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PONASTEROSIDE A, A GLYCOSIDE OF INSECT METAMORPHOSING SUBSTANCE FROM *PTERIDIUM AQUILINUM* VAR. *LATIUSCULUM*: STRUCTURE AND ABSOLUTE CONFIGURATION*

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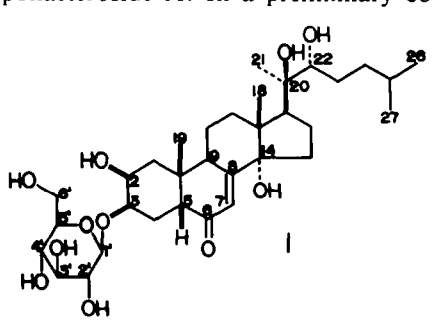
Abstract—A novel glycoside of an insect-metamorphosing substance, ponasteroside A, has been isolated from *Pteridium aquilinum* var. *latiusculum* (Pteridaceae). Chemical and physico-chemical properties of ponasteroside A and its hexaacetate (IV) and, in particular, its enzymatic hydrolysis into ponasterone A (II) and glucose have established the structure and absolute configuration of ponasteroside A as represented by formula I.

IN THE past few years, an amazing number of ecdysterols, active substances controlling the moulting and metamorphosis of arthropods, have been discovered in plants. We now wish to report the occurrence in Nature of a new type of the ecdysterol—a glycoside.

During our survey on ecdysone derivatives in the plant kingdom, we have been undertaking the wide screening tests on vegetable materials by means of bioassay and found that the crude extracts of ferns show insect-metamorphosing hormone activity in high frequency. In fact, a number of phytoecdysones such as ecdysterone, ecdysone, ponasterone A, pterosterone, polypodine B, lemmasterone and shidasterone have been isolated from ferns.[1-11] *Inter alia*, from the American bracken, *Pteridium aquilinum* Kuhn (Pteridaceae), Kaplanis *et al.* have isolated ecdysone and ecdysterone,[2] while from the Japanese bracken, *Pteridium aquilinum* Kuhn var. *latiusculum* Underw. (Pteridaceae), we have isolated ponasterone A, pterosterone and a novel substance with metamorphosing activity for which the term warabisterone was initially proposed.[8] However, since further study has revealed it to be a glycoside, we have changed the term to ponasteroside A. In a preliminary communication,[12] we have



I

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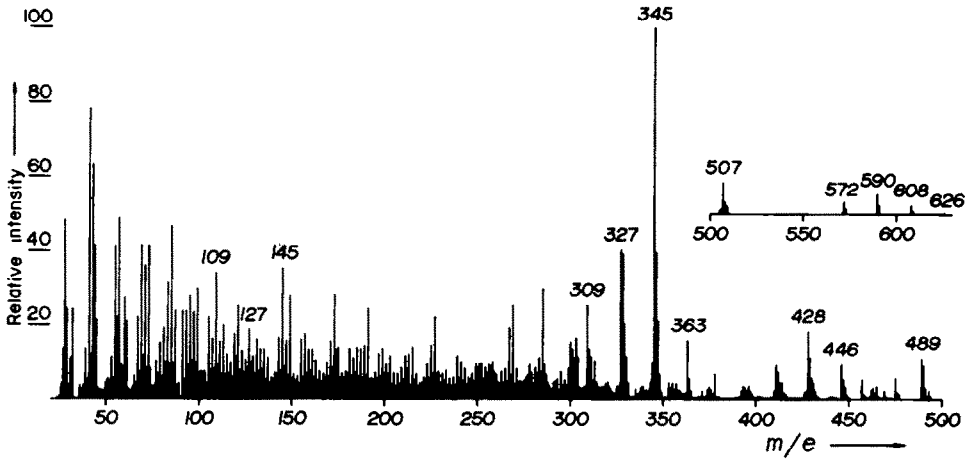


FIG. 1 Mass spectrum of ponasteroside A (70 eV).

