Free and Bound Volatile Composition and Characterization of Some Glucoconjugates as Aroma Precursors in Melón de Olor Fruit Pulp (*Sicana odorifera*)

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Free and glycosidically bound volatiles obtained from the fruit pulp of *Sicana odorifera* by liquid−liquid extraction and by chromatography, followed by enzymatic hydrolysis with Rohapect D5L, respectively, were analyzed by capillary gas chromatography (HRGC), HRGC−mass spectrometry (HRGC−MS), and HRGC−Olfatometry (HRGC−O) analyses. A total of 37 free volatiles was detected, with the major components being 3-methyl-2-butanol, 3-hydroxy-2-butanone, ethyl 3-hydroxybutanoate, and (*Z*)-3-hexenol . Among the 22 detected glycosidically bound compounds, 4-hydroxybenzyl methyl ether, 4-hydroxybenzyl alcohol, and 2-phenylethanol were found to be the major constituents. Additionally, two glucoconjugates were isolated in pure form by multilayer coil countercurrent chromatography (MLCCC) of the glycosidic extrac and further purification. Their structures were elucidated by MS and NMR analyses to be the novel [4-(β-D-glucopyranosyloxy)benzyl] 2,3-dihydroxy-3-methylbutanoate **2**, and the known 4-(β-D-glucopyranosyloxy)benzyl alcohol **1**. Compounds **1** and **2** are precursors of 4-hydroxybenzyl alcohol, one of the major volatiles generated by enzymatic hydrolysis of the glycosidic fraction.

Keywords: *Volatiles; Cucurbitaceae; Sicana odorifera; melón de olor; glycosidically bound volatiles; aroma precursors; glucoconjugates; [4-(β-D-glucopyranosyloxy)benzyl] 2,3-dihydroxy-3-methylbutanoate; 4-(β-D-glucopyranosyloxy)benzyl alcohol*

INTRODUCTION

Melón de olor (*Sicana odorífera*) plant is a tropical member of the Cucurbitaceae found at 0−1400 m above sea level from Mexico to Brazil. Its ripe fruit has a pleasant and intense aroma and a sweet and slightly acid taste, and these fruits are consumed fresh or prepared in desserts, conserves, etc. Local people use this fruit to perfume clothes and houses. In addition, the plant is used as an insect repellent (Lira, 1991). Despite the pleasant aroma of this fruit, to date no studies on its flavor are known to have been carried out. Although this fruit is not yet widely cultivated in Colombia its exotic aroma makes it promising as a raw material for the flavor industry.

As a part of our continuing studies on the flavor of Colombian fruits (Morales et al., 1996; Parada and Duque, 1998; Osorio et al., 1999), this paper describes for the first time the nature of the free and glycosidically bound aroma compounds present in the fruit pulp of Melón de olor. Furthermore, this work also describes the isolation and spectroscopic identification of 4-(β-D-glucopyranosyloxy)benzyl alcohol **1,** and the novel [4-(β-D-glucopyranosyloxy)benzyl] 2,3-dihydroxy-3-methylbutanoate **2**, precursors of 4-hydroxybenzyl alcohol, the major glycosidically bound volatile present in the fruit pulp.

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MATERIALS AND METHODS

General. NMR spectra were taken on a Fourier transform JEOL JNM-LA400 spectrometer (Akishima, Japan) and on a Bruker AC 500 spectrometer (Karlsruhe, Germany) with $CDCl_3$ as solvent and Me_4Si as internal standard. UV spectra were obtained with a Beckman 25 instrument (Fullerton, CA). Fast atom bombardment mass spectrometry (FABMS) was carried out with a JEOL JMS-AX505HA spectrometer (Akishima, Japan) using a glycerol matrix and an ion source temperature at 305 °C.

Materials. Ripe melón de olor (*Sicana odorifera*) fruits were harvested in Anolaima, Cundinamarca, Colombia. Fruits were carefully selected according to the degree of ripeness as determined by measuring pH (6.3) and peel color (completely red). All solvents employed were of analytical grade quality and redistilled before use.

Extraction of Free Volatile Compounds. Fruit pulp (ca. 1 kg), free of peel and seeds, was blended and the homogenate was centrifuged at 10 000*g* for 30 min. After addition of 2-undecanol (300 μg/kg) as internal standard, the supernatant was continuously extracted with pentane/dichloromethane (2:1, v/v) for 48 h (Morales et al., 1996). The organic phase was dried over anhydrous sodium sulfate, concentrated through a Vigreux column (36 °C) to 0.2 mL, and subjected to capillary gas chromatography (HRGC), capillary gas chromatography−mass spectrometry (HRGC−MS), and capillary gas chromatography−olfactometry (HRGC−O) analyses.

