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POLYPODOSIDE A, AN INTENSELY SWEET CONSTITUENT OF THE RHIZOMES OF *POLYPODIUM GLYCYRRHIZA*[1]

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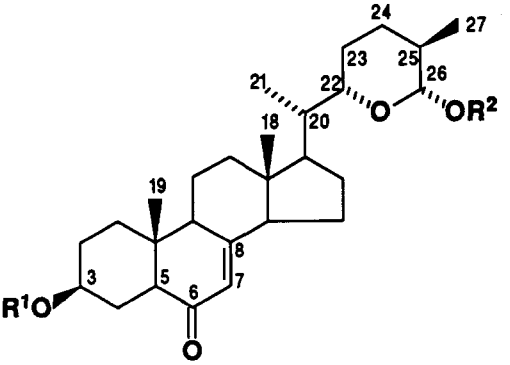
ABSTRACT.—Polypodoside A, a novel intensely sweet constituent of the rhizomes of *Polypodium glycyrrhiza*, was established by spectral and chemical methods as 26-*O*-α-L-rhamnopyranosyl-polypodogenin-3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside [**1**]. At the doses tested, this isolate was not acutely toxic for mice and was nonmutagenic with *Salmonella typhimurium* strain TM677. This compound was rated by a human taste panel as exhibiting 600 times the sweetness intensity of a 6% w/v aqueous sucrose solution.

As part of a research program directed towards the discovery of intensely sweet naturally occurring molecules, we have investigated the North American species *Polypodium glycyrrhiza* DC. Eaton (Polypodiaceae) (licorice fern). The rhizomes of this fern exhibit a bittersweet taste and have a history of human use in the Pacific northwest region, both as a foodstuff and as a medicinal agent (2,3). In an initial study in 1930, the fluid extract of *P. glycyrrhiza* rhizomes was found to be nontoxic for rats, and the triterpene glycoside, glycyrrhizin, was identified as a highly sweet constituent of this plant part in 2.36% w/w yield, as a result of a quantitative assay procedure (2). In a later investigation, glycyrrhizin was disputed as a *P. glycyrrhiza* constituent, and the sweet taste of the rhizomes was attributed to the presence of sugars such as sucrose and small amounts of an uncharacterized substance (4). No further report to resolve this issue has appeared subsequently in the literature. Recently, we have established that the bitterness of *P. glycyrrhiza* rhizomes is due to the flavonoid, (+)-afzelechin-7-*O*-β-D-apioside (5). In the present communication we report the structure of polypodoside A, the major intensely sweet constituent of this species, as 26-*O*-α-L-rhamnopyranosyl-polypodogenin-3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside [**1**]. Preliminary safety assessment and sensory evaluation data have also been obtained for this isolate.



	R^1	R^2
1	β-D-glc[2]-α-L-rha	α-L-rha
2	H	H

glc = glucopyranosyl, rha = rhamnopyranosyl

[1]Paper No. XV in the series, "Potential Sweetening Agents of Plant Origin." See Compadre *et al.* (1) for part XIV.

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RESULTS AND DISCUSSION

The mol wt of polypodoside A [**1**] was determined as *m/z* 884 as a result of observations of protonated and sodium-cationized molecular ions at *m/z* 885 and 907, respectively, in the low-resolution fast-atom bombardment (fab) mass spectrum of this compound. In addition, the elemental formula of **1** was confirmed as $C_{45}H_{72}O_{17}$ by hrfabms. Enzymatic hydrolysis of compound **1** with hesperidinase afforded D-glucose and L-rhamnose, which were identified by direct comparison with authentic samples by gc-ms and tlc. A fragment peak appearing at *m/z* 739 $[M + H - 146]^+$ in the low-resolution fab mass spectrum of **1** indicated the loss of a terminal rhamnosyl unit from the intact glycoside, whereas the observation of fragmentation ions at *m/z* 577 [*m/z* 739 − 162] and 431 [*m/z* 577 − 146] suggested that the residual sugar units were glucose and rhamnose, respectively. Furthermore, a diagnostic fragment peak at *m/z* 309 in the fab mass spectrum was consistent with the presence of a dihexosyl group consisting of one unit each of glucose and rhamnose. The abundant ion at *m/z* 431 in the fab mass spectrum was representative of the protonated aglycone moiety of this glycoside, whereas sequential losses of 18 mass units from the aglycone peak suggested the presence of two hydroxy groups in the aglycone moiety.

