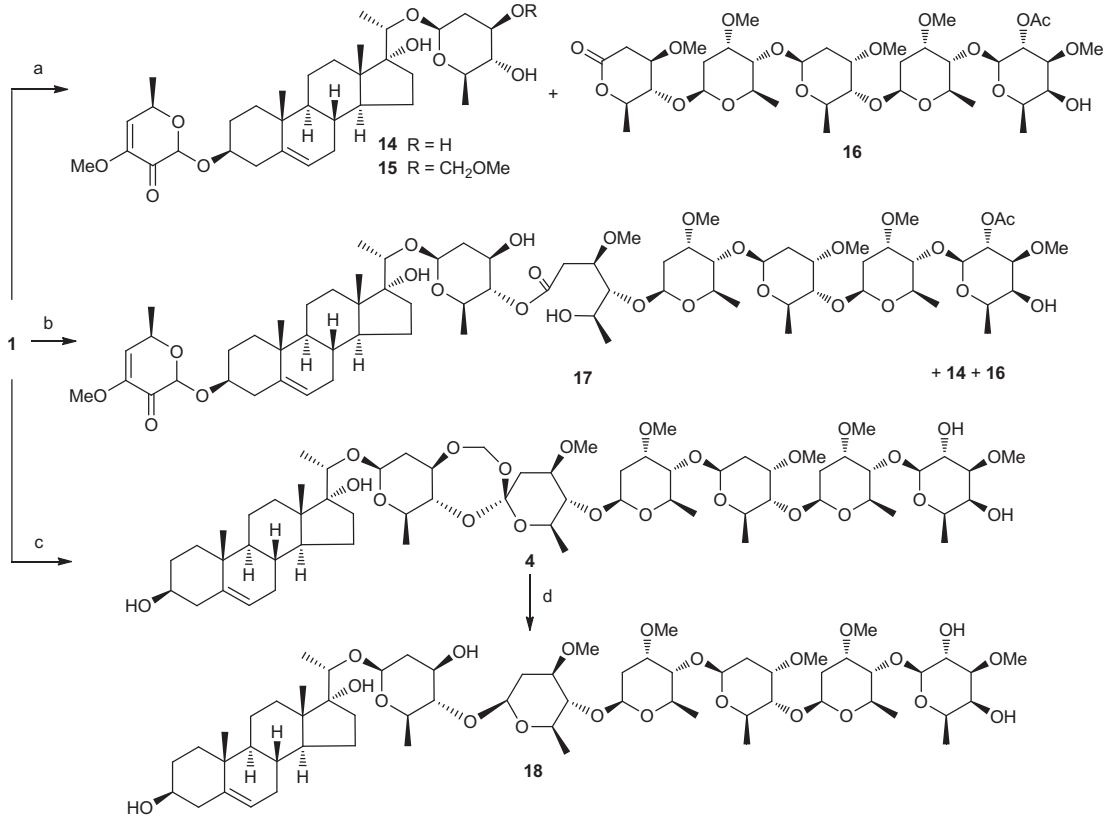
4.1. General experimental procedures

ESIMS data were obtained on a Bruker Esquire 3000 plus or Shimadzu LCMS-2020 instrument, whereas NMR spectra were run on Bruker AM 400 or INOVA-600 spectrometer with TMS as internal standard. Column chromatographic (CC) separations were carried out using silica gel H60 (300–400 mesh) (Qingdao Haiyang Chemical Group Corporation, Qingdao, People's Republic of China) as packing materials. HSGF254 silica gel TLC plates (Yantai Chemical Industrial Institute, Yantai, People's Republic of China) were used for analytical TLC. Petroleum ether with boiling range 60-90 °C was used in the experiments.

4.2. Chemical transformations of periploside C (*1*) and periploside F (*4*)

4.2.1. Partial acid hydrolysis of *1* in H_2SO_4–MeOH

A solution of **1** (141 mg, 0.10 mmol) in 0.001 N H_2SO_4–MeOH (10 mL) was stirred at room temperature until TLC analysis showed that no starting material remained. The reaction mixture was diluted with H_2O (20 mL) and extracted with EtOAc (3 × 30 mL). The organic layer was dried (anhydr. Na_2SO_4) and evaporated to dryness. The residue was subjected to silica gel CC using petroleum ether–acetone (2:1, 1:1) as eluent to give compounds **14** (31 mg), **15** (20 mg) and **16** (26 mg).



