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Steroids from herbs of *Reineckia carnea* and their anticomplement activities

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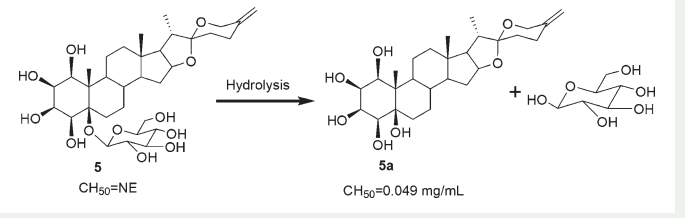
ABSTRACT

A new polyhydroxylated pregnane, named Iβ,2β,3β,4β,5β,6β-hexolhydroxy-pregn-16-en-20-one (**1**), along with nine known (**2–10**) steroidal saponins were isolated from the whole plant of *Reineckia carnea*. Structure elucidations of all compounds were established by interpretation of their NMR spectral data, HR-ESI-MS and comparing with literatures. In addition, these compounds were evaluated with anticomplement activity. The result showed that compound **1** exhibited anticomplement effects with the CH_{50} values of 0.043 mg/mL, but saponins (**2**–**10**) showed no inhibition. Interestingly, hydrolysis of steroidal saponins (**2**–**10**) resulted in its aglycones (**2a**–**10a**) correspondingly which showed anticomplement activity with the CH_{50} values of 0.049–0.156 mg/mL.



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Reineckia carnea; polyhydroxylated pregnane; steroids; anticomplement activity

1. Introduction

Reineckia carnea (Andr.) Kunth. is a monotypic plant species in the *Reineckia* genus of the Liliaceae family (Takeda et al.1961; Zhang et al. 2016). It only distributes in China and Japan (Zhang et al. 2011). In China, it mainly distributes around Yangzi valley, and is an important medical plant to Miao nationality for haemostasia, detoxification, relieving the inflammation, clearing away the lung-heat, relieving cough and inhibiting Oncomelania hupensis (Han

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et al. 2010; Xing et al. 2011; Wang, Huang et al. 2013). And modern researches have been demonstrated that the chemical components contained in the plant were saponins with spirostanol, cholestane, stigmastane, pregnane and ergostane type glycosides (Yang et al. 2010; Song, Zhang et al. 2015; Kanmoto et al. 1994; Zhang et al. 2007). As part of our research project to explore more diversity bioactive leading compounds from the Miao medicine, the chemical constituents and pharmacological studies of *R. carnea* were studied, and one new polyhydroxylated pregnane Iβ,2β,3β,4β,5β,6β- hexolhydroxy-pregn-16-en-20-one (**1**) and nine known steroidal glycosides ophiopogonin T (**2**), (25S)-5β-spirostan-1β,2β,3β,4β,5β-pentol-5-*O*-β-D-glucopyranoside (**3**),kitigenin 5-*O*-β-D-glucopyrano side (**4**), (20S, 22R)-spirost-25(27)-en-1β, 2β, 3β,4β,5β-pentaol-5-*O*-β-D-glucopyranoside (**5**), (20S, 22R)-spirost-25(27)-en-1β,3β, 4β,5β-tetraol-5-*O*-β-D-glucopyranoside (**6**), (17,20-S-*trans*)-5β-pregn-16-en-1β,3β- diol-20-one 1-*O*-β-D-xylopyranosyl-(2→1)-[α-L-rhamnopyranosyl]-3-*O*-α-L-rhamno pyranoside (**7**), (1β,3β,16β,22S)-cholest-5-ene-1,3,16,22-tetrol 1,16-di(β-D-glucopyra noside) (**8**), (1β,3β,16β,22S)-cholest-5-ene-1,3,16,22-tetrol 1-[*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside]16-(β-D-glucopyranoside) (**9**), dioscin (**10**) were acquired. This paper describes the isolation and structural elucidations of the isolated compounds and their anticomplement activities.

