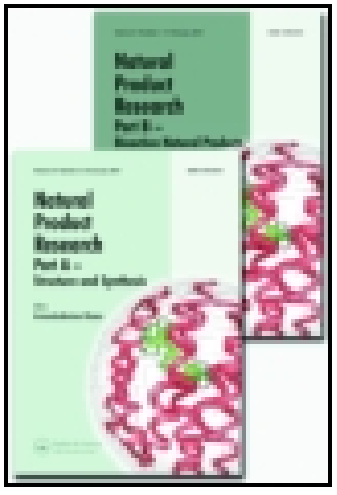

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New cytotoxic lignan glycosides from Phyllanthus glaucus

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New cytotoxic lignan glycosides from *Phyllanthus glaucus*

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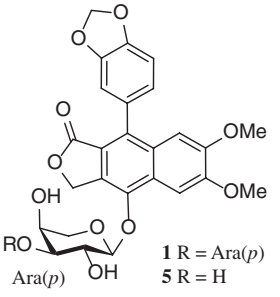

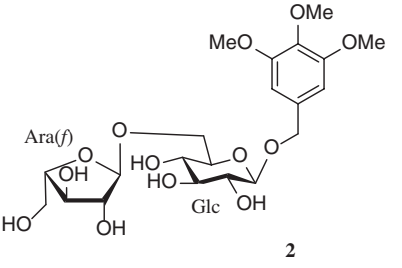

During the process of exploring bioactive lead compounds from *Phyllanthus* species, two new glycosides including an arylnaphthalene lignan, diphyllin 4-*O*-α-L-arabinopyranosyl-(1 → 3)-α-L-arabinopyranoside (**1**), and a phenolic compound, 3,4,5-trimethoxybenzyl alcohol 7-*O*-α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranoside (**2**), were isolated from the methanol extract of the whole plants of *Phyllanthus glaucus* Wall. ex Müll. Arg. In addition, 31 known compounds, including 19 lignan derivatives (**3**–**21**), four phenylpropanoids (**22**–**25**), seven simple phenolics (**26**–**32**) and one monoterpenoid (**33**) were obtained. Their structures were determined on the basis of the HR-ESI-MS, 1D and 2D NMR spectroscopic analysis, and pre-column derivative/chiral HPLC analysis in case of **1** for the absolute configurations. All these compounds were obtained from *P. glaucus* for the first time. Moreover, the known lignan glycoside, phyllanthusmin C (**5**) showed *in vitro* cytotoxicities against HL-60, MCF-7 and SW480 cells with IC_{50} values of 9.2 ± 0.2, 19.2 ± 1.7 and 20.5 ± 0.9, respectively.

Keywords: *Phyllanthus glaucus* Wall. ex Müll. Arg; glycoside; arylnaphthalene lignan; phenolics; cytotoxicity

1. Introduction

The genus *Phyllanthus* (Euphorbiaceae), comprising approximately 600 species, is distributed in the tropical and subtropical countries of the world (Zhao et al. 2013). Some *Phyllanthus* plants have been used for treating kidney and urinary bladder disturbances, intestinal infections, diabetes and hepatitis B (Calixto et al. 1998). Previous investigations resulted in the isolation of flavonoids (Zhang et al. 2000), alkaloids (Mensah et al. 1988), terpenoids (Zhang,

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Tanaka, et al. 2001), lignans (Chang et al. 2003) and tannins (Zhang, Abe, et al. 2001) from *Phyllanthus* spp.

Phyllanthus glaucus Wall. ex Müll. Arg. (Euphorbiaceae), a kind of deciduous shrub growing at elevation of 200–1000 m, is mainly found in the central parts of China. The roots were used medicinally for the treatment of rheumatoid arthritis and malnutrition in children by the local people of its growing areas. However, its chemical constituents and pharmacological principles are not clear. With the aim to search for bioactive components from *Phyllanthus* spp., the detailed phytochemical study on *P. glaucus* was carried out. This led to the isolation of 33 compounds, including two new glycosides, namely diphyllin 4-*O*-α-L-arabinopyranosyl-(1 → 3)-α-L-arabinopyranoside (**1**) and 3,4,5-trimethoxybenzyl alcohol 7-*O*-α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranoside (**2**). Herein, we report the structure elucidation of these compounds, as well as the cytotoxicities of lignan glycosides **1** and **3**–**5** against five human cancer cell lines (HL-60, SMMC-7721, A-549, MCF-7 and SW480).