Enzymatic hydrolysis of **1** also afforded the aglycone of this sweet compound, which was identified as the known steroid polypodogenin [**2**] (6), largely by interpretation of its spectral data, because no authentic polypodogenin was available for comparison purposes. It may be pointed out that this compound was characterized before the routine availability of spectral techniques such as high-field ^{1}H- and ^{13}C-nmr, thereby making comparison of data obtained for **2** with published spectral data of polypodogenin somewhat difficult. Polypodogenin [**2**] has been established as the $\Delta^{7,8}$-derivative of (22*S*,25*R*,26*R*)-22,26-epoxy-3β,26-dihydroxy-5α-cholestan-6-one, the aglycone of the sweet compound, osladin, that was isolated from a fern in the same genus as *P. glycyrrhiza*, namely, *Polypodium vulgare* L. (6–8). The stereochemistry of the aglycone of osladin was determined after its synthesis from solasodine by Havel and Černý (8).

Inferences on the functionality and stereochemistry of compound **2** that led to its assignment as polypodogenin were made on the basis of the spectral comparison of this hydrolytic product with published data for known steroids. Thus, the absorption band appearing at 1669 cm^{-1} in the ir spectrum of compound **2** indicated the presence of an α,β-unsaturated ketone in the molecule, whereas the absorption maximum at 243 nm in its uv spectrum was consistent with the presence of an enone functionality (6). The proposed identity of **2** was further supported by the observation of prominent peaks in its electron-impact mass spectrum at *m/z* 316, 261, and 115, which provided evidence for a similar structural integrity of its skeleton and side-chain to osladin aglycone (6). When the ^{13}C-nmr chemical shifts of the steroid portion of this aglycone were compared with those of the model compound, 3-acetoxy-5α-ergosta-7,22-dien-6-one (9), resonances in the two spectra were within the range calculated when substituent-induced effects are taken into account (10,11). The stereochemistry of the C-5 proton of **2** was determined as α by the comparison of its C-19 methyl group chemical shift (δ 13.21) with ^{13}C-nmr data for known 5α and 5β steroids and steroidal saponins (9, 12–15). Such resonances vary by 11–12 ppm for 5α and 5β steroids, because of shielding effects by protons attached to C-2 and C-4 (16). The C-21 methyl group stereochemistry was confirmed as α by comparison of this chemical shift with those of C-21 methylated model steroids (17). The observation of a coupling constant of 8 Hz at δ 4.32 (H-26β) in the ^{1}H-nmr spectrum of compound **2** was indicative of the diaxial relationship of the protons at C-25 and C-26 that has been demonstrated for osladin aglycone (8).

Assignments of the ^{1}H- and ^{13}C-nmr spectra of polypodoside A [**1**] were assisted by the performance of a ^{1}H-^{13}C heteronuclear chemical shift correlated nmr experiment.

Characteristic paramagnetic glycosylation shifts of C-3 (5.63 ppm) and C-26 (5.99 ppm), as well as upfield shifts of C-2, C-4, and C-25, were observed when the ^{13}C-nmr chemical shifts of polypodoside A [**1**] were compared with those of polypodogenin [**2**]. This indicated that the saccharide units of **1** were connected to C-3 and C-26 (13,18,19). The positions of the sugar linkages in **1** were differentiated by the application of the selective INEPT nmr technique (20), whose effectiveness in determining the position of the sugar attachment to a flavanoid aglycone was recently demonstrated (5).

