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Pyrazole alkaloids from watermelon (*Citrullus lanatus*) seeds

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ABSTRACT

Three new compounds, *i.e.* 1-[2-(5-hydroxymethyl-1*H*-pyrrole-2-carbaldehyde-1-yl)ethyl]-1*H*-pyrazole (**1**), 1-({[5-(α-D-galactopyranosyloxy)methyl]-1*H*-pyrrole-2-carbaldehyde-1-yl}-ethyl)-1*H*-pyrazole (**2**), and (4-hydroxyphenyl)methanol 4-[β-D-apiofuranosyl(1→2)-*O*-β-D-glucopyranoside] (**3**), were isolated from an extract of watermelon seeds. Compounds **1** and **2** were pyrazole-alkaloids with a pyrrole ring. This is the first study to show compounds with pyrrole and pyrazole rings in a molecule isolated from natural products. In the evaluation for melanogenesis inhibitory, compound **1** exhibited modest inhibitory activity on melanogenesis without cytotoxicity. Meanwhile compound **2** showed some inhibitory activity accompanied by some cytotoxicity.

1. Introduction

Citrullus lanatus (Thunb.) Matsum. and Nakai (*Citrullus vulgaris* Schrad, Japanese name: suika) is cultivated throughout the world for use as a fruit as well as a medicine. Its fruits have been used as a diuretic, its seed coat has been used to treat hematemesis and melena, and its kernel has been used as an antihypertensive and in the treatment of acute cystisis (Shanghai Scientific and Technical Publishers, Shogakukan, Inc., 1985). Previous studies detected polyphenols (Lako et al., 2006), flavonols (Lako et al., 2006), carotenoids (Lako et al., 2006), amino acids (Shanghai Scientific and Technical Publishers, Shogakukan, Inc., 1985), including citrulline and arginine, and triterpenes (Ripperger and Seifert, 1975) in the fruits of *C. lanatus*. The apoptogenic effects of cucurbitacin L 2-*O*-glucoside on colon adenocarcinoma, and anti-inflammatory activities of cucurbitacin E, both isolated from *C. lanatus*, have been reported previously (Hassan et al., 2011). We recently isolated two new cucurbitane-type triterpenes, 24-hydroperoxycucurbita-5,25-dien-3β-ol and 25-hydroperoxycucurbita-5,23-dien-3β-ol, that had hydroperoxy groups on their side chains from the Et_2O fraction of a *C. lanatus* seed MeOH extract, and examined their cytotoxic activities against the HL-60, P388, and L1210 cell lines, and melanogenesis inhibitory activities in α-MSH-stimulated B16 4A5 cells (Kikuchi et al., 2013a). In the present study, we describe the isolation and structural elucidation of compounds **1**–**3** as well as the inhibitory effects of compounds from watermelon on melanogenesis in B16 4A5 cells.

2. Results and discussion

Three new compounds (**1**–**3**) were isolated from the seeds of *C. lanatus* (Fig. 1).

Compound **1** exhibited an $[M+H]^+$ ion in the HR-FAB-MS at *m*/*z* 220.1089, which was compatible with the molecular formula $C_{11}H_{14}N_3O_2$ (calcd 220.1086). Its IR spectrum showed absorption that indicated a hydroxy group (ν_{max} 3424 cm^{-1}) and carbonyl group (ν_{max} 1649 cm^{-1}). ^1H and ^{13}C NMR spectra indicated the presence of an oxymethylene [δ_H 4.02 (s); δ_C 55.9 (t)], pyrrole ring [δ_H 6.16 (d, J = 4.1 Hz), 7.03 (d, J = 4.1 Hz); δ_C 111.3 (d), 126.8 (d), 133.4 (s), 145.3 (s)], pyrazole ring [δ_H 6.19 (t, J = 2.0 Hz), 7.16 (dd, J = 0.6, 2.0 Hz), 7.49 (dd, J = 0.6, 2.0 Hz); δ_C 106.9 (d), 131.8 (d), 140.9 (d)], and formyl group [δ_H 9.45 (s); δ_C 181.1 (d)]. The following correlations were observed in the HMBC spectrum; H-1′ [δ_H 4.52 (t, J = 5.9 Hz)]/C-5 [δ_C 131.8 (d)]; H-2′ [δ_H 4.71 (t, J = 5.9 Hz)]/C-2″ [δ_C 133.4 (s)] and C-5″ [δ_C 145.3 (s)]; H-3″ [δ_H 7.03 (d, J = 4.1 Hz)]/C-7″ [δ_C 181.1 (d)]; H-6″ [δ_H 4.02 (s)]/C-4″ [δ_C 111.3 (d)] and 5″ [δ_C 145.3 (s)] (Fig. 2). In the ^1H–^1H COSY spectrum, H-3 [δ_H 7.49 (dd, J = 0.6, 2.0 Hz)]/H-4 [δ_H 6.19 (t, J = 2.0 Hz)], H-4/H-5 [δ_H 7.16 (dd, J = 0.6, 2.0 Hz)], and H-3″ [δ_H 7.03 (d, J = 4.1 Hz)]/H-4″ [δ_H 6.16 (d, J = 4.1 Hz)] were observed (Fig. 2). Therefore, **1** was established as 1-[2-(5-hydroxymethyl-1*H*-pyrrole-2-carbaldehyde-1-yl)ethyl]-1*H*-pyrazol (Table 1).

