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3′-HYDROXYPSILOTIN, A MINOR PHENOLIC GLYCOSIDE FROM *PSILOTUM NUDUM*

FELIPE BALZA, ALISTER D. MUIR* and G. H. NEIL TOWERS†

Department of Botany, University of British Columbia, Vancouver, B.C. V6T 2B1, Canada

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Key Word Index—*Psilotum nudum*; Psilotaceae; phenolic glucosides; psilotin; 3′-hydroxypsilotin; DCCC.

Abstract—A new non-flavonoid glycoside, 3′-hydroxypsilotin {6-[4′-(β-D-glucopyranosyloxy)-3′-hydroxyphenyl]-5,6-dihydro-2-oxo-2*H*-pyran}, was isolated from *Psilotum nudum* by droplet counter current chromatography and preparative HPLC. The structure was established by spectroscopic analysis including ^{1}H and ^{13}C NMR and high resolution mass spectrometry.

INTRODUCTION

Psilotin [6-(4′-β-D-glucopyranosyloxyphenyl)-5,6-dihydro-2-oxo-2*H*-pyran, **1**] was previously isolated as a crystalline substance from *Psilotum nudum* (Psilotaceae) [1]. A re-examination of the original compound, as well as of further isolates, by analytical HPLC revealed the presence of a minor constituent identified as 3′-hydroxypsilotin {6-[4′-(β-D-glucopyranosyloxy)-3′-hydroxyphenyl]-5,6-dihydro-2-oxo-2*H*-pyran, **2**}. Separation of these two compounds on a preparative scale was achieved by droplet counter current chromatography (DCCC) and preparative HPLC. Distribution of **1** and **2** within the plant was also investigated.

RESULTS AND DISCUSSION

Besides the well-known non-flavonoid glucoside psilotin (**1**), we have isolated another simple glucoside, 3′-hydroxypsilotin (**2**), as a minor constituent from *Psilotum nudum*.

Enzymic hydrolysis of **2** with β-glucosidase gave a mixture of glucose, readily identified by PC, and the aglycone, **4**, mp 202–204°. The glucoside, **2**, as well as the aglycone, **3**, gave a dark green colour reaction with diazotized *p*-nitroaniline suggesting the presence of at least two phenolic hydroxyl groups. The high water solubility and a bathochromic shift in base in the UV spectrum of **1** also suggested a phenolic glucoside [3].

Acetylation of **1** with acetic anhydride and pyridine yielded **3**, mp 171–172°; its high resolution mass spectrum exhibited a molecular ion at *m/z* 578.1673 (578.1635 calculated for $C_{27}H_{30}O_{14}$), indicating a penta-acetate derivative. An IR band at 1757 cm^{-1} for **2** corresponding to a carbonyl group of an α,β-unsaturated δ-lactone ring was shown [4].

The ^{1}H NMR spectrum of **3** (Table 1) displayed signals in the aromatic region assignable to H-6′ (1H, *dd*, δ7.24, *J* = 8.5, 2.0 Hz), H-2′ (1H, *d*, δ7.13, *J* = 2.0 Hz) and H-5′ (1H, *d*, δ7.06, *J* = 8.5 Hz). The presence of these resonances and also that at δ2.27 (3H, *s*) corresponding to an aromatic acetyl group established the degree of substitution in the aromatic ring of the aglycone moiety of **2**, as well as providing evidence for the extra non-glucosidic phenolic hydroxyl group.

The high resolution mass spectrum of **4** revealed major fragments at *m/z* 206.0579 ([M]$^+$, $C_{11}H_{10}O_4$, calculated 206.0579), *m/z* 68 (base peak), *m/z* 138 and 137. These ions, *m/z* 68 and 138, as shown in Scheme 1, presumably arise from a retro-Diels–Alder fragmentation in the α,β-unsaturated δ-lactone ring.