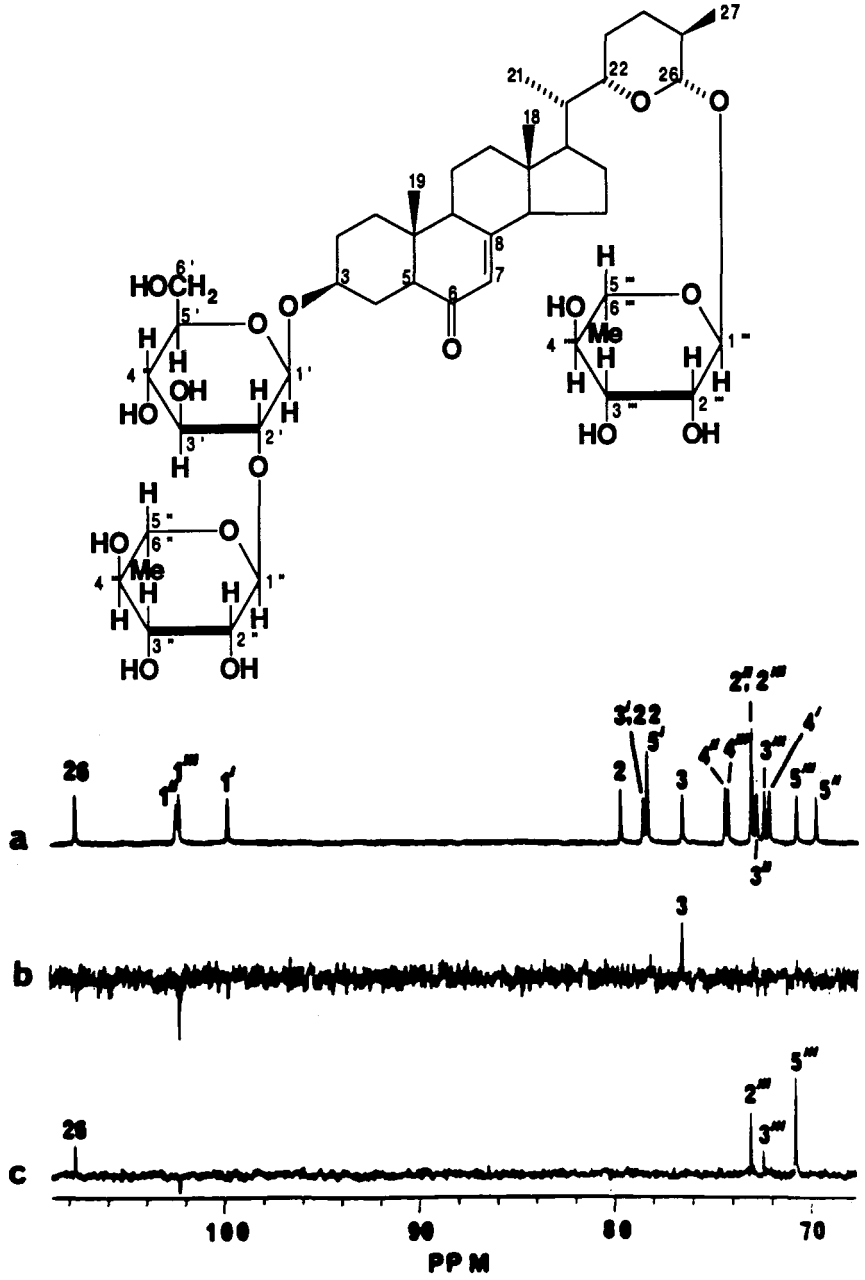

FIGURE 1. Downfield region of the decoupled ^{13}C-nmr spectrum of polypodoside A [**1**]. (a) Proton-noise decoupled spectrum; (b,c) SINEPT spectra obtained by irradiation of H-1′ (δ 5.02) and H-1‴ (δ 5.61), respectively.

