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Xanthine oxidase inhibitors isolated from *Piper nudibaccatum*

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ABSTRACT

Two new hydroxychavicol analogs nudibaccatumin A (**1**) and B (**2**), together with twenty known compounds were isolated from the methanol extract of *Piper nudibaccatum*. Their structures were elucidated by extensive spectroscopic analyses (1D and 2D NMR, HRESIMS, UV, IR and polarimetry). Hydroxychavicol is a known inhibitor of xanthine oxidase (XO). In the present study, hydroxychavicol and 5 natural analogs (**1**–**5**) were evaluated for their XO inhibitory activity. Neotaiwanensol B (**3**) (IC_{50} = 0.28 μM) showed a greater inhibitory effect than hydroxychavicol and allopurinol (the positive control). Two new compounds **1** and **2** showed a moderate inhibition activity with an IC_{50} value of 62.94 μM and 70.67 μM, respectively.

1. Introduction

Xanthine oxidase (XO) acts at the end of a catabolic sequence of purine nucleotide metabolism in humans and a few other uricotelic species (Rundles and Wyngaarden, 1969). This enzyme catalyzes the oxidation of hypoxanthine to xanthine and then to uric acid (Krenitsky et al., 1986). Overproduction of uric acid, termed hyperuricemia, is the underlying cause of gout (Harris et al., 1999).

XO inhibitors can be broadly classified as purine or non-purine analogs based on their structural similarities with natural purines (Kumar et al., 2011). The best known XO inhibitor is allopurinol, i.e., 1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one. Allopurinol is a substrate for XO (Massey et al., 1970), and its oxidation product, oxypurinol, is also an effective inhibitor. However, allopurinol may induce hypersensitivity in patients with renal insufficiency and concomitant administration of thiazide diuretics (Star and Hochberg, 1993). These side effects have led researchers to search for novel XO inhibitors. Febuxostat (TEI-6720; TMX-67), which is a synthetic non-purine selective inhibitor of xanthine oxidase, is being developed by Teijin for treatment of hyperuricemia in gout (Yu, 2007). Phenolics such as flavonoids are considered the primary chemical classes with XO inhibitory properties (Nagao et al., 1999; Van Hoorn et al., 2002; Lespade and Bercion, 2010). Hydroxychavicol (**6**, Fig. 1), a phenolic compound from *Piper* plants, shows potent inhibitory activity against XO (Murata et al., 2009).

In our previous studies, hydroxychavicol was found to be a major constituent of *Piper nudibaccatum* Tseng (Liu et al., 2013), and six alkenylphenols, which are structural homologues of hydroxychavicol, were isolated from *P. sarmentosum* Roxb (Yang et al., 2013). In this study, we report the isolation and structural elucidation of two hydroxychavicol derivatives (**1** and **2**) from the aerial parts of *P. nudibaccatum*. The naturally occurring compounds (**1**–**6**) were evaluated for their XO inhibitory activity.

2. Results and discussion

Two new compounds (**1** and **2**) and 20 known compounds were isolated from the MeOH extract of the aerial parts of *P. nudibaccatum* (synonym name *P. psilocarpum* C. DC). The new

