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FURTHER STEROIDAL AND FLAVONOID CONSTITUENTS OF THE SWEET PLANT, *POLYPODIUM GLYCYRRHIZA**

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Key Word Index—*Polypodium glycyrrhiza*, Polypodiaceae, steroidal constituents, polypodoside B, polypodoside C, sweetness assessment, flavonoids

Abstract—Two novel steroidal glycosides, polypodosides B and C, as well as three known compounds, polypodine B, (+)-catechin and (+)-afzelechin, were isolated from the rhizomes of the sweet plant, *Polypodium glycyrrhiza* By the application of various spectral methods, polypodosides B and C were assigned the structures, 26-*O*-α-L-rhamnopyranosyl-polypodogenin-3-*O*-β-D-glucopyranoside and 26-*O*-α-L-acofriopyranosyl-polypodogenin-3-*O*-β-D-glucopyranoside, respectively. Polypodoside B tasted sweet, although polypodoside C was devoid of this effect

INTRODUCTION

In previous work, we have established that the sweetness of the rhizomes of the North American fern, *Polypodium glycyrrhiza* D.C. Eaton (Polypodiaceae), is mainly due to the presence of the steroidal glycoside, polypodoside A [**1**, 26-*O*-α-L-rhamnopyranosyl-polypodogenin-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside] [1]. The structure and stereochemistry of polypodogenin (**2**), the known aglycone of **1**, were established as (22*S*, 25*R*, 26*R*)-3β,26-dihydroxy-22,26-epoxy-6-oxo-5α-cholest-7-ene by Czechoslovakian workers [2–4] Compound **1** was converted to its aglycone, **2**, using the enzyme, hesperidinase, and polypodogenin (**2**) was fully characterized using modern spectroscopic methods [1] Polypodoside A (**1**) was found to be nonmutagenic and not acutely toxic for mice, and was rated by a taste panel as being 600 times sweeter than a 6% w/v sucrose solution [1]. Other documented constituents of this plant part are the flavonoids, (+)-afzelechin-7-*O*-β-D-apioside and polydin, which proved to be bitter and neutral-tasting, respectively [5], as well as sucrose [6] and several aromatic acids [7]. In the present communication, we wish to report the isolation of additional steroidal and phenolic constituents of *P. glycyrrhiza* rhizomes, inclusive of two novel analogues of polypodoside A, which we have named polypodoside B (**3**) and polypodoside C (**4**), and the known compounds, polypodine B, (+)-catechin and (+)-afzelechin. A preliminary account of structure–sweetness relationships within the polypodogenin glycoside class of intense sweeteners is presented.

RESULTS AND DISCUSSION

As a result of the observation of a lithium-catalysed molecular ion occurring at *m/z* 745 in its low-resolution FABMS, the M_r of polypodoside B (**3**) was determined as 738 The molecular formula of this isolate was confirmed as $C_{39}H_{62}O_{13}$ by high-resolution FABMS The ^{13}C NMR chemical shifts of this compound were assigned with the assistance of a ^{1}H–^{13}C heteronuclear chemical shift correlated (HETCOR) NMR experiment, and, after comparison with the ^{13}C NMR spectrum of polypodoside A (**1**) obtained in our earlier investigation [1], it was apparent that polypodoside B (**3**) was also a glycoside of polypodogenin (**2**), that varied structurally from **1** only its saccharide moieties, and that these sugar units were also affixed to the C-3 and C-26 positions.

The sugar units obtained after acid hydrolysis of compound **3** were identified by GC-MS and TLC as D-glucose and L-rhamnose The linkages of the saccharide units to the aglycone unit of **3** were conveniently established using the selective INEPT NMR technique [1, 5, 8, 9]. The signals appearing at δ5.05 and 5 64, which in turn were found to correspond to carbon signals appearing at δ102.19 and 101.87, were assignable, respectively, to the anomeric protons of the glucose and rhamnose units of polypodoside B (**3**). Thus, when the proton at δ5 05 was irradiated ($^{3}J_{CH}$=6 Hz), only C-3 (δ76 89) in the aglycone moiety was enhanced, although an enchancement was also observed for C-3′ (δ78 44) of the glucose unit Similarly, when the C-1″ proton of the rhamnose unit of **3** was irradiated at δ5.64 ($^{3}J_{CH}$ = 6 Hz), C-26 (δ107.25) of the aglycone was enhanced, as were C-2″ (δ72.70), C-3″ (δ72.04), and C-5″ (δ70 36) of the rhamnose unit. In this manner, it was established that the D-glucose and the L-rhamnose units were affixed, respectively, to positions C-3 and C-26 in the molecule of **3**. The configurations of the anomeric protons of the sugar units of polypodoside B (**3**) were determined as 3β- and 26α-, by comparison of the ^{13}C NMR spectrum of this isolate with analogous data for sugars of known configurations [10, 11] Therefore, polypodoside B (**3**) was assigned the structure, 26-*O*-α-L-rhamnopyranosyl-polypodogenin-3-*O*-β-D-glucopyranoside

