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CHEMICAL ASSAY OF PTAQUILOSIDE, THE CARCINOGEN OF *PTERIDIUM AQUILINUM*, AND THE DISTRIBUTION OF RELATED COMPOUNDS IN THE PTERIDACEAE

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Key Word Index—*Pteridium aquilinum*; Pteridaceae; analysis, TLC-densitometry; distribution; norsesquiterpene glycoside; ptaquiloside; pterosin B.

Abstract—A chemical assay of ptaquiloside, the major carcinogen of *Pteridium aquilinum*, based on two-dimensional TLC-densitometry, was devised. The contents of ptaquiloside and related compounds in the fern collected at four places at different seasons were compared and the epidemiological implications of the results are discussed. The behaviour of ptaquiloside on treatment with acid, alkali, light and heat was examined and the instability of the compound, especially under alkaline conditions, was confirmed. The distribution of ptaquiloside and ptaquiloside-like substances in the Pteridaceae was examined by the use of the chemical assay and the modified Ames' test, and widespread occurrence of such compounds was revealed.

INTRODUCTION

Since bracken fern (*Pteridium aquilinum*) was noticed to induce tumours in animals, extensive surveys have been conducted to characterize the carcinogenic principle [1–3]. Recently Yamada *et al.* [2, 4, 5] succeeded in isolating a novel norsesquiterpene glucoside, named ptaquiloside (**1**), which was found to be the major carcinogen and the component responsible for bovine bracken poisoning [2, 6–8]. A mutagenic compound named aquilide A, assumed to be identical with ptaquiloside, was also isolated by van der Hoeven *et al.* [9]. The nature of ptaquiloside (aquilide A) (**1**) readily accounts for the difficulties encountered in the isolation of the compound. It is quite unstable; it forms an unstable conjugated dienone (**2**) under alkaline conditions and, under acidic conditions, it is transformed to pterosin B (**3**), a 1-indanone derivative isolated and characterized by us [10] (Chart 1). In order to assess the epidemiological significance of the carcinogen as a human and animal hazard, establishment of an assay method for ptaquiloiside was necessary. We have already developed a novel mutation test using a preincubation at pH 8.5 with *Salmonella typhimurium* tester strains, and have examined the content of ptaquiloside and related compounds in the fern [11]. We have now developed a chemical assay of these compounds and, using the bioassay and the chemical assay, the behaviour of the carcinogen was further examined. Using the same methods, the distribution of ptaquiloside (**1**) and the related compounds in the Pteridaceae was also studied.

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RESULTS AND DISCUSSION

Chemical assay of ptaquiloside (**1**) *and the related compounds*

Since ptaquiloside (**1**) is unstable to acid, base, light and heat, such treatments should be avoided in clean-up stages of the analysis. Several attempts using HPLC were abandoned for this reason and a TLC-densitometry procedure was adopted. As shown in Chart 1, ptaquiloside easily decomposes into pterosin B (**3**). If compounds analogous to ptaquiloside exist, similar transformations to pterosins [10] would be expected. In the modified Ames' test [11], formation of the dienone (**2**) under alkaline condition was utilized. In this chemical assay, decomposition by heating to pterosins was applied as follows. An aqueous extract of the fern was freeze-dried and the extract was developed first on a silica gel TLC plate (20 × 10 cm) with benzene–acetone (3 : 7), where free 1-indanones (pterosins [10]) show higher R_f values than the glycosides, ptaquiloside (**1**) and pterosides, the glycosides of pterosins [10]. The plate was heated at 110° for 2 hr to decompose ptaquiloside (**1**) into pterosin B (**3**), and then developed in the second dimension with benzene–ethyl acetate (1 : 1). The spots of pterosins and pterosides in the plants appeared on the diagonal line, while pterosins formed from ptaquiloside or ptaquiloside-like compounds, if any, appeared as shown in Fig. 1. They were assayed by TLC-densitometry at 250 nm, employing pterosin B (**3**), other pterosins and pterosides as the standards. For determination of other pterosins and pterosides, a slight modification of the developing solvent was required. For the determination of pterosins, the ethyl acetate layer after water–ethyl acetate partitioning of the methanol extract of the fern was used.