Isolation of Glycosidic Extract. The pulp (0.3 kg), separated from the peelings and seeds, was homogenized in 0.2 M phosphate buffer (pH 7.0) and centrifuged at 10 000*g* for 30 min. The supernatant was subjected to liquid chromatography on Amberlite XAD-2 adsorbent (glass column, 25 × 500 mm) according to Gunata et al. (1985). After washing with 1.0 L of water, elution was performed with 1.0 L of methanol.

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The eluate was concentrated to dryness under reduced pressure, redissolved in 50 mL of 0.2 M citrate-phosphate (pH 5.0), and extracted with diethyl ether to remove remaining volatiles.

Enzymatic Hydrolysis. A nonselective pectinase (300 μL of Rohapect D5L, Röhm, Darmstadt, Germany) was added to the glycosidic extract together with phenyl β-D-glucopyranoside as internal standard (800 μg/kg), and the mixture was incubated at 37 °C overnight. The liberated aglycones were extracted with diethyl ether, and the organic phase was dried over anhydrous sodium sulfate, concentrated (Vigreux column, 36 °C) to 0.2 mL, and subjected to HRGC, HRGC−MS, and HRGC−O analyses.

Capillary Gas Chromatography (HRGC). Experiments were performed on a Carlo Erba Fractovap 4160 Gas Chromatograph (Milano, Italy), equipped with a split-splitless injector and a flame ionization detector (FID), both operating at 250 °C. A J&W fused-silica DB-Wax capillary column (30 m × 0.25 mm i.d.; film thickness 0.25 μm) was used with the following temperature program: 3 min isothermal at 50 °C, then raised to 240 °C at 4 °C/min, and finally held at 240 °C for 10 min. The flow rate for the carrier gas was 1.6 mL/min He. Volumes of 1 μL were injected with a split ratio of 1:50. Temperature programmed retention indices were estimated using normal paraffins (C_9−C_{32}) as standards. Quantitative data for free and bound volatiles were obtained by the internal standard method using 2-undecanol and phenol (released by enzymic hydrolysis of phenyl β-D-glucopyranoside), respectively, as reference substances without considering calibration factors, i.e., $F = 1.00$ for all compounds.

Capillary Gas Chromatography−Mass Spectrometry (HRGC−MS). A Varian Aerograph 1440 gas chromatograph (Palo Alto, CA) directly coupled to a Finnigan MAT 44 mass spectrometer (Bremen, Germany) was used with the same type of column and temperature conditions as mentioned above for HRGC; electron energy 70 eV; mass range 30−300.

Results of qualitative analyses (mass spectral data studies) were verified by comparing the retention indices and mass spectral data with those of authentic reference substances and/or with other published spectra (EPA/NIH mass spectral library).

Capillary Gas Chromatography−Olfactometry (HRGC−O). HRGC with a simultaneous flame ionization detector (FID) and an odor evaluation port was carried out using a HP 5890 gas chromatograph (Palo Alto, CA) operated under the same conditions as mentioned above for HRGC. Approximately 50% of the effluent was diverted through a heated, glass-lined capillary (60 °C) to a sniffing mask, where it was mixed with humidified air. Each sample was sniffed three times by people trained in that technique. FID was used as monitor detector.

Isolation of Glycoconjugates. To isolate the intact conjugate derivatives of 4-hydroxybenzyl alcohol, 18 g of glycosidic extract was obtained by adsorption chromatography on XAD-2 resin as described above but using 19 kg of fruit pulp. Then, portions of 1 g of glycosidic extract were fractionated by multilayer coil countercurrent chromatography (MLCCC) with an Ito multilayer coil separator-extractor (PC Inc., Potomac, MD) equipped with a 75 m × 2.6 mm i.d. PTFE tubing for separation of glycosides. The instrument was operated using $CHC1_3$/MeOH/H_2O (7:13:8, v/v/v) as solvent system at a flow rate of 1.0 mL/min, and at a rotational speed of 800 rpm. Elution mode head to tail was used. Fifty fractions of 5 mL each were collected. Combined fractions (6−7) containing a precursor of 4-hydroxybenzyl alcohol as revealed by enzymatic hydrolysis were refractionated by MLCCC as described above and finally purified by RP-HPLC (Eurospher 100 C-18 column, 5 μm, 250 × 46 mm) with MeOH/H_2O (1:3, v/v) as mobile phase at a flow rate of 5.0 mL/min, to obtain 15 mg of glycoconjugate **1**. Additionally, 5 mg of compound **1** was acetylated (Ac_2O/pyridine) and further purified by silica gel flash chromatography to obtain peracetylated glycoconjugate **1a**.

