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New Ecdysteroids from *Polypodium vulgare*

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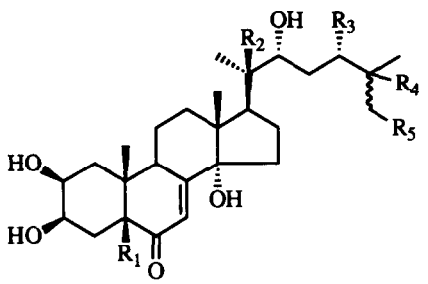
Abstract: Five phytoecdysteroids not previously reported in *Polypodium vulgare* have been isolated from methanol extracts of *in vitro* cultures of prothalli of this species. Among them, inokosterone, pterosterone and abutasterone had already been identified in other plants, whereas 24-hydroxyecdysone and 5-hydroxyabutasterone are described for the first time. The structure of these compounds was inferred from the corresponding ^{1}H and ^{13}C NMR and thermospray-HPLC-MS spectral data. The complete description of the structures includes the stereochemical assignment at C-24, which was 24*S* for all compounds. This assignation was carried out by ^{13}C NMR spectroscopic studies of 22,24-benzylidene acetal derivatives.

Ecdysteroids are a family of polyhydroxylated steroids structurally related to ecdysone (**1**), the biosynthetic precursor of the insect moulting hormone 20-hydroxyecdysone (**2**). Over 100 different ecdysteroids have been isolated so far from plants. Apparently, these compounds seem to provide some degree of protection against phytophagous insects. In addition to this postulated activity, it has been shown that ecdysteroids exhibit interesting pharmacological effects on mammals, including the stimulation of protein synthesis and the reduction of blood glucose and cholesterol levels [1].



	R_1	R_2	R_3	R_4	R_5
1: Ecdysone	H	H	H	OH	H
2: 20-Hydroxyecdysone	H	OH	H	OH	H
3: Polypodine B	OH	OH	H	OH	H
4: Inokosterone	H	OH	H	H	OH
5: Pterosterone	H	OH	OH	H	H
6: 24-Hydroxyecdysone	H	H	OH	OH	H
7: Abutasterone	H	OH	OH	OH	H
8: 5-Hydroxyabutasterone	OH	OH	OH	OH	H

Figure 1. Ecdysteroids isolated from *Polypodium vulgare*, including those reported in this paper.

In general, concentrations of ecdysteroids vary depending on the plant part, the season and the habitat. For this reason, *in vitro* cultures constitute a valuable technique in plant research, since they permit the mass production of secondary metabolites without seasonal or individual variations. In this context, *in vitro* cultures of small foliaceous structures (prothalli) produced from spores of the fern *Polypodium vulgare* showed the presence of three main

ecdysteroids [ecdysone (**1**), 20-hydroxyecdysone (**2**) and polypodine B (**3**), Figure 1] in a total amount of 0.8% dry weight [2]. In the present paper we report on the structural elucidation of five other minor ecdysteroids isolated from these *in vitro* cultures. Structures **4-8** have been inferred mainly from mass spectral data and by comparison of their NMR spectra with those published for related phytoecdysteroids [3].

A constant problem that emerges in the structural elucidation of natural products is the correct assignment of the configuration of chiral carbon atoms. Owing to the small amount of compound that usually can be isolated, crystallization and further X Ray diffraction studies are difficult to carry out. Phytoecdysteroids do not constitute an exception to that problem, specially as regards to the absolute configuration at C-24. In this respect, although the structure of abutasterone (**7**) has been reported [4], its stereochemistry at C-24 remained hitherto unknown. In this paper we also establish this configuration for **7** and related compounds **6** and **8**.

Results and Discussion

During the course of our research on secondary metabolites of *P. vulgare*, we decided to identify the minor ecdysteroids in this species to clarify some aspects of the biosynthesis of these compounds. Although all ecdysteroids reported in this paper were also found in the wild plant [5], for the reasons mentioned above *in vitro* micropropagated prothalli were used as starting material for the present study. The extraction and isolation of phytoecdysteroids from lyophilized ground prothalli was carried out as described in the experimental part. Final purification of minor compounds carried out by preparative TLC or semipreparative HPLC led to the isolation of three known ecdysteroids (**4**, **5** and **7**), and two new structures that were identified as 24-hydroxyecdysone (**6**) and 5-hydroxyabutasterone (**8**) as described below.