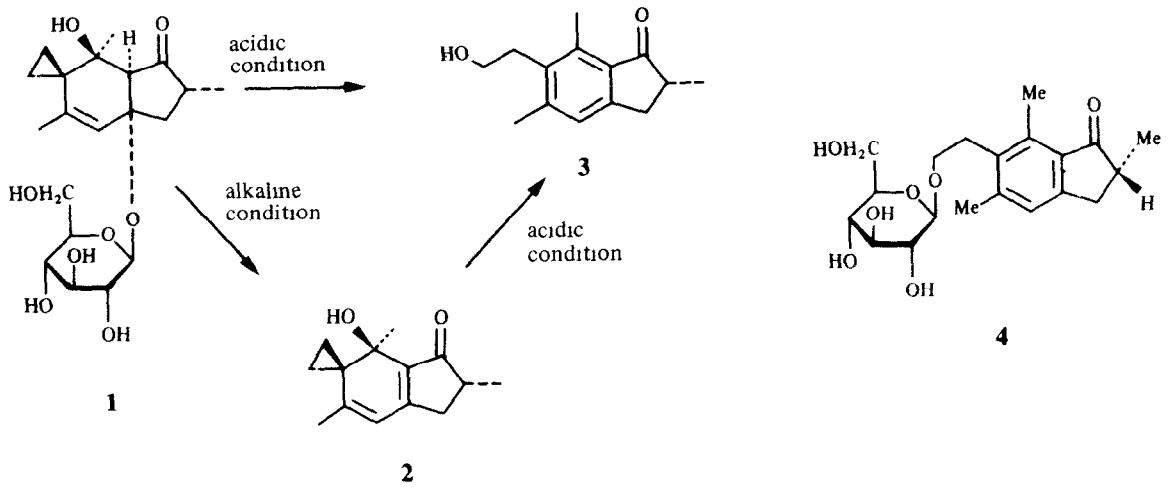

Chart 1 Structures of ptaquiloside and related compounds

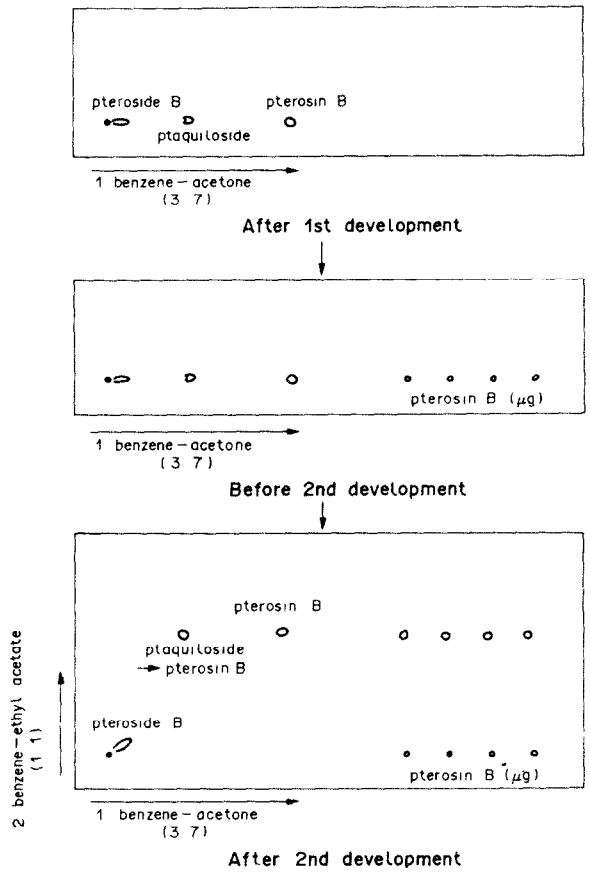

Fig 1 Two dimensional TLC for ptaquiloside and related compounds

By this method, ptaquiloside (**1**), pterosin B (**3**) and pteroside B (**4**) equivalent to 0.1–0.5 μg of pterosin B (**3**) could be quantitatively assayed. The content of ptaquiloside (**1**) obtained by the new chemical method was in good agreement with the value obtained by the modified Ames' test reported in the previous paper [11]