The coupling constant of the anomeric proton at δ5.10

Table 1. ^{1}H NMR spectral data of compound **3** (400 MHz, deuterochloroform, TMS as int. standard, δ)

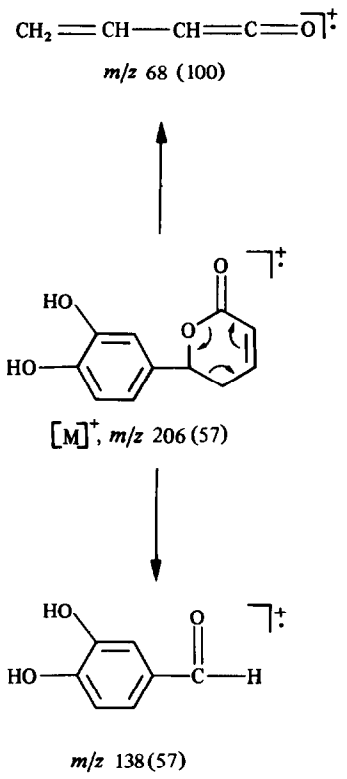
H-2′	7.13 *d* (2.0)†
H-6′	7.24 *dd* (8.5, 2.0)
H-5′	7.06 *d* (8.5)
H-4	6.96 *ddd* (10.0, 5.5, 2.5)
H-3	6.14 *ddd* (10.0, 2.5, 1)
H-6	5.41 *dd* (11.0, 5.0)
H-3″	5.31 *t* (9.5)
H-2″	5.28 *t* (9.0)
H-4″	5.14 *t* (10.0)
H-1″	5.10 *d* (7.2)
H-6$_S$″*	4.29 *dd* (12.5, 5.5)
H-6$_R$″*	4.18 *dd* (12.5, 2.5)
H-5″	3.90 *m*
H-5	2.60 *ddd* (18.0, 5.0, 1)
OAc-3′	2.27 *s*
OAc	2.08 *s*
	2.07 *s*
	2.04 *s*
	2.02 *s*

*The *S* and *R* configuration assignments are from ref. [11].

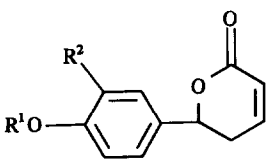
†Figures in parentheses are coupling constants or line separations in Hz.

*Present Address: Agriculture Canada, 3015 Ord Road, Kamloops, B.C. V2B 8A9, Canada.

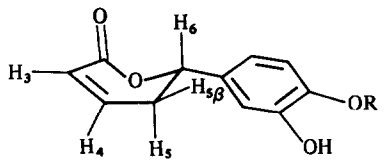
†To whom all correspondence should be addressed.



Scheme 1. Mass spectrum of 3'-hydroxypsilotinin (4).



1 R^1 = Glc, R^2 = H
2 R^1 = Glc, R^2 = OH
3 R^1 = Glc(Ac)$_4$, R^2 = OAc
4 R^1 = H, R^2 = OH
5 R^1 = R^2 = H



6 R = Glc

(J = 7.2 Hz) in the ^{1}H NMR spectrum of **3** showed that the glucose unit was in the β-configuration [5]. The ^{13}C NMR signal for C-1", the anomeric carbon of the glucose moiety in **2**, was also consistent with the above data; at δ102.8 it corresponds to a β-glucosidic linkage. The other five remaining signals of β-D-glucose at δ77.7, 77.1, 74.4, 71.0 and 62.1 were unequivocally assigned to C-3", C-5", C-2", C-4" and C-6", respectively [6]. A total of 17 carbon signals were identified by ^{1}H-decoupled ^{13}C NMR analysis, therefore confirming the structure of **2**. In **2**, weak singlets at δ147.3, 146.4 and 135.5 were assigned to the quaternary carbon atoms at positions C-3', C-4' and C-1', respectively [7]. The very low intensities of these signals as well as that of the carbonyl at δ165.1 suggest that these carbons relax particularly slowly besides having lower nuclear Overhauser enhancements. This is typical of carbons which are partially isolated from protons [8]. Assignments of ^{13}C NMR chemical shifts of the remaining signals were made with the aid of the single frequency off-resonance decoupled spectrum (SFORD). The optical rotation for **2** was found to be $[\alpha]_D^{23}$ −154.3°; however, despite the fact that 3'-hydroxypsilotinin (**4**) contains an asymmetric centre, it was established that **4** is not optically active. The same phenomenon has been observed in the hydrolysis of **1** where the aglycone psilotinin (**5**) is optically inactive [1]. Racemization takes place during enzymic hydrolysis. Nevertheless, synthesis and CD measurements of **1** have indicated that the configuration should be assigned as 6-*S* [9].

Measurements of spin–spin coupling (^3J) between H-6 and the H-5 methylene protons in the ^{1}H NMR spectra of **1–5** gave a consistent set of data where H-6 corresponds to a doublet of doublets with a large coupling of 11.0 Hz typical of *trans*-coplanar vicinal protons and a small coupling of 5.0 Hz for a *gauche* H-5β–H-6 type of interaction as depicted in **6**.