2. Results and discussion

Compound **1** was obtained as white amorphous powder, the HR-ESI-MS gave a pseudomolecular ion at *m/z* 395.2067 $[M - H]^-$ that together with NMR spectroscopic data were consistent with the molecular formula of $C_{21}H_{32}O_7$. The ^{1}H NMR spectrum of **1** (Figure S4) showed two tertiary methyl signals at δ_H 1.45 (3H, s, H-19), 0.94 (3H, s, H-18), one methyl δ_H 2.24 (3H, s) linking with carbonyl, and the presence of an olefinic proton at 6.92 (1H, dd, J = 1.8, 3.1 Hz, H-16). According to carbon signals (Figure S5), **1** was the derivatives of C_{21}-steroids. Moreover, ^{13}C NMR displayed one carbonyl carbon at δ_C 199.6 (C-20) and two olefinic carbons at δ_C 147.2 (C-16) and 156.5 (C-17), which indicated the presence of an α,β-unsaturated carbonyl group (Pan et al. 2003) (Table S1). The HMBC (Figure S8) correlations of Me-18 to C-12, C-13, C-14 and C-17, H-16 to C-13, C-15, C-17 and C-20 were observed. The above data inferred that C and D rings of **1** were almost consistent with those of 3β,5α,6β-trihydroxypregn-16-en-20-one (Wang et al. 2004).

However, ^{13}C- DEPT^{135} (Figure S6) displayed five hydroxy-methine at δ_C 67.4, 70.1, 70.7, 75.9 and 80.1, and one oxygen-quaternary carbon 79.3, and corresponding protons at δ_H 3.78, 3.92, 4.21, 4.11, 3.77, which indicated A ring of **1** was polyhydroxylated. In HMBC spectrum (Figure S8), the correlations of δ_H 1.45 (Me-19) to C-1, C-10 and C-9, δ_H 3.77 (H-1) to C-2, C-3 and C-9, δ_H 4.11 (H-3) to C-1, C-4 and C-5, δ_H 4.21 (H-6) to C-4, C-7 and C-8 were observed. Moreover, the ^{1}H-^{1}H COSY correlations (Figure S9) between H-1 and H-2 , H-2 and H-3 , H-3 and H-4 , H-6 and H_{2-7} (1H, m, H-7α) /1.45 (1H, m, H-7β) were also observed. According to these data, C-1 to C-6 was hydroxylated. The relative configuration of **1** based on the NOESY correlations (Figure S10) of H-9α/H-1/H-2/H-3/H-4/H-6, Me-19β/H-8β and chiral carbon NMR data. Thus, **1** was determined as Iβ,2β,3β,4β,5β,6β–hexolhydroxy -pregn-16-en-20-one.

Additionally, the known steroidal saponins were identified by comparing their spectra (NMR and MS) and physicochemical data with those reported in the literature as ophiopogonin T (**2**) (Lee et al. 2016), (25S)-5β-spirostan-1β,2β,3β,4β,5β-pentol-5-*O*-β-D-glucopyranoside (**3**) (Han et al. 2013), kitigenin5-*O*-β-D-glucopyranoside (**4**) (Wang, Hou

et al. 2013), (20S, 22R)-spirost-25(27)-en-1β,2β,3β,4β,5β-pentaol-5-*O*-β -D-glucopyranoside (**5**) (Song, Li et al. 2015), (20S, 22R)-spirost-25(27)-en-1β, 3β,4β,5 β-tetraol-5-*O*-β-D-glucopyranoside (**6**) (Song, Li et al. 2015), (17,20-S-*trans*)-5β-pregn- 16-en-1β,3β-diol-20-one1-*O*-β-D-xylopyranosyl-(2→1)-[α-L-rhamnopyranosyl]-3-*O*-α-L-rhamnopyranoside (**7**) (Zhang et al. 2016), (1β,3β,16β,22S)-cholest-5-ene-1,3, 16,22-tetrol 1,16-di(β-D-glucopyranoside) (**8**) (Zhang et al. 2007), (1β,3β,16β,22S) -cholest-5-ene-1,3,16,22-tetrol 1-[*O*-α-L-rhamnopyranosyl-(1→2)-β-D- glucopyrano side]16-(β-D-glucopyranoside) (**9**) (Zhang et al. 2007), dioscin (**10**) (Shu et al. 2017) (Figure 1).

