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RESEARCH ARTICLE

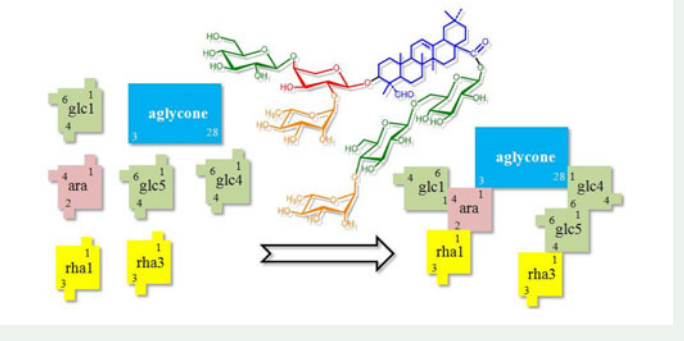
A new triterpenoid saponin from *Pulsatilla cernua* predicted by NMR-based mosaic method

An-Dong Wang[a,b,*], Ying Bao[b,c,*], Xia Wang[a,b], Mei-Chen Li[a,b], Xu-Hong Ren[b,c], Jian-Yu Liu[b,c], and Yong-Nan Xu[b,c]

[a]School of Traditional Chinese Medicine, Shenyang Pharmaceutical University, Shenyang, China; [b]Key Laboratory of Structure-Based Drug Design & Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang, China; [c]School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Shenyang, China

ABSTRACT

A saponin (**1**) with gypsogenin as aglycone was isolated from the roots of *Pulsatilla cernua*. The aglycone of compound **1** was considered as gypsogenin which was rarely found in this genus. Its structure was predicted by NMR-based "mosaic" method rapidly, and further confirmed on the basis of spectroscopic data, including 2D NMR spectra and chemical evidence. This work suggested that NMR-based mosaic method is suitable for most of saponins from common species of genus *Pulsatilla*.



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1. Introduction

As a traditional herbal medicine, the roots of *Pulsatilla cernua* (Ranunculaceae) are used for treatment of amoebic dysentery, malaria and chills for a long time. Modern researches reported that the chemical components of *P. cernua* showed strong biological activities, and especially the saponin-enriched fraction presented remarkable effects in the treatment of Alzheimer's disease (Han et al. 2007; Seo et al. 2009;

CONTACT Jian-Yu Liu burningice@126.com; Yong-Nan Xu ynxu@syphu.edu.cn
*These two authors contributed to this work equally.

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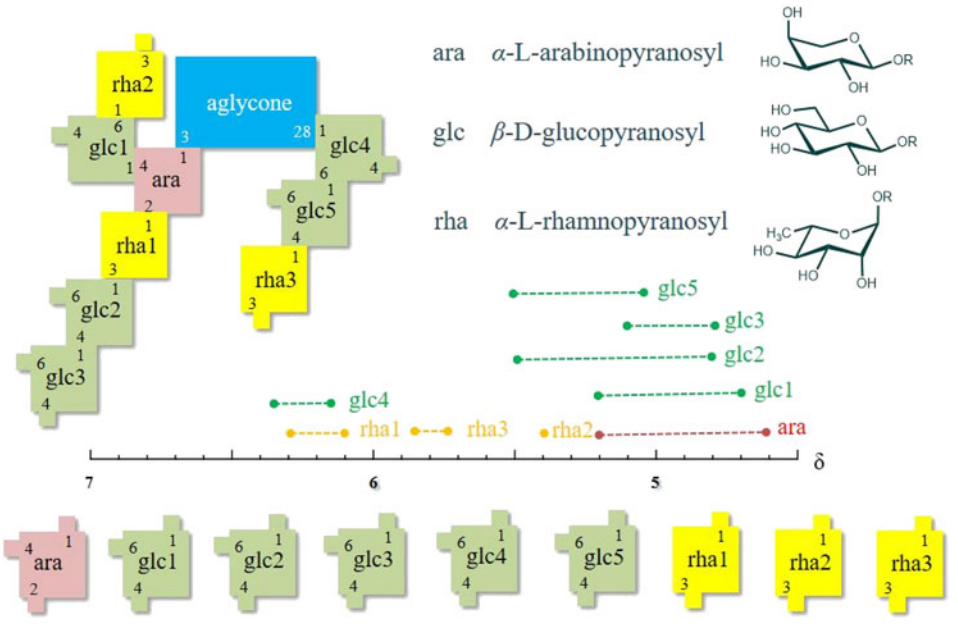

Figure 1. Mosaics of saccharide residues of most typical saponins from *P. cernua* as well as their anomeric protons ^{1}H NMR chemical shifts reigns.

