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PFAFFOSIDES AND NORTRITERPENOID SAPONINS FROM *PFAFFIA PANICULATA*

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Key Word Index—*Pfaffia paniculata*, Amaranthaceae, Brazil ginseng, nortriterpenoid saponin, pfaffic acid, pfaffosides

Abstract—Three new nortriterpene glucuronides named pfaffosides A, B and C have been isolated from roots of *Pfaffia paniculata* Their structures have been established as 3β-*O*-[β-D-xylopyranosyl-(1 → 2)-β-D-glucuronopyranosyl]-pfaffic acid, 3β-*O*-[β-D-xylopyranosyl-(1 → 2)-β-D-glucuronopyranosyl]-pfaffic acid-(28 → 1)-β-D-glucopyranosyl ester and 3β-*O*-[β-D-glucuronopyranosyl]-pfaffic acid-(28 → 1)-β-D-glucopyranosyl ester, respectively, based on their chemical and spectroscopic properties

INTRODUCTION

The roots of *Pfaffia paniculata* Kuntze, known in Brazil as 'Brazil ginseng', have been used as a tonic, an aphrodisiac and as a folk medicine for antidiabetic purposes [1] We have investigated the constituents of this plant, and isolated, besides the new nortriterpene pfaffic acid [2], three new pfaffic acid saponins named pfaffosides A, B and C and established their structures as **1**–**4** respectively Furthermore a mixture of stigmasterol and sitosterol, their glycosides and also allantoin were identified The inhibitory effects of **1**–**4** on the growth of cultured tumor cells have been investigated

RESULTS AND DISCUSSION

The roots of *Pfaffia paniculata*, collected in the Goias area of Brazil, were treated with hot methanol and partitioned in an *n*-butanol–water mixture The water insoluble portion of the *n*-butanol layer was chromatographed on silica gel to yield pfaffic acid and the mixture of stigmasterol and sitosterol The water soluble portion of the *n*-butanol layer was passed through a column of charcoal and purified by chromatography on silica gel to give pfaffosides A, B and C, allantoin and a mixture of stigmasteryl-β-D-glucoside and sitosteryl-β-D-glucoside

Pfaffic acid (**1**), $C_{29}H_{44}O_3$, mp 285–286°, $[\alpha]_D^{22}$ + 109 2° (*c* 0 72, $CHCl_3$) exerted inhibitory effects on the growth of cultured tumor cells (Fig 1) The structure was finally established as being 3β-hydroxy-16,20-cyclo-30-norolean-12-en-28-oic acid by means of X-ray crystallographic analysis of the methylate We have reported these properties and the structure in a previous communication [2]

Pfaffoside A (**2**), $C_{40}H_{60}O_{13}$ $3H_2O$, mp 268°, $[\alpha]_D^{22}$ + 14 8° (*c* 1 85, MeOH), contained hydroxyl groups

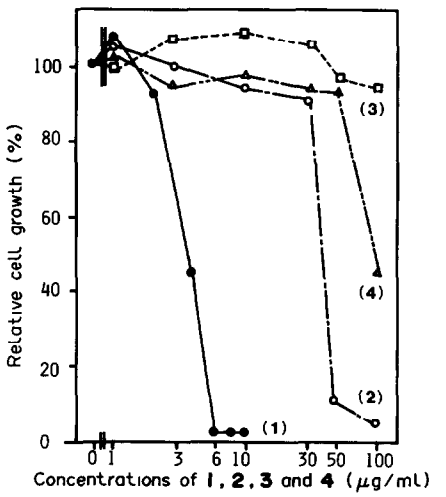
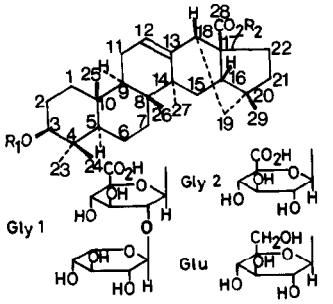