In addition, combined fractions (9−10) from the first MLCCC were refractionated by this chromatographic technique using the same conditions as described above and finally purified using RP-HPLC (Eurospher 100 C-18 column, 5 μm, 250 × 46 mm) with MeOH/H_2O (1:1, v/v) as a mobile phase at a flow rate of 5.0 mL/min, to yield 29 mg of semipure glucoconjugate **2**. The semipure compound **2** was further acetylated (Ac_2O/pyridine) and purified by silica gel flash chromatography (SiO_2 60 Merck, 0.032−0.063 μm) with ethyl ether as mobile phase, to finally yield 4 mg of pure peracetylated glycoconjugate **2a**.

4-(β-D-glucopyranosyloxy)benzyl alcohol **1**: UV (MeOH) λ_{max} 272 nm; ^1H and ^{13}C NMR data were in good agreement with those published by Taguchi et al. (1981).

Peracetylated 4-(β-D-glucopyranosyloxy)benzyl alcohol **1a**: UV (MeOH) λ_{max} 270 nm; FAB−MS, *m/z* (%) 519 (8, $[M+Na]^+$), 497 (1, $[M+H]^+$), 331 (86), 271 (4), 169 (64); ^1H and ^{13}C NMR data were in good agreement with those published by Taguchi et al. (1981).

Peracetylated [4-(β-D-glucopyranosyloxy)benzyl] 2,3-dihydroxy-3-methylbutanoate **2a**: UV (MeOH) λ_{max} 270 nm; HR-FAB−MS, *m/z* 613.2139 $[M+H]^+$ ($C_{28}H_{37}O_{15}$ requires 613.2132); FAB−MS, *m/z* (%) 635 (26, $[M+Na]^+$), 613 (27, $[M+H]^+$), 331 (52), 271 (39), 211 (41), 169 (100);^1H and ^{13}C NMR, see Table 2 below.

Biomimetic Reactions. Compounds **1** and **2** were submitted to enzymatic hydrolysis with β-glucosidase in the same conditions as described for Rohapect D5L enzyme (Röhm, Darmstadt, Germany). The liberated aglycones were extracted with diethyl ether and the organic phase was dried over anhydrous sodium sulfate, concentrated (Vigreux column, 36 °C) to 0.2 mL, and subjected to HRGC and HRGC−MS.

RESULTS AND DISCUSSION

Free and Glycosidically Bound Volatiles. Either the free volatile extract or the volatiles generated by enzymatic hydrolysis from the glycosidic fraction obtained from melón de olor (*Sicana odorifera*) fruit pulp showed aroma notes resembling the flavor of fresh fruit, described as fruity-watery-green-fatty-sweet and watery-fatty-sweet, respectively.

Table 1 shows the free and bound volatile compounds identified in the above-mentioned extracts by HRGC and HRGC−MS analyses, the retention indices found experimentally for each compound and those obtained for authentic reference substances or those reported in the chemical literature, as well as the concentration of each aroma compound calculated on the basis of the standard added and the odor description of each GC-separated volatile.

As can be seen in Table 1, 37 compounds (94.8% of total extract) were identified as free volatiles with 3-methyl-2-butanol, 3-hydroxy-2-butanone, ethyl 3-hydroxybutanoate, and (*Z*)-3-hexenol being the major components. Aliphatic alcohols (61.1%) predominated in the melón de olor free volatiles profile, followed by hydroxyketones (14.6%), aliphatic acids (7.5%), hydroxyesters (4.8%), terpenes (2.7%), aromatic compounds (2.6%), aldehydes (1.5%), and unidentified compounds (5.2%). The free volatile profile found in melón de olor is quite different from that reported for melón (*Cucumis melo*) (Homatidou et al., 1992), its close relative which also belongs to the Cucurbitaceae family. In melón fruit, sulfur compounds constitute the major part of volatiles compared with the flavor of other tropical fruits (Shibamoto and Tang, 1990) such us banana, mango, papaya, passion fruit, and guava. In contrast, the aroma spectrum here reported for melón de olor is unique.