2. Results and discussion

The MeOH extract of the whole plants of *P. glaucus* was subjected to repeated column chromatography (CC) over Diaion HP20SS, Sephadex LH-20, MCI-gel CHP-20P and Rp-18, Silica gel column, followed with preparative thin layer chromatography (p-TLC) and preparative high performance liquid chromatography (p-HPLC), led to the isolation of two new glycosides (**1** and **2**). In addition, 31 known compounds (Figure 1), referring to 19 lignan derivatives, reticulatuside A (**3**), arabelline (**4**), phyllanthusmin C (**5**), (−)-secoisolariciresinol (**6**), (−)-isolariciresinol (**7**), (−)-isolarisiresinol 9′-*O*-β-D-glucopyranoside (**8**), (−)-isolariciresinol 9-*O*-β-D-xylopyranoside (**9**), (−)-lyoniresinol (**10**), rourinoside (**11**), 7*S*,8*S*-*threo*-4,7,9,9′-tetrahydroxy-3,3′-dimethoxy-8-*O*-4′-neolignan (**12**), buddlenol D (**13**), ficusequilignan A (**14**), (−)-lariciresinol (**15**), 8*S*,8*S*′-5,5′-dimethoxy-lariciresiol (**16**), (−)-pinoresinol (**17**), (−)-syringaresinol (**18**), (7*S*,8*R*)-cedrusin (**19**), (7*R*,8*S*)-dihydrodehydrodiconiferyl alcohol 4-*O*-β-D-glucopyranoside (**20**), and (7*R*,8*S*)-dihydrodehydrodiconifenyl alcohol (**21**), four phenylpropanoids, (*E*)-isoconiferin (**22**), salidroside I (**23**), coniferin (**24**) and 1-*O*-(β-D-glucopyranosyl)-2-[2-methoxy-4-(ω-hydroxypropyl)-phenoxyl]-propan-3-ol (**25**), seven simple phenolics, 3,4-dimethoxy-benzyl alcohol-7-*O*-β-D-glucopyranoside (**26**), 3,4,5-trimethoxybenzyl alcohol-7-*O*-β-D-glucopyranoside (**27**), 3,4,5-trimethoxyphenyl-β-D-glucopyranoside (**28**), 3,4,5-tri-hydroxybenzoic acid methyl ester (**29**), ficusol (**30**), bergenin (**31**), and 11-*O*-(4′-*O*-methoxygalloyl)-bergenin (**32**), and one monoterpenoid, alangionoside J (**33**) were isolated and identified by comparison of their spectroscopic data with those reported in literatures (details shown in supplementary material). All of the isolates were obtained from the titled plant for the first time.

Compound **1** was obtained as a white amorphous powder. Its molecular formula $C_{31}H_{32}O_{15}$ was deduced from the negative HR-ESI-MS (*m/z* 643.1657 $[M-H]^-$), indicating 16 degrees of unsaturation. The ^1H and ^{13}C NMR spectra displayed the presence of two anomeric signals at δ_H 4.80 (1H, d, $J = 7.7$ Hz, H-1″) and 4.53 (1H, d, $J = 7.1$ Hz, H-1‴), and δ_C 107.2 (C-1″) and 106.5 (C-1‴). Combining with the ESI-MS fragment ion peak at *m/z* 379 $[M-H-132-132]^-$, it indicated the existence of two pentosyl moieties in **1**. Apart from the two pentosyl units, the left 21 carbon signals were attributable to two methoxyls (δ_C 56.7, 56.0), one methylenedioxy group (δ_C 102.6), one oxygenated methylene (δ_C 69.2), one carboxyl (δ_C 172.2) and 16 sp^2 resonances (δ_C 102.8–153.4) in the ^{13}C NMR spectrum of **1**. The ^1H NMR spectra of **1** showed the occurrence of one 1,3,4-trisubstituted benzene ring [δ_H 6.78 (1H, dd, $J = 7.8$ and 1.6 Hz), δ_H 6.96 (1H, d, $J = 7.8$), δ_H 6.80 (1H, d, $J = 1.6$ Hz)], two singlet aromatic protons [δ_H 7.07 and δ_H 8.16 (each 1H, s)] and one methylenedioxy group [δ_H 6.05 (2H, s)]. The above NMR features of the aglycone part of **1** were identical to those of diphyllin, a 2,3-naphtalide cytotoxic lignan