Compounds **1–10** were evaluated for anticomplement activity *in vitro*. The results were summarised in Table S2. The compound **1** exhibited anticomplement effects with the CH_{50} values of 0.043 mg/mL. However, compounds **2–10** which are saponins showed no activity, which was consistent with previous reports (Wen et al. 2017). Interestingly, hydrolysis of steroidal saponins (**2–10**) resulted in its aglycones (**2a–10a)** (Figure S1) correspondingly, identified by HR-ESI-MS data (Table S2), which exhibited anticomplement effects with CH_{50} values ranging from 0.049 to 0.156 mg/mL. Furthermore, the anticomplement activity was positively correlated with the numbers of hydroxyl in steroids on the basis of comparing compound **3** with **4**, **5** with **6**.

According to the relevant literatures (Wang, Huang et al. 2013; Han et al. 2010) , oral saponins of *R. carnea* have good effect on anti-rheumatoid arthritis, anti-inflammatory and pneumonia, while excessive complement activation may result in various inflammatory diseases, including systemic lupus erythematosus (Morgan et al. 2005), rheumatoid arthritis (Barilla-LaBarca et al. 2013), acute lung injury (Ballanti et al. 2011) and pneumonia. Therefore,

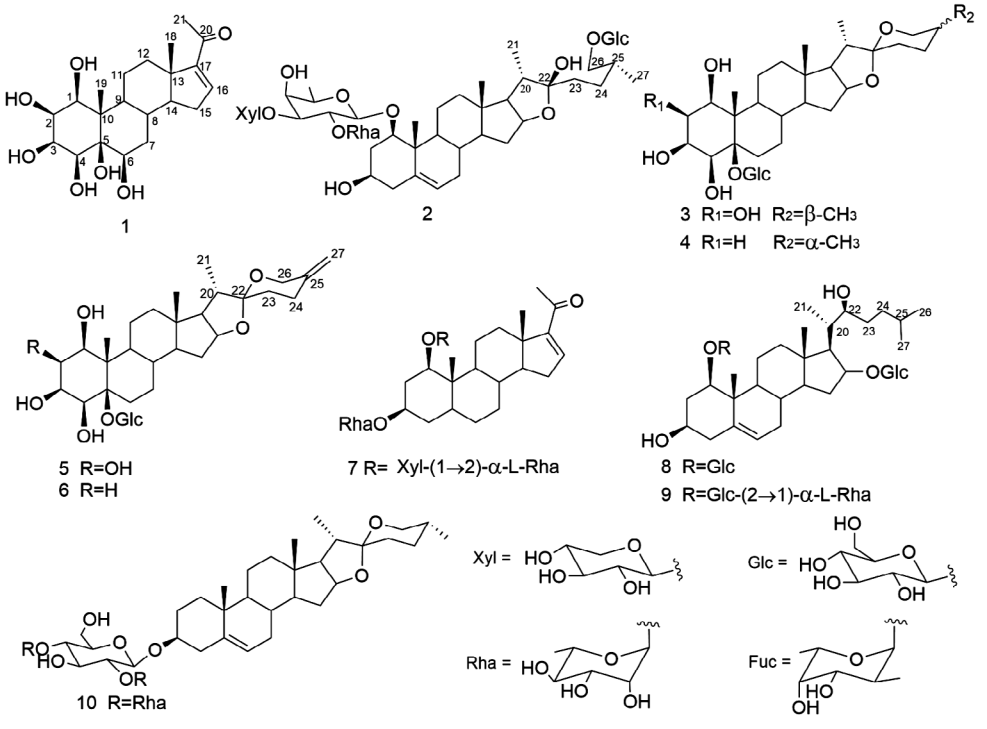

Figure 1. Structures of compounds **1–10**.

