*Phytochemistry*, Vol. 25, No. 6, pp. 1301–1304, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00


# BLECHNOSIDES A AND B: ECDYSTEROID GLYCOSIDES FROM *BLECHNUM MINUS**

APICHART SUKSAMRARN, JOHN S. WILKIE† and DENIS H. S. HORN†

Department of Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok 10240, Thailand; †Division of Applied Organic Chemistry, CSIRO, P.O. Box 4331, G.P.O. Melbourne, Victoria 3001, Australia

(*Received* 14 *August* 1985)

**Key Word Index**—*Blechnum minus*; Blechnaceae; fern; ecdysteroid glycosides; 2-deoxyecdysone; blechnosides A and B.

**Abstract**—The structures of two new ecdysteroid glycosides from *Blechnum minus* have been shown, on the basis of chemical, mass spectral, $^1$H NMR and $^{13}$C NMR spectral evidence, to be 2-deoxyecdysone 3-$\beta$-D-glycopyranoside (blechnoside A) and 2-deoxyecdysone 25-$\beta$-D-glucopyranoside (blechnoside B).

## INTRODUCTION

The fern *Blechnum minus* (R.Br.) Ettingsh. (soft water fern) occurs along many Victorian lowland streams and in damp places throughout the highlands [1]. In an earlier communication [2] we reported that this fern is a rich source of 2-deoxyecdysone (3), ecdysone, 2-deoxy-20-hydroxyecdysone and 20-hydroxyecdysone. Unlike *Blechnum volcanicum* which is endemic in New Zealand, this species does not contain significant amounts of 2-deoxy-3-epiecdysone [3]. We now report the isolation of two new ecdysteroid glycosides from the fronds of this plant.

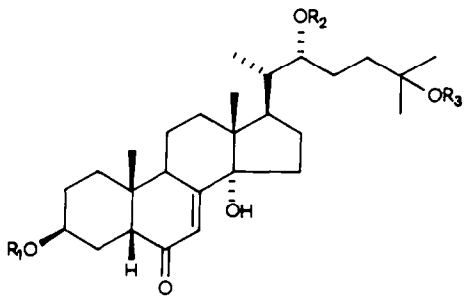
## RESULTS AND DISCUSSION

The two new glycosides, named blechnoside A (1) and blechnoside B (2), were obtained as described in the Experimental. They were closely similar in chromatographic properties but could be separated by repeated HPLC.

Blechnoside A was obtained as an amorphous solid, which on enzymic hydrolysis with $\beta$-glycosidase afforded, from TLC, D-glucose and 3. To check that the aglycone was not 2-deoxy-3-epiecdysone, which has been found in a related species [3] and could be confused with 2-deoxyecdysone on the basis of $R_f$, the aglycone was acetylated, since the 3,22-diacetates of these epimers are reported [3] to be readily distinguished by TLC. The $R_f$ of the diacetate obtained was found to be identical with that of 2-deoxyecdysone 3,22-diacetate. Desorption chemical ionization mass spectrometry (DCIMS) of 1 showed a parent ion at $m/z$ 611$[M+1]^+$ expected of a glycoside composed of 2-deoxyecdysone and one glucose unit. The $^1$H NMR spectrum of 1 (see Table 1) was closely similar to 3 except that the signals for 19-$H_3$ and H-3$\alpha$ were at higher field suggesting that the glucose unit was attached at the C-3 position [4]. This was further supported by the $^{13}$C NMR spectrum in which the C-3 signal of 1 was deshielded by 8.4 ppm [5] as compared with the parent aglycone 3 (see Table 2). In accordance with this assignment the $^1$H NMR spectrum (Table 1) of the acetylated glycoside (4) showed the presence of five acetyl groups and the H-3$\alpha$ did not show an acylation shift. The C-14 and C-25 hydroxyl groups would not undergo acetylation under the mild conditions used ($Ac_2O$–pyridine at 20° for 16 hr). The presence of a glucose unit and its $\beta$-configuration were further supported by the chemical shift values found in its $^{13}$C NMR spectrum for the resonances of the sugar carbons which were in close agreement with those given for methyl-$\beta$-D-glucoside [6] but different from other hexose aldopyranoses [7, 8]. The large coupling constant of the anomeric proton in both 1 ($J$ = 7.5 Hz, H-1′ and H-2′, axial–axial coupling) and its acetate (4) ($J$ = 8 Hz) supports the assignment of the $\beta$-configuration for the glucose unit of blechnoside A [4]. Aso the chemical shift value ($\delta$103.3) found for the anomeric carbon (C-1′) in its $^{13}$C NMR spectrum supports the assignment of the $\beta$-configuration for the glucose in blechnoside A [6, 9]. It is thus shown that blechnoside A is 2-deoxyecdysone 3-$\beta$-D-glucopyranoside (1).

