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1-*O*-β-D-GLUCOPYRANOSYL ANTHRANILATE FROM PIÑUELA (*BROMELIA PLUMIERI*) FRUIT

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Key Word Index—*Bromelia plumieri*; Bromeliaceae; piñuela fruit; 1-*O*-β-D-glucopyranosyl anthranilate; HPLC–MS/MS; electrospray ionization.

Abstract—From the glycosidic extract of piñuela fruit, 1-*O*-β-D-glucopyranosyl anthranilate was isolated by adsorption chromatography on XAD-2, followed by rotation locular counter-current chromatography, multilayer coil counter-current chromatography and HPLC. Identification was carried out by NMR (^{1}H, ^{13}C, ^{1}H–^{1}H and ^{13}C–^{1}H COSY) and HPLC–MS/MS under electrospray ionization.

INTRODUCTION

Anthranilic acid, a key metabolite in the biosynthesis of alkaloids or antibiotics, may either be formed *de novo* from chorismic acid or is a product of the degradation of L-tryptophan [1]. Anthranilic acid esters are constituents of certain essential oils, e.g. methyl anthranilate occurs in a number of blossoms (neroli, ylang-ylang, bergamot and jasmin) and citrus oils. Methyl *N*-methylanthranilate dominates the odour of petitgrain oil from mandarin leaves [2]. In addition, methyl anthranilate has been found in grape, wine, strawberry, cocoa and tea [3]. In the course of investigations on anthranilic acid metabolism the existence of a glucoconjugate (ester or *N*-glucoside) has been proposed [4–7]. Continuing our studies on flavour precursors in tropical fruits [8–10] we report the isolation and first complete structural elucidation of 1-*O*-β-D-glucopyranosyl anthranilate from piñuela fruit.

RESULTS AND DISCUSSION

During the course of our studies on glycosidic flavour precursors in tropical plants, anthranilic acid was identified as a major aglycone (*ca* 2 mg kg^{-1} fruit) in wild growing piñuela fruit (*Bromelia plumieri* Karstens). In order to isolate the intact conjugated derivative of anthranilic acid, glycosidic extracts were obtained by adsorption chromatography on Amberlite XAD-2 resin using ethyl acetate and methanol as eluents. The ethyl acetate extract was fractionated by rotation locular counter-current chromatography (RLCC) and multilayer coil counter-current chromatography (MLCCC). Subsequent HPLC yielded 40 mg of highly purified **1**. The ^{1}H and ^{13}C NMR spectra of **1** (Tables 1 and 2) revealed the ester bonding of a β-D-glucosyl residue with anthranilic acid, deduced from the shift and coupling constant of the anomeric proton (δ 5.64, 7.8 Hz) and carbon (δ 95.4, C-1′), respectively [11–13]. The assignments of signals were confirmed by 2D ^{1}H, ^{1}H-homonuclear and 2D ^{13}C,^{1}H-heteronuclear correlation spectroscopy (COSY). HPLC–tandem mass spectrometric analysis under electrospray ionization (ESI) conditions (Table 3) showed the protonated molecular ion $[M+H]^+$ *m/z* 300.1, together with other characteristic adduct ions, thus confirming the empirical formula of the glucose ester $C_{13}H_{17}NO_7$. The base peak *m/z* 138.1 observed within the corresponding daughter ion spectrum demonstrates the proton affinity of the N-containing aglycone. Enzymatic hydrolysis of **1** yielded both anthranilic acid and glucose, the latter confirmed as its methoxyoxime acetate by means of GC–mass spectrometry [14].

Additionally, an aliquot of the MLCCC fraction II (see Experimental) was peracetylated and purified by flash chromatography, yielding the peracetate **1a**. The adduct ions *m/z* 510.1 $[M+H]^+$ and 532.3 $[M+Na]^+$ obtained by ESI HPLC–mass spectrometry confirmed the pentaacetate $C_{23}H_{27}NO_{12}$. Unlike the underivatized glucose ester **1** the daughter ion spectrum of *m/z* 510.1 was dominated by fragments typical for peracetylated hexoses resulting from the neutral loss of the acetylated aglycone (Table 4). The ^{1}H and ^{13}C NMR spectra of **1a** (Tables 1 and 2) revealed four glucose-linked acetyl groups (^{1}H: δ 2.02–2.07; ^{13}C: δ 20.5–20.6). Furthermore, the presence of an unsubstituted amino group in **1** was confirmed by the signals of one additional

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Table 1. ^{1}H NMR data for compounds **1** and **1a** (400 MHz; δ in ppm; TMS, coupling constants in Hz)