Scheme 1. Chemical transformations of **1**. (a) 0.001 N H_2SO_4–MeOH, r.t.; (b) 1 N aq. HCl, THF, r.t., 39%; (c) 1% NaOH–MeOH, r.t., 88%; (d) 1 M DIBAL-H, Ar, toluene, 0 °C, 1.5 h, 17%.

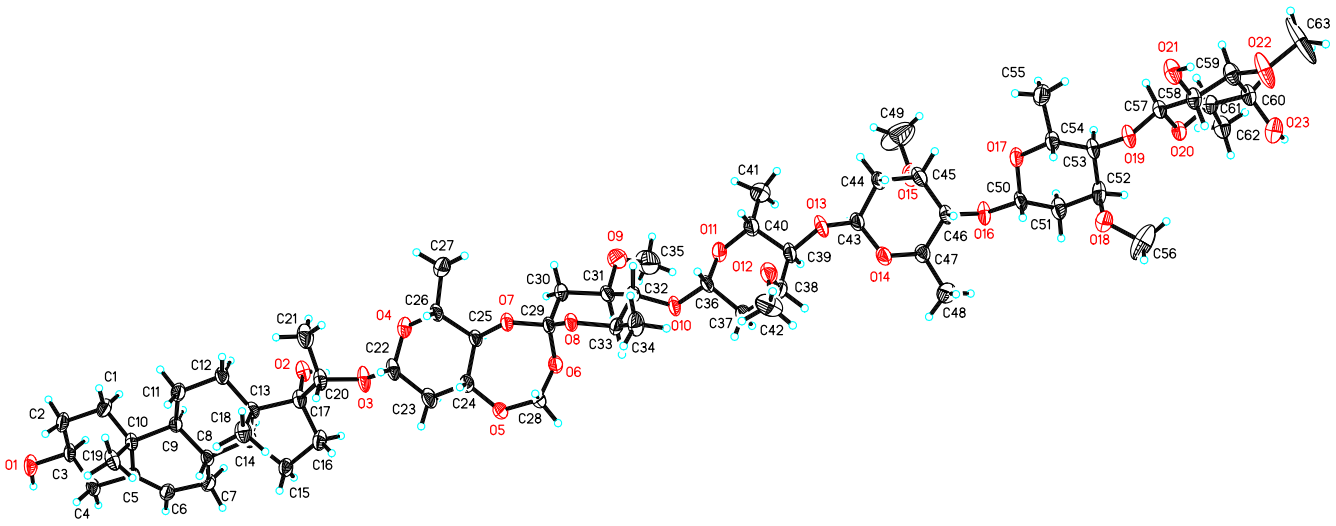


Fig. 5. Single-crystal X-ray structure of **4**.

*Compound **14**:* ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.70, 0.98 (each 3H, *s*), 1.28 (3H, *d*, *J* = 6.2 Hz), 1.31 (3H, *d*, *J* = 6.0 Hz), 1.49 (3H, *d*, *J* = 6.9 Hz), 3.61 (3H, *s*), 3.72 (1H, *q*, *J* = 6.1 Hz), 4.58 (1H, *dd*, *J* = 9.4, 1.7 Hz), 4.70 (1H, *dq*, *J* = 6.8, 2.8 Hz), 5.03 (1H, *s*), 5.34 (1H, *br s*), 5.77 (1H, *d*, *J* = 2.9 Hz); ESIMS *m/z* 627.5 $[M+Na]^+$. The ^{1}H NMR and MS data were identical with those of periploside B and periplocoside M (Oshima et al., 1987; Itokawa et al., 1988b).