Liu et al. 2012). Phytochemical investigations of this plant led to a number of triterpenoid saponins (Bang et al. 2005a,b; Fu et al. 2008; Kang 1989; Li et al. 1990; Xu et al. 2007a,b; Xu et al. 2010; Yang et al. 2010; Zhang et al. 2000a,b; Liu et al. 2012; Fan et al. 2013; Liu et al. 2015; Wang et al. 2018) and several of them are often comprised of numerous saccharides (Liu et al. 2012; Shu et al. 2013) of which ^{1}H-NMR signals are overlapped seriously. As a result, the elucidation of the structure of a saponin by NMR spectroscopy commonly involves 2D-NMR experiments (HMBC, HSQC, COSY, NOESY, and TOCSY, etc.) and comparison with previously reported data, as well as chemical experiment (acid and/or alkaline hydrolysis). Therefore the elucidation of structure of a saponin is not only a challenging but also a high-cost and time-consuming work. The mass fragmentation pattern is usually considered as an efficient method for identification of saponins. However, it is an experience required work for identification of the fragments of sugar residues. As a powerful technique for structural elucidation, NMR contains a wealth of structural information. In our present study, an NMR-based mosaic method was proposed based on our previous work and literatures. This approach is exemplified in the structural prediction of a new saponin with gypsogenin as aglycone, as well as three reported ones.

2. Results and discussion

Based on our study and previous literatures, the types and sequence of saccharide residues of most typical saponins from *P. cernua* (and even some other species of genus Pulsatilla) are distinctive and this might be closely related to their biosynthetic pathways (Table S1). The frequent saccharide residues are α-*L*-arabinopyranosyl

