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Pteridanoside, the First Protoilludane Sesquiterpene Glucoside as a Toxic Component of the Neotropical Bracken Fern *Pteridium aquilinum* var. *caudatum*

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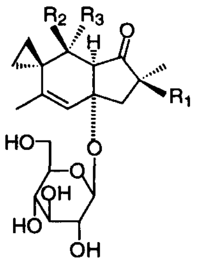
***Abstract*:** Chromatographic fractionation, monitored by brine shrimp bioassay, led to the isolation of the first protoilludane-type sesquiterpene glucoside, pteridanoside (**5**), from a bioactive hot-water extract of the neotropical bracken fern *Pteridium aquilinum* var. *caudatum* (Dennstaedtiaceae). The enzymatic hydrolysis of **5** yielded its aglycon, pteridanone (**6**), and D-glucose. The absolute stereostructure of **5** was confirmed by the CD spectroscopic analysis of **6**. Pteridanoside (**5**) exhibited toxicity (LC_{50} of 250 µg/ml at 24 h and 62.5 µg/ml at 48 h) toward brine shrimp, while the aglycon **6** did not. © 1999 Elsevier Science Ltd. All rights reserved.

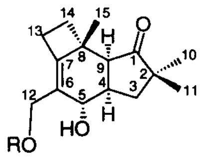
Keyword: biologically active compound, circular dichroism, phytotoxin, terpene and terpenoid.

Introduction

The phytochemistry of more than 30 species of the Dennstaedtiaceae has been reported.[1] Among them, *Pteridium aquilinum* (L. Kuhn) stands out as one of the most successful and widely distributed organisms of the plant kingdom.[2,3] It is known that the consumption of bracken fern by cattle induces bladder and intestinal carcinomas[4,5] and a number of diseases in various farm animals.[6,7] An active principle responsible for most of the carcinogenic and mutagenic activity was isolated from the aqueous fraction and characterized as the unstable norsesquiterpene glucoside ptaquiloside (**1**).[8,9] More recently, three additional compounds, **2**- **4**, sharing the same illudane skeleton, have been identified.[10,11] Their mild acid- or base-induced decomposition lead invariably to various stable indanones, such as pterosins B and Z, a number of which have been identified in the



R_1 = H, R_2 = OH, R_3 = CH_3 : Ptaquiloside (**1**)
R_1 = H, R_2= CH_3, R_3 = OH : Isoptaquiloside (**2**)
R_1 = CH_2OH, R_2= OH, R_3 = CH_3 : Caudatoside (**3**)
R_1 = CH_3, R_2= OH, R_3 = CH_3 : Ptaquiloside Z (**4**)



R = β-D-glucosyl : Pteridanoside (**5**)
R = H : Pteridanone (**6**)

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extracts of *P. aquilinum* and other related ferns.[1] It has been postulated that the illudane skeleton is biosynthesized from the protoilludane skeleton.[1] Although several protoilludane-type compounds such as illudol[12] are known, none of them have ever been isolated from the Dennstaedtiaceae. Presently, we report the isolation of one such protoilludane-type derivative, pteridanoside (**5**), from the young croziers of *P. aquilinum* var. *caudatum*, the most commonly found neotropical variety of bracken fern in the Northern Andes and Central America.

Results and Discussion

Bioassay-guided fractionation[13] of a hot-water extract of *P. aquilinum* var. *caudatum* croziers on ODS, using a water-to-acetonitrile gradient, yielded 16 fractions, of which Nos. 8, 9, and 10 were the most toxic in the assay. From an HPLC analysis, fractions No. 9 and 10 were found to contain the known compounds ptaquiloside (**1**) and ptaquiloside Z (**4**), respectively. Fraction No. 8 was separated on silica gel and an active fraction was further purified by reverse-phase HPLC to yield pteridanoside (**5**) (0.0003% from fresh weight) as a toxic component.