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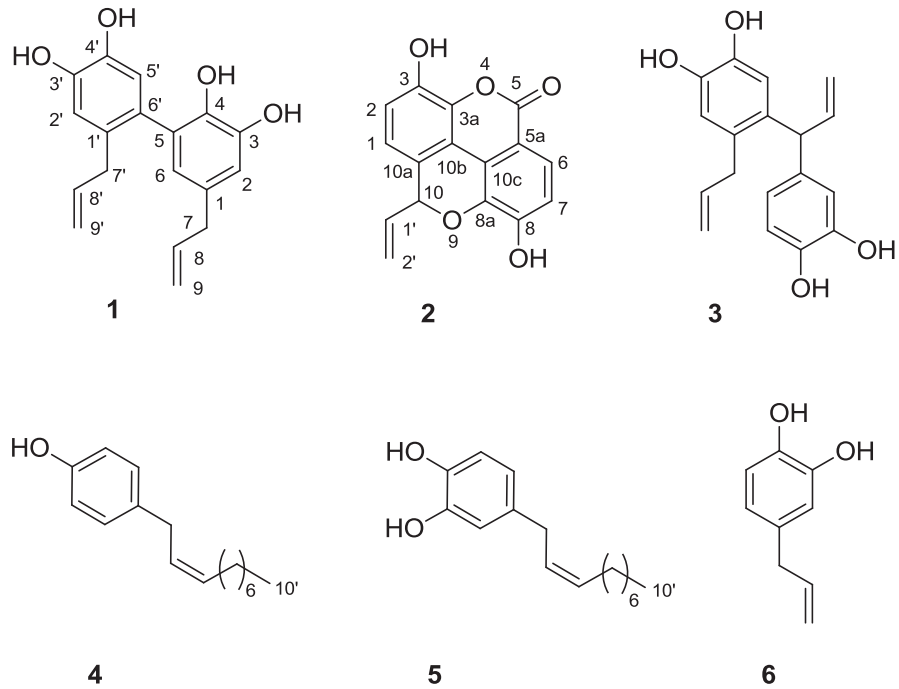


Fig. 1. Chemical structures of the compounds (**1**–**6**) under study.

compounds included a neolignan (**1**) and an ellagic acid analog (**2**). The known compounds included the neolignan, neotaiwanensol B (**3**) (Chen et al., 2013); two alkenylphenols, sarmentosumols A (**4**) and B (**5**) (Yang et al., 2013); 10 amide alkaloids, (2*E*,4*E*)-*N*-isobutylhexadeca-2,4-dienamide (**7**) (Reisch et al., 1985), (2*E*,4*E*)-*N*-isobutyl-2,4-decadienamide (**8**) (Yasuda et al., 1981), 1-[7-(1,3-benzodioxol-5-yl)-1-oxo-2,4-heptadienyl]piperidine (**9**) (Araujo-Junior et al., 1997), retrofractamide A (**10**) (Park et al., 2002), dehydropipernonaline (**11**) (Shoji et al., 1986), (2*E*,4*E*)-*N*-isobutyl-7-(3,4-methylenedioxyphenyl)hepta-2,4-dienamide (**12**) (Huang et al., 2010), (2*E*,8*E*)-*N*-[9-(3,4-methylenedioxyphenyl)-2,8-nonadienoyl] piperidine (**13**) (Tabuneng et al., 1983), retrofractamide C (**14**) (Banerji et al., 1985), piperolein B (**15**) (Tabuneng et al., 1983), and 5,6-dihydropiperlonguminine (**16**) (Navickiene et al., 2000); a monoterpene, pressafonin A (**17**) (Cheng et al., 2010); a diterpene, *trans*-phytol (**18**) (Sims and Pettus, 1976); a steroid, ergosta-4,6,8(14),22-tetraen-3-one (**19**) (He and Feng, 2000); a quinone, tocopherylquinone (**20**) (Zhao et al., 2010); a phenylpropanoid, 4-allyl-1,2-phenylene diacetate (**21**) (Villegas et al., 2011); and a phenol derivative, monomethyl olivetol (Mcclanahan and Robertson, 1985) (**22**). The structures of the known compounds were determined by comparing their spectroscopic data with previously reported data.