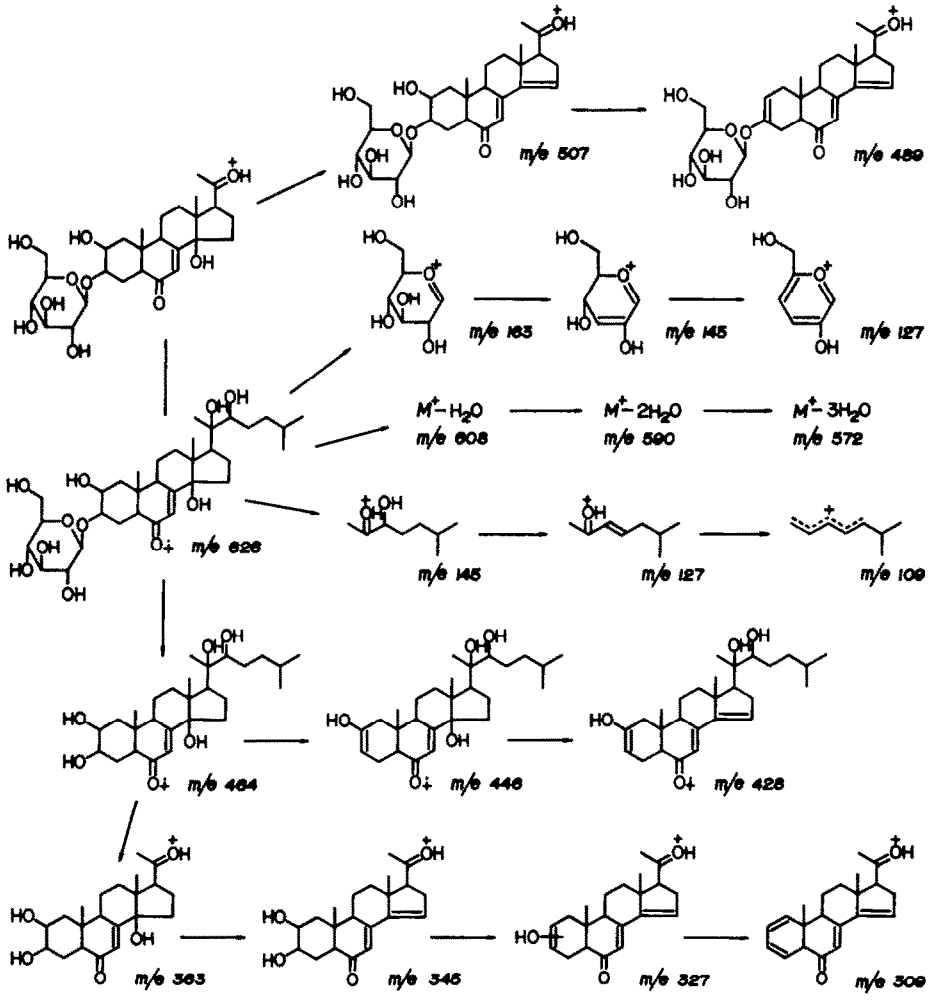


FIG. 2 Principal mass fragmentation of ponasteroside A.

outlined the evidence for the stereostructure of ponasteroside A as shown in formula I. The present paper describes full detail of this work.

Ponasteroside A shows positive colour reactions for steroids. The molecular formula was established as $C_{33}H_{54}O_{11}$ by elemental analysis and the appearance of the peaks in the mass spectrum at *m/e* 626 (0·2% relative to the base peak at *m/e* 345), 608 (3%), 590 (5%) and 572 (3%) originating from the molecular ion and the ions formed by loss of one to three molecules of water from it (Fig. 1), the compositions of the fragment ions being confirmed by high resolution mass spectroscopy. The spectral properties of ponasteroside A are very similar to those of the common phytoecdysones, e.g. ponasterone A (II).[13-15] Thus the IR spectrum shows strong bands at 3430 and 1650 cm^{-1} which are ascribable to many OH groups and an enone group, respectively (Fig. 3).