Blechnoside B (2) was obtained crystalline, mp 218–220°. Its DCIMS showed a fragment ion at $m/z$ 593 $[M+1-H_2O]^+$ indicating that it could be an isomer of 1. However, the enzymic hydrolysis was very slow, suggesting that the sugar was attached at a sterically hindered position [10]. The $^1$H NMR spectrum of 2 was similar to that of 3 (see Table 1) but the signals of the C-26/27 protons were not equivalent. Also the signal of the C-25 carbon in the $^{13}$C NMR spectrum of 2 (see Table 2) was deshielded by 7.7 ppm as compared with that of 3 indicating [5] that the sugar was attached at the C-25 position. The assignment was further supported by the observation that the $^{13}$C signal of the anomeric carbon was shielded (4.5 ppm) as compared with that of 1 as expected if the sugar residue is attached to the sterically hindered C-25 hydroxyl group [9]. Otherwise it is seen that the $^{13}$C NMR spectra of 2 and 3 are closely similar, clearly indicating that 2 is a glycoside of 3. In contrast the chemical shift values for the C-3 carbon of 2-deoxy-3-epiecdysone ($\delta$69.1) [3] is quite different from those of 2

* Part 56 in the series "Moulting Hormones". For Part 55 see *Aust. J. Chem.* (1985) **38**, 475.



1 $R_1$ = β-D-Glucopyranosyl; $R_2$ = $R_3$ = H

2 $R_1$ = $R_2$ = H; $R_3$ = β-D-Glucopyranosyl

3 $R_1$ = $R_2$ = $R_3$ = H

4 $R_1$ = Tetraacetoxy-β-D-glucopyranosyl; $R_2$ = Ac; $R_3$ = H

5 $R_1$ = $R_2$ = Ac; $R_3$ = Tetraacetoxy-β-D-glucopyranosyl

and 3 (δ64.1). The $^{13}$C NMR spectrum of 2 (Table 2) is closely similar to that of 1 and from the values for the sugar carbons it is clear that the sugar unit is again β-D-glucose [6–8]. Acetylation of 2 afforded, as shown by its $^1$H NMR spectrum, the hexaacetate (5), as expected if both the C-3 and C-22 hydroxyl groups were acetylated and the sugar was attached at the C-25 position. Also the $^1$H NMR signals of the H-3α and H-22 of 5 showed the characteristic acylation shifts. Accordingly blechnoside B is formulated as 2-deoxyecdysone 25-β-D-glycopyranoside (2).

## EXPERIMENTAL

$^1$H NMR and $^{13}$C NMR spectra were measured on a Bruker WM 250 instrument. DCIMS were determined with a Finnigan 3300 instrument at 70 eV with a desorption chemical ionization probe and $CH_4$ as ionizing gas. Plates for TLC were prepared with Kieselgel $HF_{254}$ (Merck, Darmstadt)and developed twice in the following solvent systems: A, $CHCl_3$–EtOH (80:20); B, $CHCl_3$–EtOH (96:4) and C, $CHCl_3$–MeOH–$H_2O$ (65:60:5). The spots were visualized with UV light or by spraying with vanillin–$H_2SO_4$ reagent and heating at 120° for 3–4 min. HPLC was carried out on a column (50 cm × 8 mm i.d.) of silica gel (Spherisorb S GP, 8 μm, 15 g). Mps were determined on a Kofler hot stage and are uncorr.