Fig 1 Inhibitory effects of pfaffic acid (**1**), pfaffoside A (**2**), pfaffoside B (**3**) and pfaffoside C (**4**) on the growth of melanoma (B-16)

(3400 cm^{-1}) and carboxyl groups (1730 and 1700 cm^{-1}), as judged from the IR spectrum Acid hydrolysis of **2** yielded **1** as the aglycone and xylose and glucuronic acid as the sugar moieties The hepta-*O*-methyl derivative of **2**, prepared by the Kuhn method [3], exhibited a molecular ion peak at *m/z* 846, peaks due to methyl pfaffate at *m/z* 246 and 437, and the characteristic peaks due to per-methylated terminal pentose and pentose–hexuronic acid moieties at *m/z* 175 and 393, respectively, in the mass spectrum The ^{13}C NMR spectrum of **2** showed 40 carbon signals (Table 1) Of these, 29 were assigned to the



1 R_1 = H R_2 = H
2 R_1 = Gly 1 R_2 = H
3 R_1 = Gly 1 R_2 = Glu
4 R_1 = Gly 2 R_2 = Glu

aglycone **1** in consideration of the glycosylation shift of the α- and β-carbon atoms of the aglycone alcohol [4, 5] The remaining 11 signals were attributable to the two sugar moieties, whose anomeric carbon signals appeared at δ 105 4 and 107 0 (Table 1) A comparison of the ^{13}C NMR chemical shifts due to the sugar moieties of **2** with those due to C-1, C-2 and C-3 of the inner glucopyranosyl unit of ginsenoside Rb_2 [6], C-4, C-5 and C-6 of the glucuronopyranosyl unit of methyl 3β-*O*-[β-D-glucuronopyranosyl]-oleanate [7] and the xylopyranosyl unit linked to the glucopyranosyl unit of hovenoside G [8] isolated from the seeds of *Zizyphus jujuba* revealed that the signals due to the sugar moiety of **2** were attributable to the 3-*O*-[β-D-xylopyranosyl-(1 → 2)-β-D-glucuronopyranosyl] group (Table 1) In the ^{1}H NMR spectrum of **2**, two anomeric proton signals were observed at δ 5 01 (*d*, *J* = 6.8 Hz) and 5 27 (*d*, *J* = 5 6 Hz), supporting the β-configurations of the glucuronic acid and xylose moieties In addition, consideration of molecular optical rotation by the application of Klyne's rule [9] supported the β-anomeric configurations of all sugar linkages (found $[M]_D = +111°$, calc $[M]_D = +168°$) Accordingly, the structure of **2** was established as 3β-*O*-[β-D-xylopyranosyl-(1 → 2)-β-D-glucuronopyranosyl]-pfaffic acid

Pfaffoside B (**3**), $C_{46}H_{70}O_{18}$ $3H_2O$, mp 255–260°, $[\alpha]_D^{22}$ −1 8° (*c* 1 05, MeOH), contained hydroxyl groups (3400 cm^{-1}), carboxyl and ester groups (1730 cm^{-1}), as judged from the IR spectrum Acid hydrolysis of **3** yielded **1** as the aglycone and xylose, glucuronic acid and glucose as the sugar moieties On hydrolysis with 1 N potassium hydroxide, **3** yielded **2** The results suggested that one glucose residue was attached to the carboxyl group of either pfaffic acid or glucuronic acid in the ester form The deca-*O*-methyl derivative of **3**, prepared by the Kuhn method [3], exhibited a molecular ion peak at *m/z* 1050, the characteristic peaks due to permethylated terminal pentose, pentose–hexuronic acid and terminal hexose moieties at *m/z* 175, 393 and 219, respectively, the peak due to the retro-Diels–Alder fragmentation of ring C at *m/z* 450, and the peaks due to the elimination of pentose–hexuronic acid and hexose–carboxyl moieties from the molecular ion at *m/z* 641 and 787, respectively, in the mass spectrum A comparison of the ^{13}C NMR spectrum of **3** with that of **2** revealed that the chemical shift of C-28 was displaced upfield by 3 6 ppm (Table 1) The ^{13}C NMR spectrum of the glucose moiety of **3** was superimposable with that of chikusetsu saponin IV

