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PURIFICATION OF PTAQUILOSIDE, A CARCINOGEN FROM *PTERIDIUM AQUILINUM*

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Key Word Index—*Pteridium aquilinum*; Pteridaceae; ptaquiloside; unstable carcinogen; purification.

Abstract—Ptaquiloside, the major carcinogen of bracken fern (*Pteridium aquilinum*), was isolated in high yield from milled freeze-dried plant material. Because the compound is unstable, a new method was devised to avoid steps which caused loss of activity. Modern multipulse and 2D NMR techniques allowed confirmation of the previously proposed structure, although some assignments were shown to be incorrect.

INTRODUCTION

It has been recognised for some time that the consumption of bracken fern (*Pteridium aquilinum*) by cattle induces bladder and intestinal carcinomas [1, 2]. More recently, laboratory tests have shown that rats are susceptible [3] and this was later confirmed [4, 5]. Also, epidemiological studies in Japan have provided evidence that oesophageal cancer in that country can be correlated with consumption of bracken fronds [6]. An active principle responsible for mutagenic activity was isolated simultaneously by Niwa *et al.* in Japan [7] and van der Hoeven *et al.* [8] in The Netherlands, and the structure elucidated as an unstable norsesquiterpene glycoside. The trivial name ptaquiloside used by the former authors is used in this study because it has become more generally accepted. In subsequent studies, methods have been used to isolate pure ptaquiloside from bracken fern [9, 10] giving yields of the active compound of 0.014–0.15%. The compound was found to be responsible for > 50% of the mutagenic activity observed after incubation of the methanol extract at alkaline conditions, and found to be a potent genotoxic compound in mammalian cells *in vitro* [8]. Because our work involved mechanistic studies of ptaquiloside when administered to rats, a large quantity (1–2 g) of pure compound was needed. Analytical procedures using a pure standard of ptaquiloside revealed that bracken fern growing in northern Queensland contained a high concentration of the active principle, 0.035–1.29% [11], but the task of recovering this from the plant appeared formidable given that most reported recoveries were very low and the methods used lengthy. The best reported recovery of ptaquiloside is given by van der Hoeven *et al.* [8] at 0.25%, hence, the method we developed followed to some extent these authors work. The recovery of ptaquiloside using our method was close to that estimated to be in the plant using a HPLC procedure [11].

The use of modern multipulse and 2D NMR techniques allowed confirmation of the structure proposed by Ojika *et al.* [9], although some previous assignments are shown to be incorrect.

RESULTS AND DISCUSSION

A number of properties of ptaquiloside became apparent in the course of our method development. Apart from its instability under acid and alkaline conditions [7, 8, 12], a high proportion was lost by concentration in water, particularly when the temperature was raised above 40°. To resort to concentration of large volumes of aqueous solution by freeze-drying was found to be time-consuming. Ptaquiloside was found to be unstable to storing at −5° in aqueous solutions even for short periods (2 days), but stable for at least 6 months in methanol at the same temperature. Some ptaquiloside was lost by drying at 40°, hence, in our work, solutions were never dried completely except at low temperatures. To avoid loss of ptaquiloside by the action of light all flasks and columns were shielded with a layer of aluminium foil where possible.

Initial continuous chloroform extraction of dried bracken fern for 2 days removed most of the green-coloured matter and other substances which interfered with the purification procedure. Our tests showed that ptaquiloside was effectively removed from the plant by extracting for a further 2 days with chloroform–methanol

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azeotrope. Any trace left in the plant could not be recovered by further extraction with this solvent or methanol (the only other solvent considered for initial extraction). The reason that the azeotrope was used as an extracting solvent rather than methanol were, first, the azeotrope boiled at an appreciably lower temperature than methanol (*ca* 11°) and, second, it extracted less weight of total solid from the plant than methanol.

A problem in the purification of ptaquiloside was its instability in water. An aqueous solution of ptaquiloside was used in the polyamide step, the compound being recovered from water by adsorption on XAD-2 resin and subsequent elution with methanol. A trace of water left from the XAD-2 was removed at lower temperature if butanol was added before evaporating under reduced pressure (20°). No apparent loss of ptaquiloside occurred if the procedure was carried out in a short time (0.5 – 1 hr). The clear methanol solution was concentrated to give a white solid which readily dissolves in a small volume (20 ml) of methanol. High flow super cell (HFSC, an inert material) was used as a carrier for dried ptaquiloside because loss of the compound occurred when silica gel was used for the same purpose. Gradient elution silica gel column chromatography, followed by LH-20 gel filtration, yielded ptaquiloside at least 90% pure. This estimation was based on data from analytical HPLC using a UV detector and a pure ptaquiloside standard. Fractions from both columns were monitored using TLC followed by spraying with a sugar reagent. At least three compounds other than ptaquiloside (as revealed by blue spots on TLC) were effectively excluded by using these two steps. Preparative HPLC on a silica gel column was used as a final step to obtain pure ptaquiloside (0.1% yield). To achieve good resolution, 100 mg per injection or less was used. Earlier attempts to purify ptaquiloside by preparative HPLC after the XAD-2 step were unsuccessful because it could not be resolved from large quantities of impurities. No attempt was made to identify these impurities, but evidence from the LH-20 gel filtration step indicated that they had a smaller M_r than ptaquiloside.