*Plant material.* The leaves and stems of the fern *Blechnum minus* were collected in the Dandenong ranges about 50 km east of Melbourne, with the assistance of the Victorian National Herbarium.

*Extraction and separation.* The dried and ground material (about 15 kg) was extracted × 5 with EtOH and the extract evaporated to a concentrate (11 l.). EtOH (2.5 l.) and $H_2O$ (4.5 l.)

Table 1. $^1$H NMR spectral data* of compounds 1–5

| | 1† | 2† | 3† | 4‡ | 5‡ |
|---|---|---|---|---|---|
| H-3 | 3.84 | § | 4.10 | 3.97 | 5.08 |
| H-5 | 2.89 | 2.95 | 2.96 | 2.20‖ | 2.39 |
| H-7 | 6.17 *s* | 6.17 *d* | 6.20 *d* | 5.48 *d* | 5.87 *d* |
| | | (1) | (1.5) | (1.5) | (1.5) |
| H-9 | 3.44 | 3.46 | 3.49 | 3.05 | 3.12 |
| H-18 | 0.68 *s* | 0.70 *s* | 0.73 *s* | 0.67 *s* | 0.68 *s* |
| H-19 | 0.83 *s* | 1.05 *s* | 1.05 *s* | 0.93 *s* | 1.00 *s* |
| H-21 | 1.27 *d* | 1.25 *d* | 1.30 *d* | 0.95 *d* | 0.94 *d* |
| | (6.5) | (6.5) | (6.5) | (6.5) | (6.5) |
| H-22 | 4.12 | 4.10 | 4.05 | 4.89 | 4.83 |
| H-26/27 | 1.37 *s* | 1.29 *s*, 1.39 *s* | 1.38 *s* | 1.23 *s*; 1.25 *s* | 1.23 *s* |
| OAc | — | — | — | 2.02, 2.03, 2.06, 2.07, 2.18 | 2.00, 2.03, 2.06‖ 2.09, 2.10 |
| H-1′ | 4.86 *d* | 5.02 *d* | — | 4.57 *d* | 4.63 *d* |
| | (7.5) | (7.5) | | (8) | (8) |
| H-2′ | | | | 5.01 *dd* | 5.00 *dd* |
| | | | | (10, 8) | (10, 8) |
| H-3′ | | | — | 5.23 *dd* | 5.21 *dd* |
| | | | | (10, 9.5) | (10, 9.5) |
| H-4′ | 4.0–4.5 | 3.9–4.6 | — | 5.09 *dd* | 5.04 *dd* |
| | | | | (10, 9.5) | (10, 9.5) |
| H-5′ | | | — | 3.68 | 3.69 |
| H-6′ | | | — | 4.11 *dd*; 4.26 *dd* | 4.10 *dd*; 4.25 *dd* |
| | | | | (12.5, 2; 12.5, 4.5) | (12.5, 2; 12.5, 5.5) |

*Measured at 250 MHz with TMS as internal standard. Shifts are in δ values (ppm). The figures in parentheses give the coupling constants (*J*) in Hz.

†Run in $C_5D_5N$.

‡Run in $CDCl_3$.

§Superimposed by signals at δ4.0–4.5.

‖Partially obscured by signal at δ2.18, 6H, *s*.