Contents of ptaquiloside and related compounds in Pteridium aquilinum

The carcinogenicity and the toxicity to calf of the fern have been reported to vary depending on the season and locality of the collection of the plant [2, 3] The dried

Table 1. Contents of ptaquiloside in bracken (% calculated based on dried materials*)

Part	Date and place of collection	Ptaquiloside (1)	Pterosin B(3)	Other pterosins	Pteroside B(4)	Other pterosides
Fronds	Nayoro May 29, 1985	0.02	0.21	0.08	0.10	0.05
	Nayoro June 9, 1985	0.08	0.05	0 03	0.04	0.06
	Nayoro August 6, 1985	0.06	0.002	0.003	0.006	0.04
	Nayoro September 2, 1985	0.05	0.001	0.004	0.01	0.08
	Dejima May 30, 1985	0.16	0.02	0.01	0.03	0.04
	Dejima July 17, 1985	0.11	0.01	0.01	0.02	0.05
	Dejima August 29, 1985	0.11	0.004	0.01	0.03	0.02
	Akishima August 8, 1985	0.13	0.009	0.01	0.03	0.06
	Akishima May 31, 1986	0.14				
	Akishima July 13, 1986	0.13				
	Akishima September 4, 1986	0.10			not determined	
	Yatsushiro May 5, 1986	0.12				
	Yatsushiro July 5, 1986	0.09				
	Yatsushiro October 19, 1986	0 11				
Rhizomes	Nayoro September 2, 1985	0.03	0.001	0.002	0.29	0.22
	Dejima July 17, 1985	0.12	0.009	0.01	0.47	0.04
	Dejima August 29, 1985	0.05	0.005	0.006	0 95	1.25

*Loss on drying of the materials ranged from 46.5 to 91 2% for fronds and from 67.3 to 78.8% for rhizomes

rhizomes exhibited stronger carcinogenicity than the dried fronds [12]. To clarify these phenomena, the contents of ptaquiloside and the related compounds were assayed using the fresh plant materials collected at four places, Nayoro (Hokkaido), Dejima (Ibaraki), Akishima (Tokyo) and Yatsushiro (Kumamoto), covering the north, middle, and west parts of Japan,* from May to October. Rhizomes were also analysed in some cases for comparison. Although some of the results obtained have been reported in the previous paper [11], the cumulative data are shown in Table 1.

The yield of ptaquiloside was reported to be 0.1% by the improved isolation method [13]. The content of aquilide in freeze-dried material was reported to range up to 0.25% [9]. As shown in Table 1, the content of ptaquiloside ranged from 0.02 to 0.16% (calculated for dried material) for the fronds and from 0.03 to 0.12% for the rhizomes. Seasonal variation of the contents was not remarkable except in the materials collected at Nayoro, where the hot summer season is shorter than other three places. Bovine bracken poisoning has been reported only in a few localities. However, samples collected at four different places from the north to the west of the Japanese Islands were all found to contain the toxin and the restricted occurrence of the outbreaks of bracken poisoning is assumed to be due to other factors such as the circumstances leading to intake of bracken by grazing cattle.

Although the contents of the pterosides, glucosides of pterosins, in rhizomes are much higher than those in fronds, as reported in the previous paper [14], the contents of ptaquiloside in rhizomes are lower than those in the fronds. The result was contrary to our expectation based on the carcinogenicity and, since the dried materials had been used for the feeding experiments,

*Some taxonomists identify the Japanese species as *P aquilinum* var. *latiusculum*.

