Phytochemistry, Vol. 27, No. 6, pp. 1665–1668, 1988.
Printed in Great Britain.

0031-9422/88 $3.00+0.00

THREE ECDYSTEROID GLYCOSIDES FROM *PFAFFIA IRESINOIDES**

NOBUSHIGE NISHIMOTO, YOSHINORI SHIOBARA, SHUN-SUKE INOUE, MASUMI FUJINO, TSUNEMATSU TAKEMOTO, CHEOW LIN YEOH,† FERNANDO DE OLIVEIRA,‡ GOKITHI AKISUE,‡ MARIA KUBOTA AKISUE‡ and GORO HASHIMOTO§

Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770, Japan; †Japan Bio Institute Co., Ltd, Minamioi, Shinagawa-ku, Tokyo 140, Japan, ‡Department de Farmácia da Faculudade de Ciências Farmacêuticas da Universidade de São Paulo, São Paulo 30786, Brasil; §Centro de Pesquisas História Natural, Vila Leopoldina, São Paulo 05318, Brasil

(*Received* 27 *July* 1987)

Key Word Index—*Pfaffia iresinoides*; Amaranthaceae; Brazil ginseng; ecdysteroid glycosides.

Abstract—From the crude drug 'Brazil ginseng', the roots of *Pfaffia iresinoides*, three new ecdysteroid glycosides, ecdysterone 25-*O*-β-D-glucopyranoside, pterosterone 24-*O*-β-D-glucopyranoside and podecdysone B 25-*O*-β-D-glucopyranoside have been isolated. The structures were elucidated on the basis of chemical and extensive spectral analyses. The moulting hormone activities of the former two compounds have also been examined.

INTRODUCTION

In the course of the investigation of the chemical constituents of 'Brazil ginseng', the dried roots of *Pfaffia iresinoides* Spreng., we have isolated a large amount of ecdysterone (**1**) together with pterosterone (**2**) and polypodine B[1]. Further attempts to separate the minor components led to the isolation of three new ecdysteroid glycosides.

RESULTS AND DISCUSSION

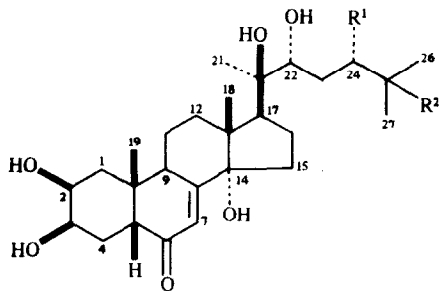
A combination of column chromatographic separation on silica gel and on Sephadex LH-20 of the methanol extract of the roots of *Pfaffia iresinoides* resulted in the isolation of ecdysterone 25-*O*-β-D-glucopyranoside (**3**), pterosterone 24-*O*-β-D-glucopyranoside (**4**) and podecdysone B 25-*O*-β-D-glucopyranoside (**5**).

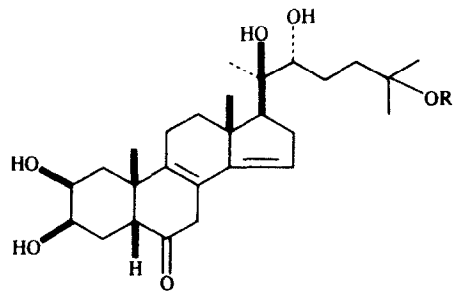
Compound **3** was obtained as a white powder. Its fast atom bombardment mass spectrum (FABMS) showed a parent ion at m/z 643 $[M+H]^+$. Enzymatic hydrolysis of **3** proceeded very slowly to afford ecdysterone (**1**) and glucose. In the ^{1}H NMR spectrum the signals ascribed to 26- and 27-methyl groups were observed unequivalent as compared with that of **1**. The location of the glucose was examined by NOE difference spectroscopy. When each of the methyl signals at δ1.31 and 1.37 was irradiated, the anomeric proton signal at δ5.00 (*d*, $J=7.6$ Hz) was enhanced. These results indicated that the glucose was located at the 25 position. This assignment was further supported by the comparison of ^{13}C NMR spectrum of **3** with that of **1** (Table 1). On going from **1** to **3**, carbon signals due to C-24, 25, 26/27 were displaced by -2.5, $+7.6$, $-2.1/-2.2$ ppm, respectively, while other carbon signals remained almost unshifted. These glycosylation shifts were consistent with those observed for blechnoside B isolated from *Blechnum minus* [2]. The large coupling constant of the anomeric proton described above and the shift of the anomeric carbon (δ98.8) indicated the β-configuration of glycosyl linkage [3, 4]. Thus the structure of **3** was established as ecdysterone 25-*O*-β-D-glucopyranoside.