The molecular formula of $C_{18}H_{18}O_4$ was assigned to compound **1** as determined by an HREIMS ion at *m*/*z* 298.1208 $[M]^+$ (calc. 298.1205) and ^{13}C NMR data (Table 1). The IR spectrum showed absorption bands for hydroxy (3441 cm^{-1}) and phenyl (1631, 1512, and 1484 cm^{-1}) groups. The ^1H NMR data of **1** (Table 1) showed resonances for two tetrasubstituted phenyl rings [δ_H 6.61 (1H, d, *J* = 2.0 Hz, H-2) and 6.34 (1H, d, *J* = 2.0 Hz, H-6); δ_H 6.68 (1H, s, H-2′) and 6.60 (1H, s, H-5′)] and two allyl groups [δ_H 5.94 (1H, m, H-8), 5.03 (1H, m, H-9a), 4.99 (1H, m, H-9b), and 3.23 (2H, d, *J* = 6.6 Hz, H_2-7); δ_H 5.80 (1H, m, H-8′), 4.86 (2H, m, H_2-9′), and 3.08 (2H, br s, H_2-7′)]. The spectral characteristics of **1** were similar to those of the known neolignan neotaiwanensol B (**3**), indicating that compound **1** also contained two hydroxychavicol moieties (Chen et al., 2013). In the HMBC spectrum of **1** (Fig. 2), the correlations from H-6 to C-6′ and from H-5′ to C-5 indicated that the two substructures were linked through C-5–C-6′. Thus, the structure of **1** was elucidated as 5,6′-diallyl-[1,1′-biphenyl]-2,1,1′,4′-tetraol, and **1** was given the trivial name nudibaccatumin A.

Nudibaccatumin B (**2**) was obtained as a yellowish oil and was determined to have a molecular formula of $C_{16}H_{10}O_5$ with 12 double bond equivalents based on the $[M]^+$ ion at *m*/*z* 282.0510 (calc. 282.0528) in its HREIMS spectrum. The IR spectrum of **2** indicated the presence of hydroxy (3432 cm^{-1}), conjugated lactone (1771 cm^{-1}), and phenyl moieties (1610, 1581, and 1438 cm^{-1}). The ^1H NMR data of **2** (Table 2) showed resonances for two 1,2,3,4-tetrasubstituted phenyl rings [δ_H 7.72 (1H, d, *J* = 8.6 Hz, H-6) and 7.04 (1H, d, *J* = 8.6 Hz, H-7); δ_H 6.99 (1H, d, *J* = 8.2 Hz, H-2) and 6.94 (1H, br d, *J* = 8.2 Hz, H-1)] and one terminal olefinic methylene resonance [δ_H 6.06 (1H, ddd, *J* = 16.8, 10.5, 6.1 Hz, H-1′), 5.25 (1H, ddd, *J* = 10.5, 1.3, 1.3 Hz, H-2′a), and 5.23 (1H, ddd, *J* = 16.8, 1.3, 1.3 Hz, H-2′b)]. Sixteen carbon resonances were observed in the ^{13}C NMR and DEPT spectra (Table 2), including a conjugated lactone carbonyl (δ_C 162.2). The NMR data of compound **2** were

Table 1
^1H and ^{13}C NMR data of 1 in CD_3OD (δ in ppm, *J* in Hz).

No.	δ_C (125 MHz)	δ_H (500 MHz)
1	132.0, C	
2	115.1, CH	6.61, d (2.0)
3	146.1, C	
4	142.3,[a] C	
5	130.0, C	
6	123.3, CH	6.34, d (2.0)
7	40.7, CH_2	3.23, d (6.6)
8	139.5, CH	5.94, m
9a	115.3, CH_2	5.03, m
9b		4.99, m
1′	131.4, C	
2′	117.1, CH	6.68, s
3′	145.6, C	
4′	144.1, C	
5′	118.4, CH	6.60, s
6′	130.7, C	
7′	38.2, CH_2	3.08, br s
8′	140.0, CH	5.80, m
9′	115.0, CH_2	4.86, m

[a] Detected by HMBC.