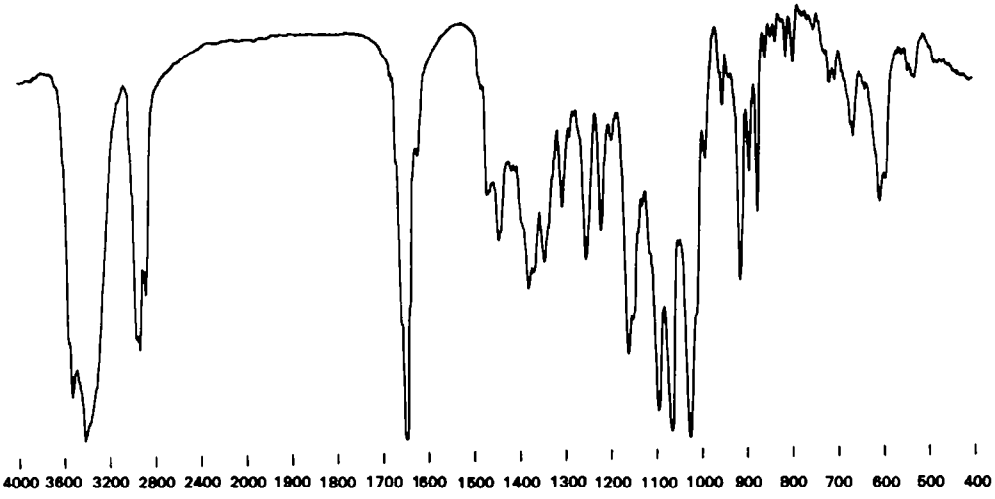


FIG. 3 IR spectrum of ponasteroside A (KBr).

The latter band together with a UV max at 245 mμ and an NMR signal at 6·18 ppm indicate the presence of a β,β-disubstituted α,β-unsaturated ketone system. When ponasteroside A was heated with ethanolic hydrochloric acid, the product exhibited UV maxima at 298 and 244 mμ attributable to a 7,14-dien-6-one chromophore and a 8,14-dien-6-one chromophore in the steroid nucleus, respectively. These observations strongly suggest that ponasteroside A possesses the 14-hydroxy-7-en-6-one structure in the steroid skeleton as the other known ecdysterols.

The NMR spectrum (Fig. 4) shows five Me signals whose chemical shifts and splitting patterns were compared with those of the common ecdysterols, and found to be in good agreement with those of ponasterone A (II) with the exception that the C-19 Me proton signal of ponasterone A is shifted upfield by 0·15 ppm in the spectrum of ponasteroside A (Table 1). At this point it was expected that ponasteroside A is closely related to ponasterone A. However, the composition indicates the presence of $C_6H_{10}O_5$ more than in ponasterone A, and this increment may correspond to the

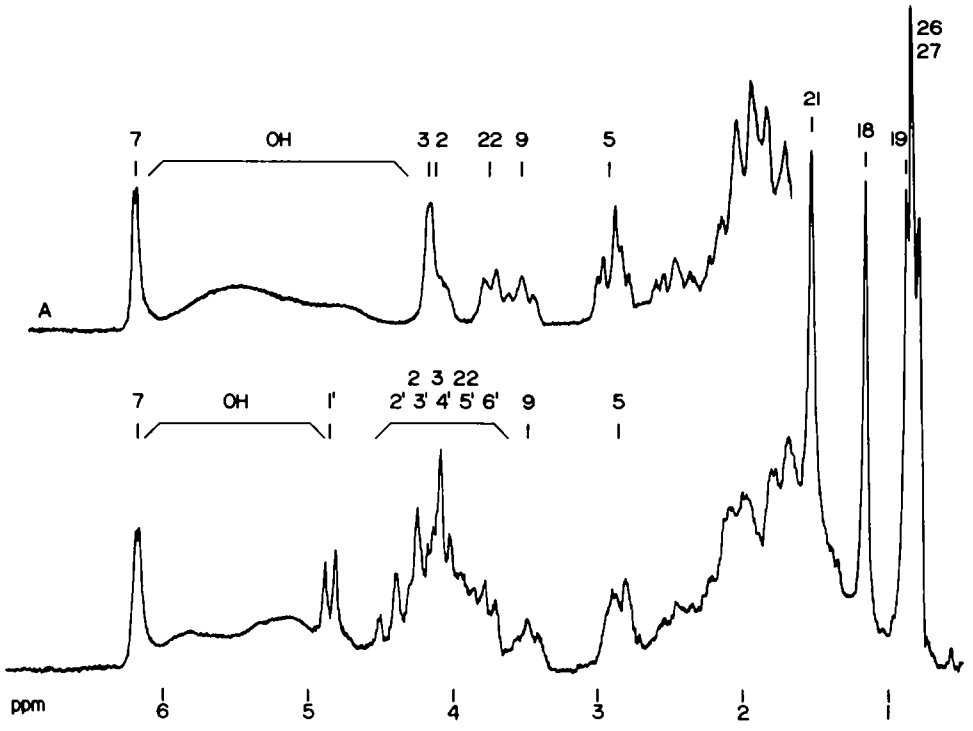
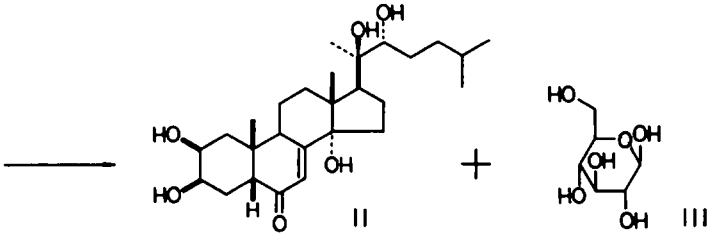