Table 1 ^{13}C NMR chemical shifts of pfaffic acid (**1**), pfaffoside A (**2**), pfaffoside B (**3**) and pfaffoside C (**4**)

Carbon	(1)	(2)	(3)	(4)
1	39 1 *t*	38 7 *t*	38 7 *t*	38 7 *t*
2	28 2 *t*	26 6 *t*	26 6 *t*	26 6 *t*
3	78 3 *d*	89 4 *d*	89 4 *d*	89 2 *d*
4	39 5 *s*	39 6 *s*	39 6 *s*	39 6 *s*
5	56 1 *d*	56 0 *d*	56 0 *d*	55 9 *d*
6	18 9 *t*	18 5 *t*	18 7 *t*	18 5 *t*
7	33 9 *t*	33 8 *t*	33 3 *t*	33 3 *t*
8	39 5 *s*	39 6 *s*	39 6 *s*	40 1 *s*
9	48 0 *d*	47 7 *d*	47 7 *d*	47 8 *d*
10	37 5 *s*	36 9 *s*	36 9 *s*	37 0 *s*
11	23 5 *t*	23 3 *t*	23 4 *t*	23 4 *t*
12	120 2 *d*	120 3 *d*	121 1 *d*	121 1 *d*
13	145 6 *s*	145 6 *s*	144 7 *s*	144 7 *s*
14	40 9 *s*	40 7 *s*	40 9 *s*	40 9 *s*
15	29 2 *t*	29 1 *t*	29 0 *t*	29 0 *t*
16	52 2 *d*	52 1 *d*	51 8 *d**	51 9 *d**
17	56 5 *s*	56 4 *s*	56 2 *s*	56 2 *s*
18	52 2 *d*	52 1 *d*	52 2 *d**	52 0 *d**
19	41 6 *t*	41 6 *t*	41 4 *t*	41 4 *t*
20	44 5 *s*	44 4 *s*	44 4 *s*	44 5 *s*
21	39 5 *t*	39 5 *t*	39 0 *t*	39 1 *t*
22	32 4 *t*	32 2 *t*	32 2 *t*	32 2 *t*
23	30 3 *q*	30 2 *q*	30 1 *q*	30 1 *q*
24	16 4 *q*	16 2 *q*	16 2 *q*	16 8 *q*
25	15 5 *q*	15 3 *q*	15 4 *q*	15 4 *q*
26	17 0 *q*	16 7 *q*	17 3 *q*	17 3 *q*
27	28 8 *q*	27 8 *q*	27 8 *q*	28 2 *q*
28	177 5 *s*	177 8 *s*	174 2 *s*	174 2 *s*
29	18 7 *q*	18 7 *q*	18 5 *q*	18 5 *q*
Glucuronic acid				
1	(105 0)† [6]	105 4 *d*	105 4 *d*	107 4 *d*
2	(83 0)† [6]	83 6 *d*	83 7 *d*	75 6 *d*
3	(78 1)† [6]	77 4 *d**	77 5 *d**	78 3 *d*
4	(72 7)† [7]	73 2 *d*	73 2 *d*	73 6 *d*
5	(76 8)† [7]	77 8 *d**	77 8 *d**	77 9 *d*
6	(170 0)† [7]	172 9 *s*	172 9 *s*	173 3 *s*
Xylose				
1	(106 7)† [8]	107 0 *d*	107 1 *d*	—
2	(75 9)† [8]	76 6 *d*	76 6 *d*	—
3	(78 0)† [8]	78 2 *d*	78 2 *d*	—
4	(70 6)† [8]	71 1 *d*	71 2 *d*	—
5	(67 5)† [8]	67 5 *t*	67 6 *t*	—
Glucose				
1	(95 7)† [7]	—	95 7 *d*	95 8 *d*
2	(75 0)† [7]	—	74 2 *d*	74 2 *d*
3	(78 5)† [7]	—	79 0 *d*	79 1 *d*
4	(71 1)† [7]	—	71 4 *d*	71 4 *d*
5	(78 7)† [7]	—	78 9 *d*	79 0 *d*
6	(62 3)† [7]	—	62 4 *t*	62 5 *t*