EXPERIMENTAL

^{1}H NMR spectra were determined with a 400 MHz spectrometer. Chemical shifts are reported in δ-units relative to TMS. ^{13}C NMR spectra were obtained at 100.6 MHz. Samples were prepared in D_2O containing two drops of 1,4-dioxane as an int. standard; chemical shifts referenced to 1,4-dioxane are expressed relative to external TMS [δ(1,4-dioxane) 68.1]. Mass spectra were recorded at 70 eV. Mps are uncorr.

Isolation of **1** *and* **2**. *P. nudum* was grown in pots in a greenhouse under natural lighting. Fresh aerial shoots were macerated in hot 80% EtOH in a Waring blender. The hot extract was filtered and the residue re-suspended twice in 80% EtOH. The filtrates were combined and concd to a syrup. A crude psilotin fraction was obtained by continuous EtOAc extraction of the aq. phase obtained from the dil. syrup.

An aliquot of the plant extract of the crude psilotin fraction (taken to dryness) was dissolved in 10 ml of the DCCC solvent (1:1 ratio of the two phases formed from $CHCl_3$–MeOH–H_2O, 7:12:8). The DCCC (300 tubes) was charged with the lower (satd $CHCl_3$) phase. The sample was loaded into a 10 ml sample loop and then pumped in an ascending mode, using the upper phase (MeOH–H_2O) as the mobile solvent. The column effluent was monitored at 254 and 280 nm. Separation was monitored by TLC on cellulose (*n*-BuOH–EtOH–H_2O, 20:5:11) and the resulting chromatograms examined under UV. Psilotin gave a green fluorescent colour, while 3'-hydroxypsilotin gave a yellow colour, when sprayed with the vanillin reagent [2].

HPLC separation. A HPLC equipped with a variable wavelength detector set at 275 nm was used. Separation was achieved using a pre-packed normal phase (amino–propyl bonded) NH_2-10 columns; analytical separation was carried out on a 30 cm × 4 mm and preparative separation on a 50 cm × 8 mm

column. Columns were eluted isocratically for 6 min with 35% MeOH in MeCN and then with a gradient to 70% MeOH at 6% /min. Flow rates were 1.0 ml/min (analytical) and 4.0 ml/min (preparative).

Distribution of **1** *and* **2** *in shoots of* P. nudum. A young spore bearing shoot (35 cm long) including part of the rhizome, was cut into 5 cm segments, each of which was extracted in 2 ml of hot 95% EtOH. The extracts were recovered by filtration and reduced to 1 ml. $CHCl_3$ (1 ml) was added to form two phases and aliquots of the upper phase (containing the glycosides) were analysed by HPLC. Psilotin and 3'-hydroxypsilotin were found in all parts of the shoot including the rhizome, with maximum levels in the top 5 cm (172 μg/mg dry wt psilotin) and lowest concns (14–15 μg/mg) in the rhizome and stem immediately above the rhizome. A similar distribution was also observed for 3'-hydroxypsilotin.

Characterization of **2**. IR ν_{max}^{KBr} cm^{-1}: 3300–3600, 1702, 1437, 1386, 1259, 1069, 907, 817; UV λ_{max}^{MeOH} nm (log ε): 276 (3.3) shifted to 292 (3.4) in 0.1 N NaOH; $[\alpha]_D^{23}$ −154.3° (MeOH; *c* 0.4); ^1H NMR (400 MHz, CD_3OD): δ7.21 (1H, *d*, J = 8.5 Hz, H-5'), 7.10 (1H, *m*, H-4), 6.96 (1H, *d*, J = 2.5 Hz, H-2'), 6.87 (1H, *dd*, J_1 = 8.5 Hz, J_2 = 2.5 Hz, H-6'), 6.08 (1H, *ddd*, J_1 = 10.0 Hz, J_2 = 2.5 Hz, J_3 = 1 Hz, H-3), 5.42 (1H, *dd*, J_1 = 10.0 Hz, J_2 = 6.0 Hz, H-6), 4.9 (*br*, OH and H-1'), 3.90 (1H, *dd*, J_1 = 12.0 Hz, J_2 = 2.0 Hz, H-6_S), 3.72 (1H, *dd*, J_1 = 12.0 Hz, J_2 = 4.5 Hz, H-6_R), 3.35–3.55 (4H, *m*), 2.64 (2H, *m*, H-5); ^{13}C NMR (100.6 MHz, 1,4-dioxane): δ169.1 (C-2), 150.6 (C-4), 147.3 (C-3'), 146.4 (C-4'), 135.5 (C-1'), 120.8 (C-5'), 120.5 (C-6'), 118.5 (C-2'), 116.2 (C-3), 102 8 (C-1''), 81.2 (C-6), 77.7 (C-3''), 77.1 (C-5''), 74.4 (C-2''), 71.0 (C-4''), 68.1 (1, 4-*d*), 62.1 (C-6''), 31.8 (C-5). (Found. C, 54.04; H, 5.68. $C_{17}H_{20}O_9$. 1/2 H_2O requires: C, 54.11; H, 5.5%.)