FIG. 4 NMR spectrum of ponasteroside A (C_5D_5N, 100 MHz). (A: that of ponasterone A).

TABLE 1. METHYL CHEMICAL SHIFTS (PYRIDINE)

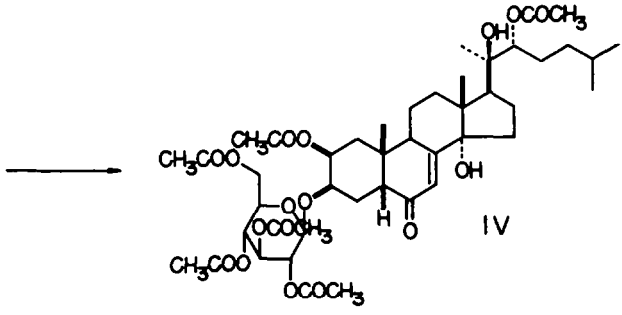
	C-18	C-19	C-21	C-26	C-27
Ponasterone A (II)	1·16	1·03	1·51	0·82	0·82
Ponasteroside A (I)	1·17	0·88	1·54	0·84	0·84

linkage of a common hexose. In keeping with this view, the IR spectrum of ponasteroside A shows much stronger bands at 1030, 1073 and 1105 cm^{-1} attributed to the C—O stretching vibrations (Fig. 3), and the NMR spectrum of ponasteroside A exhibits the signals due to carbinyl hydrogens much more than those of ponasterone A (Fig. 4). Based on the above observations, ponasteroside A is probably a glycoside of ponasterone A. Enzymatic hydrolysis of ponasteroside A was then carried out to give



ponasterone A (II) and glucose (III), establishing that ponasteroside A is an O-glucoside of ponasterone A.

As in the NMR spectrum of ponasteroside A, the chemical shift of the C-19 Me signal is displaced as compared with that of ponasterone A, the glucose must be linked with ponasterone A at ring A. In agreement with this, in the mass spectrum of ponasteroside A, the peaks attributed to the nucleus fragments formed by the cleavage of the C-20:C-22 bond followed by subsequent loss of one to two molecules of water appear at *m/e* 507 (9%) and 489 (11%) (Fig. 1), indicating that the fragment remaining after loss of the side-chain (the nucleus fragment) bears the glucose residue (Fig. 2). In order to establish the environment of the glucoside linkage, ponasteroside A was acetylated to give the hexaacetate (IV). The signals of the NMR spectrum of the