^{13}C NMR were recorded on a JEOL FX-100 FT-NMR spectrometer (25 15 MHz) The chemical shifts were expressed in δ-values in ppm relative to TMS used as internal standard

*These values are interchangeable within their respective columns

†The chemical shifts in parentheses were those of corresponding position in ginsenoside Rb_2 [6], methyl 3β-*O*-[β-D-glucuronopyranosyl]-oleanate [7], hovenoside G [8] and chikusetsu saponin IV methyl ester [7]

methyl ester [7] (Table 1) The fragmentation pattern in the mass spectrum and the ^{13}C NMR spectrum of **3** suggested that D-glucopyranose was attached at C-28 of **2** In the ^1H NMR spectrum of **3**, three anomeric proton signals were observed at δ 4 99 (*d*, J = 6 8 Hz), 5 25 (*d*, J = 6 1 Hz) and 6.18 (*d*, J = 7 1 Hz) supporting the β-configurations of the glucuronic acid, xylose and glucose moieties Further, the β-D-glucopyranosyl ester linkage was supported by application of Klyne's rule [9] $[M]_D$ (3) − $[M]_D$ (2) = −127°, $[M]_D$ (methyl α-D-glucopyranoside) = +276° [10], $[M]_D$ (methyl β-D-glucopyranoside) = −62° [10] Based on the above results, the structure of **3** was established as 3β-*O*-[β-D-xylopyranosyl-(1 → 2)-β-D-glucuronopyranosyl]-pfaffic acid-(28 → 1)-β-D-glucopyranosyl ester

Pfaffoside C (**4**), $C_{41}H_{62}O_{14}$ $3H_2O$, mp 255–226°, $[\alpha]_D^{22}$ +19 7° (*c* 0 60, MeOH), contained hydroxyl groups (3400 cm^{-1}), carboxyl and ester groups (1730 cm^{-1}), as judged from the IR spectrum Acid hydrolysis of **4** yielded **1** as the aglycone and glucuronic acid and glucose as the sugar moieties Enzymatic hydrolysis of **3** using crude naringinase yielded **4**, designated now as the structure lacking β-D-xylose from **3** Further, a comparison of the ^{13}C NMR spectrum of **4** with that of **3** revealed that the signals due to C-1, C-2 and C-3 of the β-D-glucuronopyranosyl moiety were shifted by +2 0, −8 1 and +0 8 ppm, respectively, while other corresponding signals were almost unshifted (Table 1) Therefore, it is suggested that β-D-xylose is absent from C-2 of the β-D-glucuronopyranosyl moiety in **3** In the ^1H NMR spectrum of **4**, two anomeric proton signals were observed at δ 5 04 (*d*, J = 6 6 Hz) and 6 22 (*d*, J = 6 6 Hz) supporting the β-configurations of the glucuronic acid and glucose moieties In addition, the consideration of the molecular optical rotation by the application of Klyne's rule [9] supported the β-anomeric configurations of all sugar linkages (Found $[M]_D$ = +153°, Calc $[M]_D$ = +213°) The above results led to the formulation of **4** as 3β-*O*-[β-D-glucuronopyranosyl]-pfaffic acid-(28 → 1)-β-D-glucopyranosyl ester

It is interesting that **2**, **3** and **4** are novel structures based on nortriterpene glucuronides and that **2** and **4** show inhibitory effects on the growth of cultured tumor cell melanomas (B-16) at concentrations of *ca* 50 and *ca* 100 μg/ml, respectively, using the method devised by Takemoto *et al* [11] (Fig 1)