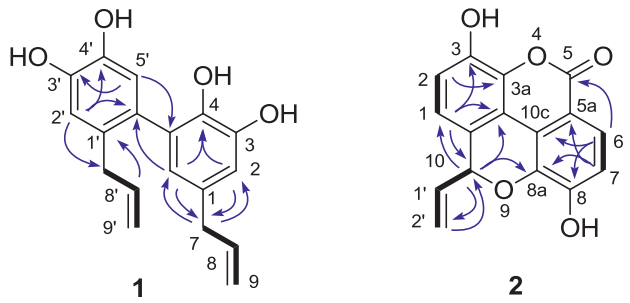


Fig. 2. Key ^{1}H–^{1}H COSY (bold) and HMBC (arrows, H → C) correlations of **1** and **2**.

Table 2
^{1}H and ^{13}C NMR data of 2 in CD_3OD (δ in ppm, J in Hz).

No.	δ_C (150 MHz)	δ_H (600 MHz)
1	122.1, CH	6.94, br d (8.2)
2	118.4, CH	6.99, d (8.2)
3	145.4, C	
3a	139.0, C	
5	162.2, C	
5a	111.7, C	
6	124.8, CH	7.72, d (8.6)
7	119.5, CH	7.04, d (8.6)
8	152.4, C	
8a	138.0, C	
10	79.2, CH	5.96, br d (6.1)
10a	122.8, C	
10b	113.2, C	
10c	122.1, C	
1′	137.8, CH	6.07, ddd (16.8, 10.5, 6.1)
2′a	118.0, CH_2	5.25, ddd (10.5, 1.3, 1.3)
2′b		5.23, ddd (16.8, 1.3, 1.3)

similar to those of the known ellagic acid-related derivative nigricanin (Tan et al., 2004), except that resonances for a vinyl group [δ_H 6.07 (1H, ddd, J = 16.8, 10.5, 6.1 Hz, H-1′), 5.25 (1H, ddd, J = 10.5, 1.3, 1.3 Hz, H-2′a), and 5.23 (1H, ddd, J = 16.8, 1.3, 1.3 Hz, H-2′b); δ_C 117.8 (CH, C-1′) and 118.0 (CH_2, C-2′)] were observed in the spectra of **2** rather than those for the methoxy group [δ_H 3.57 (3H); δ_C 56.0 (CH_3)] in nigricanin. In addition, the resonance for C-10 (δ_C 99.9) in nigricanin was replaced with one at δ_C 79.2 (C-10) in **2**, implying that the vinyl group was located at C-10; this location was supported by the HMBC correlations from H_2-2′ to C-10 and from H-10 to C-2′ in **2** (Fig. 2). The findings described above were also supported by HMBC correlations from H-10 to C-1, C-8a and C-10b, from H-1 to C-10, and from H-6 to C-5. Thus, the structure of **2** was elucidated as 1,8-dihydroxy-10-vinyl[1]benzopyrano[5,4,1-*cde*][1]benzopyran-5(10*H*)-one, and **2** was given the trivial name nudibaccatumin B.

The six isolated compounds (**1**–**6**) were evaluated for inhibitory activity against XO. The data are listed in Fig. 3. Hydroxychavicol (**6**, IC_{50} = 0.38 ± 0.78 μM) and neotaiwanensol B (**3**, IC_{50} = 0.28 ± 0.09 μM) showed the most potent inhibitory effects on XO compared to allopurinol (IC_{50} = 7.06 ± 1.53 μM). Previous results have shown that the two hydroxy groups in the structure of hydroxychavicol are important for the XO inhibitory activity. The activity of 3-*O*-methyl or 3,3-di-*O*-methyl derivatives of hydroxychavicol against XO are much lower than for the unmethylated substance (Murata et al., 2009). Therefore, the decrease in the activity of compound **2** is reasonable. According to our results, the longer side chain in **5** at the phenyl ring led to the disappearance of this activity. Dimers connected by a C-6–C-7′ bond (**3**) of hydroxychavicol increased the activity, but dimers connected at C-5–C-6′ bond (**1**) decreased the activity. To characterize the binding region of xanthine oxidase, a kinetic assay was performed and Lineweaver–Burk double reciprocal plots are shown in Fig. 4. Compounds **3** and **6** displayed competitive inhibition with a K_i value of 1.4 μM and 38.6 μM, respectively.