*Compound **15**:* ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.71, 0.98 (each 3H, *s*), 1.29, 1.34 (each 3H, *d*, *J* = 6.2 Hz), 1.50 (3H, *d*, *J* = 6.7 Hz), 3.42, 3.62 (each 3H, *s*), 3.74 (1H, *q*, *J* = 6.5 Hz), 4.56 (1H, *dd*, *J* = 9.7, 1.8 Hz), 4.70 (3H, overlapped), 5.04 (1H, *s*), 5.34 (1H, *br s*), 5.77 (1H, *d*, *J* = 2.9 Hz); ESIMS *m/z* 671.4 $[M+Na]^+$. The ^{1}H NMR and MS data were identical with those of periplocoside O (Itokawa et al., 1988c).

*Compound **16**:* ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.15, 1.18, 1.29 (each 3H, *d*, *J* = 6.1 Hz), 1.35, 1.42 (each 3H, *d*, *J* = 6.2 Hz), 2.06 (3H, *s*), 2.71 (2H, *m*), 3.36, 3.39, 3.41, 3.42, 3.43 (each 3H, *s*), 4.37 (1H, *d*, *J* = 8.0 Hz), 4.74 (2H, *m*), 4.87 (1H, *dd*, *J* = 9.7, 1.9 Hz), 5.06 (1H, *dd*, *J* = 9.9, 8.1 Hz); ESIMS *m/z* 817.5 $[M+Na]^+$. The ^{1}H NMR and MS data were identical with those of 2-*O*-acetyl-β-D-digitalopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-oleandronic-δ-lactone (Oshima et al., 1987; Itokawa et al., 1988b).

4.2.2. Hydrolysis of **1** with 1 N aqueous HCl in THF

A solution of 1 N aqueous HCl (0.1 mL) was added to a solution of compound **1** (71 mg, 0.050 mmol) in THF (3 mL) and the reaction mixture was stirred at room temperature until TLC analysis showed that no starting material remained. The mixture was then partitioned between EtOAc (15 mL) and H_2O (15 mL). The organic phase was washed with brine, dried (anhydr. Na_2SO_4), filtered, and concentrated. The residue was subjected to silica gel CC to give compound **17** (27 mg, 39% yield) as the expected product, and compounds **14** and **16** as the byproducts.

*Compound **17**:* ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.71, 0.98 (each 3H, *s*), 1.15, 1.17, 1.18, 1.21, 1.22 (each 3H, *d*, *J* = 6.2 Hz), 1.28, 1.35 (each 3H, *d*, *J* = 6.4 Hz), 1.49 (3H, *d*, *J* = 7.0 Hz), 2.06 (3H, *s*), 2.62 (1H, *dd*, *J* = 15.8, 8.0 Hz), 2.84 (1H, *dd*, *J* = 15.8, 5.2 Hz), 3.39, 3.40, 3.41, 3.42, 3.43, 3.61 (each 3H, *s*), 4.17 (1H, *m*), 4.36 (1H, *d*, *J* = 8.0 Hz), 4.50 (1H, *t*, *J* = 9.1 Hz), 4.59 (1H, *dd*, *J* = 10.0, 1.5 Hz), 4.72 (3H, overlapped), 4.79 (1H, *dd*, *J* = 9.5, 1.0 Hz), 5.03 (1H, *s*), 5.06 (1H, *dd*, *J* = 9.8, 8.0 Hz), 5.34 (1H, *br s*), 5.76 (1H, *d*, *J* = 3.0 Hz); for ^{13}C NMR (100 MHz, $CDCl_3$) spectrum, see supplementary data; ESIMS *m/z* 1421.8 $[M+Na]^+$.

4.2.3. Basic hydrolysis of **1**

A solution of **1** (284 mg, 0.20 mmol) in 1% methanolic NaOH (20 mL) was stirred at room temperature until TLC analysis showed that no starting material remained. The resultant solution was diluted with H_2O (60 mL) and extracted with EtOAc (3 × 80 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness to afford compound **4** (216 mg) in 88% yield.