Compound **2** exhibited an $[M+H]^+$ ion in the HR-FAB-MS at *m*/*z* 382.1617, which was compatible with the molecular formula

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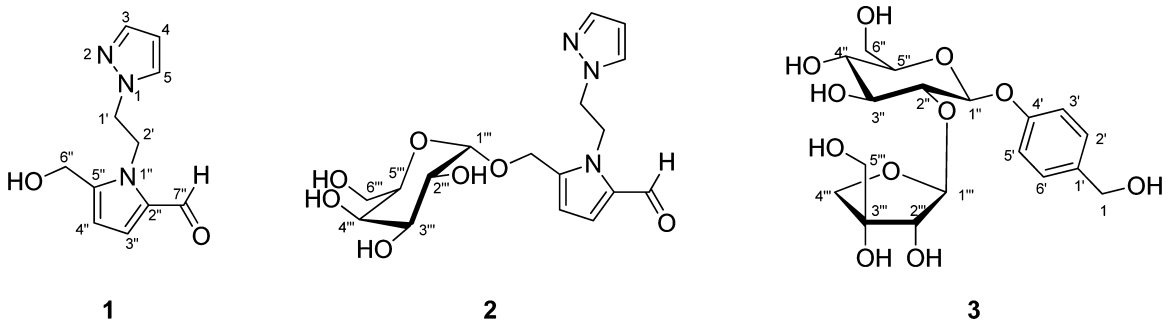

Fig. 1. Structures of compounds **1**–**3** from watermelon seeds.

$C_{17}H_{24}N_3O_7$ (calcd for 382.1609). Its IR spectrum showed absorption that indicated hydroxy groups (3408 cm^{-1}) and a carbonyl group (1656 cm^{-1}). ^1H NMR and ^{13}C NMR spectra suggested compound **2** was a glycoside of **1** since the signals of **2** were similar to those of **1**, except for the sugar moiety. This was confirmed by HMBC and ^1H–^1H COSY spectra (Fig. 2). The ^1H and ^{13}C NMR signals of the sugar moiety [δ_H 4.79 (d); δ_C 62.8 (t), 70.0 (d), 71.1 (d), 71.3 (d), 72.9 (d), 99.5 (d)] suggested the presence of a terminal D-galactopyranose, and its configuration at C-1‴ was established as the α-orientation due to the coupling constant of H-1‴ (J = 4.1 Hz). This was confirmed by acid hydrolysis, derivatization with L-cysteine methyl ester hydrochloride and *o*-tolylisothiocyanate, and HPLC analysis. Therefore, compound **2** was established as 1-({[5-(α-D-galactopyranosyloxy)methyl]-1*H*-pyrrole-2-carbaldehyde-1-yl}-ethyl)-1*H*-pyrazol (Table 1). Compounds **1** and **2** are rare pyrazole-alkaloids. Kumar et al. (2013) isolated L-α-amino-β-(pyrazolyl-*N*)-propanoic acid, a naturally occurring amino acid with a pyrazole ring, from the juice of watermelons. The pyrazole rings of **1** and **2** may be derived from this amino acid. This is the first study to show compounds with pyrrole and pyrazole rings in a molecule isolated from natural products; however, pyrrole–imidazole alkaloids have been isolated from the Mediterranean sponge (Fattorusso and Taglialatela-Scafati, 2000).