The M_r of polypodoside C (**4**) was proposed as 752, as protonated and sodium-cationized molecular ions were

*Part 16 in the series 'Potential Sweetening Agents of Plant Origin' For part 15, see ref. [1]

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apparent at *m/z* 753 and 775, in its low-resolution FABMS, and the elemental formula of this compound was confirmed as $C_{40}H_{64}O_{13}$ by high-resolution FABMS After the ^1H and ^{13}C NMR spectra of **4** were correlated by a ^1H–^{13}C HETCOR experiment, it was apparent that this compound was again based on the aglycone, polypodogenin (**2**), and differed from **3** in only one of its saccharide moieties

Enzymatic hydrolysis of compound **4** with β-glucosidase afforded compound **5** and D-glucose, with this sugar being identified by GC-MS and TLC The D-glucose unit was determined as being attached to C-3 in **4**, as a result of the observation of an upfield shift from δ 76 83 in the C-3 resonance in the ^{13}C NMR spectrum of **4** to δ70 67 in that of **5**. This was confirmed by a selective INEPT experiment, in which irradiation of the anomeric proton at C-1′ (δ 5 07, $^3J_{CH}$ = 6 Hz) of **4** resulted in the enhancement of C-3 (δ76.83). Therefore, polypodoside C (**4**) was identical to polypodoside B (**3**) in terms of the sugar unit attached to C-3

That the other sugar unit of polypodoside C (**4**) was attached to the C-26 position was confirmed by a selective INEPT NMR experiment When the anomeric proton at C-1″ was irradiated (δ5 65, $^3J_{CH}$ = 6 Hz), carbon C-26 was the only carbon on the aglycone of **4** to be enhanced In the ^1H NMR spectrum of compound **4**, a methoxy group signal appeared at δ3 56, and the position of this functionality was investigated by a further selective INEPT experiment Irradiation of the methoxy group at δ3 56 ($^3J_{CH}$ = 8 Hz) led to the enhancement of C-3″ (δ82.62) on the C-26-affixed saccharide unit, and showed unambiguously that the methoxy group was not directly attached to the aglycone of **4**, as in the case of the known compound 26-*O*-methylpolypodosaponin [2] The methoxy group of this sugar unit was confirmed as occurring at the 3″-position, since the ^{13}C NMR data observed for this sugar unit closely correlated with those obtained for the L-acofriose (=3-*O*-L-rhamnose) unit of the compound, digitoxigenin α-L-acofrioside [12]. The identity of this sugar was confirmed after hydrolysis of compound **5** using methanolic HCl and direct comparison with an authentic sample of methyl L-acofrioside Acofriose is of apparently limited distribution in the plant kingdom [13] The configuration of this sugar unit at C-26 was assigned as α- on the basis of ^{13}C NMR chemical shift comparison of the respective anomeric carbons of compounds **1** [1] and **3** The structure of compound **4** was therefore assigned as 26-*O*-α-L-acofriopyranosyl-polypodogenin-3-*O*-β-D-glucopyranoside

Three nonglycosidic isolates were also obtained in this investigation The ecdysterol, polypodine B, was identified by interpretation of its spectral characteristics, and confirmed direct by comparison with the authentic substance Also isolated were the flavonoids, (+)-catechin and (+)-afzelechin, which were both identified by comparison with authentic standards Herout and coworkers obtained polypodine B and (+)-catechin as constituents of another species in the genus *Polypodium*, namely, *P. vulgare* [14, 15]