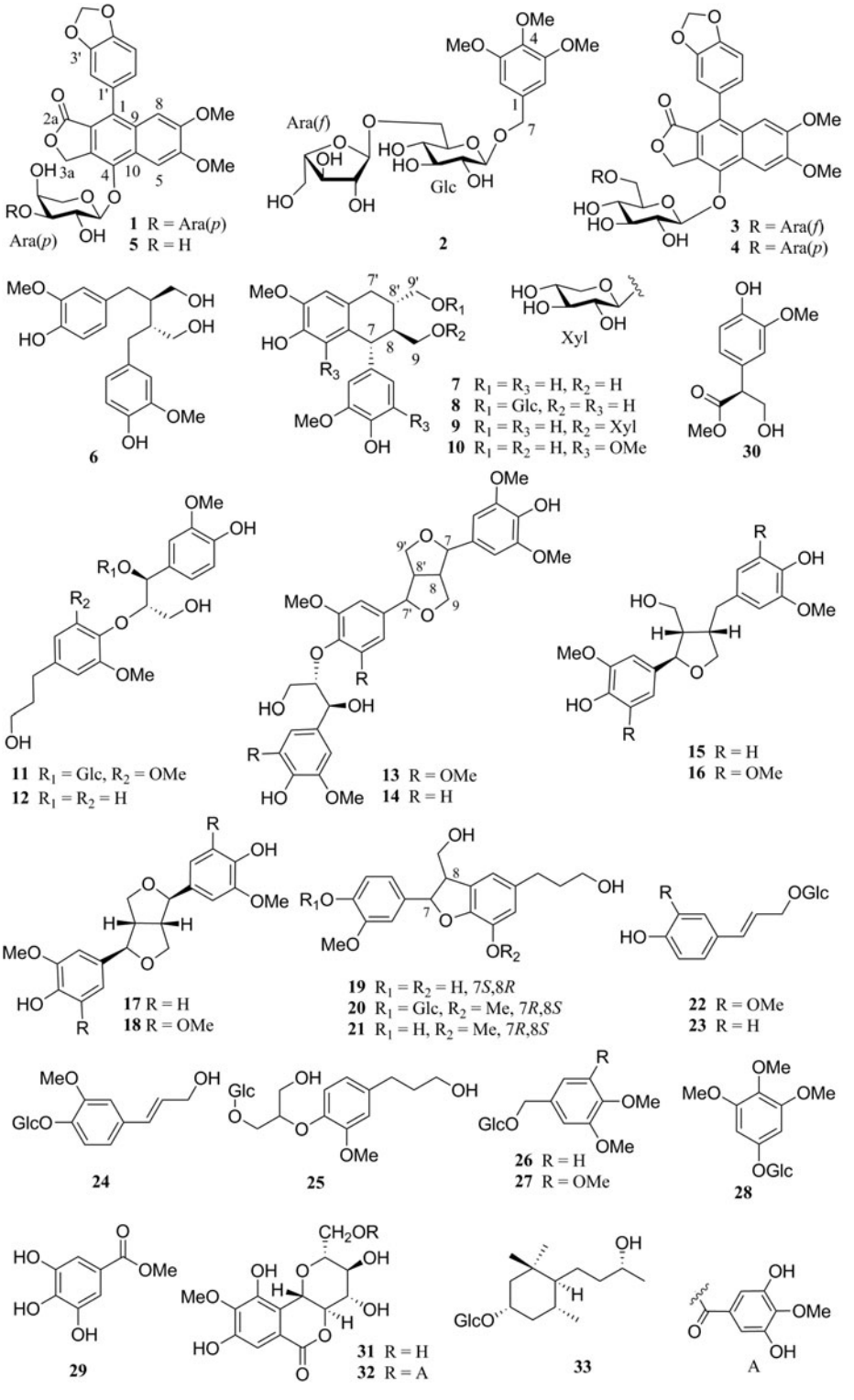

Figure 1. Structures of compounds **1**–**33**.

reported from *Justicia procumbens* (Fukamiya & Lee 1986), indicating that **1** was a diglycoside of diphyllin.