3. Experimental

3.1. General experimental procedures

Optical rotations were determined on a Jasco-DIP-370 automatic digital polarimeter. UV spectra were measured on a Shimadzu double-beam 210A spectrometer. IR spectra were recorded on a Bio-Rad FTS-135 infrared spectrophotometer with KBr disks. 1D and 2D NMR spectra were recorded on BRUKER AM-400, DRX-500 and Bruker Avance III 600 spectrometers with TMS as an internal standard. EIMS and HREIMS were performed on a Waters Autospec Premier P776 mass spectrometer. Semi-preparative HPLC was performed on an Agilent 1200 series pump equipped with a diode array detector and a Zorbax SB-C_{18} column (5.0 μm; 9.4 × 250 mm). Silica gel G (80–100 mesh, Qingdao Makall Group Co., Ltd.), C_{18} silica gel (40–75 μm, Fuji Silysia Chemical Ltd.), silica gel H (10–40 μm, Qingdao), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB) were used for column chromatography, and silica gel GF_{254} (Qingdao) was used for preparative TLC as precoated plates. The TLC spots were visualized under UV light and by dipping into 5% H_2SO_4 in alcohol followed by heating.

3.2. Plant material

The aerial parts of *P. nudibaccatum* were collected from Xishuangbanna of Yunnan Province, P. R. China in May 2011 and identified by one of the authors, namely Prof. C. L. Long at the Kunming Institute of Botany. A voucher specimen (No. HGW-00705) was deposited at the Key Laboratory of Economic Plants and Biotechnology, Kunming Institute of Botany.

3.3. Extraction and isolation

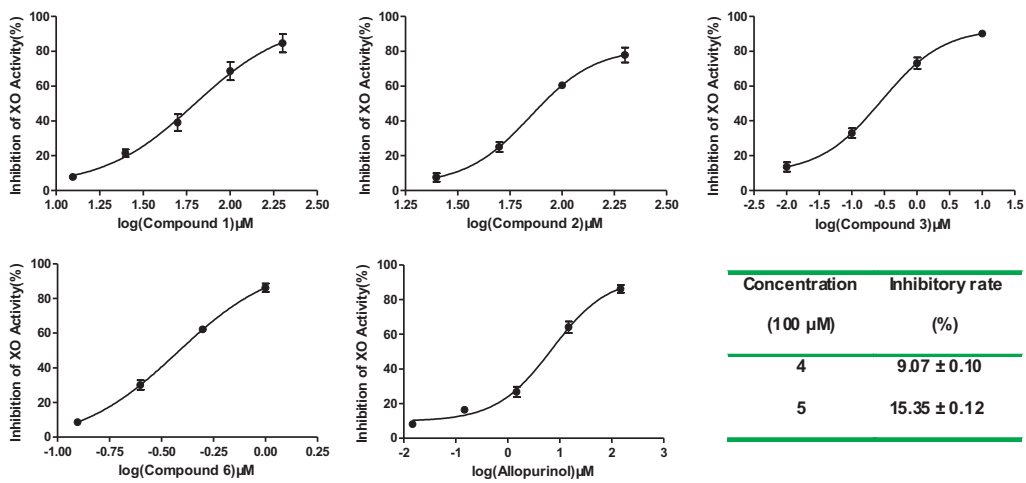