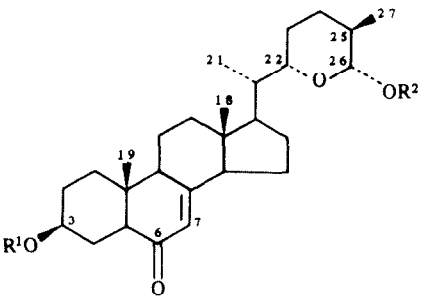
In the present investigation, polypodosides B (**3**) and C (**4**) were obtained as minor polypodogenin glycoside constituents of *P. glycyrrhiza* rhizomes During the fractionation procedure that led to the isolation of polypodoside A (**1**) as the major intensely sweet principle of this plant part, it was found that polypodoside B (**3**) was slightly sweet, while the corresponding 3″-*O*-methyl derivative, polypodoside C (**4**) was devoid of this effect Although a detailed sensory evaluation of polypodoside B (**3**) was not carried out, it was apparent that it was less intensely sweet than polypodoside A (**1**) It may be pointed out that the known monodesmosidic polypodogenin analogue, polypodosaponin (**6**), was not associated with the sweetness of its plant of origin, *P. vulgare*, since this taste sensation was attributed entirely to the steroidal saponin, osladin, a compound based on a different aglycone to polypodosides A and B [1–4, 15] Therefore, these observations indicate that sweet polypodogenin glycosides must be bidesmosidic to exhibit a sweet taste, with saccharide substitution occurring at the C-3 and C-26 positions Any alteration in either of these sugar units apparently profoundly affects sweetness

EXPERIMENTAL

Mps uncorr UV EtOH, IR KBr disc ^1H and ^{13}C NMR spectra were recorded on 300 or 360 MHz instruments with TMS as int std EIMS (70 eV) data were measured with a direct probe

Plant material The rhizomes of *Polypodium glycyrrhiza* D C Eaton (Polypodiaceae) were collected in south Oregon in the autumn of 1983 Specimens documenting these collections have been deposited in the herbarium of the Field Museum of Natural History, Chicago, Illinois

Extraction and isolation procedure The extraction of the air-dried, milled plant material with 80% MeOH–H_2O, partition of the residue on drying between *n*-BuOH and H_2O, and CC of the *n*-BuOH extract over silica gel, have been described previously [1] A portion (1 38 g) of a fraction eluted by $CHCl_3$–MeOH (7 1) was further purified by gel filtration chromatography over Sephadex® LH-20 (100 g) using MeOH as eluent An initial fraction (0 97 g) was purified by low-pressure CC (silica gel, 100 g, 230–400 mesh, eluent $CHCl_3$–MeOH–H_2O (13 7 2, lower layer) to afford polypodoside B (**3**, 280 mg, 0 05% w/w), while a second fraction (0 34 g) yielded (+)-catechin (15 mg, 0.0028% w/w) and (+)-afzelechin (5 mg, 0 0010% w/w) after prep TLC using $CHCl_3$–MeOH–H_2O (13.7 2, lower layer) as developing solvent A portion (3 53 g) of a fraction eluted by $CHCl_3$–MeOH (8 1) after gel filtration chromatography was



	R¹	R²
1	β-D-glc²-α-L-rha	α-L-rha
2	H	H
3	β-D-glc	α-L-rha
4	β-D-glc	α-L-rha-3-Me
5	H	α-L-rha-3-Me
6	β-D-glc²-α-L-rha	H

further purified by low-pressure CC (silica gel, 300 g, 230–400 mesh, eluent, $CHCl_3$–MeOH–H_2O (6:3:1, lower layer), and reversed-phase CC (RP-8, Merck, size B, gradient elution from H_2O to H_2O–MeOH mixture) to provide polypodoside C (**4**, 1.2 g, 0.06% w/w) and polypodine B (350 mg, 0.02% w/w)