we deduced that saponins of *R. carnea* exerting pharmacological effect which may via anticomplement. However, steroid saponins as the most abundant constituent in *R. carnea* exhibited no activity *in vitro*. Moreover, most herbal medicines are orally administered, and their components are brought into contact with intestinal microflora in the alimentary tract, followed by intestinal bacteria transformation before exerting biological activities (Kim et al. 1998; Xing et al. 2014). Therefore, we deduced that saponins of *R. carnea* may exert pharmacological effect via anticomplement, and experiment of steroid saponins incubated with intestinal bacteria will requires further investigation and confirmation.

3. Experimental

3.1. General experimental procedures

The IR spectra were recorded on a Bruker TENSOR-27 instrument. The HR-ESI-MS was measured on a Triple TOF™ 5600^+ system with a Duo Spray source (AB SCIEX, Foster City, CA, USA). 1D and 2D NMR spectra were recorded on a Bruker-AVANCE600 instrument with TMS as an internal standard. Optical rotations were recorded on a PerkinElmer Model 343 polarimeter. The analytical HPLC was performed on a Shimadzu HPLC system (Kyoto, Japan) consisting of a LC-20AD solvent delivery system, a SIL-20AC autosampler, a CTO-30A column oven, a DGU-20A3 degasser and a CBM-20A controller, separation was performed on a Cosmosil C_{18} column (4.6 × 250 mm, 5.0 μm). Semipreparative HPLC was performed on a system comprising a Waters 550 pump equipped with a Waters 2487 detector and a Cosmosil C_{18} column (10 × 250 mm, 5.0 μm). Sephadex LH-20 gel was purchased from GE Health care Bio-Sciences AB (Uppsala, Sweden). Macroporous adsorption resin HP20 as purchased from Mitsubishi Chemical Corporation (Tokyo, Japan). Silica gel was purchased from Qingdao Haiyang Chemical Group Corporation (Qingdao, China).

3.2. Plant material

Reineckia carnea (Andr.) Kunth. is a monotypic plant species in the *Reineckia* genus of the Liliaceae family, which was identified by Vice Director of Pharmacists Bei Wu, Nanchang Institute for Food and Drug Control. The material was collected from An'shun city in Guizhou province, in March 2014. A voucher specimen (No. Z-140310-01) has been deposited at Jia ngxi University of Traditional Chinese Medicine, Nan chang, China.

3.3. Animals and reagents for the anticomplement assay

Guinea pigs were purchased from Beijing Vital River Laboratory Animal Technology Corporation Ltd. (Beijing, China). Sheep red blood cell (SRBC), hemolysin, and barbital buffer solution (BBS) were purchased from Shanghai yuanye Biotechnology Corporation Ltd. (Shanghai, China). Heparin sodium salt (≥140 IU/mg, dry basis) is a polyanionic glycosaminoglycan from porcine intestinal mucosa and was purchased from Solarbio (Beijing, China).