Table 2. $^{13}C$ NMR spectral data*

| Carbon No. | 1 | 2 | 3 | 2-Deoxy-3-epiecdysone† |
|---|---|---|---|---|
| 1 | 29.9 (a)‡ | 33.2 | 33.2 | 35.6 |
| 2 | 29.5 (a) | 29.1 (c) | 29.1 (c) | 31.4 |
| 3 | 72.5 | 64.1 | 64.1 | 69.1 |
| 4 | 27.6 (a) | 29.5 (c) | 29.5 (c) | 34.3 |
| 5 | 51.5 | 51.7 | 51.6 | 57.2 |
| 6 | 203.0 | 203.3 | 203.2 | 201.9 |
| 7 | 121.3 | 121.4 | 121.3 | 121.3 |
| 8 | 165.9 | 166.0 | 166.0 | 165.8 |
| 9 | 34.7 | 34.6 | 34.3 | 34.0 |
| 10 | 36.8 | 37.0 | 37.0 | 36.8 |
| 11 | 21.6 | 21.5 | 21.3 | 20.8 |
| 12 | 31.8 | 31.7 | 31.7 | 31.6 |
| 13 | 48.2 | 48.1 | 48.1 | 47.6 |
| 14 | 84.1 | 84.1 | 84.0 | 83.8 |
| 15 | 31.8 | 31.7 | 31.7 | 31.9 |
| 16 | 26.8 | 26.7 | 26.7 | 26.7 |
| 17 | 48.4 | 48.3 | 48.3 | 48.3 |
| 18 | 15.9 | 15.8 | 15.8 | 15.8 |
| 19 | 24.0 | 24.4 | 24.3 | 23.9 |
| 20 | 43.0 | 43.2 | 43.0 | 43.0 |
| 21 | 13.7 | 13.6 | 13.6 | 13.7 |
| 22 | 74.1 | 73.9 | 73.9 | 74.0 |
| 23 | 25.8 | 24.7 | 25.5 | 25.6 |
| 24 | 42.5 | 39.6 | 42.5 | 42.5 |
| 25 | 69.8 | 77.4 | 69.7 | 69.7 |
| 26 | 30.0 | 27.4 | 30.0 | 30.0 |
| 27 | 30.3 | 27.6 | 30.2 | 30.3 |
| 1′ | 103.3 | 98.8 | | |
| 2′ | 75.3 | 75.4 | | |
| 3′ | 78.3 (b) | 78.6 | | |
| 4′ | 71.9 | 72.1 | | |
| 5′ | 78.7 (b) | 78.6 | | |
| 6′ | 63.0 | 63.4 | | |

*Measured at 62.9 MHz with pyridine-$d_5$ as solvent.

†Data from ref. [3].

‡Signals followed by bracketed letters (a, b, c or d) may be interchanged.

were added and the mixture extracted twice with *n*-hexane (18 l.). The hexane layers were extracted each in turn with a mixture of EtOH–$H_2O$ (3:1, 18 l.). The combined aq. EtOH extracts (36 l.) were then diluted with $H_2O$ and EtOH to give an extract (50 l.) containing EtOH(33%). This extract was extracted twice with 50 l. portions of the lower phase of a mixture of $CHCl_3$–EtOH–$H_2O$ (1:1:1). The $CHCl_3$ phases were then extracted, each in turn, with two portions (50 l.) of the upper aq. phase of the $CHCl_3$–EtOH–$H_2O$ (1:1:1) mixture. The aq. layers were discarded and the $CHCl_3$ layers were combined and concd to a viscous oil (estimated dry wt 480 g).

*Isolation of ecdysteroids.* The above oil was mixed with EtOH (1.3 l.) and EtOAc (2.7 l.) and filtered. The filtrate was transferred to a column of neutral $Al_2O_3$ (1.5 kg, 10% $H_2O$) and eluted with EtOAc–EtOH 2:1). The eluate was collected in three 2 l. fractions which were evaporated to dryness (5.6, 28.3 and 47.8 g respectively). Each fraction was subjected to a 30 transfer countercurrent distribution (total withdrawal) using $CHCl_3$–MeOH–$H_2O$ (2:1:1) and 150 ml of each phase. In the case of the last fraction the material was loaded into the first two tubes of a Craig CCD apparatus. The combined less polar fractions afforded crude 2-deoxyecdysone (4 g), 2-deoxy-20-hydroxyecdysone (1 g), ecdysone (5 g), and crude 20-hydroxyecdysone together with blechnosides A and B (8 g). This latter fraction was dissolved in MeOH and mixed with Celite (30 g) and dried on a rotary evaporator. The dried powder was transferred to a column of silica gel (250 g, 10% $H_2O$) and eluted first with a mixture of $CHCl_3$–EtOH–$H_2O$ (86:14:0.35), to remove a variety of substances including 20-hydroxyecdysone (1 g), and finally $CHCl_3$–EtOH–$H_2O$ (80:20:0.35) to yield the crude mixture of **1** and **2** (1.18 g).