EXPERIMENTAL

Mps are uncorr ^1H NMR and ^{13}C NMR spectra were taken in pyridine-d_5, using TMS as internal standard EIMS (direct inlet) was at 70 eV Crude naringinase (Lot No N-8631) was commercially available TLC was conducted on Kieselgel 60 F_{254} (Merck) using solvent A $CHCl_3$–MeOH–H_2O (65 35 10, lower phase), solvent B $CHCl_3$–MeOH (12 1), solvent C *n*-BuOH–AcOH–H_2O (4 1 1), spots were detected by spraying with 10% H_2SO_4, followed by heating

Plant material Roots of *Pfaffia paniculata* were collected in the Goias area of Brazil and identified by Prof G Akisue and Prof F Oliveira at the University of São Paulo in Brazil

Isolation of pfaffic acid (**1**), *the mixture of stigmasterol and sitosterol, their glucosides, allantoin and pfaffosides* (**2**, **3** *and* **4**) The air-dried roots (18 kg) were crushed and treated with hot MeOH (3 × 200 l) Evaporation of the solvent under red pres yielded a brown syrup (2 kg) The MeOH extract (820 g) was suspended in H_2O (500 ml) and treated with *n*-BuOH (5 × 500 ml) The organic layer was evaporated under red pres to yield a brown, gummy mass (140 g), which was divided into water soluble and insoluble portions The water insoluble portion (27 g) was chromatographed on a silica gel (Wakogel C-200) column (700 g) with a gradient of hexane–AcOEt–MeOH Removal of the solvent from the elute with hexane–AcOEt (1 5 → 0 5), followed by recrystallization from EtOH, gave a mixture of stigmasterol and sitosterol (280 mg) Removal of the solvent from the eluate with AcOEt–MeOH (20 0 → 20 1) then yielded the residue (15 g) containing **1** Compound **1** was isolated from the above residue by CC on silica gel (500 g) with a gradient of MeOH in $CHCl_3$ The separation was monitored by TLC (R_f = 0 44, solvent B) Pure **1** was obtained as colourless needles (180 mg) after recrystallization from MeOH The water soluble portion was adsorbed on a charcoal (Wako) column (350 g) Elution was performed with H_2O (500 ml), EtOH (500 ml), EtOH–AcOEt (3 7, 7 1), EtOH–AcOEt (2 8, 5 1), EtOH–AcOEt (1 9, 3 1) and AcOEt (2 1) Evaporation of the solvent from the elute with EtOH–AcOEt (3 7 → 0 7) afforded the crude saponin (19 g) as a pale yellow powder. The crude saponin (5 g) was chromatographed on a silica gel column (150 g) and eluted with $CHCl_3$–MeOH (20 1, 2 1), $CHCl_3$–MeOH (10 1, 1 1), $CHCl_3$–MeOH–H_2O (8 2 0 5, lower phase, 3 1) to afford besides non-investigated pfaffosides a mixture of stigmasteryl-β-D-glucoside and sitosteryl-β-D-glucoside (460 mg) which were purified by recrystallization from EtOH Further elution with $CHCl_3$–MeOH–H_2O (7 3 1, lower phase, 6 1) afforded crude **2**, **4** and **3**, other non-investigated pfaffosides and allantoin (23 mg), purified by recrystallization from MeOH–AcOEt Crude **2**, **4** and **3** were repeatedly subjected to CC on silica gel and eluted with $CHCl_3$–MeOH–H_2O (7 3 1, lower phase) or *n*-BuOH–AcOEt–H_2O (4 1 2, upper phase) to afford chromatographically pure pfaffosides Since the pfaffosides obtained as above were still contaminated with the carboxylate form (IR ν_{max}^{KBr} cm^{-1} 1610), they were dissolved in H_2O, acidified to *ca* pH 4 or treated with a cation-exchange resin (Amberlite IR-120B), and extracted with *n*-BuOH satd with H_2O The *n*-BuOH layer was washed with H_2O and evaporated *in vacuo* to dryness Pure samples of **2**, **4** and **3** were obtained from MeOH–AcOEt as needles (85 mg), amorphous solid (35 mg) and fine crystals (170 mg), respectively