3.4. Extraction and isolation

The air-dried whole plant of *R. carnea* (20 kg) was powdered and extracted with 80% EtOH under reflux for three times at 80 °C. After removing the solvent, the concentrated residue was successively partitioned with petroleum ether, EtOAc and *n*-BuOH . The *n*-BuOH-soluble extract (812 g) was subjected to HP20 macroporous resin column eluted successively with 20–95% EtOH to give six fractions (Fr.1–Fr.6). Fr. 3 (78 g) was subjected to column chromatography on ODS gel and further separated by repeated preparative HPLC to give compound **1** (10.2 mg; $t_R = 37$ min). and compound **5** (5.8 mg; $t_R = 32$ min), compound **6** (34.4 mg; $t_R = 45$ min), compound **7** (11.0 mg; $t_R = 28$ min), compound **10** (5.8 mg; $t_R = 25$ min). Fr. 4 (62 g) was also subjected to column chromatography on ODS gel and was purified by preparative HPLC to obtain compound **2** (10.2 mg; $t_R = 18$ min), compound **3** (4.8 mg; $t_R = 46$ min), compound **4** (4.5 mg; $t_R = 50$ min), compound **8** (230.2 mg; $t_R = 40$ min) and compound **9** (28.0 mg; $t_R = 26$ min).

3.5. Iβ,2β,3β,4β5β,6β-hexolhydroxy-pregn -16-en-20-one

A white amorphous powder; $[\alpha]_D^{20}$– 26.5° ($c = 0.15$, MeOH); IR (KBr) v_{max}: 3501, 3413, 2918, 1688, 1459, 1112 cm^{-1}(Figure S3), ^1H NMR (600 MHz, in CD_3OD) and ^{13}C NMR (150 MHz, in CD_3OD) spectral data, see Table S1; *m/z* 395.2067 $[M - H]^-$(calcd for $C_{21}H_{31}O_7$ 395.2148).

3.6. Acid hydrolysis of compounds (2–10)

Compound **2–10** (4.0 mg, respectively) were hydrolysed with 1.5 mol/L HCl (CH_3OH:H_2O = 9:1) and heated to 70 °C in a water bath for 2 h, respectively. The hydrolysed saponnins were tested by TLC and HR-ESI-MS data (Table S2), and the corresponding aglycones (Figure S2) were obtained from ethyl acetate extracting and evaluated with anticomplement activity.

3.7. Anticomplement assay through the classical pathway

On the basis of methods described previously by Song et al. (2014), sensitised erythrocytes (EA) were prepared by incubation of sheep erythrocytes (4.0×10^8 cells/mL) with rabbit anti-sheep erythrocyte antibodies in GVB^{2+}, each compound and heparin (the positive control) were dissolved in GVB^{2+}. Guinea pig serum (1:32), according to estimation of serum potency, was chosen to give submaximal lysis in the absence of complement inhibitors. In brief, various dilutions of test samples (200 µL) were preincubated with 200 µL of guinea pig serum at 37 °C for 10 min. then, EA (200 µL) was added, and the mixture was incubated at 37 °C for 30 min. Controls [blank (200 µL of EA in 400 µL of GVB^{2+}), 100% lysis (200 µL of EA in 400 µL of H_2O), and sample control (200 µL dilution of each sample in 400 µL of GVB^{2+})] were incubated under the same conditions. The mixture was centrifuged (4 °C, 4000 rpm), and the optical density of the supernatant (200 µL) was measured at 405 nm with a spectrophotometer (Thermo, Multiscan, MK3). Anticomplement activity was determined as the mean of triplicate measurements at each concentration and expressed as 50% inhibitory concentration (CH_{50} value).

4. Conclusion

In conclusion, A new polyhydroxylated pregnane, named Iβ,2β,3β,4β,5β,6β-hexolhydr oxy-pregn-16-en-20-one, along with nine known steroidal saponins were isolated from the whole plant of *R. carnea*. Compound **1** exhibited anticomplement effects with the CH_{50} values of 0.043 mg/mL, and saponins(**2–10**) showed no inhibition. However, hydrolysis of steroidal saponins (**2–10**) resulted in its aglycones (**2a–10a**) correspondingly which exhibited anti-complement activity with the CH_{50} values of 0.049–0.156 mg/mL.

Supplementary material

This consists of the abstract, TableS1–S2 and Figures S1–S10.

Disclosure statement

No potential conflict of interest was reported by the authors.

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