*Separation of blechnosides A and B.* The crude mixture of **1** and **2** was triturated with $CHCl_3$–EtOH (80:20, 30 ml), filtered from an unidentified yellow crystalline aromatic compound and the filtrate evaporated to dryness. The residue (1.02 g) was dissolved in $CHCl_3$–EtOH (85:15, 25 ml) and filtered through a column of Sephadex LH20 (Pharmacia, 100 g) made up in the same solvent. Middle fractions afforded a mixture of **1** and **2** (664 mg). This material was subjected to HPLC in 50 mg batches using $CHCl_3$–EtOH (88:12) and the two main peak fractions were each rechromatographed until completely separated. The first peak fractions afforded almost pure **2** (22 mg) while the later peak fractions afforded pure, 2-deoxyecdysone 3-β-D-glucopyranoside (**1**, 12 mg); UV $\lambda_{max}^{EtOH}$ nm (log ε): 243 (4.02); IR $\nu_{max}^{KBr}$ $cm^{-1}$; 3400 (OH), 1648 (CO); DCIMS *m/z* (rel. int. %): 611 $[M+1]^+$ (0.4), 431 $[M+1-glucose]^+$ (10). $^1H$ NMR and $^{13}C$ NMR data are given in Tables 1 and 2. TLC solvent A, $R_f$ 0.2 (blue/green spot), cf. 20-hydroxyecdysone $R_f$ 0.3 (yellow/green spot).

*Hydrolysis of blechnoside A* (**1**). Blechnoside A (**1**, 4 mg) was incubated at 37–38° in citrate buffer (pH 4.5, 10 ml) with β-glucosidase (Sigma, 20 mg). After 6 days TLC indicated 50% hydrolysis and the mixture was extracted with $CHCl_3$–EtOH (1:1, 20 ml). The $CHCl_3$ layer was evaporated to dryness and the residue chromatographed on silica gel (HPLC) with gradient from $CHCl_3$–EtOH (86:14 to 84:16) and afforded 2-deoxyecdysone (*ca* 1 mg). Its $^1H$ NMR spectrum and TLC were identical with those of an authentic specimen. Blechnoside A (3.6 mg) was also hydrolysed by refluxing for 4 hr with 1 M HCl (1 ml) and the mixture extracted with *n*-BuOH. The aq. layer was subjected to TLC in solvent C and showed a spot identical to D-glucose.

*2′,3′,4′,6′,22-Pentaacetoxy-2-deoxyecdysone 3-β-D-glucopyranoside* (**4**). Compound **1** (15 mg) was acetylated with $Ac_2O$ (0.5 ml) and pyridine (1 ml) at 20° for 16 hr. MeOH was added and the mixture evaporated to dryness. Purification of the crude acetate on silica gel (HPLC) using $CHCl_3$–EtOH (96:4) as eluting solvent afforded the pure **4** as a colourless powder. DCIMS *m/z*: 821 $[M+1]^+$ (32), 473 $[M+1-glucose\ tetraacetate]^+$ (8). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3460 (OH), 1755 (OAc), 1655 (CO). $^1H$ NMR data given in Table 1. TLC solvent B $R_f$ 0.45 (blue spot).