^1H and ^{13}C NMR data for inokosterone (**4**), pterosterone (**5**) and abutasterone (**7**), summarized in Tables 1 and 2, are consistent with those previously published [3]. It is important to remark that only one of the stereoisomers at C-25 of inokosterone is obtained in our case, in contrast to the epimeric mixture described by Hikino et al. [6]; however, we have not assigned the absolute configuration for this epimer.

Table 1. Selected ^1H NMR spectral data of inokosterone (**4**), pterosterone (**5**), 24-hydroxyecdysone (**6**), abutasterone (**7**) and 5-hydroxyabutasterone (**8**). Chemical shifts are given in ppm referred to TMS (C_5D_5N).

	4	5	6	7	8
2-Ha	4.18	4.15	4.13	4.20	4.26
3-He	4.23	4.23	4.20	4.22	4.17
5-H	3.02(dd 15,4.5)	3.05(dd 13.5,4.3)	3.01 (dd 15,3.8)	3.0	--
7-H	6.26(d 2.0)	6.27(d 2.3)	6.22 (d 2.4)	6.24(d 2.2)	6.26 (d, 2.4)
9-Ha	3.66(d 6.3)	3.58	3.54 (m $w_{1/2}$ 24.38)	3.59	3.61 (d 9.6)
17-H	2.96(t 9.0)	2.93(t 9.1)	2.98 (t 8.4)	3.0	2.98 (t 8.4)
22-H	3.86(d 9.6)	4.15	4.08	4.25	4.25 (d 10.5)
24-H	--	3.95	4.41 (d 8.4)	4.08(d 9.1)	4.09 (d 9.3)
18-Me	1.22	1.22	0.68	1.20	1.19
19-Me	1.05	1.06	1.05	1.07	1.15
21-Me	1.57	1.60	1.26 (d 6.6)	1.61	1.61
26-	3.62(m),3.76(m)	1.01(d 6.9)	1.49&	1.47§	1.47¶
27-Me	1.03(d 6.9)	1.01(d 6.9)	1.43&	1.52§	1.52¶

Assignments marked with &, § or ¶ can be exchanged.

Likewise, two new compounds identified as 24-hydroxyecdysone (**6**) and 5-hydroxyabutasterone (**8**), were isolated from *P. vulgare* . These structures were inferred from mass spectral data, which indicated the presence

of six and eight hydroxyl groups, respectively, as shown by the corresponding peaks at m/z: 481 $[M + 1]^+$ and 513 $[M + 1]^+$. The location of these hydroxyl groups in the cholest-7-en-6-one skeleton was assigned by comparison of the ^{13}C NMR spectra of **6** and **8** with those of related ecdysteroids [ecdysone (**1**), 20-hydroxyecdysone (**2**), polypodine B (**3**), pterosterone (**5**) and abutasterone (**7**)]. In the case of **6**, it appeared clearly that six signals attributable to hydroxyl substituted carbon atoms were present, as it occurs in 20-hydroxyecdysone and two of them corresponded to quaternary carbon atoms. As shown in Table 2 the main part of the signals from the ring framework were very close to those shown by ecdysone, which indicated a similar structure for both compounds (*cf.* chemical shifts of carbons C-5,C-16,C-17,C-18 and C-19). Therefore, the difference (with respect to ecdysone and 20-hydroxyecdysone) should arise from the side chain hydroxyl substitutions. The comparative analysis of the ^{13}C chemical shift changes at C-22,C-23,C-24,C-25,C-26 and C-27 among the pair of products 20-hydroxyecdysone (**2**) vs. abutasterone (**7**) led us to assign one hydroxy substitution at C-24 and consequently the structure of the new compound as 24-hydroxyecdysone (**6**). The corresponding ^1H-NMR spectrum reinforced the above assignation (Table 1).

Table 2. ^{13}C NMR spectral data of ecdysone (**1**), 20-hydroxyecdysone (**2**), polypodine B (**3**), inokosterone (**4**), pterosterone (**5**), 24-hydroxyecdysone (**6**), abutasterone (**7**) and 5-hydroxyabutasterone (**8**). Chemical shifts are given in ppm referred to TMS (C_5D_5N).