The molecular formula of **5** was determined as $C_{21}H_{32}O_8$ by a combination of NMR and high-resolution FABMS. The IR spectrum exhibited a broad absorption centered at 3407 and a band at 1717 cm^{-1} due to the hydroxyl and ketone groups, respectively. The UV spectrum (λ_{max} 197 nm) led us to conclude that **5** has no conjugated systems. The ^{13}C NMR data (Table 1) revealed the presence of a ketone (δ 211.2), a tetrasubstituted double bond (δ 128.4, 149.1), and two quaternary carbons (δ 46.7, 47.9). The remaining carbon signals could be attributed, with the aid of the DEPT spectrum, to three methyls, three methylenes, two oxygenated methylenes, two methynes and six oxygenated methyne carbons. The anomeric resonance of carbon (δ 102.8) and a proton (δ 4.31) unveiled the presence of a sugar moiety, which turned out to be D-glucose based on a degradation experiment (vide infra). The assignment of the protonated carbons was performed by an HMQC experiment. The above information and analysis of a DQF-COSY experiment let to the partial structures shown in Fig. 1. These partial structures were connected by the long-range C-H correlations observed in an HMBC spectrum to give the gross structure of **5** shown in Fig. 2. Thus, the HMBC correlations establishing the gross structure of the aglycon portion were as follows: the angular methyl H-15 to C-7, C-9 and C-14; H-12 to C-5, C-6 and C-7; H-5 to C-6 and C-7; H-9 to C-1 and C-7; H-3 to C-1, C-2, C-10 and C-11; H-4 to C-8; H-13 to C-6 and C-8; H-14 to C-7 and C-9; H-11 to C-10; H-10 and H-11 to C-1 and C-2. The correlation from the anomeric H-1' to C-12 indicated that glucose was connected to C-12. The β configuration of the glucoside bond was deduced by the coupling constant $J_{1'\text{-}2'}$ of 7.9 Hz.

The relative stereochemistry of the aglycon part of **5** was ascertained from NOESY data (Fig. 3). The correlation from H-10, assumed to be α-orientated, to H-3α, H-4 and H-9 suggested that these hydrogens should be on the same α side of the molecule. The correlations from H-3β to H-5, H-11 and H-15 established the β orientation of these hydrogens. These correlations indicated that the A/B ring fusion was *cis*, although the NOE correlation between H-4 and H-9 was unclear because of their close chemical shift. The coupling constant between H-4 and H-9 of $J_{4,9}$ = 11.8 Hz revealed a near eclipsed conformation. The orientation (β or α) of the methylene protons of H-13 and H-14 was also determined by the NOE correlations from H-15 to H-13β (δ 2.91) and H-14β (δ 2.02), and from H-9 to H-14α (δ 2.12), confirming the relative stereochemistry of the aglycon of **5** (Fig. 3).

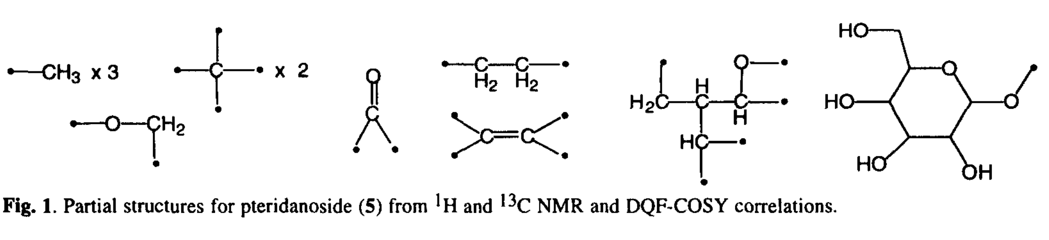

Fig. 1. Partial structures for pteridanoside (**5**) from ^{1}H and ^{13}C NMR and DQF-COSY correlations.

Table 1. NMR data for pteridanoside (**5**) and pteridanone (**6**).[a]