Based on the $^{1}H-^{1}H$ COSY and QC-TOCSY experiments, two arabinopyranosyl units could be constructed, which were further confirmed by the acid hydrolysis followed with pre-column derivative/chiral separation/DAD detection analysis. 1-Phenyl-3-methyl-5-pyrazolone (PMP) derivative of monosaccharide mixture from acid hydrolysis was carried out. After chiral separation, the monosaccharide of **1** was determined to be L-arabinose, by comparing its retention times (rt) with the corresponding standard L-arabinose (rt = 11.3 min). The large coupling constants (7.7 and 7.1 Hz) of the two anomeric protons revealed the α configuration for both L-arabinopyranosyl units. In the ROESY spectrum of **1** (Figure 2), correlations of the terminal arabinosyl H-1‴ (δ_H 4.53) with the inner arabinosyl H-3″ (δ_H 3.71), and the inner arabinosyl H-1″ (δ_H 4.80) with aglycone H-5 (δ_H 8.16) confirmed the arabinosyl-(1 → 3)-arabinosyl linkage, which was further attached to the aglycone C-4. Thus, compound **1** was determined to be diphyllin 4-*O*-α-L-arabinopyranosyl-(1 → 3)-α-L-arabinopyranoside.

Compound **2** was obtained as a white amorphous powder and had a molecular formula $C_{21}H_{32}O_{13}$, as designated from the HR-ESI-MS (*m/z* 515.1736 [M + Na] $^{+}$), indicating six degrees of unsaturation. The ^{1}H, ^{13}C NMR and DEPT spectra indicated the existence of one hexosyl and one pentosyl units [anomeric signals: δ_H 4.33 (1H, d, $J_{1',2'} = 8.1$ Hz, H-1′) and 5.00 (1H, brs, H-1″), δ_C 103.1 (C-1′) and 110.2 (C-1″)]. In addition, signals attributable to one 1,3,4,5-tetrasubstituted symmetric benzene ring [δ_C 154.6 (2C), 138.4 (C), 135.2 (C), 106.3 (2CH)], three aromatic methoxyls (δ_C 56.7, 56.7 and 61.2) and one oxymethylene (δ_C 71.8) were observed, suggesting that **2** was a phenolic glycoside. Based on the HSQC-TOCSY experiment, the sugar moieties in **2** were determined unambiguously to be an inner β-glucopyranosyl and a terminal α-arabinofuranosyl (Hanniffy et al. 1999) units. Their absolution configurations were assumed to be D and L, respectively, based on biogenetic considerations. In the HMBC spectrum of **2**, correlations from the oxymethylene H-7 to the aromatic methines of C-2 and C-5 indicated the aglycone part of **2** to be a benzyl alcohol, on which three methoxyl groups were located at the C-3, C-4 and C-5 positions, respectively (Figure 2). The sugar linkage and sequence were determined on the basis of the HMBC correlations of the inner glucosyl H-1′ and H-6′ with the oxymethylene C-7 (δ_C 71.8) and the terminal arabinosyl C-1″, respectively. On the basis of the above evidences, the structure of **2** was determined to be as 3,4,5-trimethoxybenzyl alcohol 7-*O*-α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranoside.

The arylnaphthalene lignan glycosides **1** and **3**–**5** were evaluated for their cytotoxicities against five human cancer cell lines, e.g. lung cancer (A-549), human myeloid leukemia (HL-60), breast cancer (MCF-7), hepatocellular carcinoma (SMMC-7721) and colon cancer

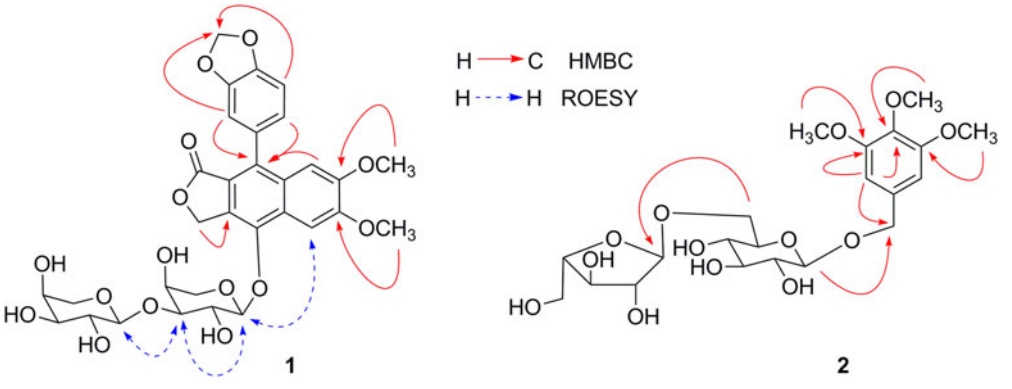

Figure 2. Key HMBC and ROESY correlations of **1** and **2**.