	1	2	3	4	5	6	7	8
C-1	38.08	38.09	34.77	38.02	38.02	37.97	37.93	34.85
C-2*	68.10	68.33	67.89	68.11	68.17	68.14	68.06	67.99
C-3*	68.10	68.23	69.81	68.19	68.10	68.08	67.95	69.90
C-4	32.45	32.53	35.90	32.52	32.51	32.50	32.41	35.99
C-5	51.41	51.48	79.83	51.47	51.45	51.44	51.38	79.93
C-6	203.36	203.56	200.89	203.61	203.57	203.69	203.45	201.01
C-7	121.61	121.79	119.86	121.72	121.73	121.61	121.60	119.92
C-8	165.53	166.11	166.74	166.24	166.16	165.7	166.08	166.85
C-9	34.63	34.67	38.20	34.47	34.46	34.48	34.41	38.29
C-10	38.76	38.80	44.69	38.74	38.72	38.73	38.60	44.77
C-11	21.20	21.29	22.02	21.15	21.14	21.09	21.08	22.01
C-12	31.49	32.19	31.97	32.10	32.03	31.36	31.65	32.02
C-13	47.70	48.27	48.06	48.15	48.11	47.56	48.03	48.12
C-14	83.97	84.42	83.92	84.22	84.16	83.77	84.02	83.96
C-15	32.03	31.88	31.62	31.83	31.83	31.95	31.91	31.65
C-16	26.74	21.61	21.34	21.53	21.53	26.64	21.46	21.44
C-17	48.28	50.28	59.90	50.10	50.05	48.17	49.98	49.92
C-18	15.89	17.99	17.82	17.97	17.95	15.81	17.85	17.91
C-19	24.55	24.55	17.13	24.52	24.51	24.52	24.45	17.23
C-20	43.04	77.09	76.73	76.82	76.97	42.54	76.66	76.74
C-21	13.74	21.77	21.64	21.72	21.70	13.80	21.70	21.79
C-22	74.07	77.75	77.48	77.30	77.66	74.93	78.18	78.33
C-23	25.69	27.59	27.43	30.29	35.86	30.67	32.83	32.95
C-24	42.55	42.64	42.61	32.10	76.72	80.85	80.22	80.37
C-25	69.80	69.86	69.46	36.82	34.01	72.31	72.13	72.27
C-26	30.09#	30.10⌘	29.95¶	67.35	17.00$	26.74&	26.87§	27.0¶
C-27	30.01#	30.15⌘	30.10¶	17.92	19.64$	25.44&	25.19§	25.27¶

Assignments marked with *, #, ⌘, ¶, $, &, § or ¶ can be exchanged.

Similarly, the analysis of the ^{13}C NMR spectral data for the other compound led us to assign its structure as **8**. In this case, the DEPT spectrum showed two new hydroxylated carbons when compared to that of 20-hydroxyecdysone, one of them at a quaternary carbon and the other at a tertiary together with the corresponding

reduction of CH_2 and CH moieties in the aliphatic region. The comparison of chemical shifts with those of reference compounds **1**, **2** and **3** showed that the signals due to the carbon atoms in the ring frame appeared at positions close to those of polypodine B (**3**), including also those corresponding to C-18, C-19, and in the side chain, C-20 and C-21. Thus, compound **8** should have a ring system identical to polypodine B and, on the other hand, the ^{13}C signals corresponding to the side chain resembled to those of abutasterone (**7**). These facts led to assign the structure of **8** as 5-hydroxyabutasterone. The corresponding ^{1}H-NMR spectrum fully supported this assignation.

As we have mentioned above the configuration at C-24 of abutasterone (**7**) was not defined by previous authors [4]. In our case, compounds **6** and **8** have a hydroxyl group at C-24, and from carbon and proton NMR data it was not possible to establish that configuration. To solve this problem we applied the methodology described by Blunt et al. These authors established the 24*S* configuration for ponasterone C by ^{13}C- NMR studies of its 22,24-benzylidene derivative. The same configuration was inferred for pterosterone (**5**) by comparison of the ^{13}C-NMR of both underivatized ecdysteroids [7].

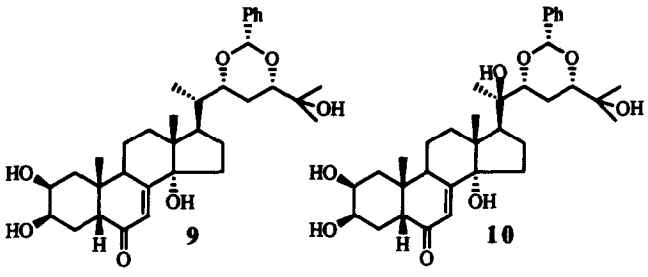


Table 3. ^{13}C NMR selected signals of benzylidene derivatives **9** and **10***.