IV

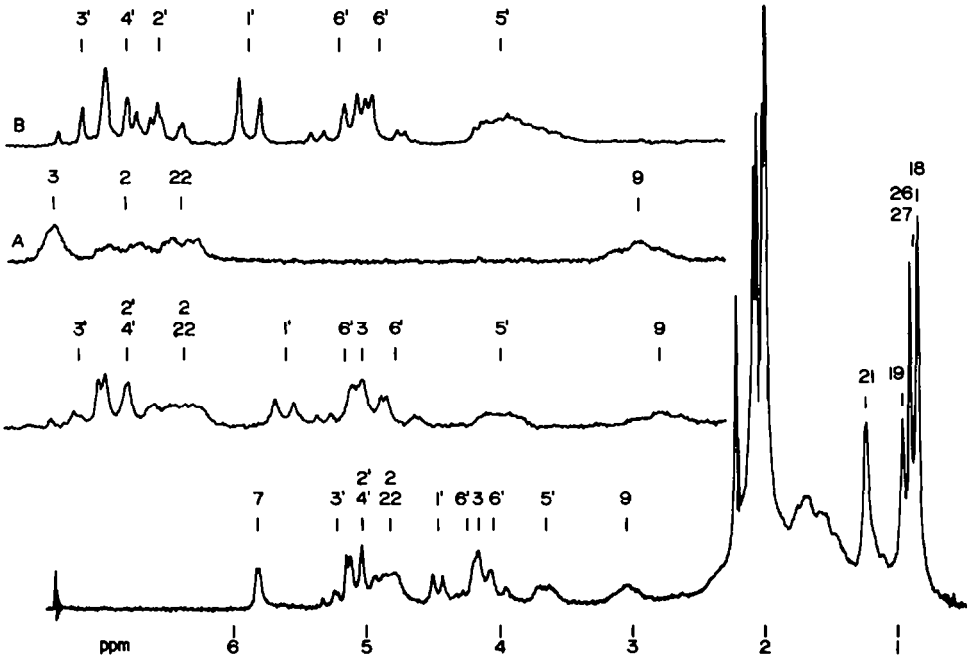


FIG. 5 NMR spectrum of ponasteroside A hexaacetate ($CDCl_3$, 100 MHz). (A: that of ponasterone A triacetate, B: that of stigmastanyl-β-D-glucopyranoside tetraacetate).

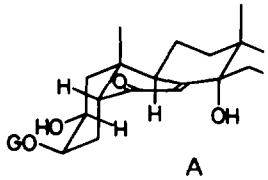
hexaacetate (IV) in deuteriochloroform were assigned by comparison with the spectra of the reference substances, ponasterone A 2,3,22-triacetate and stigmastanyl β-D-glucopyranoside tetraacetate, coupled with the aid of the double resonance experiments (Fig. 5 and Table 2). Two signals originating from the two carbinyl hydrogens

TABLE 2. PROTON SIGNALS ($CDCl_3$)

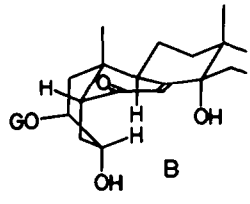
	C-2α	C-3α	C-7	C-9	C-18	C-19	C-21	C-22	C-26	C-27
Ponasterone A 2,3,22-tri-acetate	5·05 ddd	5·32 ddd	5·86 d	3·12 ddd	0·85 s	1·02 s	1·24 s	4·82 dd	0·88 d	0·88 d
Ponasteroside A 2,22,2′-3′,4′,6′-hexaacetate	~4·8 +	~4·15 +	5·82 d	3·05 ddd	0·85 s	0·96 s	1·24 s	~4·8 +	0·88 d	0·88 d
	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′				
Stigmastanyl β-D-glucoside 2′,3′,4′,6′-tetraacetate	4·59 d	4·93 dd	5·22 dd	5·04 dd	3·64 ddd	4·10 dd	4·24 dd			
Ponasteroside A 2,22,2′-3′, 4′,6′-hexaacetate	4·46 d	~5·02 +	5·22 dd	~5·02 +	3·65 ddd	4·05 dd	4·24 dd			

+Patterns are unclear due to overlapping of the signals.