*Hydrolysis of blechnoside B* (**2**). Compound **2** and β-glucosidase (20 mg) were incubated in the same manner as with compound **1**. After 20 days TLC revealed only a trace of **3**.

*2-Deoxyecdysone 25-β-D-glucopyranoside* (**2**). The compound **2** fractions (see above) from three runs (83.5 mg), still contaminated with some unidentified impurities, were rechromatographed on silica gel HPLC and finally on Sephadex LH20 from $CHCl_3$–EtOH (88:12). The middle fractions (53 mg) were recrystallized from EtOAc–MeOH affording blechnoside B (**2**), mp 218–220° (23 mg). (Found: C, 62.9; H, 8.8. $C_{33}H_{55}O_{10}\cdot H_2O$ requires: C, 63.0; H, 9.0%.) UV $\lambda_{max}^{EtOH}$ nm (log ε): 243 (4.03); IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3440 (OH), 1660 (CO), DCIMS *m/z*: 593 $[M+1-H_2O]^+$ (0.19), 431 $[M+1-glucose]^+$ (10). $^1H$ NMR and $^{13}C$ NMR data are given in Tables 1 and 2.

*2′,3′,4′,6′,3,22-Hexaacetoxy-2-deoxyecdysone 25-β-D-glucopyranoside* (**5**). Compound **2** (15 mg), purified by HPLC, was

acetylated for 18 hr at 20° with $Ac_2O$ (0.5 ml) and pyridine (1.0 ml). MeOH was added and the mixture evaporated to dryness. Chromatography (HPLC) of the residue on silica gel and eluting with $CHCl_3$ afforded pure **5** as a colourless gum; IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3460 (OH), 1755, 1735, (OAc) 1667 (CO); DCIMS *m/z*: 863 $[M+1]^+$ (0.02), 497 $[M+1-$glucose tetraacetate $-H_2O]^+$ (0.1). $^1H$ NMR data are given in Table 1.

*Biological activity.* The mixture of blechnosides A and B showed high biological activity in the *Calliphora* bioassay, possibly following enzymic hydrolysis of the glycosides in the insect during incubation.

*Acknowledgements*—We are indebted to the Victorian National Herbarium for assistance with the collection and identification of the plant material, to Alan Faux for the large scale extractions, Ian Willing and Roland Nearn for the NMR spectra and Graeme Heath for the mass spectra. One of us (AS) acknowledges the support of the Network for the Chemistry of Biologically Important Natural Products; which is an activity of the International Development Program of Australian Universities and Colleges.

## REFERENCES

1. Willis, J. H. (1962) *A Handbook to Plants in Victoria*, 1st edn, Vol. 1, p. 44. Melbourne University Press, Australia.
2. Chong, Y. K., Galbraith, M. N. and Horn, D. H. S. (1970) *J. Chem. Soc. Chem. Commun.* 1217.
3. Russell, G. B., Greenwood, D. R., Lane, G. A., Blunt, J. W. and Munro, M. H. G. (1981) *Phytochemistry* **20**, 2407.
4. Takemoto, T., Arihara, S. and Hikino, H. (1968) *Tetrahedron Letters* 4199.
5. Hylands, P. J. and Kosugi, J. (1982) *Phytochemistry* **21**, 1379.
6. Usui, T., Yamaoka, N., Matsuda, K., Tuzimura, K., Sugiyama, H. and Seto, S. (1973) *J. Chem. Soc. Perkin Trans.* 1, 2425.
7. Perlin, A. S., Casu, B. and Koch, H. J. (1970) *Can. J. Chem.* **48**, 2596.
8. Dorman, D. E. and Roberts, J. D. (1970) *J. Am. Chem. Soc.* **92**, 1355.
9. Tori, K., Seo, S., Yoshimura, Y., Arita, H. and Tomita, Y. (1977) *Tetrahedron Letters* 179.
10. Baumann, H. and Pigman, W. (1957) in *The Carbohydrates, Chemistry, Biochemistry, Physiology* (Pigman, W., ed.) p. 583. Academic Press, New York.