Enzymic hydrolysis of **2**. A mixture of **2** (22 mg) and β-glucosidase (2 mg) in H_2O (3 ml) was incubated at 35° for 24 hr, then extracted with EtOAc. The EtOAc layer was concd and applied to a silica gel column to afford the aglycone (**4**) (10 mg), mp 202–204°. R_f 4: 0.40 in $CHCl_3$–Me_2CO (7:3) located by short-wave UV light and visualized by the green colour formed with the vanillin reagent. UV λ_{max}^{MeOH} nm (log ε): 283 (3.2). IR ν_{max}^{KBr} cm^{-1}. 3200–3400, 1681, 1610, 1532, 1446, 1384, 1301, 1263, 1191, 813. MS *m/z* (rel. int.): 206.0579 $[M]^+$ (66), ($C_{11}H_{10}O_4$, calcd 206.0579); 161 $[C_{10}H_9O_2]^+$ (30); 160 $[C_{10}H_8O_2]^+$ (13), 138 $[C_7H_6O_3]^+$ (60); 137 $[C_7H_5O_3]^+$ (73), 136 $[C_8H_8O_2]^+$ (38), 115 $[C_9H_7]^+$ (37), 110 $[C_6H_6O_2]^+$ (40), 109 $[C_6H_5O_2]^+$ (32), 68 $[C_4H_4O]^+$ (100). ^1H NMR [400 MHz, $(CD_3)_2CO$]: δ8.28 (2H, *br*, OH's), 7.37 (1H, *m*, H-4), 7.26 (1H, *d*, J = 2.5 Hz), 7.12 (2H, *m*, H-6' and H-5'), 6.01 (1H, *ddd*, J_1 = 10.0 Hz, J_2 = 2.5 Hz, J_3 = 1 Hz, H-3), 5.35 (1H, *dd*, J_1 = 11.0 Hz, J_2 = 5.0 Hz, H-6), 2.64 (2H, *m*, H-5). Glucose was identified by PC in the hydrolysates using EtOAc–HOAc–pyridine–H_2O (12:3:3:2) as solvent [10].

Penta-acetate of **2** (**3**). Compound **2** (24 mg) was acetylated with Ac_2O–pyridine (1:1, 2 ml) in the usual manner to give the penta-acetate, **3** (30 mg), a solid which crystallized from EtOAc–petrol, mp 171–172°. R_f (**3**) = 0.69 in $CHCl_3$–Me_2CO (7:3), located by short-wave UV light and by the greenish colour formed after spraying with vanillin followed by heating [2]. IR ν_{max}^{KBr} cm^{-1}: 1757, 1665, 1622, 1434, 1375, 1226, 1043, 907, 817. MS *m/z* (rel. int.): 578.1673 $[M]^+$ (0.02), 331 $[C_{14}H_{19}O_9]^+$ (32), 271 $[C_{12}H_{15}O_7]^+$ (16), 229 $[C_{10}H_{13}O_6]^+$ (9), 211 $[C_{10}H_{11}O_5]^+$ (15), 206 $[C_{11}H_{10}O_4]^+$ (16), 187 $[C_8H_{11}O_5]^+$ (9), 169 $[C_8H_9O_4]^+$ (88), 145 $[C_6H_9O_4]^+$ (28), 139 $[C_7H_7O_3]^+$ (34), 127 $[C_6H_7O_3]^+$ (55), 115 $[C_5H_7O_3]^+$ (32), 109 $[C_6H_5O_2]^+$ (70), 103 $[C_4H_7O_3]^+$ (28), 97 $[C_5H_5O_2]^+$ (37), 68 $[C_4H_4O]^+$ (42), 43 $[CH_3CO]^+$ (100).

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