Compound **4**, $C_{33}H_{54}O_{12}$, was isolated as colourless prisms, mp 276–277°, which gave pterosterone (**2**) and glucose on enzymatic hydrolysis. In the ^{1}H NMR spectrum the signals due to 26- and 27-methyl groups were observed unequivalent. This suggested that the glucose was located in the side chain of **2**. In the ^{13}C NMR spectrum the anomeric carbon signal was observed at δ105.9, suggesting that the glucose was attached at a secondary hydroxyl group and had the β-configuration of glycosyl linkage [3, 4]. A comparison of the ^{13}C NMR data of **4** with that of **2** (Table 1) revealed that one of the tertiary oxygen-bearing carbons in the side chain was displaced downfield at δ84.7. These results indicated that the glucose was located at 22 or 24 position. To confirm the location of the glucose, the assignment of the ^{13}C signal described above was performed by the long-range ^{13}C–^{1}H shift correlation spectroscopy (COSY) (Table 2). The observation of the long-range couplings between each of the 26- and 27-methyl protons and the ^{13}C signal at δ84.7 clearly demonstrated that the glucose was attached at the 24 position. Thus, the structure of **4** was established as pterosterone 24-*O*-β-D-glucopyranoside.

Compound **5** was obtained as a white powder, the ^{1}H NMR spectrum of which showed five tertiary methyl signals similar to those of compound **3**. However, the IR absorption at 1710 cm^{-1} (ascribed to a saturated carbonyl group) indicated that it lacks a 7-en-6-one system which is characteristic of ecdysteroids [5]. The UV spectrum (244 nm) suggested the presence of a conjugated

*This work was presented at the 107th Annual Meeting of Pharmaceutical Society of Japan, Kyoto, Japan, April 1987, Abstract Papers, p. 333.





1 R^1 = H, R^2 = OH
2 R^1 = OH, R^2 = H
3 R^1 = H, R^2 = Oglc
4 R^1 = Oglc, R^2 = H

5 R = glc
6 R = H

glc: β-D-glucopyranosyl

Table 1. ^{13}C NMR data* of compounds **1**–**6**

C	3	1†	4	2†	5	6
1	37.9	37.5	38.0	37.7	38.5	38.6
2	68.1	68.0	68.3	68.1	69.6	69.6
3	68.0	67.9	68.2	68.0	68.3	68.3
4	32.4	32.2	32.6	32.4	32.9	32.9
5	51.4	51.2	51.5	51.3	53.6	53.6
6	203.7	204.0	203.6	203.8	212.4	212.4
7	121.7	121.5	121.7	121.6	39.4	39.4
8	166.1	166.4	166.3	166.3	122.8	122.9
9	34.4	34.2	34.5	34.3	136.3	136.4
10	38.6	38.5	38.8	38.6	43.6	43.6
11	21.1	20.9	21.2	21.0	23.0	23.0
12	32.0	31.8	32.1	31.9	37.4	37.5
13	48.1	47.9	48.1	48.0	46.7	46.6
14	84.3	84.1	84.2	84.1	148.9	148.9
15	31.7	31.5	31.8	31.6	119.6	119.5
16	21.4	21.3	21.6	21.4	31.5	31.5
17	50.0	49.9	49.9	49.9	56.9	57.0
18	17.9	17.8	18.0	17.9	18.7	18.7
19	24.4	24.2	24.6	24.4	29.5	29.6
20	76.9	76.9	76.8	76.8	76.3	76.2
21	21.7	21.5	21.6	21.6	20.8	20.8
22	77.7	77.5	75.4	77.4	77.7	77.7
23	26.6	27.2	36.1	35.7	26.6	27.4
24	39.8	42.3	84.7	76.7	40.0	42.7
25	77.4	69.8	32.1	33.8	77.4	69.7
26	27.5	29.6	17.3	17.0	27.5	30.0
27	27.6	29.8	19.5	19.5	27.6	30.7
1′	98.8		105.9		98.8	
2′	75.5		75.5		75.4	
3′	78.7‡		78.7‡		78.8‡	
4′	72.1		72.1		72.1	
5′	78.5‡		78.2‡		78.6‡	
6′	63.4		63.3		63.5	