The air-dried powder of the plant material (13 kg) was exhaustively extracted with MeOH (3 × 20 L) at room temperature. The resulting MeOH extract (2.3 kg) was suspended in H_2O and further partitioned into three fractions as follows: petroleum ether (a, 358 g), $CHCl_3$ (b, 300 g), and H_2O (c). Subsequently, fractions (Fr.) a and b were combined and subjected to column chromatography (silica gel; petroleum ether/EtOAc, 100:5 → 0:100, v/v) to afford Fr. A–F. Fr. C (15 g) was purified by chromatography over RP-18 (MeOH/H_2O, 50:50 → 90:10, v/v), to give Fr. C_1–C_4. Fr. C_1 was subjected to column chromatography over silica gel (petroleum ether/EtOAc, 15:1, v/v) to give **6** (30 g). Fr. C_2 was fractionated by CC (Sephadex LH-20, MeOH; silica gel, petroleum ether/EtOAc, 20:1 → 10:1, v/v) to give **4** (11.5 mg) and **21** (11.0 mg). Fr. C_3 was fractionated by CC (Sephadex LH-20, MeOH; silica gel, petroleum ether/EtOAc, 50:1, v/v) to give **20** (9.4 mg). Fr. C_4 was fractionated by CC (Sephadex LH-20, MeOH; silica gel, petroleum ether/EtOAc, 50:1, v/v) to give **17** (32.0 mg). Fr. D (24 g) was purified by RP-18 (MeOH/H_2O, 50:50 → 90:10, v/v), to give *Fr.* D_1–D_5. Fr. D_2 was subjected to column chromatography over Sephadex LH-20 to give two subfractions (D_{21} and D_{22}). Compound **7** (20.0 mg) and **8** (3.5 mg) were obtained from subfr. D_{21} by prep. TLC (petroleum ether/EtOAc, 3:1, v/v). Fr.D_4 was fractionated by CC (Sephadex LH-20, MeOH; silica gel, petroleum ether-EtOAc, 15:1, v/v) to give **5** (10.0 mg) and **22** (19.0 mg). Fr.D_5 was fractionated by CC (Sephadex LH-20, MeOH; silica gel, petroleum ether-EtOAc, 20:1, v/v) and preparative TLC to give **18** (22.0 mg) and **19** (18.6 mg). Fr. E (18 g) was purified by RP-18 (MeOH/H_2O, 50:50 → 90:10, v/v, Sephadex LH-20, MeOH and semipreparative HPLC, MeCN/H_2O, 70:30, 2 ml/min) to give **9** (4.0 mg, t_R = 13.0 min), **10** (4.2 mg, t_R = 14.0 min), **11** (3.5 mg,