	9	**10**
C-2#	68.1	68.1
C-3#	68.1	68.2
C-5	51.3	51.4
C-6	203.2	203.2
C-8	166.0	167.0
C-13	47.6	47.8
C-14	83.8	84.0
C-17	47.5	48.9
C-20	39.7	74.4
C-22	79.7	81.5
C-24	84.2	85.1
C-25	71.1	72.4
C-2 Acetal	101.1	102.0

*Chemical shifts are given in ppm referred to TMS (C_5D_5N).
Assignments can be exchanged.

Preparation of the 22,24-benzylidene acetals of **6** and **7** by using the procedure reported by Blunt et al. led to derivatives **9** and **10**, respectively. The ^{13}C NMR spectra of these compounds showed chemical shifts characteristic of a six membered ring benzylidene acetal (Table 3). The formation of this acetal at C-22 and C-24, and not at C-20 was deduced from comparison of the respective chemical shifts with those of the corresponding underivatized compounds. Thus, a downfield shift was observed for the C-22 and C-24 signals, whereas the absorption at C-20 exhibited a slight upfield change. On the other hand, it has been established that a phenyl group at C-2 in a 1,3-dioxane ring prefers an equatorial conformation [8]. The ^{13}C chemical shift of this carbon depends on the substitution at C-4 and C-6 positions of the 1,3-dioxane ring. One axial substituent at these positions induces an upfield shift of approximately 7 ppm with respect to the all-equatorial substituted derivative, appearing the C-2 signal in a range from 100 to 104 ppm [7,9]. In our case, from the observed absorptions at 101.1 and 102.0 ppm for this carbon in compounds **9** and **10**, respectively, and the absence of signals in the 99-90 ppm region, it can be inferred that all substituents in the 1,3-dioxane ring are equatorial. Consequently, the configurations of these derivatives and those corresponding to their respective parent

compounds 24-hydroxyecdysone (**6**) and abutasterone (**7**) should be 24*S*. Finally, from the analogies observed for the ^{13}C NMR spectra of compounds **7** and **8** it can be also concluded that 5-hydroxyabutasterone (**8**) has the (*S*) configuration at C-24. As we have mentioned above, these configurational assignments agree with those previously established for related phytoecdysteroids, such as pterosterone (**5**) and ponasterone C [7].

Experimental Section

^1H-NMR (300 Hz) and ^{13}C-NMR (75Hz) were recorded on a Varian Unity 300 spectrometer. Chemical shifts are given in ppm using tetramethylsilane as internal reference. The ^{13}C-NMR multiplicities were determined by DEPT experiments. All the coupling constants and width at half height ($w_{1/2}$) are given in Hz. Due to the instrumental intrinsic low sensitivity we optimize all the factors that had some influence in sensitivity, such as sample concentration in the coil volume and acquisition parameters. All spectra were recorded by dissolving the sample (2-4 mg) in 250 µl (sample concentration approx. 25 mM) of dry deuteropyridine and using special low volume NMR tubes with reduction and antivortex glass plugs with magnetic susceptibility similar to the value of pyridine to avoid heterogeneities of magnetic field inside the sample volume. Due to the concentration problems, it is important to establish the best acquisition time, pulse width and exponential decay filter, we used an average value of T_1 and T_2 relaxation times 20-hydroxyecdysone in deuteropyridine (T_1=2 s, T_2=0.2 s)to obtain a correct optimization of those values (Adq.t=1s, pw=50º, lb=2Hz). Under the above conditions an overnight acquisition renders a ^{13}C spectra with an acceptable signal to noise ratio (4:1 rms) to the lower intensity signals (consequence of their short T_2 and high linewidth, approx. 6Hz, signal of C-4, C-9 and C-11). All NMR data are summarized in Tables 1, 2 and 3.

Mass spectra were obtained by HPLC-TSP-MS with a HP-5988A quadrupole apparatus (direct flow injection with HCO_2NH_4 50 mM/CH_3CN (50:50) at 1 mL/min, positive mode, TSP tip 180 ºC, stem 96 ºC and ion source 260 ºC).