in ring A of the hexaacetate (IV) appear at ~4·15 and ~4·8 ppm. From the line positions, the former signal must be ascribable to a hydrogen on a carbon attached to an OH group participating the glycoside linkage, and the latter signal attributable to a hydrogen on a carbon bearing an acetylated OH group. However, the two signals in question are overlapped with the other signals and, consequently, complete analysis of their spin–spin coupling patterns could not be achieved. Therefore, in the hope that the two signals would be separated from the other signals on using benzene instead of chloroform, the NMR spectrum of ponasteroside A hexaacetate (IV) in deuteriobenzene was measured and compared with the spectra of ponasterone A triacetate and stigmastanyl β-D-glucopyranoside in the same solvent. However, more overlapping of the signals occurred in the spectrum of the hexaacetate (IV) in deuteriobenzene solution. Although the overlapping does not permit the exact determination of the band width at half height of each signal in the spectrum of the hexaacetate (IV) recorded in deuteriochloroform, the shape of the carbinyl signal at ~4·15 ppm indicates that it is associated with an equatorial hydrogen, while that of the carbinyl signal at ~4·8 ppm shows that it is due to an axial hydrogen. The combined evidence demonstrates that the structure and conformation of the A-ring of ponasteroside A hexaacetate (IV) is as shown in the perspective A or B. In accordance with this conclusion, an intramolecular nuclear Overhauser effect was observed between the



A



B

hydrogen on an acetoxyl-bearing carbon at ~4·8 ppm (i.e. the C-2 hydrogen in conformation A or the C-3 hydrogen in conformation B) and the C-9 allylic hydrogen at 3·05 ppm in the spectrum of the hexaacetate (IV). On the other hand, the conformation of the A-ring of ponasterone A and its triacetate has been shown to be the same as that of ecdysone which has a chair form (X-ray analysis[16]). A chair conformation of the A-ring of ponasteroside A and its hexaacetate (IV) was indicated by (i) the ORD curves of ponasteroside A and its acetate (IV), these being similar to those of ponasterone A (II) and its triacetate (Table 3); and (ii) the splitting pattern of the C-5 hydrogen

TABLE 3. AMPLITUDES (*a*) OF ORD CURVES (MeOH).

	at ca. 240 mμ ($\pi \rightarrow \pi^*$)	at ca. 340 mμ ($n \rightarrow \pi^*$)
Ponasteroside A	−288	+107
Ponasterone A	−228	+68
Ponasteroside A hexaacetate	−233	+94
Ponasterone A triacetate	−344	+101

signal in the NMR spectrum of ponasteroside A is essentially identical with that of ponasterone A (Fig. 4). Furthermore, conformational analysis demonstrates that formation of the glycoside linkage between glucose and the C-2 equatorial hydroxyl in the chair-formed A-ring of ponasterone A, makes the hydroxyl quasi-axial and the A-ring boat-formed as represented by perspective B for ponasteroside A, this conformation being energetically much less advantageous. The combined evidence leads to the conclusion that in ponasteroside A the A-ring is in the chair conformation and, consequently, the aglycone is linked through the C-3 oxygen to glucose.

The remaining uncertainties in the structure of ponasteroside A are the size of the ring, and the stereochemistry and, in particular, the configuration at the anomeric C atom of the glucose residue. In the NMR spectrum of the hexaacetate (IV), the C-6′, methylene proton signals occur at 4·05 and 4·24 ppm which are coupled to each other (J = 12 Hz) and to the C-5′ methine proton signal at 3·65 ppm (J = 5 and 2 Hz, respectively). The line position of the last signal must be ascribable to a hydrogen on carbon attached to an ethereal oxygen but not with that on a carbon bearing an acetoxyl group, showing that the glucose residue in ponasteroside A has the 1,5-(pyranose) ring structure. Since ponasteroside A is hydrolyzed by an enzyme which specifically assists hydrolysis of β-glucosides, the glucose residue is inferred to be present as a β-anomer. This was confirmed by the observation that the C-1′ anomeric hydrogen signals in the NMR spectra of ponasteroside A and its acetate (IV) appear as doublets with the large coupling constants 7 Hz, corresponding to those between vicinal H atoms in diaxial relationship. The rotation contribution of glucose component in ponasteroside A ($[M]_D$ of ponasteroside A − $[M]_D$ of ponasterone A = −92 (pyridine)). This indicates that the glucose involved in ponasteroside A is of the D series.[17]

On the basis of the above results, ponasteroside A is ponasterone A 3-β-D-glucopyranoside (I).

The mass spectral data of ponasteroside A fully support the deduced structure I as summarized in Fig. 2.