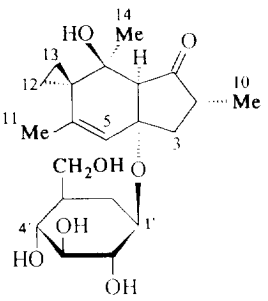
Multi-pulse and 2D NMR techniques confirmed the structure proposed for ptaquiloside by Ojika *et al.* [9], but some previous assignments are shown to be incorrect. The ^{13}C NMR spectrum of ptaquiloside (**1**) exhibited only 19 of the expected 20 signals but all were at chemical shifts consistent with the proposed structure. Hydrogen multiplicities deteremined by DEPT indicate the presence of three Me, four CH_2 and eight CH groups, Furthermore, the overlap of a CH and a CH_2 signal at δ45.2 in the DEPT spectrum accounted for the missing signal. The remaining ^{13}C signals correspond to give five quaternary carbon atoms. The following features of the ^1H NMR are also consistent with the proposed structure: a three-proton singlet at δ1.27 (MeC–O), a three-proton doublet at δ1.07 (MeCH–, adjacent to a carbonyl group) and a three-proton signal at δ1.52, for which long-range coupling to H-5 is observed (Me attached to a trisubstituted alkene). The one-proton doublet at δ4.59 is characteristic of H-1 of a glycoside and the presence of only one signal in this region, together with the H-1/H-2 coupling constant of 8 Hz, suggested the presence of the β-isomer only.

Homonuclear correlation spectroscopy (COSY) established the hydrogen connectivities and allows correction of some previously misassigned signals. For example, cross-peaks were observed between H-10 and H-2, H-2 and H-3_α, H-2 and H-3_β, and, H-3_α, and H-3_β. These peaks, together with the multiplicities and integrated intensities of the signals, permitted assignments in ring B to be established unequivocally. The only cross-peaks associated with the cyclopropane ring (H-12_a and H-12_b, H-13_a and H-13_b, and, H-12 and H-13) confirmed that C-7 had no attached proton. A weak cross-peak between H-11 and H-5 also established the position of the double bond in ring A. Surprisingly, the H-9 hydrogen was observed as a doublet (J = 1.5 Hz) even though it was an isolated hydrogen (a fact reported by Ojika *et al.* [9] but not commented upon). However, the cross-peak between H-9 and H-5 revealed the source of the extra coupling and molecular modelling (MM2) confirms the favourable "W" formation between these two hydrogens. Although some overlap of proton signals in the glycoside ring is evident, the cross-peak between H-1^1 and H-2^1, clearly indicated that the multiplet at δ3.19 was due to H-2^1 (not H-4^1 as previously reported). Other cross-peaks in this area enabled assignment of the remaining signals within this ring.

Heteronuclear correlation spectroscopy (HETCOR) permitted the assignment of all the protonated carbon signals. In particular, unequivocal assignment of C-12 and C-13 (which were previously incorrectly assigned [7] can now be made as follows: a NOESY cross-peak was observed between H-14 (at δ1.2) and the signal at δ0.68. Since C-14 had α-stereochemistry, the signal at δ0.68 must be due to the hydrogen attached to C-12 (the carbon of α-stereochemistry) on the surface of the cyclopropane ring which faces C-14. The HETCOR spectrum indicated that the proton at δ0.68 was attached to the carbon at δ5.9 along with one of the protons at δ0.85. The remaining ^{13}C signal in this region (at δ10.6), and its attached protons, must, therefore, belong to C-13. Furthermore, the assignment in the glycoside ring suggested by the COSY spectrum can be confirmed (see Experimental).

The stereochemistry of the chiral sites has been determined by the X-ray crystallography for ptaquiloside tetracetate [13]. Cross-peaks between H-10/H-3_α and H-14/H-9 in the NOESY spectrum supported these assignments as they suggest that the hydrogens involved had the same stereochemistry. Furthermore, the presence of an H-5/H-3_β cross-peak suggested that the glycoside link to the C-4 had α-stereochemistry. However, since the crystallographic analysis was performed on acetylated material, the orientation of the glucoside ring in native ptaquiloside is still uncertain. The observation of a NOESY cross-peak between H-5 and H-1^1 suggested the close proximity of these hydrogens—at least in methanol solution. This is consistent with the fact that an energy minimized structure (MM2 force-field) suggested

that the distance between these two protons should be 0.152 nm. Thus, the orientation of the glucoside ring in the native compound appeared to be similar to that suggested by the crystallographic analysis.