*Measured at 100 MHz with C_5D_5N as solvent. Chemical shifts are given in δ values in ppm from TMS.

†Data from ref. [1].

‡These assignments may be reversed.

Table 2. Long-range couplings observed in the long-range ^{13}C–^{1}H COSY spectra* of compounds **4** and **6**

Compound	Proton (δ)	Correlated carbon $^{2}J_{CH}$ coupling	Correlated carbon $^{3}J_{CH}$ coupling
4	H-7 (6.22)		C-14
	H-18 (1.17)	C-13	C-14
	H-19 (1.04)	C-10	C-5
	H-21 (1.56)	C-20	C-22
	H-26, 27 (1.05, 1.14)	C-25	C-24
6	H-15 (5.43)		C-13
	H-18 (1.35)	C-13	C-12, 14, 17
	H-19 (1.07)	C-10	C-1, 5, 9
	H-21 (1.55)	C-20	C-17
	H-26, 27 (1.43, 1.44)	C-25	C-24

* Measured under the conditions of $\Delta_2 = 20$ msec ($J_{CH} = 12.5$ Hz) for **4** and $\Delta_2 = 25$ msec ($J_{CH} = 10.0$ Hz) for **6**.

Table 3. Assay of compounds **3**, **4** and **1** on isolated abdomens of *Lucilia cuprina*

Compound	Dose* (μg)	Number of test animals	Number of pupation 24 hr	Number of pupation 48 hr
3	5	20	3	8
4	5	20	6	13
1	0.05	20	12	16
Control	0	20	0	0

*Each animal was injected with 5 μl of compound dissolved in 10% EtOH

diene system. Further, the ^{13}C NMR spectrum displayed the presence of a trisubstituted double bond, a tetrasubstituted double bond and carbonyl group, along with the disappearance of C-14 quaternary oxygen-bearing carbon signal which was also characteristic of ecdysteroids [5]. The above data along with the M_r (FABMS: m/z 625 $[M+H]^+$) indicated that the aglycone of **5** might be podecdysone B which had 8,14-dien-6-one system in the molecule [6].

The UV absorption alterations by acid treatment have been utilized for characterization of ecdysteroids. This reaction was attributed to the formation of 8,14-dien-6-one system by dehydration of allylic hydroxyl group (14-OH) and subsequent migration of double bond [7]. In the case of ecdysterone (**1**), dehydration products have never been isolated [8]. We attempted this reaction under mild conditions (see Experimental). The major product **6** was obtained in 37% yield, the physico-chemical properties of which were identical with those of podecdysone B[6]. Further, the aglycone obtained by enzymatic hydrolysis of compound **5** was identified with **6** by comparison of ^{1}H NMR spectrum. The present assignments of ^{1}H (see Experimental) and ^{13}C (Table 1) NMR spectra of **6** were performed by means of ^{1}H–^{1}H and ^{13}C–^{1}H COSY spectra. Quaternary carbon signals were assigned by means of the long-range ^{13}C–^{1}H COSY spectrum. The observed long-range couplings are shown in Table 2.