We are now of the opinion that glycosides of ecdysterols are also widely distributed in the plant kingdom. Ponasteroside A presently characterized constitutes the first example.

Of particular interest biologically is the fact that ponasteroside A, a glycoside, also exhibits high insect-metamorphosing hormone activity in the insect (*Sarcophaga*) test, and shows stimulating effect on protein synthesis in the mouse,[18] though the possibility that ponasteroside A reveals the activities only after enzymatic hydrolysis in animals, cannot be excluded at present. This phenomenon will provide an interesting subject in the biochemical field.

EXPERIMENTAL

M.ps are uncorrected. NMR spectra were determined on a Varian HA-100 spectrometer. Chemical shifts are given in ppm downfield from internal TMS and coupling constants (J) in Hz. Abbreviations: s = singlet, d = doublet, m = multiplet and dd = doublet of doublets.

Isolation of ponasteroside A. The dried whole plants of *Pteridium aquilinum* Kuhn var, *latiusculum* Underw, (Pteridaceae) (37 kg) were extracted 3 times with refluxing MeOH (200 l. each) for 5 hr (each extraction). The combined MeOH soln was concentrated to yield an extract (1·9 kg), which on extraction with AcOEt and evaporation gave a residue (360 g). After chromatography of the residue over alumina (700 g), the fraction eluted with AcOEt: MeOH (5 : 2) were combined (10 g) and chromatographed on silica gel twice. Fractions containing a substance more polar than ecdysterone were combined and crystallized from EtOH to furnish *ponasteroside A* (I) as colourless needles (52 mg), m.p. 278–279°, $[\alpha]_D$ +28·5° (*c* 5·2, pyridine), ORD (*c* 0·0980, MeOH): $[\phi]_{336}^{peak}$ +4300, $[\phi]_{300}^{trough}$ −6500, $[\phi]_{290}^{peak}$ −6400, $[\phi]_{266}^{trough}$ −9600, $[\phi]_{235}^{peak}$ +19200, CD (*c* 0·0962, dioxan): $[\theta]_{339}^{max}$ +4·5 × 10^3; UV λ_{max}^{EtOH} mμ (log ε): 245 (4·14); IR ν_{max}^{KBr} cm^{-1}: 3430 (OH), 1650 (cyclohexenone); NMR (C_5D_5N): 6H d at 0·82 (J = 6, $C_{(26)}\underline{H}_3$, $C_{(27)}\underline{H}_3$), 3H s at 0·88 ($C_{(19)}\underline{H}_3$), 3H s at 1·17 ($C_{(18)}\underline{H}_3$), 3H s at 1·54 ($C_{(21)}\underline{H}_3$), 1H dd at 2·86 (J = 4, 13, $C_{(5)}\underline{H}$), 1H m at 3·48 ($C_{(9)}\underline{H}$), 9H m in the region 3·7–4·5 ($C_{(2)}\underline{H}$, $C_{(3)}\underline{H}$, $C_{(22)}\underline{H}$, $C_{(2')}\underline{H}$, $C_{(3')}\underline{H}$, $C_{(4')}\underline{H}$, $C_{(5')}\underline{H}$, $C_{(6')}\underline{H}_2$), 1H d at 4·85 (J = 7, $C_{(1')}\underline{H}$), 1H d at 6·18 (J = 2, $C_{(7)}\underline{H}$). (Found: C, 63·51; H, 8·72. $C_{33}H_{54}O_{11}$ requires: C, 63·21; H, 8·69%).

Acid treatment of ponasteroside A. Ponasteroside A (0·24 mg) in dil ethanolic HCl (1%, 2 ml) was heated under reflux for 1 hr to give the mixture of the 8,14-dien-6-one and the 7,14-dien-6-one, UV $\lambda_{max}^{EtOH(HCl)}$ mμ; 245, 298.

Enzymatic hydrolysis of ponasteroside A. Ponasteroside A (5 mg) in water (3 ml) was stirred in the presence of cellulase containing β-glucosidase (2 mg) at 30° for 24 hr.

The ppt was collected by filtration and crystallized from AcOEt-MeOH to give II as colourless needles, m.p. 277–278°, IR ν_{max}^{KBr} cm^{-1}: 3350 (OH), 1640 (cyclohexenone). The identity was confirmed by the usual criteria.