Signals resonating at δ 5.02 and δ 99.53 ppm in the ^{1}H- and ^{13}C-nmr spectra of compound **1**, respectively, were assignable to C-1′ of the glucose unit, which is attached to the C-3 position of the aglycone moiety. When the anomeric proton at δ 5.02 ppm (H-1′) was irradiated ($^3J_{CH}$ = 8 Hz), the carbon at position 3 of the aglycone moiety was selectively enhanced (Figure 1b). The anomeric resonances appearing at δ 6.26 and δ 102.17 ppm in the ^{1}H- and ^{13}C-nmr spectra of **1** were assigned to C-1″ of the rhamnopyranosyl unit attached to the 2′-position of glucopyranose (21). Therefore, the configuration of this dihexosyl unit, β-neohesperidose, was determined as α-L-rhamnopyranosyl-(1↦2)-β-D-glucopyranose by comparison of its carbon chemical shifts with those of published ^{13}C-nmr data (22,23). The signals appearing at δ 5.61 and δ 102.03 ppm in the ^{1}H- and ^{13}C-nmr spectra of polypodoside A [**1**] were assigned to H-1‴ and C-1‴, respectively, of the rhamnose unit, which was attached to the C-26 position of the aglycone moiety. When the proton signal at δ 5.61 ($^3J_{CH}$ = 8 Hz) was irradiated, the resonance corresponding to C-26 was selectively enhanced at δ 107.32 ppm, which clearly indicated the linkage of this saccharide unit to the C-26 position (Figure 1c). A comparison of the ^{13}C chemical shifts of the sugar carbons in the rhamnose attached to C-26 with those of methyl-α-L-rhamnopyranoside and methyl-β-L-rhamnopyranoside suggested an α configuration of this sugar unit (19).

The structure of **1** was, therefore, determined as 26-*O*-α-L-rhamnopyranosylpolypodogenin-3-*O*-α-L-rhamnopyranosyl-(1↦2)-β-D-glucopyranoside. On biogenetic grounds, it may be postulated that compound **1** represents the $\Delta^{7,8}$-derivative of the known sweet compound, osladin, although the configuration of the C-26-affixed rhamnosyl unit of osladin was not determined at the time of its isolation (7) and has not been investigated in the intervening period. It is because of this remaining structural uncertainty that a trivial name based on osladin has not been chosen for compound **1**. The sweetness potency of osladin relative to sucrose has been placed in the range 300–3000× (24), and it has been shown in the present investigation that polypodoside A [**1**] is 600× sweeter than 6% w/v aqueous sucrose.

While compound **1** is, therefore, an extremely sweet substance and occurs in its plant of origin in about 10× the yield of osladin in *P. vulgare* rhizomes (25), it is unlikely that this compound will be suitable for commercial application as a sucrose substitute for several reasons. Polypodoside A [**1**] exhibits undesirable hedonic effects when tasted and is only sparingly soluble in H_2O. The rhizomes of *P. glycyrrhiza* have been shown to grow extremely slowly during cultivation (4) and are now very difficult to collect from natural populations. Also, while polypodoside A [**1**] was determined as nontoxic and nonmutagenic in preliminary safety studies in this investigation, it contains an α,β-unsaturated ketone functionality in its algycone moiety, which conceivably could produce undesirable associated biological effects. For example, compounds bearing this functionality have previously been shown to mediate a mutagenic response (1,26,27). Thus, although compound **1** does not appear to be active in this capacity, the potential of its metabolic products to mediate a toxic response would need to be carefully examined. Further, we have recently found that stizophyllin, a novel steroid derivative isolated from *Stizophyllum riparium* (H.B.K.) Sandw. (28), mediates a cytotoxic response by virtue of its α,β-unsaturated carbonyl group and specifically blocks cultured P-388 cells in the G_2 phase of the cycle (manuscript in preparation). A variety of possible adverse biological effects would, therefore, have to be taken into account.