Based on the above data, the assignments of ^{13}C NMR spectrum of compound **5** were made (Table 1). The signals corresponding to the side chain (C-22–C-27) and the glucose moiety were almost superimposable over compound **3**. Thus the structure of **5** was established as podecdysone B 25-*O*-β-D-glucopyranoside. It is unlikely that **5** is an artefact formed by dehydration of **3** during isolation, since (i) throughout the purification of **3**, dehydration was not observed and (ii) despite a large amount of **1** being isolated from the same material, **6** could not be detected [6].

Finally, compounds **3**, **4** and **1** were assayed for their activities upon the induction of puparium formation of isolated larval abdomens of the sheep blowfly (*Lucilia cuprina* Wiedemann) (Table 3) [9]. As a result, it was found that **3** and **4** showed weak activities (*ca* 1/100) compared to **1** [10].

EXPERIMENTAL

Mps: uncorr. TLC was performed on Kiesel gel $60F_{254}$ precoated silica gel plates (Merck) using solvent A: $CHCl_3$–MeOH–H_2O (13:7:2, lower phase); solvent B: *n*-BuOH–HOAc–H_2O (4:1:1); solvent C:$CHCl_3$–MeOH (4:1); spots were visualized by UV light or by spraying with 10% H_2SO_4 and heating at 150°. LC was carried out on a column (50 cm × 22 mm i.d.) of silica gel (TLC-Kieselgel 60 H, 15 μm, Merck).

Plant material was the same as described in the preceding paper [1].

Extraction and isolation of ecdysteroid glycosides. The dried roots (60 kg) of *P. iresinoides* were treated by the same procedure as described in the preceding paper [1]. The *n*-BuOH extract (470 g) obtained chromatographed on silica gel (2.5 kg) and eluted successively with EtOAc (20 l), EtOAc–MeOH (49:1, 20 l), EtOAc–MeOH (19:1, 40 l), EtOAc–MeOH (17:3, 20 l). The eluant was collected as 1 l fractions. Fractions 35–80 were concd and the residual solid was recrystallized from EtOAc–MeOH to give ecdysterone (**1**, 95 g). The residue (40 g) obtained from fractions 95–130 was rechromatographed on silica gel (400 g) using $CHCl_3$–MeOH–H_2O (8:2:1, lower phase, 24 l) and $CHCl_3$–MeOH–H_2O (15:5:2, lower phase, 15 l) and 300 ml fractions were collected. Fractions 31–48 were concd and the residue (1.3 g) was subjected to medium pressure liquid chromatography using $CHCl_3$–MeOH–EtOAc–H_2O (2:2:4:1, lower phase) and 20 ml fractions were collected. The residue obtained from fractions 46–52 was reprecipitated from EtOAc–MeOH to afford compound **5** (27 mg). Fractions 88–110 were concd and the residue (1.7 g) was subjected to LC using $CHCl_3$–MeOH–EtOAc–H_2O (2:2:4:1, lower phase) and 20 ml fractions were collected. The crystalline solid obtained from fractions 95–101 was recrystallized from EtOH to give compound **4** (160 mg). The residue obtained from fractions 110–115 was passed through a column of Sephadex LH-20 using $CHCl_3$–MeOH (1:1) as an eluent, followed by reprecipitation from EtOAc–MeOH to give compound **3** (75 mg).

Ecdysterone 25-O-β-D-glucopyranoside (**3**). A white powder; $[\alpha]_D^{15}$ +26.0° (MeOH, *c* 1.5); UV λ_{max}^{EtOH} nm (log ε): 243 (4.02); IR ν_{max}^{KBr} cm^{-1}: 3450, 1650; FABMS: *m/z* 643 $[M+H]^+$; ^{1}H NMR (400 MHz, C_5D_5N): δ 1.04 (3H, *s*, H-19), 1.18 (3H, *s*, H-18), 1.31 and 1.37 (each 3H, *s*, H-26 and H-27), 1.56 (3H, *s*, H-21), 2.42 (1H, *q*, *J* = 10.4 Hz, H-16), 2.52 (1H, *dt*, *J* = 13.2 and 4.2 Hz, H-12), 2.94 (1H, *t*, *J* = 8.8 Hz, H-17), 2.99 (1H, *dd*, *J* = 13.2 and 4.0 Hz, H-5), 3.55 (1H, *br t*, *J* = 9.0 Hz, H-9), 3.83 (1H, *br d*, *J* = 9.4 Hz, H-22), 5.00 (1H, *d*, *J* = 7.6 Hz, H-1′), 6.21 (1H, *d*, *J* = 2.2 Hz, H-7); TLC: solvent A, R_f 0.20.