*Compound **4**:* ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.71, 0.99 (each 3H, *s*), 1.19, 1.21, 1.26, 1.28, 1.29 (each 3H, *d*, *J* = 6.2 Hz), 1.30 (3H, *d*, *J* = 5.9 Hz), 1.34 (3H, *d*, *J* = 6.6 Hz), 3.42 (×3), 3.43, 3.50 (each 3H, *s*), 4.27 (1H, *d*, *J* = 7.7 Hz), 4.58 (1H, *dd*, *J* = 9.6, 1.5 Hz), 4.73 (1H, *d*, *J* = 7.7 Hz), 4.75, 4.76 (each 1H, *dd*, *J* = 9.5, 1.5 Hz), 4.91 (1H, *dd*, *J* = 9.5, 1.2 Hz), 5.12 (1H, *d*, *J* = 7.7 Hz), 5.34 (1H, *br s*); for ^{13}C NMR (100 MHz, $CDCl_3$) spectrum, see Supplementary data; ESIMS *m/z* 1251.8 $[M+Na]^+$. The NMR and MS data were identical with those of periplocoside F (Itokawa et al., 1988c).

4.2.4. DIBAL-H reduction of **4**

To a stirred, cooled (0 °C) solution of compound **4** (190 mg, 0.15 mmol) in dry toluene (5 mL) under Ar was added DIBAL-H (5 mL, 1.0 M in toluene) dropwisely via a syringe. The reaction mixture was stirred at 0 °C for 1.5 h and then quenched at 0 °C by the dropwise addition of MeOH over a period of 10–15 min, following which iced water (20 mL) was added. The resulting gelatinous mixture was extracted with EtOAc (3 × 25 mL). The combined organic layer was washed with brine, dried with (anhydr. Na_2SO_4) and concentrated under reduced pressure. Purification of the crude mixture by silica gel CC ($CHCl_3$–MeOH, 21:1) afforded compound **18** (32 mg, 17% yield).

*Compound **18**:* ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.70, 0.98 (each 3H, *s*), 1.18, 1.19, 1.21, 1.24, 1.26, 1.29 (each 3H, *d*, *J* = 6.2 Hz), 1.33 (3H, *d*, *J* = 6.6 Hz), 3.40 (×2), 3.41 (×2), 3.49 (each 3H, *s*), 4.26 (1H, *d*, *J* = 8.1 Hz), 4.41 (1H, *dd*, *J* = 9.2, 1.5 Hz), 4.56 (1H, *dd*, *J* = 9.6, 1.5 Hz), 4.72, 4.74, 4.90 (each 1H, *dd*, *J* = 9.5, 1.5 Hz), 5.33 (1H, *br s*); for ^{13}C NMR (100 MHz, $CDCl_3$) spectrum, see Supplementary data; ESIMS *m/z* 1246.2 $[M+COOH]^-$.

4.3. X-ray crystallography data of **4**

$C_{63}H_{104}O_{23}$, MW 1229.46, triclinic space group *P*1, *a* = 11.5609 (13) Å, *b* = 11.6537(12) Å, *c* = 14.7887(16) Å, α = 94.035(2)°, β = 109.436(2)°, γ = 112.512(2)°, *V* = 1690.4(3) Å^3, *Z* = 1, D_{calcd} = 1.208 mg/m^3; *F*(0 0 0) = 666, μ = 0.091 mm^{-1}, reflection collected 8955, reflection unique 7498(R_{int} = 0.0192), final *R* indices for $I > 2\sigma(I)$,