Polypodoside B (**3**) Colourless needle-shaped crystals from MeOH; mp 207–209°, $[\alpha]_D$ −27.0° (MeOH, *c* 0.1), UV λ_{max} nm 244 (log ε, 4.08); IR, ν_{max} cm^{-1} 3420, 1666, 1382, 1142, 1094, 1062, 1031, 984, ^1H NMR (360 MHz, pyridine-d_5) δ 5.85 (1H, *br s*, H-7), 5.64 (1H, *br s*, H-1''), 5.05 (1H, *d*, *J* = 7.6 Hz, H-1'), 4.47 (1H, *d*, *J* = 8 Hz, H-26), 3.46 (1H, *m*, H-22), 1.68 (3H, *d*, *J* = 6 Hz, H-6''), 1.02 (3H, *d*, *J* = 6 Hz, H-21), 0.91 (3H, *d*, *J* = 6 Hz, H-27), 0.74 (3H, *s*, H-19), 0.49 (3H, *s*, H-18), ^{13}C NMR 90.8 MHz, pyridine-d_5) δ 198.72 (*s*, C-6), 162.99 (*s*, C-8), 123.19 (*d*, C-7), 107.25 (*d*, C-26), 102.19 (*d*, C-1'), 101.87 (*d*, C-1''), 78.44 (*d*, C-3'), 78.35 (*d*, C-5'), 78.10 (*d*, C-22), 76.89 (*d*, C-3), 75.24 (*d*, C-2'), 73.87 (*d*, C-4''), 72.70 (*d*, C-2''), 72.04 (*d*, C-3''), 71.69 (*d*, C-4'), 70.36 (*d*, C-5''), 62.88 (*t*, C-6'), 55.03 (*d*, C-14), 53.18 (*d*, C-5), 52.85 (*d*, C-17), 49.92 (*d*, C-9), 44.68 (*s*, C-13), 40.35 (*d*, C-20), 38.83 (*t*, C-12), 38.28 (*t*, C-1), 36.79 (*s*, C-10), 36.40 (*d*, C-25), 31.47 (*t*, C-24), 29.32 (*t*, C-2), 27.30 (*t*, C-4), 26.95 (*t*, C-16), 23.99 (*t*, C-23), 22.85 (*t*, C-15), 21.88 (*t*, C-11), 18.37 (*q*, C-6''), 16.65 (*q*, C-27), 13.84 (*q*, C-21), 13.02 (*q*, C-19), 12.12 (*q*, C-18), EIMS, 70 eV, *m/z* (rel int.) 738 $[M^+]$ (missing), 574 $[M-162]^+$ (26), 430 [aglycone] (11), 413 [aglycone−OH] (49), 395 (94), 97 (100), FABMS, (DTE/DTT), *m/z* 761 $[M+Na]^+$, FABMS, (LiI/3-NBA), *m/z* 745 $[M+Li]^+$, HR-FABMS, mass measurement, found, 745.4320, calcd for $C_{39}H_{62}O_{13}Li$, 745.4359 Polypodoside B (**3**, 3 mg) was dissolved in 1 M HCl dioxane–H_2O (1:1) (3 ml), and hydrolysed for 4 hr at 100° The reaction mixture was partitioned between $CHCl_3$ and H_2O after neutralization utilizing Ag_2CO_3. The sugar fraction 1 mg) was found to contain D-glucose and L-rhamnose, which were identified by GC-MS and TLC.

Polypodoside C (**4**) Colourless needle-shaped crystals from MeOH, mp 200–202°; $[\alpha]_D$ −26.3° (MeOH, *c* 0.3), UV λ_{max} nm 244 (log ε 4.16), IR ν_{max} cm^{-1}. 3450, 1659, 1384, 1143, 1102, 1074, 1058, 1048, 1033, 987, ^1H NMR (360 MHz, pyridine-d_5) δ 5.86 (1H, *br s*, H-7), 5.65 (1H, *br s*, H-1''), 5.07 (1H, *d*, *J* = 7 Hz, H-1'), 4.50 (1H, *d*, *J* = 8 Hz, H-26), 3.56 (3H, *s*, 3''-OMe), 3.49 (1H, *m*, H-22), 1.67 (3H, *d*, *J* = 6 Hz, H-6''), 1.05 (3H, *d*, *J* = 6 Hz, H-21), 0.99 (3H, *d*, *J* = 6 Hz, H-27), 0.74 (3H, *s*, H-19), 0.50 (3H, *s*, H-18); ^{13}C NMR (90.8 MHz, pyridine-d_5) δ 198.78 (*s*, C-6), 163.04 (*s*, C-8), 123.21 (*d*, C-7), 107.19 (*d*, C-26), 102.17 (*d*, C-1'), 101.74 (*d*, C-1''), 82.62 (*d*, C-3''), 78.48 (*d*, C-3'), 78.43 (*d*, C-5'), 78.15 (*d*, C-22), 76.83 (*d*, C-3), 75.26 (*d*, C-2'), 72.14 (*d*, C-4''), 71.67 (*d*, C-4'), 70.38 (*d*, C-2''), 67.85 (*d*, C-5''), 57.07 (*q*, 3''-OMe), 55.02 (*d*, C-14), 53.15 (*d*, C-5), 52.82 (*d*, C-17), 49.90 (*d*, C-9), 44.67 (*s*, C-13), 40.36 (*d*, C-20), 38.81 (*t*, C-12), 38.28 (*s*, C-10), 36.78 (*t*, C-1), 36.42 (*d*, C-25), 31.48 (*t*, C-24), 29.31 (*t*, C-2), 27.30 (*t*, C-4), 26.97 (*t*, C-16), 23.95 (*t*, C-23), 22.85 (*t*, C-15), 21.87 (*t*, C-11), 18.37 (*q*, C-6''), 16.72 (*q*, C-27), 13.85 (*q*, C-21), 13.02 (*q*, C-19), 12.11 (*q*, C-18), EIMS, 70 eV, *m/z* (rel int.) 752 $[M^+]$ (missing), 430 [aglycone] (23), 413 [aglycone−OH] (63), 395 (40), 43 (100); FABMS, (3-NBA), *m/z* 775 $[M+Na]^+$, 753 $[M+H]^+$, HR-FABMS, mass measurement, found, 753.4437, calcd for $C_{40}H_{65}O_{13}$, 753.4425