Pterosterone 24-O-β-D-glucopyranoside (**4**). Mp 276–277°; $[\alpha]_D^{15}$ +40.9° (MeOH; *c* 1.5); Found: C, 61.72; H, 8.52. Calc for $C_{33}H_{54}O_{12}$:C, 61.66; H, 8.47. UV λ_{max}^{EtOH} nm (log ε): 243 (4.04); IR ν_{max}^{KBr} cm^{-1}: 3400, 1650; ^{1}H NMR (400 MHz, C_5D_5N): δ 1.04 (3H, *s*, H-19), 1.05 and 1.14 (each 3H, *d*, *J* = 6.6 Hz, H-26 and H-27), 1.17 (3H, *s*, H-18), 1.56 (3H, *s*, H-21), 2.42 (1H, *q*, *J* = 10.2 Hz, H-16), 2.51 (1H, *dt*, *J* = 13.2 *and* 5.0 Hz, H-12), 2.89 (1H, *t*, *J* = 9.0 Hz, H-17), 2.98 (1H, *dd*, *J* = 13.4 and 4.0 Hz, H-5), 3.55 (1H, *br t*, *J* = 9.0 Hz, H-9), 4.96 (1H, *d*, *J* = 7.6 Hz, H-1′), 6.22 (1H, *d*, *J* = 2.2 Hz, H-7); TLC: solvent A, R_f 0.24.

Podecdysone B 25-O-β-D-glucopyranoside (**5**). A white powder; $[\alpha]_D^{15}$ −18.9° (MeOH; *c* 1.1); UV λ_{max}^{EtOH} nm: 244 (4.12); IR ν_{max}^{KBr} cm^{-1}: 3400, 1710, 1650; FABMS: *m/z* 625 $[M+H]^+$; ^{1}H NMR (400 MHz, C_5D_5N): δ 1.06 (3H, *s*, H-19), 1.34 (3H, *s*, H-18), 1.42 and 1.47 (each 3H, *s*, H-26 and H-27), 1.54 (3H, *s*, H-21), 2.89 and 3.40 (each 1H, *br d*, *J* = 21.0 Hz, H-7), 3.02 (1H, *br dd*, *J* = 16.0 and 10.4 Hz, H-16), 3.05 (1H, *dd*, *J* = 12.4 and 4.0 Hz, H-5), 3.84 (1H, *br d*, *J* = 7.8 Hz, H-22), 4.47 (1H, *br s*, H-3), 5.02 (1H, *d*, 7.4 Hz, H-1′), 5.44 (1H, *br s*, H-15); TLC: solvent A, R_f 0.32.

Enzymatic hydrolysis of compound **3**. Compound **3** (10 mg) was incubated at 37° in M/10 acetate buffer (pH 5, 10 ml) containing a few drops of Tween 80 with β-glucosidase (Toyobo, 80 mg). After a week, TLC indicated 20% hydrolysis. The reaction mixture was evapd and the residue was chromatographed on silica gel (20 g) using $CHCl_3$–MeOH–H_2O (8:2:1, lower phase), $CHCl_3$–MeOH–H_2O (15:5:2, lower phase) and $CHCl_3$–MeOH (7:3). From the first eluate ecdysterone (**1**, *ca* 1 mg) was obtained, the ^{1}H NMR spectrum and TLC (solvent A, R_f 0.44) of which were identical with those of an authentic specimen. From the second eluate compound **3**(6 mg) was recovered. The third eluate showed a spot identical to glucose on TLC (solvent A, R_f 0.08; solvent B, R_f 0.26).

