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Grayanane and leucothane diterpenoids from the leaves of *Rhododendron micranthum*

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ABSTRACT

Eleven grayanane diterpenoids, 1-*epi*-grayanotoxin IV, 1-*epi*-grayanotoxin II, 6-deoxy-1-*epi*-grayanotoxin XVII, 6-deoxygrayanotoxin XVII, 16-acetylgrayanotoxin II, 3-oxograyanotoxin IX, 14-deoxygrayanotoxin VIII, 14-acetylisograyanotoxin II, rhodomicranols C–E, and a leucothane diterpenoid, rhodomicranol F, together with eleven known diterpenoids were isolated from leaves of *Rhododendron micranthum*. Their structures were elucidated by spectroscopic analyses, with the absolute configurations of 1-*epi*-grayanotoxin IV and rhodomicranol C determined by single-crystal X-ray diffraction with Cu Kα radiation, and the structures of 14-acetylisograyanotoxin II and known grayanotoxins IX and X confirmed by single-crystal X-ray diffraction. All twenty-three diterpenoids were evaluated for their *in vitro* immunomodulatory activities, and none showed significant immunomodulatory activities in a dose-dependent manner. In addition, they are non-toxic to the murine lymphocytes in the general cytotoxicity assay.

1. Introduction

Plants of the family Ericaceae, comprising of about 125 genera and 4000 species, are widely distributed in temperate regions and mountainous regions of the tropics (Fang et al., 2005). Several genera and many species are ornamentals. *Rhododendron* L. with about 1000 species is the largest genus of the Ericaceae, and is widely distributed in Asia, Europe, and North America (Fang et al., 2005). Many *Rhododendron* species have been used as traditional medicines in Asia, North America, and Europe to treat asthma, common cold, gastro-intestinal disorders, inflammation, pain, and skin disease (Popescu and Kopp, 2013). Phytochemical studies on the genus of *Rhododendron* established the presence of flavonoids, phenolic compounds, diterpenoids, triterpenoids, iridoid glycosides, and magestigmane sesquiterpenoids, and some isolates showed acetylcholinestherase inhibitory, anti-HIV, antiinflammatory, antimicrobial, antinociceptive, antiprotozoal, antiviral, immunomodulatory, and tyrosinase inhibitory activities (Li et al., 2013; Popescu and Kopp, 2013; Qiang et al. 2011).

Rhododendron micranthum Turcz, a semi-evergreen shrub, is wide-spread in regions of North China, Northeast China, and Henan, Shandong, Hunan, Hubei, and Sichuan provinces of China, as well as in North Korea (Fang et al., 2005). In Mainland China, the branches and leaves of *R. micranthum* have been used as a traditional Chinese medicine for treatment of chronic tracheitis, dysmenorrhea, fracture, hypertension, menoxenia, puerperal arthrodynia, and rheumatoid arthritis (Editorial Committee of Chinese Materia Medica, 1999), and a Chinese patent medicine, known as "Zhaoshanbai Jingao Pian", has been developed. However, the reported chemical constituents of *R. micranthum* are limited to flavonoids (Yang et al., 2010; Xia et al. 1999) and triterpenoids (Chang and Luo, 2011). In a previous phytochemical investigation on *R. micranthum*, three novel compounds were isolated: a novel diterpene with an unprecedented micranthane carbon skeleton, micranthanone A, two new grayanane diterpenoids possessing an unprecedented 5,6-(3,4-dihydroxybenzylidene acetal) motif, rhodomicranols A and B, and three known grayanane diterpenoids (grayanotoxins II, IV, and IVIII) (Zhang et al., 2013). These results inspired the search for more novel diterpenoids from *R. micranthum*. As a continuation of that work, eleven new grayanane diterpenoids were isolated: 1-*epi*-grayanotoxin IV (1), 1-*epi*-grayanotoxin II (2), 6-deoxy-1-*epi*-grayanotoxin XVII (3),