Extraction and isolation of phytoecdysteroids: Lyophilized and ground prothalli derived from spores of *P. vulgare* collected in Ahrensburg (Germany) or Montseny (Spain) [10] were extracted with MeOH (1/4 w/v, fourfold) in an ultrasonic bath (45 min). The combined extracts were concentrated to 250 mL, diluted with water to a 7:3 (MeOH:H_2O) ratio and treated as described by Camps et al. [2]. Final purification of minor ecdysteroids was carried out by preparative TLC (silica gel, 0.5 mm, 44:9:1, $CHCl_3$:MeOH:H_2O) or semipreparative HPLC (ODS2, 5 µm; 15 x 1 cm; CH_3CN:H_2O 9:40 at 3 mL/min). The purification procedure was monitored by TLC (eluting with 4:1, $CHCl_3$:MeOH) and by HPLC (ODS2, 5 µm; 15 x 0.46 cm; maintained at 55 ºC and eluted with CH_3CN:*i*-PrOH:H_20 3:4:53 at 1.2 ml/min). Under these HPLC conditions the retention times (min) for the ecdysteroids present in *P. vulgare* were: 7.02 (5-hydroxyabutasterone, **8**), 7.78 (abutasterone, **7**), 9.84 (polypodine B, **3**), 10.98 (20-hydroxyecdysone, **2**), 13.58 (inokosterone, **4**), 21.4 (24-hydroxyecdysone, **6**), 26.78 (pterosterone, **5**) and 30.72 (ecdysone, **1**).

2β,3β,14α,20R,22R,26-Hexahydroxy-5β-cholest-7-en-6-one (4, inokosterone). Starting from 12 g of lyophilized prothalli, 10 mg of this compound was obtained. MS (TSP) m/z: 498 $[M+NH_4]^+$, 481 $[M+1]^+$, 463 $[M+1-H_2O]^+$, 445 $[M+1-2H_2O]^+$, and 427 $[M+1-3H_2O]^+$.

2β,3β,14α,20R,22R,24S-Hexahydroxy-5β-cholest-7-en-6-one (5, pterosterone). Starting from 12 g of lyophilized prothalli, 5 mg of this compound was obtained. MS (TSP) m/z: 498 $[M+NH_4]^+$, 481 $[M+1]^+$, 463 $[M+1-H_2O]^+$, 445 $[M+1-2H_2O]^+$, and 427 $[M+1-3H_2O]^+$.

2β,3β,14α,22R,24S,25-Hexahydroxy-5β-cholest-7-en-6-one (6, 24-hydroxyecdysone). Starting from 12 g of lyophilized prothalli, 6 mg of this compound was obtained. MS (TSP) m/z: 498 $[M+NH_4]^+$, 481 $[M+1]^+$, 463 $[M+1-H_2O]^+$, 445 $[M+1-2H_2O]^+$, and 427 $[M+1-3H_2O]^+$.

2β,3β,14α,20R,22R,24S,25-Heptahydroxy-5β-cholest-7-en-6-one (7, abutasterone). Starting from 12 g of lyophilized prothalli, 5 mg of this compound was obtained. MS (TSP) m/z: 514 $[M+NH_4]^+$, 497 $[M+1]^+$, 479 $[M+1-H_2O]^+$, 461 $[M+1-2H_2O]^+$, and 443 $[M+1-3H_2O]^+$.

2β,3β,5β,14α,20R,22R,24S,25-Octahydroxy-5β-cholest-7-en-6-one (8, 5-hydroxyabutasterone). Starting from 100 g of lyophilized prothalli, 2 mg of this compound was obtained. MS (TSP) m/z: 530 $[M+NH_4]^+$, 513 $[M+1]^+$, 495 $[M+1-H_2O]^+$, 477 $[M+1-2H_2O]^+$, 459 $[M+1-3H_2O]^+$ and 379 [M-side chain cleavage C20-C22]$^+$.

24-Hydroxyecdysone-22,24-benzylidene acetal (9) and abutasterone-22,24-benzylidene acetal (10). Following the general procedure described by Blunt et al.[7], a solution of anhydrous zinc chloride (3 mg) in benzaldehyde (0.2 mL) was added to a suspension of 5 mg of the corresponding ecdysteroid in 0.3 mL of benzaldehyde. The mixture was stirred at room temperature for 10 min and then benzaldehyde was evaporated under vacuum. The residue obtained was redissolved in chloroform and loaded onto a silica gel column. The benzylidene acetal derivatives (at the 22,24 position as major compound) were eluted with 3 ml of 4:1 $CHCl_3$: EtOH solvent mixture.

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