Nonetheless, the current study provides further information on the nature of the intensely sweet principles produced by the genus *Polypodium* and demonstrates the use of contemporary nmr techniques in establishing the saccharide-moiety configurations and

linkages in a sweet glycoside. Further investigation of the relationship between the structure and sweetness of steroidal saponins such as polypodoside A [**1**] is underway.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined using a Kofler hot-stage instrument and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. The uv spectra were obtained on a Beckman DU-7 spectrometer and ir spectra measured on a Nicolet MX-1 FT-ir (AgCl or KBr) interferometer. ^{1}H-Nmr spectra were recorded in $CDCl_3$, using TMS as internal standard, employing either Nicolet NT-360 or Varian XL-300 instruments (360 MHz or 300 MHz, respectively). ^{13}C-Nmr spectra were recorded in $CDCl_3$ with a Nicolet NT-360 instrument operating at 90.8 MHz. Low-resolution mass spectra were obtained with a Varian MAT 112S instrument operating at either 80 or 20 eV. Droplet counter-current chromatography was performed on a Model-A instrument (Tokyo Rikakikai, Tokyo, Japan).

PLANT MATERIAL.—The rhizomes of *P. glycyrrhiza* were collected in south Oregon in the autumn of 1983 by one of us (F.A.L.). Specimens documenting this collection have been deposited in the Herbarium of the Field Museum of Natural History, Chicago, Illinois.

EXTRACTION AND FRACTIONATION.—The air-dried, milled plant material (6.87 kg) was extracted with 80% MeOH/H_2O to afford an initial MeOH/H_2O extract (1.26 kg), on removal of solvent in vacuo. A portion (584.5 g) was partitioned between H_2O (3 liters) and *n*-BuOH (6 × 1 liter), which yielded, on drying, 218.2 g of an *n*-BuOH residue and 360.3 g of an H_2O residue. Sweetness was found to concentrate in the *n*-BuOH extract, and a portion (218.2 g) was dissolved in MeOH (100 ml), impregnated in Si gel (200 g, 230–400 mesh, Merck, Darmstadt, W. Germany), and dried completely under an N_2 stream. This impregnated *n*-BuOH extract was subjected to cc over Si gel (800 g), using as eluents $CHCl_3$ and $CHCl_3$/MeOH mixtures of increasing polarity. A total of 107 fractions (600 ml each) were collected. Fractions showing similar tlc profiles were pooled to give nine combined fractions.

ISOLATION AND CHARACTERIZATION OF POLYPODOSIDE A [**1**].—Fractions 50–80 (57.4 g) eluted from the column with $CHCl_3$-MeOH (6:1) were combined, and a portion (37.09 g) was further purified in a batchwise manner, using Sephadex® LH-20 (100 g portions, Sigma, St. Louis, MO). In a representative separation, 1.5 g was dissolved in MeOH and was eluted with this same solvent. A total of 43 fractions (15 ml each) were collected. Polypodoside A [**1**] was present in fractions 1–30 (0.81 g), and a further 19.3 g of similar combined fractions containing this isolate was prepared by repeated chromatography. An aliquot (9.37 g) of this material was dissolved in $CHCl_3$-MeOH-H_2O (6:3:1, lower layer) and separated using this same solvent as eluent by low-pressure cc over Si gel (300 g, 230–400 mesh). Elution was conducted at a flow rate of 1.5 ml/min. Fractions 53–65 from this separation were finally purified by dccc using an equilibrated mixture of $CHCl_3$-MeOH-iPrOH-H_2O (5:6:1:4), where the upper layer was applied as a mobile phase in ascending-mode separation. A portion (500 mg) of fractions 53–65 from the low-pressure column was dissolved in equal amounts (3.5 ml) of a mixture of the mobile and stationary phases and was injected into a 7-ml sample chamber. Fractions (300 drops each) were collected at a pressure of 2–4 kg/cm^2 on an automatic fraction collector and were monitored by tlc. This procedure was repeated twice. Dccc fractions 70–77 contained a homogeneous compound as shown by tlc, and recrystallization from EtOH afforded pure polypodoside A [**1**] (1.35 g, 0.29% w/w).