Pfaffic acid (**1**) Mp 285–286°, $[\alpha]_D^{22}$ +109 2° (*c* 0 72, $CHCl_3$) (Found C, 79 3, H, 10 1 $C_{29}H_{44}O_3$ requires C, 79 0, H, 10 1%) IR, MS, ^1H NMR and ^{13}C NMR spectra were described previously [2]

Mixture of stigmasterol and sitosterol The mixture was identified by GC and TLC (GC detector, FID, carrier gas, N_2 at 50 ml/min, inj temp, 300°, column temp, 230°, packed column, 1 m × 3 mm, 1 5% SE-30, stigmasterol, R_t = 16 min, sitosterol, R_t = 18 5 min) (TLC solvent B, R_f = 0 61)

Pfaffoside A (**2**) Mp 268°, $[\alpha]_D^{22}$ +14 8° (*c* 1 85, MeOH) (Found C, 60 1, H, 8 1 $C_{40}H_{60}O_{13}$ $3H_2O$ requires C, 59 8, H, 8 3%) IR ν_{max}^{KBr} cm^{-1} 3400 (OH), 1730 and 1700 (–CO_2H) ^{13}C NMR Table 1

Pfaffoside B (**3**) Mp 255–260°, $[\alpha]_D^{22}$ −1 8° (*c* 1 05, MeOH) (Found C, 57 5, H, 8 0 $C_{46}H_{70}O_{18}$ $3H_2O$ requires C, 57 3, H, 7 9%) IR ν_{max}^{KBr} cm^{-1} 3400 (OH), 1730 (–CO_2– and –CO_2H) ^{13}C NMR Table 1

Pfaffoside C (**4**) Mp 225–226°, $[\alpha]_D^{22}$ +19 7° (*c* 0 60, MeOH) (Found C, 58 8, H, 7 9 $C_{41}H_{62}O_{14}$ $3H_2O$ requires C, 59 1, H, 8 2%) IR ν_{max}^{KBr} cm^{-1} 3400 (OH), 1730 (–CO_2– and –CO_2H) ^{13}C NMR Table 1

Allantoin Allantoin was identified by mmp, HPLC and the comparison of IR spectra with an authentic sample (HPLC detector, 220 nm, mobile phase, 1/50 M KH_2PO_4–1/5 N HCl (pH 3 5), flow rate, 1 5 ml/min, column, 30 cm × 3 9 mm packed

with Nucleosil C_{18}, R_t 2 2 min)

Mixture of stigmasteryl-β-D-glucoside and sitosteryl-β-D-glucoside The mixture was indistinguishable from the authentic samples by TLC and the comparison of IR spectra (TLC solvent A, R_f = 0 63) Acid hydrolysis of the mixture (11 7 mg) yielded a mixture (6 7 mg) of stigmasterol and sitosterol as aglycones, identified by GC, TLC and the comparison of IR spectra with authentic samples, and D-glucose as the sugar component, identified by TLC and PC with an authentic sample (TLC solvent C, R_f, 0 30, PC Toyo Filter Paper No 50, solvent C, R_f, 0 12)

Acid hydrolysis of pfaffosides A (**2**), *B* (**3**) *and C* (**4**) Compounds **2** (15 mg), **3** (12 mg) or **4** (12 mg) were refluxed with 20% H_2SO_4–MeOH (1 1, 7 ml) for 4 hr Reaction mixtures were concd under red pres to remove MeOH Addition of H_2O gave a white ppt, which was collected by filtration and crystallized from MeOH to give colourless needles (5 mg, 3 mg and 4 mg, respectively) identical in every aspect with **1** obtained directly from *Pfaffia paniculata* (GC detector, FID, carrier gas, N_2 at 50 ml/min, inj temp, 300°, column temp, 260°, packed column, 1 m × 3 mm 1 5% SE 30; R_t, 10 6 min) The aq filtrate was concd under red pres, adjusted to pH 5–6 with aq satd $Ba(OH)_2$ and centrifuged The supernatant was further concd under red pres and subjected to TLC to identify the sugar components by comparison with authentic samples (TLC solvent A, glucuronic acid, R_f = 0 02, xylose, R_f = 0 18, glucose, R_f = 0 10)