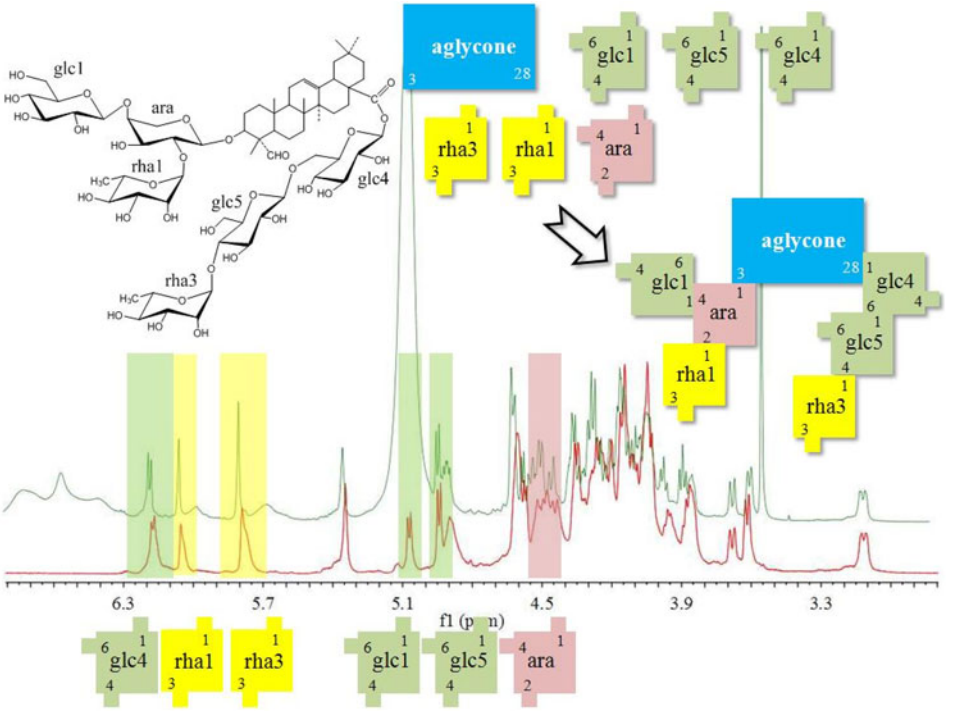

Figure 2. Stitching the mosaics of compound 1 to complete and predict the structure Glc1 signal was overlapped by water when NMR was measured in pyridine-d_5 (lower spectrum), while it can be recognized clearly when a drop of deuterated water was added (upper spectrum).

(ara), β-*D*-glucopyranosyl (glc), and α-*L*-rhamnopyranosyl (rha) groups, and their glycosylation sites are position C-2, 4 (ara), C-4, 6 (glc), and C-3 (rha), respectively. Further study indicated that the NMR profiles of anomeric hydrogens of these saccharide residues are also characteristic (Table S2). It can be seen that chemical shift of certain saccharide residue keeps in a certain region. Chemical shifts of ara H-1 linked to C-3 of aglycone are δ 4.61–5.20, and those of other saccharide residues are δ 4.70–5.20 (glc1), 4.95–5.50 (glc2), 4.85–5.15 (glc3), 6.12–6.29 (rha1), 5.37–5.39 (rha2), 6.14–6.30 (glc4), 4.90–5.50 (glc5), 5.76–5.87 (rha3), respectively (Figure 1 and Figure S1). Coupling constant of glc is 7–8 Hz, ara is 6.0 Hz and rha is broad singlet based on their dihedral angle between bonds C1-H1 and C2-H2. Furthermore, remaining signals will not change significantly when one or more saccharide residues are removed (Table S2) because of their discrete spin systems. As a result, a rapid and efficient strategy for elucidate a new or known saponin from this plant based on ^{1}H NMR is feasible. Of this method, each saccharide residue or aglycone is considered as one single mosaic, and each one can be easily recognized according to its ^{1}H NMR signal. In order to predict the structure, these mosaics could be stitched together. For structural prediction of known saponins, kalopanaxsaponin Lb (**16**), pulsatillacernuoside Kb (**38**) and pulsatillacernuoside Ka (**44**) (Supplementary material) were used as examples. Spectrum of anomeric proton of **16** was in good agreement with that of **15** which shared same sugar chain but different aglycone. Compound **37** and compound **38**, which have same anomeric protons, showed that they have same sugar chain but different

aglycone. Same to anomeric proton of **43,** compound **44** have same sugar chain (Figure S2). These mosaics were then stitched together as Figure S3 to complete the structures and they are in great agreement with our previous report (Wang et al. 2018).

Compound **1** was obtained as a white amorphous powder using D101 macroporous resin, silica gel and recycling preparative HPLC from 70% aqueous ethanol extract (Supplementary material). After analysis by submitting the ^{1}H NMR data of aglycone to an on-line structure search engine of the NMR database (Shanghai Micronmr Infor Technology Co., Ltd, Shanghai, China), the aglycone of **1** was considered as gypsogenin which was rarely found in this genus (Liu et al. 2012). From signals of anomeric protons, six mosaics of saccharide residues were recognized as ara, rha1, glc1, glc4, glc5 as well as rha3. These mosaics were then stitched together as Figure 2 to complete the structure. Compared with the signals of compound **56** which shared the same aglycone shown in Supplementary material, only glc2 was absent in compound **1** (Figure S4). In order to verify this method and confirm the structures of **1**, structure elucidation using various spectra and chemical reactions were further investigated.