Polypodoside A [**1**] exhibited the following data: colorless, needle-shaped crystals from EtOH; mp 198–200°; $[\alpha]_D$ −37° (c = 0.3, MeOH); uv (EtOH) λ max (EtOH) 244 (log ε 4.23) nm; ir ν max (KBr) 3435 (OH), 1667 (α,β-unsaturated ketone), 1460, 1453, 1225, 1036 cm^{-1}; ^{1}H nmr (360 MHz, C_5D_5N) δ 6.26 (1H, br s, H-1″), 5.84 (1H, br s, H-7), 5.61 (1H, br s, H-1‴), 5.02 (1H, d, J = 6.6 Hz, H-1′), 4.45 (1H, d, J = 8 Hz, H-26), 3.84 (1H, m, H-3), 3.46 (1H, m, H-22), 1.74 (3H, d, J = 6 Hz, 6″-Me), 1.66 (3H, d, J = 6 Hz, 6‴-Me), 1.01 (3H, d, J = 6 Hz, 21-Me), 0.91 (3H, d, J = 6 Hz, 27-Me), 0.86 (3H, s, 19-Me), 0.48 (3H, s, 18-Me) ppm; ^{13}C nmr (90.8 MHz, C_5D_5N) δ 198.69 (s, C-6), 163.02 (s, C-8), 123.21 (d, C-7), 107.32 (d, C-26), 102.17 (d, C-1″), 102.03 (d, C-1‴), 99.53 (d, C-1′), 79.47 (d, C-2′), 78.30 (d, C-3′), 78.11 (d, C-22, C-5′), 76.31 (d, C-3), 74.12 (d, C-4‴), 73.95 (d, C-4″), 72.78 (d, C-2″, C-2‴), 72.50 (d, C-3″), 72.14 (d, C-3‴), 71.87 (d, C-4′), 70.48 (d, C-5‴), 69.49 (d, C-5″), 62.74 (t, C-6′), 55.05 (d, C-14), 53.13 (d, C-5), 52.88 (d, C-17), 49.91 (d, C-9), 44.69 (s, C-13), 40.38 (d, C-20), 38.85 (t, C-12), 38.39 (s, C-10), 36.86 (t, C-1), 36.45 (d, C-25), 31.49 (t, C-24), 29.22 (t, C-2), 26.98 (t, C-4), 26.83 (t, C-16), 23.98 (t, C-23), 22.90 (t, C-15), 21.89 (t, C-11), 18.75 (q, C-6″), 18.44 (q, C-6‴), 16.68 (q, C-27), 13.85 (q, C-21), 13.10 (q, C-19), 12.09 (q, C-18) ppm ; eims (70 eV) *m/z* $[M]^+$ 884 (missing), $[aglycone]^+$ 430 (1%), $[aglycone - H_2O]^+$ 412 (23), 395 (32), 379 (5), 342 (5), 285 (9), 261 (8), 147 (12), 128 (17), 97 (37), 43 (100); fabms (DTE/DTT) *m/z* $[M + Na]^+$ 907 $[M + H]^+$ 885, 739, 721, 577, 559, 431, 413, 395, 309; hrfabms mass measurement found 885.4843, calcd for $C_{45}H_{73}O_{17}$, 885.4848, found 577.3723, calcd for $C_{33}H_{53}O_8$, 577.3740.

ENZYMATIC HYDROLYSIS OF POLYPODOSIDE A [**1**].—Compound **1** (300 mg) was dissolved in 2% aqueous EtOH (50 ml) and hydrolyzed using hesperidinase (300 mg, Sigma). The solution was incubated at 37° in a water bath for 24 h and extracted with EtOAc (3 × 50 ml). The dried organic layer (50 mg) was purified by preparative tlc with $CHCl_3$-MeOH (10:1), to afford 30 mg of the aglycone **2** (polypodogenin). The H_2O layer containing sugars was evaporated in vacuo and was subjected to gc/ms and tlc analysis. The sugar fraction was found to contain D-glucose and L-rhamnose.