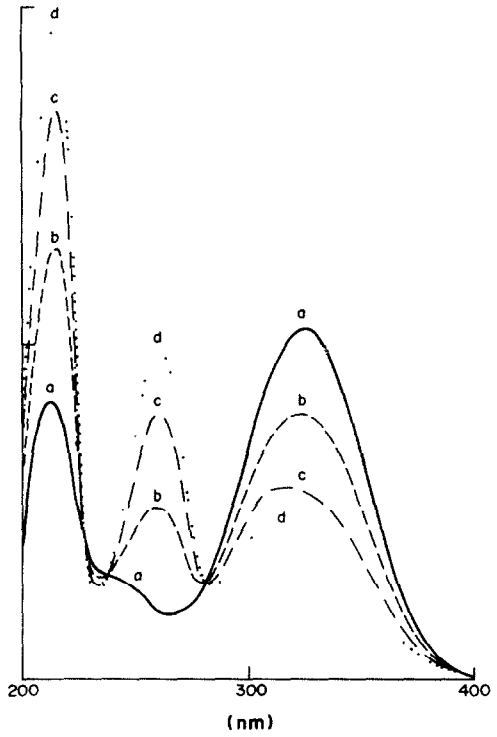

Fig 2 UV absorption changes of ptaquiloside (1) in 0 02 M Na_2CO_3 after (a) 0, (b) 5, (c) 10 and (d) 15 min.

some difference in the stability of ptaquiloside in the two parts in the course of drying might account for this.

In Japan, young fronds of bracken fern are widely eaten as a foodstuff. They are generally put into boiling water after the collection to maintain the green colour, or preserved by salting. They are treated with wood ash or sodium hydrogen carbonate to remove the harshness before cooking Six commercial products from Tokyo market, three imported from Siberia and three collected in Japan, were also analysed. None of these samples showed the presence of the carcinogen (detection limit, 0.01%). The spores and sporangia of the fern collected in North Wales, U.K., by Dr W. C Evans (deceased July 1988) and the callus developed from the root of cultured sporophyte of the fern by Dr H. W. Elmore (Marshall University) were also analysed by the same methods but neither contained a detectable amount of ptaquiloside (1).

Behaviour of ptaquiloside (1) *on treatment with acid, alkali, light and heat*

As reported in the previous paper [11], ptaquiloside quantitatively decomposes into pterosin B (3) on alkaline treatment. Figure 2 shows the UV absorption change of ptaquiloside (1) in 0.02 M Na_2CO_3 solution, indicating that decomposition through the dienone (2) (λ_{max} 321 nm) [5] to pterosin B (3) (λ_{max} 260, 304 nm) [10] took place within several minutes

To confirm such changes quantitatively at various pH values, the time course of the amount of ptaquiloside in Johnson–Lindsay buffer (pH 11.5, 11.0, 8.5, 7.0, 5.5, 4.0) at 37° was assayed by the TLC–densitometry method. As shown in Fig. 3, the compound easily decomposes under alkaline conditions, and, at pH 11.5, it completely decomposes within 20 min Under acidic conditions, it decomposes gradually at the same temperature; at pH 4.0, it decomposes by half within seven days. The compound is rather stable under neutral conditions.

These properties of ptaquiloside would explain the passage of the compound without much decomposition through the stomach, the pH of which is less acidic in animals than that in human, and the preferential induction of tumours in the rat at the terminal 20 cm of the ileum, the most alkaline region of the intestine, and urinary bladder, which is again alkaline [2, 9].

Next, to examine the decomposition of ptaquiloside under natural conditions, ptaquiloside, the extract of the fronds, fronds and the rhizomes of the fern were exposed to sunlight or heated at 60° and the decomposition of ptaquiloside was followed by chemical assay As shown in Fig 4, ptaquiloside decomposed gradually under these treatment but that in the fronds and the rhizomes was more stable. This also is consistent with the results of feeding experiments using the powder of the fern.