Polypodoside C (**4**, 300 mg) was dissolved in 2% EtOH (50 ml), and hydrolysed by the addition of β-glucosidase (500 mg) The reaction mixture was incubated at 37° for 24 hr, and extracted with EtOAc (3 × 50 ml) The EtOAc fraction was dried *in vacuo* to yield 91 mg of 26-*O*-α-L-acofriopyranosyl-polypodogenin (**5**). The water layer was found by GC-MS and TLC to contain only D-glucose. Compound **5** exhibited the following data: colourless amorphous powder from $CHCl_3$, mp 142–144°, $[\alpha]_D$ −35.0° ($CHCl_3$, *c* 0.1), UV λ_{max} nm. 243 (log ε 3.86), IR, ν_{max} cm^{-1} 3435, 1665, 1144, 1108, 1100, 1061, 1031, 1012, ^1H NMR (300 MHz, $CDCl_3$) δ 5.73 (1H, *br s*, H-7), 5.08 (1H, *br s*, H-1'), 4.24 (1H, *d*, *J* = 8 Hz, H-26), 3.49 (3H, *s*, 3'-OMe), 1.26 (3H, *d*, *J* = 6 Hz, H-6'), 0.98 (3H, *d*, *J* = 7 Hz, H-21), 0.89 (3H, *d*, *J* = 6 Hz, H-27), 0.87 (3H, *s*, H-19), 0.61 (3H, *s*, H-18), ^{13}C NMR (90.8 MHz, $CDCl_3$): δ 199.58 (*s*, C-6), 163.33 (*s*, C-8), 123.11 (*d*, C-7), 106.99 (*d*, C-26), 99.59 (*d*, C-1'), 81.21 (*d*, C-3'), 78.00 (*d*, C-22), 71.60 (*d*, C-4'), 70.67 (*d*, C-3), 68.15 (*d*, C-5'), 66.83 (*d*, C-2'), 56.95 (*q*, 3'-OMe), 55.23 (*d*, C-14), 53.32 (*d*, C-5), 52.73 (*d*, C-17), 50.05 (*d*, C-9), 44.70 (*s*, C-13), 40.12 (*d*, C-20), 38.80 (*t*, C-12), 38.19 (*s*, C-10), 36.82 (*t*, C-1), 35.87 (*d*, C-25), 31.20 (*t*, C-24), 30.40 (*t*, C-2), 30.24 (*t*, C-4), 26.89 (*t*, C-16), 23.53 (*t*, C-23), 22.62 (*t*, C-15), 21.78 (*t*, C-11), 17.27 (*q*, C-6'), 16.47 (*q*, C-27), 13.62 (*q*, C-21), 13.20 (*q*, C-19), 12.20 (*q*, C-18); EIMS, 70 eV, *m/z* (rel int.) 590 $[M^+]$ (1), 430 [aglycone] (34), 413 [aglycone-H_2O] (70); HRMS, mass measurement, found, 590.3811, calcd for $C_{34}H_{54}O_8$, 590.3819. Compound **5** (3 mg) was dissolved in a 1 M MeOH–HCl soln (3 ml), and hydrolysed for 4 hr at 100° The reaction mixture was neutralized by Ag_2CO_3, and partitioned between $CHCl_3$ and H_2O By GC-MS and TLC, the sugar fraction (1 mg) was found to contain only methyl α-L-acofrioside

Polypodine B was recrystallized from MeOH as colourless needles, mp undepressed on mixing with polypodine B isolated from *P. vulgare* This compound exhibited closely comparable physical and UV, IR, ^1H NMR, ^{13}C NMR and EIMS data to those published previously [14, 16, 17]

(+)-Catechin was identified by comparison of its physical and spectral characteristics with those of published data [18, 19], and confirmed by direct comparison with an authentic sample purchased from Aldrich Chemical Co., Milwaukee, Wisconsin

(+)-Afzelechin was identified by comparison of its physical and spectral data with published values [20, 21], and confirmed by direct comparison with an authentic sample isolated from *Saxifraga ligulata* Wall

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