Enzymatic hydrolysis of compound **4**. Compound **4** (5 mg) was incubated at 37° in M/10 NaOAc buffer (pH 5, 10 ml) with β-D-glucosidase (20 mg). After 2 days, the reaction mixture was evapd and the residue was chromatographed on silica gel (20 g) using $CHCl_3$–MeOH–H_2O (8:2:1, lower phase) and $CHCl_3$–MeOH (7:3). From the former eluate pterosterone (**2**, *ca* 2 mg) was obtained and identified with an authentic specimen by comparison of ^{1}H NMR spectrum and TLC (solvent A, R_f 0.54). The latter eluate showed a spot identical to glucose.

Enzymatic hydrolysis of compound **5**. Compound **5** (5 mg) was treated by the same procedure as described in the hydrolysis of compound **3**. The aglycone obtained was identified with compound **6** by comparison of ^{1}H NMR spectrum and TLC (solvent C, R_f 0.45).

Dehydration of ecdysterone (**1**). To an ice-cooled soln of **1** (500 mg) in MeOH (10 ml) was added 11 M HCl (5 ml) and the resulting soln was stirred for 45 min at room temp. The reaction was quenched by the addition of $NaHCO_3$, and the reaction mixture was evapd. The residue was chromatographed on silica gel (50 g) using $CHCl_3$–MeOH (9:1) and 20 ml fractions were collected. Fractions 19–22 were combined and concd to yield compound **6** (180 mg), the physico-chemical data of which were identical with those of podecdysone B[6].

Compound **6**. Mp 122–124°; $[\alpha]_D^{15}$ −15.7° (MeOH; *c* 1.1); UV λ_{max}^{EtOH} nm (log ε) 244 (4.14); IR ν_{max}^{KBr} cm^{-1}: 3400, 1710, 1650; FABMS: *m/z* 463 $[M+H]^+$; ^{1}H NMR (400 MHz, C_5D_5N): δ 1.07 (3H, *s*, H-19), 1.35 (3H, *s*, H-18), 1.43 and 1.44 (each 3H, *s*, H-26 and H-27), 1.55 (3H, *s*, H-21), 2.90 and 3.44 (each 1H, *br d*, *J* = 21.0 Hz, H-7), 3.01 (1H, *br dd*, *J* = 16.0 and 10.6 Hz, H-16), 3.04 (1H, *dd*, *J* = 12.4 and 4.0 Hz, H-5), 3.86 (1H, *br d*, *J* = 8.2 Hz, H-22), 3.92 (1H, *br dt*, *J* = 11.4 and 3.0 Hz, H-2), 4.46 (1H, *br s*, H-3), 5.43 (1H, *br s*, H-15).

REFERENCES

1. Nishimoto, N., Shiobara, Y., Fujino, M., Inoue, S., Takemoto, T., Oliveira, F., Akisue, G., Akisue, M. K., Hashimoto, G., Tanaka, O., Kasai, R. and Matsuura, H. (1987) *Phytochemistry* **26**, 2505.
2. Suksamrarn, A., Wilkie, J. S. and Horn, D. H. S. (1986) *Phytochemistry* **25**, 1301.
3. Tori, K., Seo, S., Yoshimura, Y., Arita, H. and Tomita, Y. (1977) *Tetrahedron Letters* 179.
4. Kasai, R., Okihara, M., Asakawa, J., Mizutani, K. and Tanaka, O. (1979) *Tetrahedron* **35**, 1427.
5. Hikino, H. and Hikino, Y. (1970) *Fortschritte Chem. Org. Naturst.* **28**, 256.
6. Galbraith, M. N., Horn, D. H. S. and Middleton, E. J. (1969) *Chem. Commun.* 402.
7. Karlson, P., Hoffmeister, H., Hummel, H., Hocks, P. and Spiteller, G. (1965) *Chem. Ber.* **98**, 2394.
8. Galbraith, M. N. and Horn, D. H. S. (1969) *Aust. J. Chem.* **22**, 1045.
9. Kobayashi, M., Takemoto, T., Ogawa, S. and Nishimoto, N. (1967) *J. Insect Physiol.* **13**, 1395.
10. Heinrich, G. and Hoffmeister, H. (1970) *Z. Naturforsch.* **256**, 358.