H		**1** (CD_3OD)		**1a** ($CDCl_3$)
Aglycone				
3	6.67	1H, *dd* (8.5, 1.0)	8.69	1H, *dd* (8.4, 1.0)
4	7.18	1H, *ddd* (8.4, 6.9, 1.5)	7.58	1H, *ddd* (8.5, 7.2, 1.6)
5	6.50	1H, *ddd* (8.1, 7.1, 1.1)	7.09	1H, *ddd* (8.2, 7.1, 1.1)
6	7.83	1H, *dd* (8.1, 1.3)	7.95	1H, *dd* (8.1, 1.6)
CH_3CONH			2.23	3H, *s*
CH_3CONH			10.71	1H, *s*
Glucose				
1′	5.64	1H, *d* (7.8)	5.86	1H, *d* (7.8)
2′		3.40–3.55 *m**	5.33	2H, *m**
3′		3.40–3.55 *m**	5.33	2H, *m**
4′		3.40–3.55 *m**	5.20	1H, *dd* (9.6, 9.7)
5′		3.40–3.55 *m**	3.93	1H, *ddd* (9.4, 4.2, 2.2)
6a′	3.64	1H, *dd* (12.3, 1.6)	4.14	1H, *dd* (12.6, 2.2)
6b′	3.79	1H, *dd* (12.3, 4.3)	4.32	1H, *dd* (12.6, 4.2)
CH_3CO_2			2.02–2.07	4 × 3H, *s*

*Overlapped.

Table 2. ^{13}C NMR data for compounds **1** and **1a** (100 MHz; δ in ppm; TMS)

C	**1** (CD_3OD)	**1a** ($CDCl_3$)
Aglycone		
1	110.2 *s*	113.0 *s*
2	153.2 *s*	142.3 *s*
3	118.0 *d*	120.5 *d*
4	135.6 *d*	135.8 *d*
5	116.5 *d*	122.8 *d*
6	132.3 *d*	131.3 *d*
CO_2Glc	168.0 *s*	166.3 *s*
CH_3CONH		25.4 *q*
CH_3CONH		170.4 *s**
Glucose		
1′	95.4 *d*	92.4 *d*
2′	73.9 *d*	70.1 *d*
3′	78.0 *d*	72.6 *d*
4′	71.0 *d*	68.0 *d*
5′	78.7 *d*	72.9 *d*
6′	62.3 *t*	61.5 *t*
CH_3CO_2		20.5–20.6; 4 × *q*
CH_3CO		168.9–169.9; 4x*s**

*Assignments exchangeable.

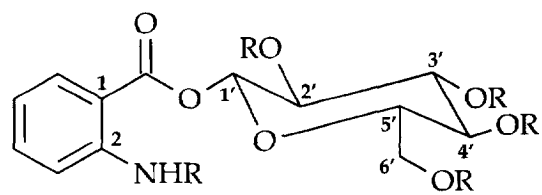
Table 3. Mass spectra of compound **1** obtained by ESI HPLC–MS/MS

m/z	Interpretation
341.3	$[M + CH_3CN + H]^+$
322.1	$[M + Na]^+$
300.1	$[M + H]^+$
Daughter ion spectrum of *m/z* 300.1 (C_{off} −12 eV; 1.8 mtorr Ar)	
300.1	$[M + H]^+$
282.9	$[M - NH_3 + H]^+$
138.1	$[Aglycone + H]^+$
120.1	$[Aglycone - H_2O + H]^+$

Table 4. Mass spectra of compound **1a** obtained by ESI HPLC–MS/MS

m/z	Interpretation
532.3	$[M + Na]^+$
510.1	$[M + H]^+$
Daughter ion spectrum of *m/z* 510.1 (C_{off} −15 eV; 1.8 mtorr Ar)	
510.1	$[M + H]^+$
450.4	$[M - HAc + H]^+$
389.9	$[450 - HAc]^+$
348.1	$[389.9 - CH_2{=}C{=}O]^+$
331.2	$[M - (aglycone\text{-}Ac) + H]^+$
288.1	$[348.1 - HAc]^+$
271.2	$[331.2 - HAc]^+$
168.9	$[271.2 - HAc - CH_2{=}C{=}O]^+$
162.1	$[(aglycone\text{-}Ac) - H_2O + H]^+$

N-acetyl group (^{1}H: δ 2.2; ^{13}C: δ 25.4) as well as by the considerable downfield shift of the deshielded H-3 in **1a** [12]. Assignments of the acetylated aglycone moiety were based on correlated homonuclear and heteronuclear NMR spectra of *N*-acetyl methylanthranilate. Thus, **1** was identified as 1-*O*-β-D-glucopyranosyl anthranilate.