A Xanthine oxidase inhibitory activity of hydroxychavicol (1) and its analogues.

compound	Source	IC_{50} (μM) [b]
1	*P. nudibaccatum*	62.94 ± 2.91
2	*P. nudibaccatum*	70.67 ± 0.01
3	*P. nudibaccatum*	0.28 ± 0.09
4	*P. nudibaccatum*	-----
5	*P. nudibaccatum*	-----
6	*P. nudibaccatum*	0.38 ± 0.78
allopurinol	SIGMA	7.06 ± 1.53

[a] Each result is performed in triplicate.

B Concentration-dependent xanthine oxidase (XO) inhibition.



Concentration (100 μM)	Inhibitory rate (%)
4	9.07 ± 0.10
5	15.35 ± 0.12

Fig. 3. Inhibition of xanthine oxidase by hydroxychavicol (**1**) and its analogs. (A) IC_{50} values of test compounds. (B) Concentration-dependent xanthine oxidase (XO) inhibition by compounds **1**, **2**, **3**, **6** and allopurinol. Data presented are the mean values ± SE from three independent experiments.

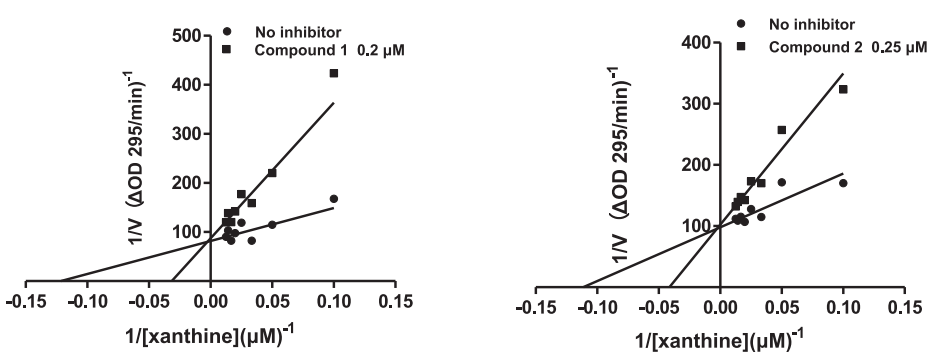


Fig. 4. Kinetic assays of xanthine oxidase inhibition by compounds **3** and **6**. Lineweaver–Burk double reciprocal plots for the inhibition of xanthine oxidase by compound **3** (right panel), and compound **6** (left panel). Plots represent 1/velocity vs. 1/xanthine (μM^{-1}) without or with inhibitors in reaction solution. Each point is the average value from three independent experiments.

t_R = 15.8 min), **12** (3.0 mg, t_R = 17.2 min), **13** (2.1 mg, t_R = 20.6 min), **14** (2.4, t_R = 25.7 min mg), **15** (2.2 mg, t_R = 32.0 min), **16** (2.0 mg, t_R = 35.8 min). Fr. F (7 g) was purified by RP-18 ($MeOH/H_2O$, 50:50 → 90:10, v/v), to give Fr. F_1 and F_2. Fr. F_1 was subjected to column chromatography over silica gel ($CHCl_3$/MeOH, 3:1, v/v) and semipreparative HPLC ($MeOH/H_2O$, 80:20, 2 ml/min) to give **3** (9.5 mg, t_R = 22.6 min). Compound **1** (7.8 mg, t_R = 18.5 min) was obtained by semipreparative HPLC (MeCN/H_2O, 80:20, 2 ml/min) from Fr. F_1. Fr. F_2 was subjected to column chromatography over silica gel ($CHCl_3$/MeOH, 5:1, v/v) and then by recrystallization to give **2** (6.0 mg).

3.4. Nudibaccatumin A (*1*)

Yellowish oil; UV (MeOH) λ_{max} (log ε) 319 (2.71), 289 (3.10), 207 (3.81) nm; IR ν_{max} (KBr) 3442, 1631, 1512, 1432, 1383, 1272, 788, 591 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; negative ESIMS *m/z* 297 $[M-H]^-$; HREIMS *m/z* 298.1208 $[M]^+$ (for $C_{18}H_{18}O_4$, calc. 298.1205).

3.5. Nudibaccatumin B (*2*)

Yellowish oil; $[\alpha]^{24}_D$ − 1.0 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 340 (3.35), 329 (3.37), 259 (3.97), 239 (3.96), 200 (3.88) nm; IR ν_{max} (KBr) 3432, 1771, 1630, 1581, 1438, 1384, 1279, 1186, 1090, 1035 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; negative ESIMS *m/z* 281 $[M-H]^-$; HRESIMS *m/z* 281.0530 $[M-H]^-$ (for $C_{16}H_9O_5$, calc. 281.0528).

3.6. Xanthine oxidase inhibition assay

The enzymatic activity was measured spectrophotometrically by continuously measuring uric acid formation at 295 nm with xanthine as the substrate (Kong et al., 2000; Niu et al., 2011). The reaction mixture contained 0.3% sodium pyrophosphate buffer (pH 8.3) and 10 U/L xanthine oxidase with or without the test compounds. Allopurinol was used as a positive control. After preincubation at 25 °C for 15 min, uric acid formation in the reaction mixture was initiated by the addition of 160 μM of xanthine dissolved in sodium pyrophosphate buffer. In the enzyme kinetics tests, the xanthine concentrations used were 10, 20, 30, 40 50, 60, 70 and 80 μM. The type of xanthine oxidase inhibition induced by the test compounds was identified from Lineweaver–Burk plots, and the K_i value was analyzed using GraphPad Prism 5.0 software.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2015.03.005.

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