Compound **3** exhibited an $[M+Na]^+$ ion in the HR-FAB-MS at m/z 441.1375, which had the molecular formula of $C_{18}H_{26}O_{11}Na$ (calcd 441.1372). Its IR spectrum revealed absorption bands indicating hydroxy groups (ν_{max} 3392 cm^{-1}). The ^{13}C NMR spectrum of **3** displayed δ_C 62.5 (t), 71.4 (d), 78.0 (d), 78.6 (d), 78.7 (d), 101.0 (d) attributed to inner glucose, and δ_C 66.1 (t), 75.5 (t), 78.1 (d), 80.8 (s), 110.8 (d) attributed to terminal apiose. In the ^1H NMR spectrum, the A_2B_2-type aromatic proton signals, [δ_H 7.04 (d, 8.8) and 7.27 (d, 8.8)] and hydroxy methylene signal [δ_H 4.53 (s); δc 64.8 (t)] suggested the presence of a hydroxy methyl phenyl moiety. The following correlations were observed in the HMBC spectrum: H-1 [δ_H 4.53 (s)]/C-1′ [δ_c 136.5 (s)]; H-2′ [δ_H 7.27 (d)]/C-1′; H-3′ [δ_H 7.04 (d)]/C-4′ [δ_c 158.4 (s)]; H-1″ [δ_H 4.95 (d)]/C-4′; H-1‴ [δ_H 5.46 (d)]/C-2″ [δ_c 78.6 (d)], C-3‴; H-5″/C-1″, C-4″; H-6″/C-4″; H-2‴/C-3‴; H-4‴/C-3‴; H-5‴/C-3‴(Fig. 2). On acid hydrolysis, **3** afforded D-glucose and D-apiose, which were identified by derivatization with L-cysteine methyl ester hydrochloride and *o*-tolylisothiocyanate, and HPLC analysis. Thus, compound **3** was established as (4-hydroxyphenyl)methanol 4-[β-D-apiofuranosyl(1→2)-*O*-β-D-glucopyranoside] (Table 1).

Compounds **1**–**3** and the positive control, arbutin were evaluated for there melanogenesis inhibitory effects against α-MSH-induced melanogenesis in B16 4A5 melanomas (Table 2). To determine safe concentrations, the cytotoxicities of three compounds were examined using the MTT assay. Compound **1** did not exhibit cytotoxicity against B16 4A5 cells at 3–100 μM. It also modestly inhibited melanogenesis in B16 4A5 cells (melanin content: 80.3% at 30 μM, 64.1% at 100 μM). This compound may be valuable in the development of skin-whitening agents. Compound **2** exhibited some melanogenesis inhibitory activity (melanin content: 86.0% at 30 μM, 48.6% at 100 μM); however, this was attributed to its cytotoxic effects (cell viability: 92.7% at 30 μM, 70.4% at 100 μM).

3. Experimental

3.1. General

Chemicals and reagents were purchased as follows: fetal bovine serum (FBS) from Invitrogen Co. (Carlsbad, CA, USA), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) from Sigma–Aldrich Japan Co. (Tokyo, Japan), Dulbecco's modified Eagle's medium (D-MEM), and antibiotics from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals and reagents were of analytical grade. Optical rotations were measured with a JASCO DIP-1000 digital polarimeter. IR spectra were recorded on a Perkin-Elmer 1720X FTIR spectrophotometer. The ^1H (600 MHz) and ^{13}C

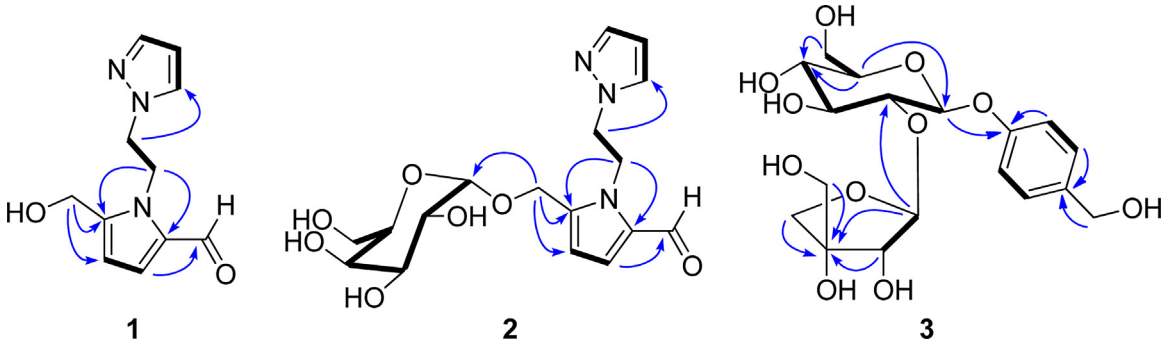

Fig. 2. Key HMBC (⟶) and ^1H–^1H COSY (▬) correlations of compounds **1**–**3**.

Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectroscopic data of compounds **1–3** (CD_3OD).[a]

No.	**1**			**2**				**3**			
	δ_C		δ_H (J in Hz)	δ_C			δ_H (J in Hz)	δ_C			δ_H (J in Hz)
1								64.8	t		4.53 s
3	140.9	d	7.49 dd (0.6, 2.0)	140.9	d		7.50 dd (0.9, 2.1)				
4	106.9	d	6.19 t (2.0)	106.9	d		6.19 t (2.1)				
5	131.8	d	7.16 dd (0.6, 2.0)	131.9	d		7.23 dd (0.9, 2.1)				
1′	53.4	t	4.52 t (5.9)	53.2	t		4.53 t (5.9)	136.5	s		
2′	47.3	t	4.71 t (5.9)	47.4	t		4.75 td (2.0, 5.8)	129.5	d		7.27 d (8.8)
3′								117.4	d		7.04 d (8.8)
4′								158.4	s		
5′								117.4	d		7.04 d (8.8)
6′								129.5	d		7.27 d (8.8)
1″								101.0	d		4.95 d (7.6)
2″	133.4	s		133.8	s			78.6	d		3.63 m
3″	126.8	d	7.03 d (4.1)	126.2	d		7.03 d (4.1)	78.7	d		3.58 t (8.5)
4″	111.3	d	6.16 d (4.1)	113.1	d		6.26 d (4.1)	71.4	d		3.39 dd (8.5, 9.7)
5″	145.3	s		141.0	s			78.0[b]	d		3.41 m
6″	55.9	t	4.02 s	60.7	t	A	4.03 d (12.9)	62.5	t	A	3.68 dd (5.3, 12.0)
						B	4.23 d (12.9)			B	3.88 dd (2.0, 12.0)
7″	181.1	d	9.45 s	181.3	d		9.47 s				
1‴				99.5	d		4.79 d (4.1)	110.8	d		5.46 d (1.5)
2‴				70.0	d		3.77 dd (4.2, 10.3)	78.1[b]	d		3.94 d (1.5)
3‴				71.3	d		3.71 brd (10.3)	80.8	s		
4‴				71.1	d		3.86 dd (1.2, 3.5)	75.5	t	A	3.78 d (9.5)
										B	4.05 d (9.5)
5‴				72.9	d		3.76 brd (11.7)	66.1	t	A	3.51 d (11.4)
										B	3.54 d (11.4)
6‴				62.8	t	A	3.68 dd (4.9, 11.1)				
						B	3.72 dd (6.7, 11.1)				

[a] Assignments were based on ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY spectroscopic data.
[b] Interchangeable.

(150 MHz) NMR spectra were recorded on an Agilent vnmrs 600 in CP_3OD with tetramethylsilane as the internal standard. HR-FAB-MS was recorded on a JEOL JMS-7000 mass spectrometer. Silica gel 60 (230–400 mesh, Nacalai Tesque, Inc., Kyoto, Japan) Diaion HP-20 (*Mitsubishi Chemical Co., Tokyo, Japan*), octadecylsilica gel (ODS;100-200 mesh; *Fuji Siliya Chemical, Ltd*., Aichi, Japan), and sephadex LH-20 *CGE healthcare Bio-Sciences AB*, Uppsala, Sweden). Were used for column chromatography. HPLC [PU-2086 pump and RI-2031 refractive index detector (Jasco, Tokyo, Japan)] was carried out on an ODS column [*Cosmosil 5C$_{18}$-PAQ column* (Nacalai Tesque, Inc., Kyoto, Japan), 25 cm × 20 mm i.d.] at 35 °C with H_2O/MeCN [20:1 (HPLC system *I*), 90: 10 (HPLC system *II*), 85: 15 (HPLC system *III*), flow rate 4.0 mL/min].

3.2. Plant material

The seeds of *C. lanatus*, obtained in 2011, in the USA, were purchased from Takada Seeds Co., Ltd. in 2011. A voucher specimen (CL-S-2011-01) was deposited in the Herbarium of the Laboratory of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences.