Distribution of ptaquiloside (1) *and related compounds in the Pteridaceae*

Chemotaxonomic work on the Pteridaceae by two of us [15, 16] has shown that many species of the family contain pterosin derivatives, sesquiterpenoids with a 1-indanone skeleton, as characteristic constituents * The aglycone part of ptaquiloside has an illudane nucleus, which is assumed as the precursor of pterosins Coexistence of ptaquiloside with pterosins and pterosides [10, 14] in bracken fern suggests the presence of ptaquiloside-type compound(s) having a cyclopropane ring in other Pteridaceae plants Aqueous extracts were prepared from 31 plant materials available and analysed by the modified Ames' test and by TLC-densitometry for the presence of such compounds As shown in Table 2, more than half of the ferns examined indicated the presence of ptaquiloside-type compounds Besides these materials, five ferns belonging to other families but customarily eaten in local areas of Japan, *Osmunda japonica*, *Osmundastrum cinnamomeum*, *O. claytonianum*, *Matteuccia orientalis*, and *M struthiopteris*, were tested, but none showed the presence of such compounds.

Among the plants of the Pteridaceae giving positive results, the constituents of three, *Pteris cretica*, *Histiopteris incisa*, and *Hypolepis punctata*, have so far been examined: ptaquiloside has been isolated from the former two species, while the third species has been proved to contain three new compounds, named hyplosides A, B and C (5–7) corresponding to pterosin Z (8) [10] (Chart 2). These new glycosides exhibited mutagenicity and caused chromosomal aberration as ptaquiloside (1) The details of the chemical and biological work will be reported in a forthcoming paper.

Although there has been no toxicological report on the other members of the Pteridaceae besides *Pteridium aquilinum*, except one on *Cheilantes sieberi* [17], our

*The chemotaxonomic work by two of the present authors [15] supports the classification by Copeland but does not agree with the classification by those, who divide the family into three or more groups

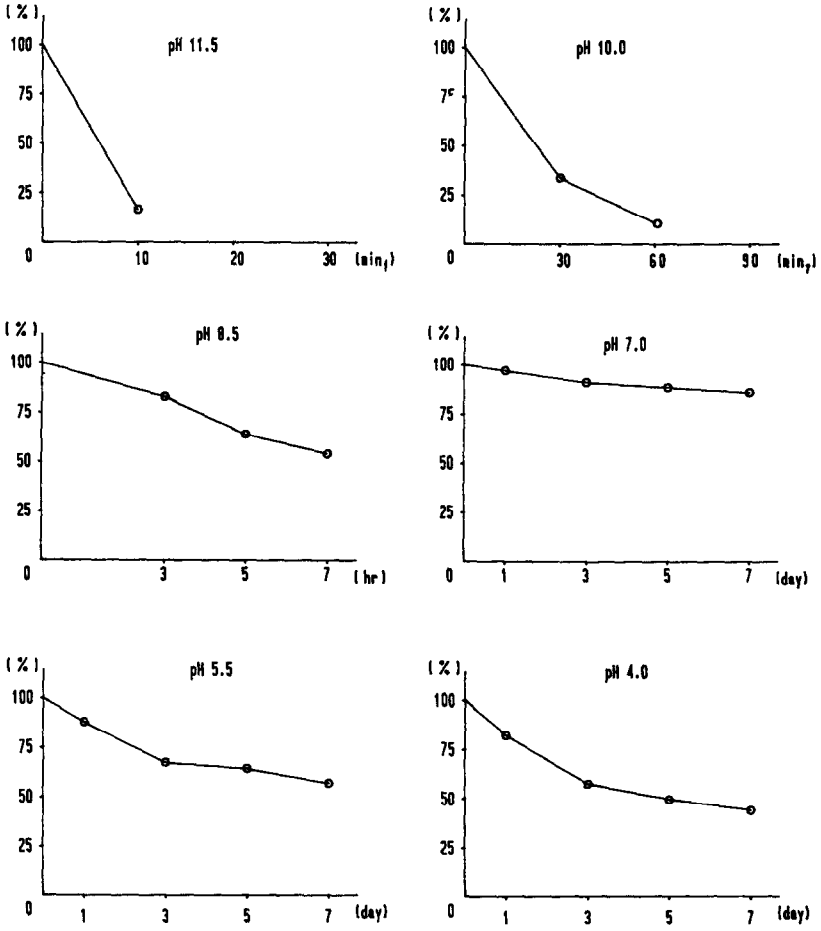

Fig. 3. Stability of ptaquiloside (1) to acid and alkali.