1 R = H

1a R = Ac

EXPERIMENTAL

General. TLC was carried out on silica gel 60 F_{254} (Merck) using the less polar RLCC layer as mobile phase (detection: 254 nm and vanillin/H_2SO_4). Flash chromatography was performed on silica gel 60 (activity grade II, 0.032–0.063 mm). RLCC was used in the ascending mode ($CHCl_3$–MeOH–H_2O, 7:13:8) and analyt. MLCCC with the same solvent system in the tail-to-head mode. Prep. HPLC was performed on Eurospher 100 C-18 (Knauer, 5 μm; 250 × 16 mm) with a flow rate of 5 ml min^{-1} and UV detection (254 nm). For analyt. HPLC the same conditions, but a 4-mm column with a flow rate of 1 ml min^{-1} and diode array detection were used. HRGC and HRGC-MS analyses were carried out using a J&W DB-Wax fused silica capillary column (30 m × 0.25 mm i.d., d_f = 0.25 μm). Split-injection (1:20) was employed. Temp. programme: 50° for 3 min, then from 50 to 240° at 4° min^{-1} and 240° for 10 min. Flow rates for carrier gas were 2.5 ml min^{-1} He, for make-up gas 30 ml min^{-1} N_2 and for detector gases 30 ml min^{-1} H_2 and 300 ml min^{-1} air. Injector and detector (FID) temps were kept at 220 and 250°, respectively. For HRGC-MS the temp. of the ion source and all connection parts was 220°; electron energy 70 eV and cathodic current 0.7 mA. 2D ^1H–^1H homonuclear and 2D ^{13}C–^1H heteronuclear COSY experiments were performed at 250 MHz for ^1H and 62.5 MHz for ^{13}C. TMS was used as int. standard. HPLC–MS/MS was under ESI: HPLC on Eurospher 100 C-18 column (Knauer; 5 μm; 100 × 2 mm) with a linear H_2O–TFA (0.05%)–MeCN gradient (20% MeCN–100% MeCN in 10 min), flow rate 200 μl min^{-1} spray capillary voltage 4 kV, temp. of heated inlet capillary serving simultaneously as repeller electrode (20 V) was 200°. N_2 served both as sheat (50 psi) and auxiliary gas (10 ml min^{-1}). Positive ions were detected by scanning from 150 to 600 mu with a total scan duration of 1.0 s and a dwell time of 2 msec. MS/MS experiments were performed at a collision pressure of 1.80 mtorr Ar, mass range 20–600 mu, collision offset $C_{off} - 12$ and -15 eV, respectively. Electron multiplier voltage was set to 1200 V in scan mode and 1800 V for MS/MS experiments.

Plant material. Piñuela fruit (*B. plumieri* Karstens), was harvested from January to June 1994 in Charalá, Santander, Colombia. A voucher specimen is deposited in the Herbario National Colombiano (Nr. 365432).

Extraction and isolation of compound **1**. A crude glycosidic extract (20 g from 17.5 kg of fruit) was obtained by MeOH elution as described previously [8–10]. It was readsorbed on Amberlite XAD-2 (Serva) resin and eluted with EtOAc (1 l) and subsequently MeOH (1 l). The EtOAc fr. (4 g) was further fractionated by RLCC resulting in combined frs I–VI, from which RLCC fr. II was subjected to analyt. MLCCC yielding MLCCC frs I–III. From MLCCC fr. II 250 mg crude **1** was obtained and further purified by prep. HPLC (yield 40 mg). During the isolation and purification steps, the frs were monitored by TLC and analyt. HPLC (H_2O–MeOH gradient: 30–100% in 30 min). Identity of the aglycone was demonstrated by HRGC-MS after enzymic hydrolysis with Rohapect D5L pectinase (Röhm) or emulsin (Sigma) [9].

Acetylation. An aliquot of the MLCCC fr. II was acetylated using standard Ac_2O–pyridine procedure at room temp. for 12 h in the dark. Purification of **1a** was carried out by flash chromatography with $CHCl_3$ under N_2 pressure followed by prep. HPLC (H_2O–MeOH gradient: 30–100% in 30 min).

Sugar analysis. Pure **1** (0.5 mg) in 1 ml 0.2 M citrate–phosphate buffer was incubated with 3 mg emulsin (12 hr, 37°). The liberated aglycone was extracted with Et_2O and the aq. phase ultracentrifuged (Ultrafree-MC 5000 NMGG, Millipore). After lyophilization, the liberated glucose was converted into the corresponding methoxyoxime acetate and analysed by HRGC-MS as described previously [14].

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