Table 2
Melanogenesis inhibitory activities of compounds **1–3** from watermelon seeds.[a,b]

	Melanin content (%)			
	3 μM	10 μM	30 μM	100 μM
1	119.3 ± 5.1*	94.1 ± 9.8	80.3 ± 9.3*	64.1 ± 5.8**
2	103.9 ± 0.7	99.7 ± 1.7	86.0 ± 2.4**	48.6 ± 0.6**
3	106.6 ± 2.3	100.9 ± 1.6	90.5 ± 1.6*	65.9 ± 2.1**
Arbutin[c]	98.6 ± 2.5	85.7 ± 6.7*	66.6 ± 6.1**	45.2 ± 2.0**

[a] Melanin content (%) were determined based on absorbances at 405 nm, and comparisons with values for vehicle (DMSO) control (100%). Each value represents the mean ± standard error (S.E.) of three determinations. The concentration of DMSO in the sample solution was 2 mL/mL. Significant differences from the control group were shown as $^{*}p < 0.05$, and $^{**}p < 0.01$.
[b] Compound **2**, **3**, and arbutin exhibited some cytotoxicities at 100 μM (cell viability **2**: 70.4 ± 1.9%; **3**; 88.4 ± 0.5%; arbutin: 86.2 ± 1.2%).
[c] Positive control.

3.3. Extraction and isolation

The seeds of *C. lanatus* (10 kg) were pulverized in juice mixer, and subjected to extraction with MeOH under reflux (10 L, 1 week 4×). The MeOH extract (250 g) was then partitioned between Et_2O and H_2O (1:1, v/v), followed by *n*-BuOH and H_2O (1:1, v/v). The *n*-BuOH fraction (17 g) was subjected to Diaion HP-20 CC [Diaion HP-20 (330 g)] resulting in H_2O Fr. (4.0 g), MeOH Fr. (12.1 g), and acetone Fr. (0.81 g) were eluted with H_2O, MeOH, and acetone, respectively]. A portion of MeOH Fr. (11.7 g) was subjected to silica-gel CC [silica-gel (200 g)] to yield 5 fractions, A–E [$CHCl_3$/MeOH (1:1) → (7:3) → (9:1) → (1:0)]. Among them, Fr. C (5.71 g) was subjected to ODS CC [ODS (50 g)] to yield 6 fractions, C1–C6 [MeOH/H_2O (1:1) → (7:3) → (9:1) → (1:0)]. C-1, eluted with MeOH/H_2O (1:1), was carried out ODS CC [ODS (50 g)] to give sixteen fractions: C1-1–C1-16. C1-5 (312.02 mg) was fractionated with preparative HPLC (*HPLC system III*) to nineteen fractions, C1-5-1–C1-5-19. Purification of C1-5-13 gave **2** (1.47 mg; t_R 232.0 min, *HPLC system II*). Preparative HPLC of C1-9 (62.6 mg), which was eluted with MeCN/H_2O (1:9), gave **1** (2.1 mg; t_R 66.4 min)(*HPLC system III*). Fr. D (4.51 g) was subjected to ODS CC [ODS (50 g)] to yield 6 fractions, D1–D7 [MeOH/H_2O (1:1) → (9:1) → (1:0)], followed by D1 (3.12 g) was carried out re-column chromatography with ODS to give D1-1–D1-9. Fr. D1-1 (1.67 g) was subjected to shephadex LH-20 CC [sephadex LH-20 (40 g)] to yield 6 fractions, D1-1-1–D1-1-7 [MeOH/H_2O (0:1) → (1:0)]. Preparative HPLC of D1-1-2 (466.6 mg), which was eluted with H_2O, **3** (3.3 mg; t_R 104.6 min) (*HPLC system I*).