	Pteridanoside (**5**)		Pteridanone (**6**)
Position	^{13}C	^{1}H[b]	^{1}H[b]
1	211.2 s		
2	47.9 s		
3α	46.4 t	2.35 dd (12.8, 7.7)	2.34 dd(12.3, 7.8)
3β		1.60 dd (12.8, 12.3)	1.57 dd (12.3, 12.1)
4	44.7 d	2.61 dddd(12.3, 11.8, 7.7, 6.5)	2.57 m
5	73.4 d	4.17 ddd (6.5, 3.0, 2.0)	4.15 m
6	128.4 s		
7	149.1 s		
8	46.7 s		
9	55.9 d	2.70 d (11.8)	2.67 d (12.1)
10	25.5 q	1.06 s	1.04[c] s
11	23.1 q	1.039 s	1.02[c] s
12α	64.8 t	4.41 d (10.7)	4.15 d (12.3)
12β		4.32 d (10.7)	4.22 d (12.3)
13α	26.3 t	2.72 m	2.75 m
13β		2.91 m	2.91 m
14α	36.5 t	2.12 ddd (11.0, 10.0, 3.4)	1.99 ddd (11.1, 10.1, 3.2)
14β		2.02 ddd (11.0, 8.8, 8.8)	2.11 ddd (10.1, 9.8, 9.0)
15	19.5 q	1.042 s	1.03[c] s
1'	102.8 d	4.31 d (7.9)	
2'	75.1 d	3.20 dd (8.9, 7.9)	
3'	78.1[c] d	3.36 dd (9.0, 8.9)	
4'	71.7 d	3.28 dd (9.0, 9.0)	
5'	78.2[c] d	3.26 ddd (9.0, 5.5, 2.0)	
6'α	62.8 t	3.67 dd (11.9, 5.5)	
6'β		3.87 dd (11.9, 2.0)	

[a] Measured in CD_3OD at 150 MHz for ^{13}C and at 600 MHz for ^{1}H.

[b] Coupling constants are in parentheses.

[c] Data are interchangeable.

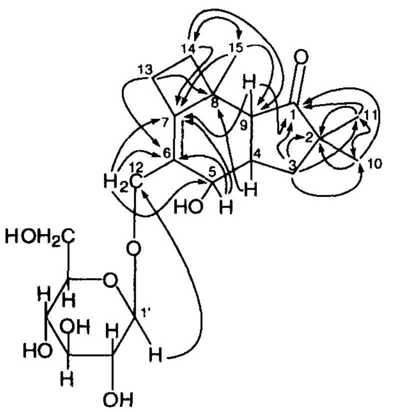

Fig. 2. Gross structure for pteridanoside (**5**) from HMBC correlations (arrows).

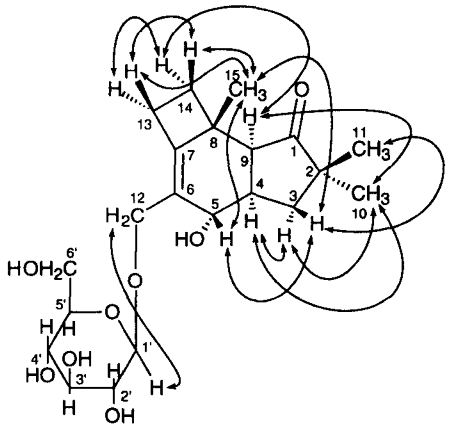

Fig. 3. Relative stereostructure for pteridanoside (**5**) from NOESY correlations (arrows).

The absolute stereochemistry of **5** was next examined as follows. The enzymatic hydrolysis of **5** using β-glucosidase led to the isolation of the aglycon named pteridanone (**6**) and its sugar moiety, which was determined as D-glucose by acidic methanolysis (H_2SO_4, MeOH) followed by acetylation to give methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside as the major product. The absolute stereochemistry of **6** was determined by a combination of conformational energy calculations and the CD spectroscopy of **6**. The energy minimization of **6** by the MM2* force field in the molecular modeling package MacroModel 6.0 gave the lowest energy conformer, which was placed in a three-dimensional octant grid in order to apply the octant rule (Fig. 4).[14] Since there were many more atoms in the minus areas of the back octants than in the plus areas (Fig. 4a), this model was expected to display a negative cotton effect. Indeed, the CD spectrum of **6** (Fig. 5) showed a negative cotton effect at ca. 300 nm, which corresponds to the n- π* transition of the ketone group (C-1). These results bode well with the absolute stereochemistry of the proposed structures of **5** and **6**.

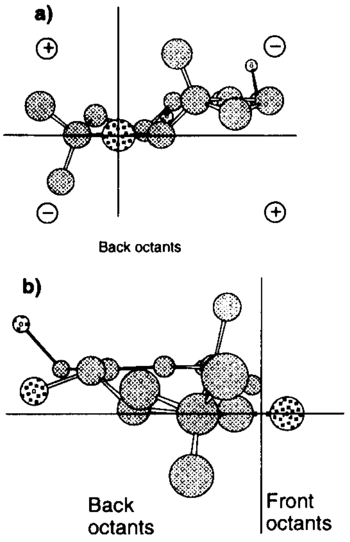

Fig. 4. The lowest energy conformation of **6** obtained by MM2* calculations. Projections were done on the plane of the ketone group, viewed from the C=O axis (a) and from the side of the C=O group (b).