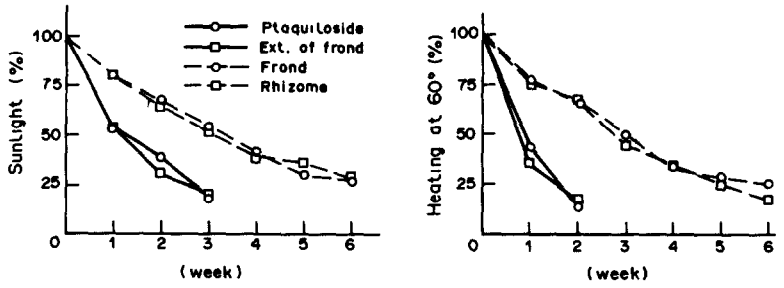

Fig. 4. Stability of ptaquiloside (1) to light and heat

observations strongly suggest the widespread occurrence of the carcinogens/mutagens and bovine poisonings in the family.

EXPERIMENTAL

Plant materials. Pteridium aquilinum used for the extraction of ptaquiloside (1) was collected at Nayoro, Hokkaido, in July, dried with a blower at 60° and stored at 4°. The plant materials used for the assay were collected at the four places in Japan shown in Table 1, frozen immediately, and stored at −20° before use. The plants of the Pteridaceae shown in Table 2 were collected at the places listed in the table and treated in the same way.

Ptaquiloside (1). The carcinogen was isolated by an improved method as follows. Dried bracken powder (500 g) was extracted with cold water (11 × 2) for 2 hr, then centrifuged, and the supernatant lyophilized The extract was dissolved in MeOH and chromatographed on a silica gel column with $CHCl_3$–MeOH (10·1) The fraction containing ptaquiloside was further purified by HPLC on Nucleosil 50–5, Develosil ODS-5, and Nucleosil 50-5 (twice) columns (250 × 10 mm) with EtOAc–MeOH (100.1), MeOH–H_2O (2:3), and CH_2Cl_2–MeOH (22:3), respectively, and employing an RI de-

Table 2 Distribution of ptaquiloside and related compounds

Species	Place of collection	Pterosins reported [15]	Pterosins identified by TLC	Ptaquiloside and ptaquiloside-like compounds TLC	Ames test
Cheilanthes myriophylla (= *Myriopteris myriophylla*)			pterosin Z	+	+
Cibotium harometz	Okinoerabu Is.		+	+	+
Coniogramme gracilis	Amami Is		pterosin Z	–	
C intermedia	Kiyosumi		–	–	–
C japonica	Kiyosumi	pterosin O, X, Y etc	–	–	–
Dennstaedtia distenta			–	–	–
D hirsta	Kushimoto		–	+	+
D scabra	Kushimoto	pterosin A, V, K, F	+	+	+
Histiopteris incisa	Yaku Is	pterosin B J, histiopterosin A, B etc.	+	+	+
Hyplepis bamleriana	Yaku Is	pterosin H, I, Z	+	+	+
H tenuifolia	Taiwan		pterosin Z, D	+	+
H. punctata	Tsukuba	pterosin A, H, I, Z, D, L etc	pterosin H, I, Z, D, L	+	+
Microlepia marginata var. *hipinnata*	Tsukuba		–	–	–
M strigosa	Kushimoto	pterosin B, C, D, F, L, O, etc.	–	+	–
Monachosorum arakii	Kyoto	mucagolactone monachosorin A, B	–	–	+
Monachosorum flagellare	Kyoto		–	–	+
Onychium japonicum	Kiyosumi, Kushimoto	pteroside M	–	+	+
Pityrogramma calomelanos	Taiwan		+	+	+
P sulphurea			pterosin Z	+	+
Pteris cretica	Tsukuba	pterosin A, B, C, F, S	pterosin B, S	+	+
P dispar	Kushimoto	pterosin C-3-glu	+	+	–
P. excelsa	Kushimoto	pterosin B, O	pterosin B	–	–
	Kiyosumi			+	–
P. fauriei	Yaku Is	pterosin W, X pteroside X, U, W, S	–	–	–
P. nipponica	Kiyosumi		–	+	+
P. oshimensis	Kushimoto	pterosin Q etc.	+	+	+
P. purpureorachis	Yaku Is		–	–	–
P ryukyuensis	Amami Is	pterosin B, C, J Q	pterosin B	–	–
P. semipinnata	Yaku Is		+	–	–
P tremula		pterosin B, F, J etc	pterosin B	+	+
P wallichiana	Kushimoto	pterosin C, D, Q	–	–	–
	Yaku Is			+	+
Sphenomeris chusana	Kushimoto, Yaku Is		–	–	–