(SW480). Only the known lignan glycoside, phyllanthusmin C (**5**) showed cytotoxicity against HL-60, MCF-7 and SW480 cells with IC_{50} values of 9.2 ± 0.2, 19.2 ± 1.7 and 20.5 ± 0.9 μM, compared with the positive control cisplatin with IC_{50} values of 1.7 ± 0.5, 10.9 ± 1.0 and 9.9 ± 0.9 μM, respectively.

3. Experimental

3.1. *General*

Optical rotations were determined with a P-1020 polarimeter (JASCO, Tokyo, Japan). UV spectra were obtained on a 210A double-beam spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were measured on a Bruker Tensor 27 spectrometer with KBr pellets. ESI-MS and HR-ESI-MS were measured at Bruker HCT/Esquire and Agilent G6230. 1D and 2D NMR spectra were run on Bruker DRX-400, 500 and AVANCE III-600 NMR spectrometers operating at 400, 500 and 600 MHz for ^{1}H, and 100, 125 and 150 MHz for ^{13}C, respectively. Semi-preparative HPLC were performed on an Agilent 1260. TLC was performed on precoated TLC plates (0.2–0.25 mm thickness, GF254 Silica gel, Qingdao Hailang Chemical Co., Ltd., Qingdao, China) with compounds visualised by spraying the dried plates with 10% aqueous H_2SO_4 followed by heating until the plate was dry. Silica gel (200–300 mesh, Qingdao Hailang Chemical Co., Ltd., Qingdao, China), Diaion HP20SS (Mitsubishi Chemical Co., Ltd., Tokyo, Japan), Lichroprep RP-18 (40–63 μm, Merck, Darmstadt, Germany), Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemical Co., Ltd., Uppsala, Sweden) and MCI-gel CHP20P (75–150 μm, Mitsubishi Chemical Co., Ltd., Tokyo, Japan) were used for CC.

3.2. *Plant material*

The whole plants of *P. glaucus* were collected from Pan'an in Zhejiang Province, China, in July, 2011, and identified by Prof. Chong-Ren Yang (Kunming Institute of Botany, Chinese Academy of Sciences). A voucher sample (KIB-Z-00004) has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

3.3. *Extraction and isolation*

The whole fresh plant (24.0 kg) of *P. glaucus* was cut into small pieces and extracted with MeOH three times (each time 3 h) under reflux at 70°C. The combined extracts were filtered and concentrated under vacuum. Then, the resulting MeOH extract (3.0 kg) was suspended in H_2O and partitioned with $CHCl_3$ and *n*-BuOH, respectively, to afford $CHCl_3$ extract (330 g), *n*-BuOH extract (1130 g) and water extract (1500 g). The *n*-BuOH extract was subjected to Diaion HP20SS CC, eluting with MeOH/H_2O (0:1–1:0), to give five fractions (Fr. A–E). Fr. B (25 g) was subjected to CC over Sephadex LH-20 (MeOH/H_2O, 0–100%) and silica gel ($CHCl_3$/MeOH/H_2O, 9:1:0.1–7:3:0.5) to give compounds **26** (8 mg), **27** (20 mg), **28** (5 mg) and **30** (14 mg). The residue of Fr. B was purified by semi-preparative HPLC (flowing rate: 3 mL/min, CH_3CN/H_2O 12:88) to afford **2** (3 mg), **20** (3 mg), **22** (8 mg), **23** (23 mg) and **24** (23 mg). Fr. D (75 g) was applied to CC over MCI gel CHP-20P (MeOH/H_2O, 20–100%) to get Fr. D1–D4. Then, Fr. D4 (25 g) was subjected to CC over Sephadex LH-20 (MeOH/H_2O, 50:50–100:0) to get Fr. D41-Fr. 44. And Fr. 43 (4.7 g) purified by PTLC ($CHCl_3$/MeOH/H_2O, 9:1:0.1) to afford **1** (24 mg), **3** (30 mg), **4** (16 mg), **5** (239 mg). Fr. D42 (5.0 g) was purified by semi-preparative HPLC with an isocratic flow of CH_3CN/H_2O (flowing rate: 3 mL/min, 25:75) to afford **13** (16 mg), **14** (6 mg), **15** (23 mg), **16** (5 mg), **19** (4 mg) and **21** (7 mg), Fr. D2 (15 g) was subjected to CC over Rp-18 (MeOH/H_2O, 30:70–100:0) and silica gel ($CHCl_3$/MeOH/H_2O, 9:1:0.10–