Polypodogenin [**2**] exhibited the following data: colorless, amorphous powder; mp 188–190°; $[\alpha]_D$ +6° (*c* = 0.1, EtOH) [lit. (6) mp 162–164°, $[\alpha]_D$ +6.6° (*c* = 0.5, MeOH)]; uv λ max (EtOH) 243 (log ε 3.97) nm; ir ν max (KBr) 3404 (OH), 1669 (α,β-unsaturated ketone), 1149, 1061, 1033 cm^{-1}; ^1H nmr (360 MHz, $CDCl_3$) δ 5.72 (1H, m, H-7), 4.32 (1H, d, *J* = 8 Hz, H-26β), 3.62 (1H, m, H-3), 3.47 (1H, m, H-22β), 1.00 (3H, d, *J* = 7 Hz, 21-Me), 0.96 (3H, d, *J* = 6 Hz, 27-Me), 0.86 (3H, s, 19-Me), 0.62 (3H, s, 18-Me) ppm; ^{13}C nmr (90.8 MHz, $CDCl_3$) δ 199.62 (s, C-6), 163.32 (s, C-8), 123.11 (d, C-7), 101.33 (d, C-26), 78.09 (d, C-22), 70.68 (d, C-3), 55.22 (d, C-14), 53.32 (d, C-5), 52.81 (d, C-17), 50.05 (d, C-9), 44.75 (s, C-13), 40.05 (d, C-20), 38.77 (t, C-12), 38.19 (s, C-10), 37.84 (d, C-25), 36.82 (t, C-1), 31.05 (t, C-24), 30.40 (t, C-2), 30.24 (t, C-4), 26.95 (t, C-16), 23.65 (t, C-23), 22.61 (t, C-15), 21.78 (t, C-11), 16.69 (q, C-27), 13.78 (q, C-21), 13.21 (q, C-19), 12.25 (q, C-18) ppm; eims, (70 eV) *m/z* $[M]^+$ 430 (5%), $[M-H_2O]^+$ 412 (13), 342 (6), 316 (71), 287 (11), 285 (17), 261 (26), 126 (37), 115 (100), 97 (37).

After the hydrolysis of compound **1** with hesperidinase, isomeric ^1H- and ^{13}C-nmr chemical shifts of compound **2** were observed, as a result of pyran-ring opening at position 26 in solution. These resonances occurred at δ 5.08 (1H, d, *J* = 3 Hz, H-26α), 3.98 (1H, m, H-22α), and 95.45 (d, C-26), 69.35 (d, C-22), respectively.

SAFETY EVALUATION OF *P. GLYCYRRHIZA* EXTRACTS AND POLYPODOSIDE A.—The initial MeOH/H_2O, *n*-BuOH, and H_2O extracts from *P. glycyrrhiza* and pure polypodoside A [**1**] were evaluated for acute toxicity with male Swiss-Webster mice at dose levels of 1 and 2 g/kg body wt. Procedures and protocols for toxicological testing were followed as published previously (29,30). None of the samples tested caused any lethality, and body weights recorded on days 0 (prior to administration), 1, 3, 7, and 14 did not differ for treated versus control animals. The *P. glycyrrhiza* MeOH/H_2O extract and polypodoside A [**1**] were evaluated at the dose ranges 1.88–15 and 0.31–5.0 mg/ml, respectively, and were found not to be mutagenic for *Salmonella typhimurium* strain TM677, both in the presence and absence of a metabolic activating system, when tested as described previously (31).

SENSORY EVALUATION OF POLYPODOSIDE A [**1**].—Polypodoside A [**1**] was tested for its sweetness intensity relative to sucrose and its sensory characteristics by an expert taste panel consisting of three persons (32). This steroidal glycoside **1** was rated as exhibiting 600× the sweetness intensity of a 6% w/v aqueous solution of sucrose and revealed a licorice-like off-taste and some lingering aftertaste.

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