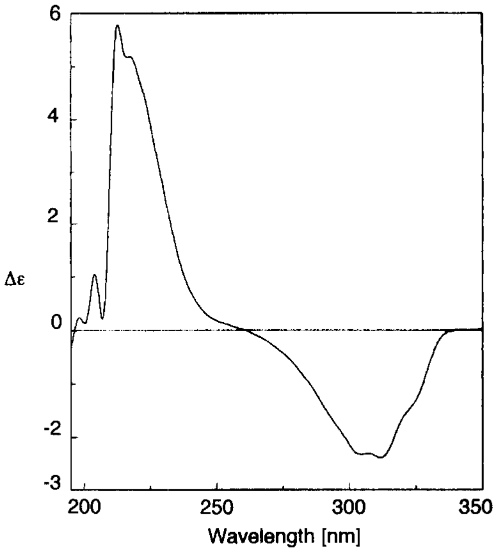

Fig. 5. CD spectrum of pteridanone (**6**) in CH_3CN (8.2×10^{-3} M)

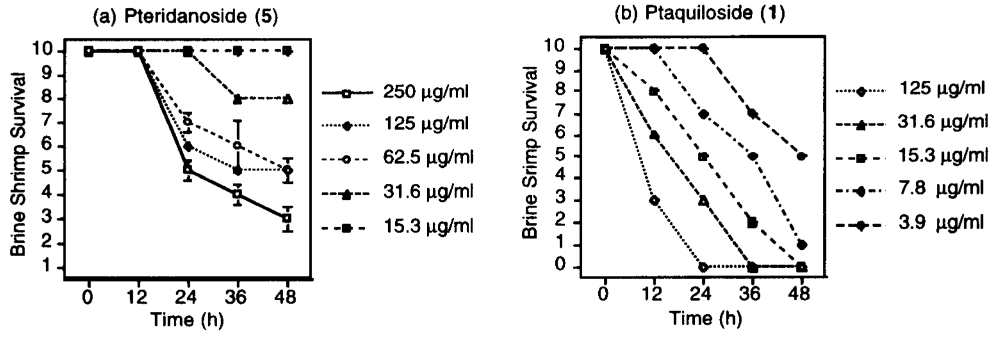

Fig. 6. Toxicity of **1** and **5** against brine shrimp.

Pteridanoside (**5**) is not only the first example of a protoilludane-type metabolite from the Dennstaedtiaceae family but also the first protoilludane-type compound holding a sugar. Pteridanoside (**5**) showed toxicity against the brine shrimp *Artemia salina* LEACH (lethal concentration LC_{50} = 250 and 62.5 μg/ml, 24 and 48 h after treatment, respectively), though 16-fold less toxic than the bracken carcinogen ptaquiloside (**1**) (Fig. 6). On the other hand, the aglycone **6** showed no toxicity in this assay, indicating that the sugar moiety of **5** plays a role in the solubility and/or better uptake.

Experimental

Instruments

HPLC was performed on a JASCO high-pressure gradient system with PU-980 pumps and detection was carried out using a JASCO UV-970 at 220 nm. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were recorded on a JASCO Ubest-50 UV/VIS spectrometer. IR spectra were recorded on a JASCO FT/IR-7000S. Mass spectra were recorded on a JEOL Mstation JMS-700 mass spectrometer using glycerol as a matrix in the positive mode. One and two-dimensional (HMBC, HMQC, DQF-COSY, and NOESY) NMR spectra were obtained for CD_3OD or $CDCl_3$ solutions using a Bruker AMX-600 spectrometer (600 MHz for ^1H). Chemical shifts are reported relative to TMS. CD spectra were recorded on a JASCO J-720 spectropolarimeter. The MM2* calculations in MacroModel ver. 6.0 were performed on a Silicon Graphics O_2 R5000SG computer.