7:3:0.5) to afford **29** (1.2 g), **31** (1.3 g) and **33** (5 mg). The residue of Fr. D2 was purified by semi-preparative HPLC with an isocratic flow of CH_3CN/H_2O (flowing rate: 3 mL/min, 15:85) to afford **6** (5 mg), **7** (3 mg), **8** (19 mg), **9** (13 mg) and **10** (22 mg). Fr. D1 (5.6 g) was chromatographed over MCI gel CHP-20P ($MeOH/H_2O$, 10–100%), then purified by semi-preparative HPLC CH_3CN/H_2O (flowing rate: 3 mL/min, 12:88) to afford **11** (7 mg), **12** (2 mg), **17** (7 mg), **18** (7 mg), **25** (50 mg) and **32** (22 mg).

3.3.1. *Diphyllin 4-O-α-L-arabinopyranosyl-(1 → 3)-α-L-arabinopyranoside (**1**)*

Colourless amorphous powder; $[\alpha]_D^{23} = -1.4$ ($c = 0.2$, MeOH); UV (MeOH) λ_{max} (log ε) 314 (3.09), 296 (3.07), 260 (3.79) 202 (3.76) nm; IR (KBr) ν_{max} 3425, 2920, 2851, 1747, 1623, 1508 and 1017 cm^{-1}; ESI-MS *m/z* 643 $[M-H]^-$, 379 $[M-H-132-132]^-$; HR-ESI-MS *m/z* 643.1657 $[M-H]^-$, calcd for 643.1663; ^1H NMR (500 MHz, CD_3OD): δ 8.16 (1H, s, H-5), 7.07/7.06[a] (1H, s, H-8), 6.96 (1H, d, $J = 7.8$ Hz, H-5′), 6.804/6.800[a] (1H, d, $J = 1.6$ Hz, H-2′), 6.78 (1H, dd, $J = 7.8$ and 1.6 Hz, H-6′), 6.05/6.04[a] (2H, s, -OCH_2O-), 5.57 (1H, d, $J = 15.3$ Hz, H-3a), 5.49 (1H, d, $J = 15.3$ Hz, H-3a), 4.01 (3H, s, 6-OCH_3), 3.72 (3H, s, 7-OCH_3), inner Ara(*p*): 4.80 (1H, d, $J = 7.7$ Hz, H-1″), 4.14 (1H, dd, $J = 7.7$ and 9.1 Hz, H-2″), 3.71 (1H, dd, $J = 4.7$ and 9.1 Hz, H-3″), 3.83 (1H, brs, H-4″), 3.96 (1H, dd, $J = 12.8$ and 4.8 Hz, H-5″b), 3.55 (1H, m, H-5″a), terminal Ara(*p*): 4.53 (1H, d, $J = 7.1$ Hz, H-1‴), 3.70 (1H, dd, $J = 7.1$ and 9.0 Hz, H- 2‴), 3.56 (1H, dd, $J = 3.5$ and 9.0 Hz, H-3‴),4.07 (1H, brs, H-4‴), 3.87 (1H, dd, $J = 12.5$ and 2.9 Hz, H-5‴b), 3.55 (1H, m, H-5‴a); ^{13}C NMR (150 MHz, CD_3OD): δ 172.2 (C-2a), 153.4 (C-6), 151.7 (C-7), 149.0 (C-3′, C-4′), 146.4 (C-4), 137.6 (C-1), 132.2 (C-3), 131.9 (C-10), 130.0 (C-1′), 128.9 (C-9), 124.7 (C-6′), 120.0 (C-2), 111.74/111.69[a] (C-2′), 109.0 (C-5′), 106.9 (C-8), 102.8 (C-5), 102.6 (-OCH_2O-), 69.2 (C-3a), 56.7 (6-OCH_3), 56.0 (7-OCH_3), inner Ara(*p*): 107.2 (C-1″), 71.9 (C-2″), 84.1 (C-3″), 69.7 (C-4″), 67.3 (C-5″), terminal Ara(*p*): 106.5 (C-1‴), 72.9 (C-2‴), 74.0 (C-3‴), 69.7 (C-4‴), 67.1 (C-5‴). ([a]Splitted signals caused by atropisomerism)