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6-deoxygrayanotoxin XVII (**4**), 16-acetylgrayanotoxin II (**5**), 3-oxograyanotoxin IX (**6**), 14-deoxygrayanotoxin VIII (**7**), 14-acetylisograyanotoxin II (**8**), rhodomicranols C (**9**), D (**10**), and E (**11**), a new leucothane diterpenoid, rhodomicranol F (**12**), and eleven known diterpenoids **13**–**23** (Fig. 1). In this paper, the isolation, structure determination, and *in vitro* immunomodulatory activities of diterpenoids **1**–**23** are described.

2. Results and discussion

The air-dried leaves of *R. micranthum* were powdered and extracted with 95% EtOH at room temperature. The extract was suspended in H_2O and partitioned with petroleum ether (PE), $CHCl_3$, EtOAc, and *n*-BuOH. The $CHCl_3$ partition fraction was separated by repeated column chromatographic procedures using silica gel, reversed phase (RP) C_{18} silica gel, and Sephadex LH-20, as well as by RP C_{18} HPLC, to yield twelve new diterpenoids 1–**12** and eleven known diterpenoids **13**–**23**. The latter were identified as leucothol A (**13**) (Furusaki et al., 1972), grayanotoxin XXI (**14**) (Zhang et al., 2005b), kalmanol (**15**) (Burke et al., 1989), grayanotoxins I (**16**) (Burke and Doskotch, 1990), III (**17**) (El-Naggar et al., 1980), IX (**18**) (Hikino et al., 1971), VII (**19**) (Hikino et al., 1970), XIX (**20**) (Sakakibara, et al., 1980a), X (**21**) (Hikino et al., 1971), and VIII (**22**) (Hikino et al., 1971), and isograyanotoxin II (**23**) (Terai et al., 2000), respectively, based on spectroscopic analyses and comparison with the data in the literature. Eleven known diterpenoids **13**–**23** were isolated from *R. micranthum* for the first time.

1-*Epi*-grayanotoxin IV (**1**) was obtained as colorless needles with mp 239–240 °C. Its molecular formula was established as $C_{22}H_{34}O_6$ by the HRESIMS ion at *m/z* 417.2235 $[M+Na]^+$ (calcd for $C_{22}H_{34}O_6Na$, 417.2253), indicating six indices of hydrogen deficiency. Its IR spectrum showed characteristic absorptions for hydroxy (3435 cm^{-1}) and ester carbonyl (1716 cm^{-1}) functionalities. Its ^1H NMR spectrum (Table 1) displayed resonances for three methyls (δ_H 1.36, s, H_3-17; 1.19, s, H_3-19; 0.94, s, H_3-18), an acetyl (δ_H 2.09, s), two exocyclic olefinic protons (δ_H 5.262, s, H-20a; 5.258, s, H-20b), an acylated oxymethine (δ_H 5.24, s, H-14), two oxymethines (δ_H 3.71, d, J = 4.6 Hz, H-3; 3.48, d, J = 10.0 Hz, H-6), and a methine (δ_H 2.78, t, J = 9.3 Hz, H-1). The ^{13}C NMR and DEPT spectra (Table 2) displayed 22 carbon resonances assignable to an ester carbonyl (δ_C 173.0, $-OCOCH_3$), an exocyclic double bond (δ_C 153.8, C-10; 112.5, C-20), four quaternary carbons including two oxygenated (δ_C 87.2, C-5; 80.4, C-16; 51.5, C-4; 50.5, C-8), six methines including three oxygenated (δ_C 84.9, C-3; 82.5, C-14; 71.5, C-6; 59.3, C-1; 58.5, C-9; 54.6, C-13), five methylenes (δ_C 56.7, C-15; 41.0, C-7; 36.9, C-2; 28.3, C-12; 26.7, C-11), and four methyls (δ_C 24.8, C-17; 23.0, C-18; 21.5, $-OCOCH_3$; 20.5, C-19). HSQC, ^1H–^1H COSY, and HMBC analyses established structure **1** (Fig. 2) as 3,5,6,16-tetrahydroxy-14-acetoxy-grayan-10(20)-ene,