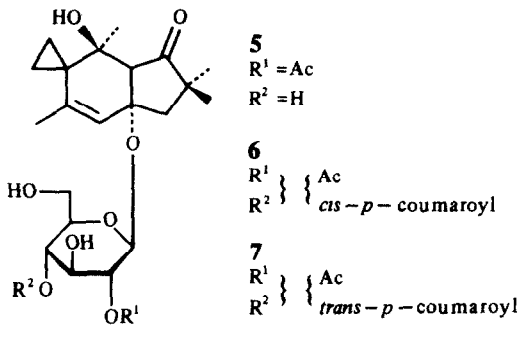

Chart 2 Structures of hypolosides

tector. Ptaquiloside thus obtained showed the same physical properties as reported [5, 11, 13]

Chemical assay of ptaquiloside (**1**) *and the related compounds by TLC-densitometry* Frozen material (2.0 g) was pulverized and extracted with water (60 ml × 2) at room temperature for 90 min, and the combined extract lyophilized The residue was dissolved in H_2O (1 ml) and an aliquot was applied to a silica gel plate (Kieselgel 60 F_{254}, Merck, 20 × 10 cm) as shown in Fig 1 The plate was first developed with C_6H_6–Me_2CO (3 7) and the developed plate heated at 110° for 2 hr After drying the plate in a desiccator, aliquots of authentic pterosin B (or other pterosins) in the range 0.1–0 5 μg were spotted on the plate as shown in Fig 1 and the plate was developed with C_6H_6–EtOAc (1 1) in the second dimension After drying, the plate was scanned by a

Shimadzu CS-910 double-beam chromatoscaner employing the zigzag scan mode (260 nm for the determination and 350 nm as the reference). The amount was determined by the use of a standard curve obtained with an authentic sample

For the determination of pterosides the same extract was used but the solvent was replaced by $CHCl_3$–MeOH–H_2O (32:8:1). The procedure was the same as above, employing authentic pterosides as standards [10] For the determination of pterosins the same fern material (2 g) was extracted with MeOH (60 ml × 3) at room temp. for 1 hr, the solvent was evapd, and the residue partitioned into EtOAc–H_2O. The organic layer was evapd and the residue dissolved in a definite amount of MeOH. The solution was used as the sample solution, developed on the TLC plate using $CHCl_3$–MeOH (15:1) and determined by the same method as above. For these analyses the same plant materials were dried at 110° for 6 hr and the loss on drying was determined. The contents of ptaquiloside, pterosins and pterosides were expressed on a dried material basis

Treatment of ptaquiloside (1) *or fronds and rhizomes of* Pteridium aquilinum *with acid, alkali, light and heat.* Ptaquiloside (1) (10 ml) in Johnson–Lindsay buffer, pH 4.0–11.5 (10 ml), was kept at 37° using a shaking incubator and the solution was treated as described above to determine ptaquiloside.

Powdered fronds or rhizomes of the fern, the MeOH extract, and 1 were kept outside on sunny days on September in Tokyo or in an incubator at 60° and, after standing, the fronds and rhizomes were treated as above and the extract and ptaquiloside were dissolved in MeOH to be used for the determination. The results (Figs 3 and 4) are given by per cent of the original amounts.

The extraction and detection of ptaquiloside (1) *and related compounds in the Pteridaceae.* The frozen sample (2 g) was extracted with H_2O (30 ml × 2). The extract, with or without sterilization by passage through a membrane filter, was freeze-dried and the residue was used for the modified Ames' test [11] and the chemical assay

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