3.3.2. *3,4,5-trimethoxybenzyl alcohol 7-O-α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranoside (**2**)*

Colourless amorphous powder; $[\alpha]_D^{22} = -58.9$ ($c = 0.2$, MeOH); UV (MeOH) λ_{max} (log ε) 270 (2.58), 204 (3.81) nm; IR (KBr) ν_{max} 3406, 1595, 1509, 1072 and 1041 cm^{-1}, ESI-MS *m/z* 515 $[M + Na]^+$; HR-ESI-MS *m/z* 515.1736 $[M + Na]^+$, calcd 515.1741; ^1H NMR (600 MHz, CD_3OD): δ 6.77 (2H, s, H-2,5), 4.83 (1H, d, $J = 12.0$ Hz, H-7b), 4.64 (1H, d, $J = 12.0$ Hz, H-7a), 3.85 (6H, s, 3,5-OCH_3), 3.75 (3H, s, 4-OCH_3), Glc: 4.33 (1H, d, $J = 8.1$ Hz, H-1′), 3.26 (1H, dd, $J = 8.1$ and 9.1 Hz, H-2′), 3.33 (1H, dd, $J = 9.1$ and 9.1 Hz, H-3′), 3.31 (1H, dd, $J = 9.1$ and 9.1 Hz, H-4′), 3.44 (1H, m, H-5′), 4.05 (1H, dd, $J = 11.3$ and 2.3 Hz, H-6′b), 3.63 (1H, m, H-6′a), Ara(*f*): 5.00 (1H, brs, H-1″), 4.01 (1H, dd, $J = 1.4$ and 3.4 Hz, H-2″), 3.83 (1H, m, H-3″), 3.97 (1H, dt, $J = 3.6$, 5.6 and 9.7 Hz, H-4″), 3.75 (1H, m, H-5″b), 3.62 (1H, m, H-5″a); ^{13}C NMR (150 MHz, CD_3OD): δ 154.6 (C-3,5), 138.4 (C-4), 135.2 (C-1), 106.3 (C-2,6), 71.8 (C-7), 56.7 (3,5-OCH_3), 61.2 (4-OCH_3), Glc: 103.1 (C-1′), 75.2 (C-2′), 78.1 (C-3′), 72.1 (C-4′), 77.0 (C-5′), 68.2 (C-6′), Ara(*f*): 110.2 (C-1″), 83.5 (C-2″), 79.0 (C-3″), 85.9 (C-4″), 63.2 (C-5″).

3.4. *Acidic hydrolysis of compounds 1 and 5*

Compounds **1** and **5** (each about 3 mg) were separately hydrolysed in HCl (3 M, 1 mL) for 6 h under 80°C water bath. After cooling down to room temperature, the reaction mixture was extracted three times with $CHCl_3$: H_2O (each 1:1, v/v, 1 mL). The aqueous layer was neutralised with Amberlite IRA-401. The monosaccharides in **1** and **5** were first identified as arabinose, by co-TLC with authentic sugar, eluting with chloroform/*n*-butanol/methanol/acetic acid/water 17:10:6:2:3 (Rf = 0.40). Then, the PMP derivative of the monosaccharide mixture was prepared

as reported by Honda et al. (1989). The resulted PMP-monosaccharide was dissolved in ethanol/*n*-hexane solution (1:4, v/v) and analysed by HPLC using CHRIALPAK AD-H column, with an elution solvent system of *n*-hexane/2-propanol (87:13), at 25°C, and detected by DAD detector. The standard sugars were operated with the same methods. The monosaccharides in **1** and **5** were determined to be L-arabinose (rention time: 11.3 min).

3.5. *Cytotoxic activity*

As previously reported (Lv et al. 2013).

Supplementary material

Supplementary material relating to this article is available online: Identification of the known compounds **3**–**33**, and 1D and 2D NMR, MS and HR-ESI-MS of compounds **1** and **2**.

Disclosure statement

No potential conflict of interest was reported by the authors.

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