The molecular formula $C_{65}H_{104}O_{31}$ was established on the basis of HR-ESI-MS at m/z 1403.6454 $[M+Na]^{+}$ (calcd for $C_{65}H_{104}O_{31}Na$, 1403.6459). The ^{1}H NMR (pyridine-d_5 with one drop of D_2O, 600 MHz) showed the present of six anomeric proton signals at δ_H 4.60 (1H, d, J = 6.0 Hz), 4.95 (1H, d, J = 7.8 Hz), 5.10 (1H, d, J = 7.8 Hz), 5.80 (1H, brs), 6.06 (1H, brs) and 6.18 (1H, d, J = 8.4 Hz) and their associated ^{13}C resonances assigned from HSQC experiment were at δ_C 104.8, 104.6, 106.2, 102.5, 101.3, and 95.4, respectively, which indicated that it is a glycoside with six sugar moieties. Acid hydrolysis of **1** with 1 M HCl gave arabinose, rhamnose and glucose, which were identified by TLC comparison with authentic samples. The β-anomeric configurations for the *D*-glucose, and the α-anomeric configurations for *L*-arabinose were determined by their $^{3}J_{H1,H2}$ coupling constants of 7–8 Hz. The α-anomeric configuration of *L*-rhamnose was judged by the chemical shift of C-5 of rhamnose (δ_C 69–70) (Mahato and Kundu 1994). The absolute configuration of the saccharides was determined by GC analysis of chiral derivatives in the hydrolysate.

Six tertiary methyl proton singlets at δ_H 0.79, 0.81, 0.81, 0.95, 1.13, and 1.39 as well as two doublets at δ_H 1.65 (3H, d, J = 6.0 Hz) and 1.66 (3H, d, J = 6.0 Hz) indicated the aglycone should be a kind of triterpene and two of the sugar moieties might be rhamnose. Signals at δ_H 5.30 (1H, s) and 9.67 (1H, s) and their associated ^{13}C resonances at δ_C 122.6 and 205.5 indicated that there should be one olefince and aldehyde group in this structure. The aglycone was then considered as gypsogenin by compared with data from our previous work (Liu et al. 2012).

The ^{13}C NMR data of C-3 (δ_C 83.8) and C-28 (δ_C 176.6) revealed that **1** was a bidesmosides saponin. The linkage of the sugar moiety at C-3 of the aglycone was established from the HMBC correlations between δ 4.60 (1H, d, J = 6.0 Hz, ara H-1) and δ 83.8 (C-3), δ 5.10 (1H, d, J = 7.8 Hz, glc1 H-1) and δ 79.3 (ara C-4), δ 6.06 (1H, brs, rha1 H-1) and δ 75.5 (ara C-2). The linkage of the sugar moiety at C-28 of the aglycone was established from the HMBC correlations between δ 6.18 (1H, d, J = 8.4 Hz, glc4 H-1) and 176.6 (C-28), δ 4.95 (1H, d, J = 7.8 Hz, glc5 H-1) and 69.0 (glc4 C-6), 5.80 (1H, brs, rha3 H-1) and 78.0 (glc5 C-4) (Figure S8). Based on the above evidence, the structure of **1** was determined

to be gypsogenin 3-*O*-α-L-rhamnopyranosyl(1→2)[β-D-glucopyranosyl(1→4)]-α-*L*- arabinopyranosyl-28-*O*-α-*L*-rhamnopyranosyl(1→4)-β-*D*-glucopyranosyl(1→6)-β-*D*-glucopyranosyl ester, and we propose the trivial name pulsatillacernuoside G. The spectra of **1** see Figures S5-S11 and data in Tables S3 and S4.

In vitro assay indicated compound **1** showed no anti-proliferative activities against five human cancer cell lines (A549, MDA-MB-231, SK-Hep-1, SNU638, and HCT116), and all IC_{50} values of **1** were above 100 μM compared with those of positive control Etoposide (4.1, 50.1, 0.6, 4.1 and 7.8 μM, respectively), see Table S5 and Figure S12.

3. Conclusion

An NMR-based mosaic method was proposed to predict typical known or even undescribed saponins from *P. cernua*. In this method, complex ^{1}H NMR profiles (such as that of **1**) can be taken apart to form several pieces of smaller mosaics which can be stitched together subsequently according to the characteristic of typical saponins from this plant. It is noteworthy that this method is also suitable for some other species of this genus such as *P. chinensis*, *P. campanella* and *P. dahurica*, while it should be used carefully for other species because saponins from these plants might contain different kind of sugar chains.

Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCID

Jian-Yu Liu http://orcid.org/0000-0003-0714-1319

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