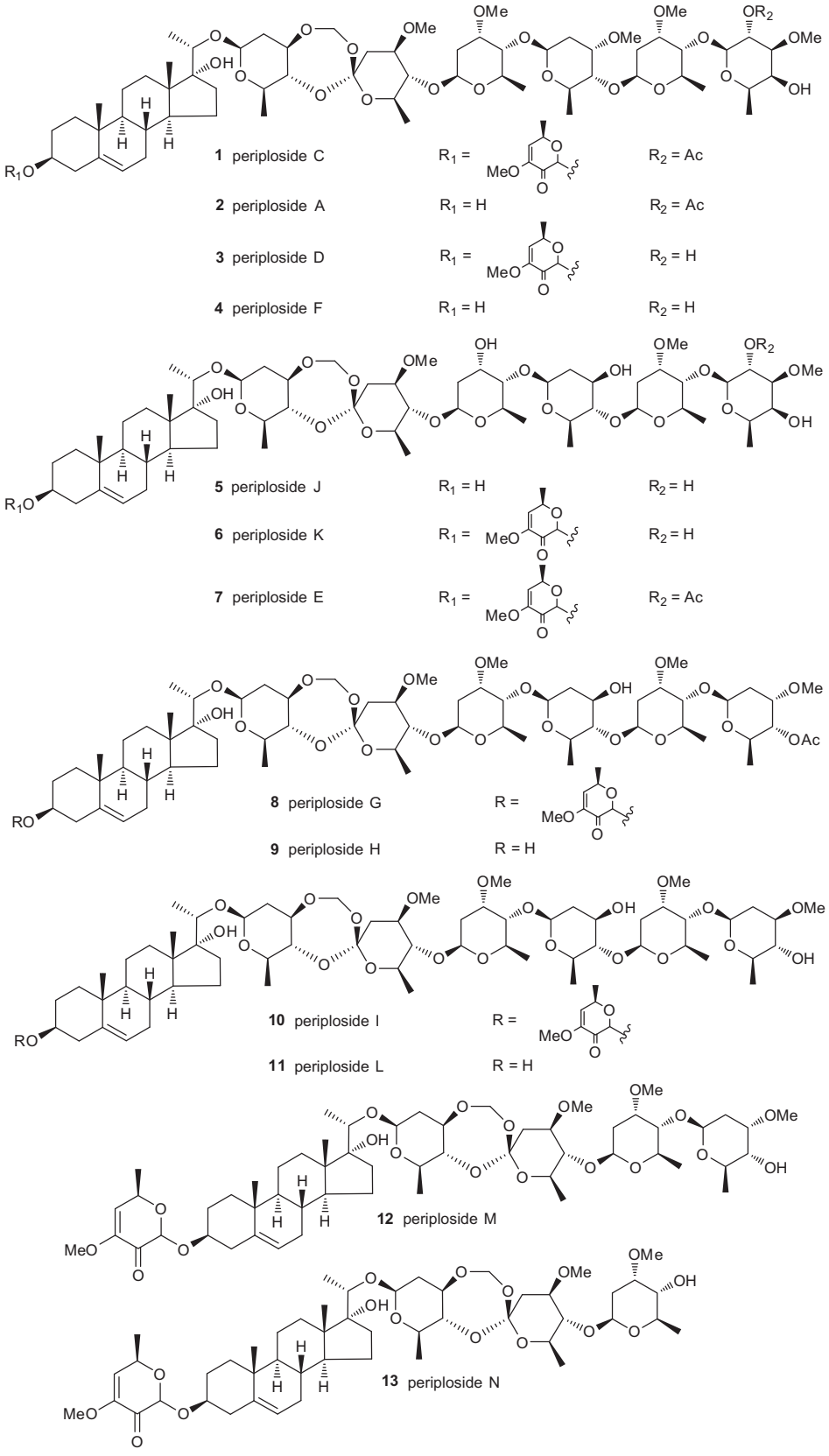


Fig. 6. Revised structures of pregnane glycosides from the genus *Periploca*.

*R*1 = 0.0508, *wR*2 = 0.1297, *R* indices for all data *R*1 = 0.0687, *wR*2 = 0.1468, completeness to θ (25.50) 98.8%, maximum transmission 1.00000, minimum transmission 0.71354. A single crystal of dimensions 0.331 × 0.279 × 0.156 mm was used

for X-ray measurements. The data collection was performed on a Bruker Smart Apex CCD diffractometer, using graphite monochromated Mo Kα ($\lambda = 0.71073$) radiation. The structure was solved by direct methods using the program SHELXS-97. Refinement method was full-matrix least-squares on F^2, and goodness-of-fit on F^2 is 1.001. CCDC 822099 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam. ac.uk).

4.4. T cell function assay

The inhibitory effects of T cell proliferation of compounds **4** and **14–18** were evaluated according to the method as described previously (Zhu et al., 2006a; Feng et al., 2008).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.07.018.

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