Alkali hydrolysis of pfaffoside B (**3**) A soln of **3** (100 mg) in 1 N KOH (7 ml) was heated under N_2 gas flow at 95° for 3 hr The reaction mixture was cooled to room temp, neutralized with 1 N HCl, and extracted with *n*-BuOH The extract was washed with H_2O and evaporated *in vacuo* The residue was repeatedly recrystallized from MeOH–AcOEt to afford colourless needles (65 mg) identical with **2** by mmp, TLC, IR and elemental analysis (TLC solvent A, R_f, 0 14)

Enzymatic hydrolysis of pfaffoside B (**3**) In accordance with the method of Kitagawa *et al* [12], enzymatic hydrolysis of **3** (147 mg), for 12 hr using crude naringinase (2 g), afforded **4** (63 mg), identified by mmp, TLC, elemental analysis and IR comparison with an authentic sample (TLC solvent A, R_f, 0 11)

Per-O-methylation of pfaffosides A (**2**) *and B* (**3**) Following the method of Kondo *et al* [10], **2** (40 mg) and **3** (200 mg) were methylated by the Kuhn procedure

Per-*O*-methyl pfaffoside A 17 mg, mp 85–87° (crystals from aq EtOH) (Found C, 66 0; H, 8 8 $C_{47}H_{74}O_{13}$ requires C, 66 6, H, 8 8%) IR ν_{max}^{KBr} cm^{-1} 1745 (–CO_2–) EIMS (probe) 70 eV, *m/z* (rel int) 846 $[M]^+$ (0 0), 437 $[M-C_{17}H_{29}O_{11}]^+$ (9 8), 393 $[C_{17}H_{29}O_{10}]^+$ (2 3), 246 $[C_{16}H_{22}O_2]^+$ (100), 175 $[C_8H_{15}O_4]^+$ (91 2)

Per-*O*-methyl pfaffoside B 85 mg, mp 230–232° (crystals from aq EtOH) (Found C, 64 0, H, 8 9 $C_{56}H_{90}O_{18}$ requires C, 64 0; H, 8 6%) IR ν_{max}^{KBr} cm^{-1} 1760 and 1750 (–CO_2–) EIMS (probe) 70 eV, *m/z* (rel int) 1050 $[M]^+$ (0 0), 787 $[M-C_{11}H_{19}O_7]^+$ (0 2), 641 $[M-C_{17}H_{29}O_{11}]^+$ (0 9), 450 $[C_{25}H_{38}O_7]^+$ (2 9), 393 $[C_{17}H_{29}O_{10}]^+$ (1 8), 219 $[C_{10}H_{19}O_5]^+$ (25 9), 175 $[C_8H_{15}O_4]^+$ (100)

Inhibitory effects of pfaffic acid (**1**) *and pfaffosides* **2**, **3** *and* **4** *on the growth of melanoma* (*B*-16) Melanoma (B-16) cells were propagated in a culture medium composed of 70% L-15 and 30% Ham's F-10, supplemented with 2% fetal bovine serum, so that the prepared medium afforded 50% growth of the melanoma (B-16) cells Cells were plated at 1×10^5 cells per 60 mm dish in 5 ml of the culture medium preparations containing **1**, **2**, **3** and **4**, supplemented with 2% fetal bovine serum and incubated at 37° in 5% CO_2 and 95% air Four days after plating, the cells were washed twice with 5 ml saline, treated with 0 5 ml of 0 125% trypsin in calcium- and magnesium-free Hank's soln, and resuspended in 4 5 ml of saline The samples were counted in a Toa Microcellcounter CC-108

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