The filtrate was concentrated under reduced press to give a syrup. PPC (n-BuOH-AcOH-water = 4 : 1 : 5): R_f 0·20 (glucose: 0·20), PPC (phenol saturated with water): R_f 0·39 (glucose: 0·39). TLC (silica gel, n-BuOH-AcOH-water = 4 : 1 : 1): R_f 0·25 (glucose: 0·25).

Acetylation of ponasteroside A. Ponasteroside A (20 mg) in pyridine (0·4 ml) and Ac_2O (0·2 ml) was set aside at room temp overnight. After isolation in the usual manner, the product (25 mg) was chromatographed over silica gel (5 g). $CHCl_3$–ether eluate (20 mg) was crystallized from EtOH-isopropyl ether to give *ponasteroside A 2,22,2',3',4',6'-hexaacetate* (IV) as colourless needles, m.p. 126–127°. ORD (*c* 0·177, MeOH): $[\phi]_{356}^{peak}$ +4200, $[\phi]_{306}^{trough}$ −5300, $[\phi]_{287}^{peak}$ −4700, $[\phi]_{260}^{trough}$ −6500, $[\phi]_{232}^{peak}$ +17900. IR ν_{max}^{KBr} cm^{-1}: 3540 (OH), 1748, 1223 (acetoxyl), 1663 (cyclohexenone); NMR ($CDCl_3$): 3H s at 0·85 ($C_{(18)}\underline{H}_3$), 6H d at 0·88 (J = 6, $C_{(26)}\underline{H}_3$, $C_{(27)}\underline{H}_3$), 3H s at 0·96 ($C_{(19)}\underline{H}_3$), 3H s at 1·24 ($C_{(21)}\underline{H}_3$), six 3H s's at 2·00, 2·00, 2·03, 2·07, 2·09, 2·23 ($C\underline{H}_3$—COO—), 1H ddd at 3·05 (J = 2, 8, 10, $C_{(9)}\underline{H}$), 1H ddd at 3·65 (J = 9, 5, 2, $C_{(5')}\underline{H}$), 1H dd at 4·05 (J = 2, 12, $C_{(6')}\underline{H}$), 1H dd at 4·24 (J = 5, 12, $C_{(6')}\underline{H}$), 1H single peak at ca. 4·15 ($C_{(3)}\underline{H}$), 1H d at 4·46 (J = 7, $C_{(1')}\underline{H}$), 2H m at ca. 4·8 ($C_{(2)}\underline{H}$, $C_{(22)}\underline{H}$), 2H m at ca. 5·02 ($C_{(2')}\underline{H}$, $C_{(4')}\underline{H}$), 1H dd at 5·22 (J = 11, 9, $C_{(3')}\underline{H}$), 1H d at 5·82 (J = 2, $C_{(7)}\underline{H}$), NMR (C_6D_6): 3H s at 0·83 ($C_{(18)}\underline{H}_3$), 6H d at 0·86 (J = 6, $C_{(26)}\underline{H}_3$, $C_{(27)}\underline{H}_3$), 3H s at 0·89 ($C_{(19)}\underline{H}_3$), 3H s at 1·26 ($C_{(21)}\underline{H}_3$), six 3H s's at 1·69, 1·72, 1·80, 1·84, 1·94, 2·32 ($C\underline{H}_3$—COO—), 1H broad peak at 3·13 ($C_{(9)}\underline{H}$), 1H ddd at 3·46 (J = 9, 5, 2, $C_{(5')}\underline{H}$), 1H dd at 4·06 (J = 2, 12, $C_{(6')}\underline{H}$), 1H dd at 4·32 (J = 5, 12, $C_{(6')}\underline{H}$), 1H single peak at 4·31 ($C_{(3)}\underline{H}$), 1H d at 4·34 (J = 7, $C_{(1')}\underline{H}$), 5H m at 4·9–5·6 ($C_{(2)}\underline{H}$, $C_{(22)}\underline{H}$, $C_{(2')}\underline{H}$, $C_{(3')}\underline{H}$, $C_{(4')}\underline{H}$), 1H d at 5·91 (J = 2, $C_{(7)}\underline{H}$).

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