Plant material

Young fronds of bracken fern were collected between 8-12 days[15] after emerging from the ground at the site of "El Cerro La Bandera" in Mérida, Venezuela, in July 1998 in an area where only the *caudatum* variety was growing. Specimens were identified using the key of Ortega[16] as *Pteridium aquilinum* var. *caudatum* . A voucher specimen (UVI 95-002) has been deposited in the herbarium of the Faculty of Pharmacy, Universidad de Los Andes, Mérida, Venezuela.

Extraction and purification procedures

Fresh fronds (2000 g) were mashed in hot boiling water for 1 h using a high-power blender that reduced the plant tissue to an amorphous mass in which no fibers could be discerned under a mid-power scope (40X). After blending, the mixture was cooled to 0 °C, filtered through a glass fiber filter and centrifuged (3000 rpm). The supernatant was freeze-dried to give 98 g of a solid material, which was stored with silica gel at -30 °C. The solid material was first fractionated by ODS (Cosmosil 140 C_{18}- OPN, 175 g, Nacalai Tesque) open-column chromatography and eluted using 100% H_2O to 100% MeOH to afford 16 fractions. Brine shrimp toxicity assays were performed on each fraction. Fraction No. 8 (892 mg), eluted with 30 % MeOH, showing high effect, was further purified by silica gel (BW 300, 40g, Fuji-Silysia) open-column chromatography eluted with 90:10 EtOAc:MeOH to 100% MeOH to afford 4 fractions. Fraction No. 3 (555 mg), eluted with 80:20 EtOAc:MeOH, was further purified by preparative HPLC [column: Develosil ODS-HG-10 (20 x 250 mm)] using 37:63 MeOH:H_2O to afford 12 fractions. Fraction No. 9 (39 mg, t_R = 110 min) was further purified by preparative HPLC [column: Develosil ODS-HG-5 (10 x 250 nm)] using 14.5:85.5 THF:H_2O to give 6.2 mg of pteridanoside (**5**) (t_R = 21 min) as a colorless powder, $[\alpha]_D^{24}$ –35° (*c* 0.12, CH_3CN), IR (KBr) 3407 (br), 1717, 1645 cm^{-1}, UV (CH_3CN) λ_{max} 197 nm (ε 7100), HRFABMS *m/z* 435.2037, calcd for $C_{21}H_{32}O_8Na$ (M + Na) 435.1995.

Pteridanoside (5) degradation

The enzymatic hydrolysis of **5** was carried out using 0.3 units of β-glucosidase (40 units/mg from sweet almond, Oriental Yeast) in 1 ml of acetate buffer (100 mM, pH 5.0) at 37 °C for 7 h. The reaction mixture was extracted with EtOAc (3 x 3 ml). The organic layer was evaporated and purified by a silica gel micro-column (Kieselgel 60, 50 mg, Merck) using 90:10 EtOAc:MeOH as an elution solvent to give the aglycon **6** (0.8 mg, 56%): $[\alpha]_D^{21}$ –21° (*c* 0.045, CH_3CN), UV (CH_3CN) λ_{max} 198 nm (ε 7200), CD (CH_3CN) λ_{ext} 312 ($\Delta\varepsilon$ –2.4), 304 (–2.3), 217 (+5.2), 213 (+5.8), 204 (+1.0) nm. ^1H NMR data are shown in Table 1. HRFABMS *m/z* 273.1432, calcd for $C_{15}H_{22}O_3Na$ (M + Na) 273.1467.

The aqueous layer was dried and subjected to methylation (H_2SO_4, MeOH) followed by an acetylation (Ac_2O, pyridine) procedure described in a previous paper[11] to give methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (0.14 mg, 9.3%), $[\alpha]_D^{24}$ +127° (*c* 0.007, $CHCl_3$) [Lit. $[\alpha]_D^{20}$ +130.5° ($CHCl_3$)],[9] and its β-anomer in a trace amount.

Bioassay

The testing samples were prepared by serial dilution from 250 to 1.9 µg/ml in each well of a 24-well micro plate in 1 ml of artificial sea water (SWING Hi-Marine, Hipet). A suspension of nauplii containing 10 organisms (10 µl) was added to each well, and the covered plate was incubated at 25-27 °C for 48 h. Plates were observed every 12 h under a binocular microscope (× 4) and the number of dead (non-motile) nauplii in each well was counted. The 50% lethal concentrations (LC_{50}) at 12, 24, 36 and 48 h were measured by serial dilution media.

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