3.4. Acid hydrolysis and determination of sugar configuration

Determination of the absolute sugar configuration was carried out according to a method reported previously with a little modification (Tanaka et al., 2007). A solution of **2** (1 mg) and **3** (1 mg), respectively, in 2 M CF_3COOH (1 mL) was heated under reflux for 3 h. The mixture was extracted with EtOAc (3 times), and

the H_2O layer was concentrated to dryness. The residue was then dissolved in pyridine (0.1 mL) and stirring with L-cystein methyl ester hydrochloride (0.5 mg) at 60 °C for 1 h. The reaction mixture was treated with *o*-tolylisothiocyanate, and heated at 60 °C for 1 h. The reaction mixture was analyzed by reversed-phase HPLC [column: *Cosmosil 5 C_{18}-PAQ column* (Nacalai Tesque), 250 mm × 4.6 mm i.d. (5 μm); mobile phase: MeCN–H_2O in 1% AcOH (2:8, v/v); detection: refractive index; flow rate: 1.0 mL/min; column temperature: 35 °C] to identify the derivatives of D-galactose in **2**, and D-glucose and D-apiose in **3** by comparison of their retention times with those of authentic samples (t_R: D-glucose 28.4 min, L-glucose 26.0 min, D-apiose 45.4 min, D-galactose 22.4 min, L-galactose 23.3 min).

3.5. Cell culture

The cell line B16 4A5 cells were grown in Dulbecco's modified Eagle medium (D-MEM). The medium was supplemented with 10% FBS and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). The cells were incubated at 37 °C in a 5% CO_2 humidified incubator.

3.6. Viability of melanoma cells

Melanoma cells proliferation was examined according to a method reported previously (Yamada et al., 2011), with a little modification. Briefly, B16 4A5 cells [obtained from Riken Cell Bank (Tsukuba, Ibaraki, Japan)] (3×10^3 cells in 100 μL), preincubated for 24 h, were treated for 72 h with test samples dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 100, 30, 10, and 3 μM, and MTT solution was added. After 3 h of incubation, 20% NDS containing 0.1 M HCl was added to dissolve the formazan produced in the cells. The absorbance of each well was read at 570 nm using a microplate reader.

3.7. Assay of melanin content

The assay of melanin content was performed as described previously (Kikuchi et al., 2013b), with a little modification. B16 4A5 cells (3×10^4 cells in 1000 μL) were pre-incubated in α-MSH (100 nM) containing medium. Test samples dissolved in DMSO were added to the medium and the cells were cultured for 72 h. The medium was removed and the cells were dissolved in 2 M NaOH containing 10% DMSO. The amount of melanin was determined spectrophotometrically by measuring absorbance at 405 nm using a microplate reader. The optical density of control cells was assumed to be 100%.

3.8. 1-[2-(5-Hydroxymethyl-1H-pyrrole-2-carbaldehyde-1-yl)ethyl]-1H-pyrazole (**1**)

Amorphous solid, $[\alpha]_D^{27}$ −2.4 (*c* = 0.055, EtOH); UV λ_{max}^{EtOH} nm (log ε): 219.0 (3.33), 255.0 (3.26), 292.5 (3.58); IR ν_{max}^{KBr} cm^{-1}: 3480, 3424, 3354, 1649, 1585, 1377, 1157, 1089; FAB-MS *m*/*z*: 220 $[M+H]^+$; HR-FAB-MS *m*/*z*: 220.1089 (calcd for 220.1086: $C_{11}H_{14}N_3O_2$).

3.9. 1-({[5-(α-D-Galactopyranosyloxy)methyl]-1H-pyrrole-2-carbaldehyde-1-yl}-ethyl)-1H-pyrazole (**2**)

Amorphous solid, $[\alpha]_D^{27}$; 62.4 (*c* = 0.062, EtOH); UV λ_{max}^{EtOH} nm (log ε): 206.5 (3.66), 256.5 (3.84), 282.0 (3.84), 294.0 (3.70); IR ν_{max}^{KBr} cm^{-1}: 3408, 2922, 1743, 1723, 1656, 1640, 1400, 1353, 1400, 1281, 1029; FAB-MS *m*/*z*: 382 $[M+H]^+$; HR-FAB-MS *m*/*z*: 382.1617 (calcd for 382.1609: $C_{17}H_{24}N_3O_7$).

3.10. (4-Hydroxyphenyl)methanol 4-[β-D-apiofuranosyl (1 → 2)-O-β-D-glucopyranoside] (**3**)

Amorphous solid; $[\alpha]_D^{27}$ −85.6 (*c* = 0.097, EtOH); UV λ_{max}^{EtOH} nm (log ε): 221.5 (3.95), 270.5 (3.11); IR ν_{max}^{KBr} cm^{-1}: 3392, 1612, 1509, 1225, 1076; FAB-MS *m*/*z*: 441 $[M+Na]^+$; HR-FAB-MS *m*/*z*: 441.1375 (calcd for 441.1372: $C_{18}H_{26}O_{11}Na$).

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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.2015.02.017.

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