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EXPERIMENTAL

Plant material. Fronds of bracken fern were gathered from an area in north Queensland where known cases of bracken fern poisoning of cattle have occurred. A sample was indentified as *P. aquilinum* var. *revolutum* and given a herbarium number AQ629215. After freeze-drying, fronds were milled to a fine powder.

Purification and isolation. Milled freeze-dried bracken (250 g) was extracted in a Soxhlet apparatus, first with $CHCl_3$ (2 days) followed by MeOH-$CHCl_3$ (1:1) (2 days). Ptaquiloside was extracted from the plant with $CHCl_3$–MeOH azeotrope, MeOH–$CHCl_3$ (13:87), bp *ca* 53°. The extract containing ptaquiloside was concd to remove solvents, H_2O (500 ml) added and the suspension extracted with Et_2O. The aq. layer was removed and the dissolved Et_2O evapd under red. pres. (20°). The aq. soln was purified by adding it to a polyamide (200 g) column and washing the column with H_2O (500 ml). Ptaquiloside was recovered from the aq. eluate by adsorption on XAD-2 resin (Serva 0.05–0.1 mm) (100 g) followed by elution with MeOH (500 ml). The MeOH soln was concd using BuOH–H_2O azeotrope to remove traces of H_2O. To the oily residue, MeOH (20 ml) was added followed by High Flow Super Cell (20 g). After evapn under red. pres. (20°) to remove MeOH, the solid was packed on top of a dry-packed silica gel column (4 × 50 cm) and eluted initially with EtOAc (500 ml) followed by a gradient (0–10%) of MeOH in EtOAc (11). The frs collected (50 ml) were monitored by TLC and those containing ptaquiloside combined and evapd under red. pres. (20°). The oily residue was further purified using a (3.5 × 80 cm) LH-20 column with MeOH as eluant. Final purification was carried out using prep. silica gel HPLC (25 × 100 mm) with 12% MeOH in CH_2Cl_2 as eluant. The final yield of pure ptaquiloside was *ca* 0.1%.

TLC. Samples were monitored using TLC aluminium sheets of silica gel 60 (Merck). Best resolution of the various compounds in the sample was given by (1) MeOH–EtOAc (1:49) and (2) toluene–Me_2CO (3:7). Resolved compounds of interest were located using aniline–diphenylamine–Me_2CO–80% H_3PO_4 (2 ml:2 g:100 ml:15 ml) spray reagent and heating to 100° for a short time. The active compound was revealed as a blue-grey spot R_f 0.3 in solvent (1) and 0.4 in (2).

NMR. Spectra were recorded in CD_3OD soln with TMS as int. standard. DEPT, COSY and HETCOR spectra were acquired with standard pulse-sequences without solvent suppression.

Ptaquiloside (**1**). ^1H NMR (300 MHz, CD_3OD): δ0.48 (1H, *m*, H-13a), 0.68 (1H, *m*, H-12b), 0.85 (2H, *m*, H-12a and H-13b), 1.05 (3H, *d*, J = 6.5 Hz, H-10), 1.27 (3H, *s*, H-14), 1.52 (3H, *d*, J = 1 Hz, H-11), 1.92 (1H, *dd*, J = 12 and 12 Hz, H-3α) 2.20 (1H, *ddq*, J = 6.5, 7.5, and 12 Hz, H-2), 2.47 (1H, *dd*, J = 8, and 12 Hz, H-3β), 2.63 (1H, *d*, J = 1.5 Hz, H-9), 3.19 (1H, *dd*, J = 8 and 8.5 Hz, H-2'), 3.30 (1H, H-4'), 3.32 (1H, H-3'), 3.32 (1H, H-5'), 3.66 (1H, *dd*, J = 12 and 5.5 Hz, H-6b'), 3.88 (1H, *dd*, J = 12 and 2 Hz, H-6a'), 4.59 (1H, *d*, J = 8 Hz, H-1'), 5.75 (1H, *dq*, J = 1 and 1.5 Hz, H-5). ^{13}C NMR(75 MHz, CD_3OD): δ5.9 (CH_2, C-12), 10.6 (CH_2, C-13), 13.6 (Me, C-10), 19.5 (Me, C-11), 27.0 (Me, C-14), 30.1 (C, C-7), 45.2 (CH_2, C-3), 45.2 (CH, C-2), 62.5 (CH, C-9), 62.9 (CH_2, C-6'), 71.92 (CH, C-4'), 71.89 (C, C-8), 75.2 (CH, C-2'), 77.7 (CH, C-3'), 78.2 (CH, C-5'), 82.0 (C, C-4), 99.3 (CH, C-1'), 123.1 (CH, C-5), 144.5 (C, C-6), 224.0 (C, C-1).

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