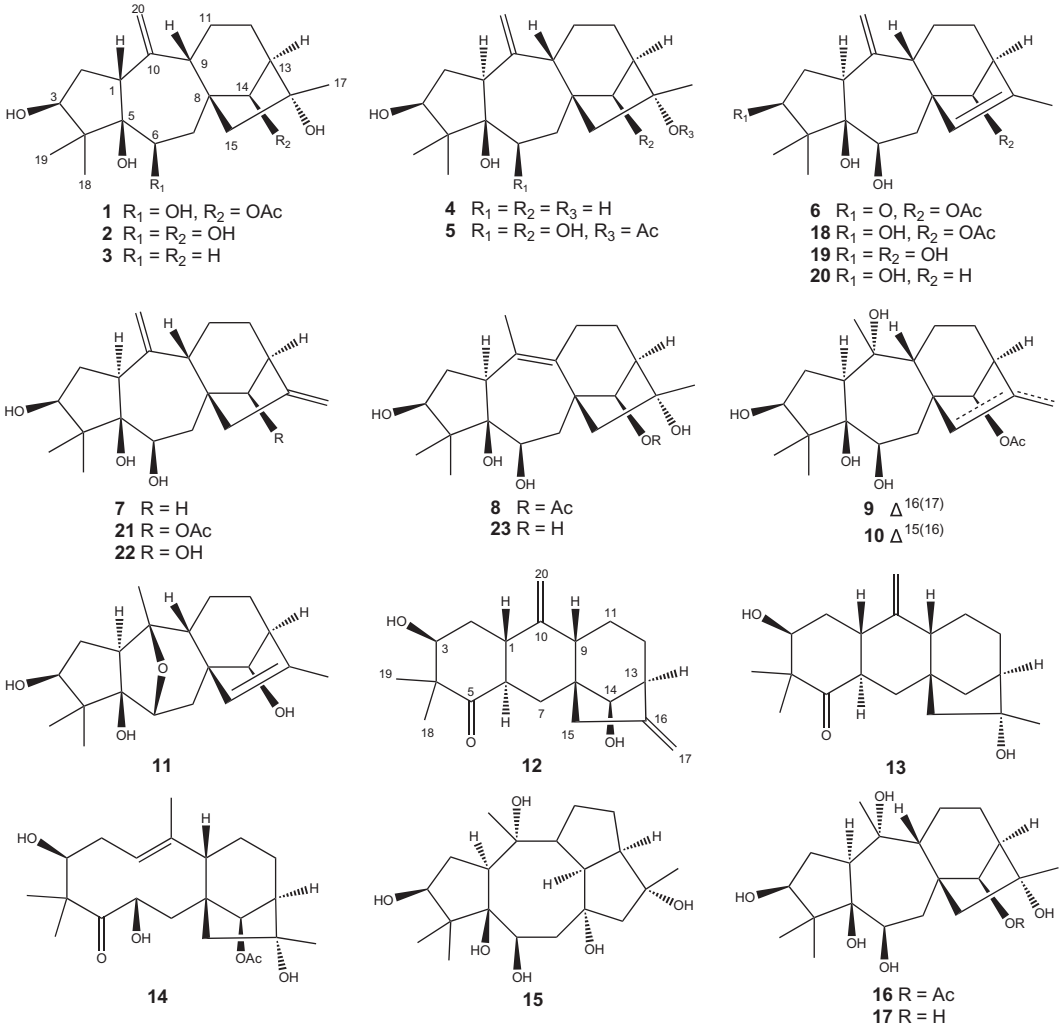

Fig. 1. Chemical structures of compounds **1**–**23**.