Table 1 also shows 22 aglycones released by enzymatic hydrolysis which were identified for the first time as bound aroma constituents in melón de olor fruit. The identified glycosidically bound compounds mainly consisted of compounds having aromatic structure (87.3%), and these were followed by aliphatic acids (5.0%),

Table 1. Free and Glycosidically Bound Volatile Compounds Identified in Melón de Olor (*Sicana odorífera*) Fruit Pulp

		retention indices[a]		amount (μg/kg)		
no.	compound	ref	exp	free	bound	odor at sniffing port
1	3-methyl-2-butanol		1089	1450	-[c]	green
2	butanol	1113	1115	21	-	green
3	3-methyl-1-butanol	1196	1199	47	237	green
4	(*E*)-2-hexenal	1202	1213	35	-	
5	3-hydroxy-2-butanone	1278	1276	426	52	fruity
6	ethyl 2-hydroxy-2-methylbutanoate	1303	1299	6	-	
7	(*Z*)-2-pentenol	1310	1314	28	-	
8	hexanol	1331	1349	26	-	
9	(*Z*)-3-hexenol	1376	1377	113	-	green
10	cyclohexanol	1375	1392	30	-	
11	acetic acid	1424	1430	33	449	acetic (pungent)
12	(*Z*)-linalool oxide, furanoid	1426	1432	58	-	
13	1-octenol	1440	1453	-	133	green-sweet
14	(*E*)-linalool oxide, furanoid	1440	1455	12	-	
15	ethyl 3-hydroxybutanoate	1490	1507	126	417	sweet-fatty
16	2-methylpropanoic acid	1535	1524	101	102	green
17	unknown (74, 45, 56, 57, 102)[b]		1539	24	-	
18	unknown (74, 45, 56, 57, 102)[b]		1557	24	-	
19	(*Z*,*E*)-2,6-nonadienal	1551	1576	10	-	watery-fatty
20	2-methylbutanoic acid	1641	1639	14	67	pungent-acid
21	salicylaldehyde	1573	1653	7	-	
22	1-nonanol	1636	1665	17	-	
23	ethyl 3-hydroxyhexanoate	1675	1671	7	-	
24	(*Z*)-6-nonenol	1696	1676	47	-	watery
25	3-(methylthio)propanol	1710	1702	-	37	
26	2-undecanol (I. S.)	1712	1716	300	-	
27	unknown (41, 67, 55, 79, 93)[b]		1742	103	-	
28	2-butenoic acid	1744	1763	-	43	
29	hexanoic acid	1848	1838	6	-	
30	unknown (43, 73, 45, 55, 75)[b]		1840	-	46	
31	benzyl alcohol	1853	1858	24	903	balsamic-earthy
32	2-phenylethanol	1899	1893	17	691	sweet
33	heptanoic acid	1900	1923	14	-	
34	phenol (I. S.)	1950	1989	-	800	
35	octanoic acid	2035	2050	13	-	green
36	*p*-cresol	2050	2080	22	135	
37	nonanoic acid	2124	2153	19	-	
38	4-vinylguaiacol	2162	2174	-	172	
39	decanoic acid	2244	2285	4	-	
40	farnesol	2323	2337	10	-	
41	4-(1-hydroxyethyl)-γ-butanolactone		2347	-	106	
42	4-vinylphenol	2325	2367	-	162	
43	benzoic acid	2390	2407	-	65	
44	dodecanoic acid	2412	2465	16	-	
45	4-hydroxybenzyl methyl ether		2475	-	1635	green-earthy
46	4-hydroxybenzyl ethyl ether		2529	-	78	
47	1-octadecanol		2569	10	-	
48	4-hydroxy-3-methoxyacetophenone		2585	4	25	
49	hydroxybenzaldehyde		2894	3	368	
50	4-hydroxybenzyl alcohol	2942	2932	-	7380	refreshing

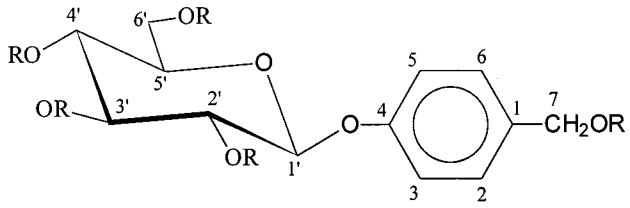
[a] Retention index for authentic reference substances measured on DB−Wax column. [b] Prominent MS peaks. [c] − = nondetected.

hydroxyesters (3.9%), aliphatic alcohols (2.8%), one hydroxyketone (0.4%), one sulfur compound (0.3%), and one unidentified compound (0.3%). As can be seen in Table 1, the profile of bound volatiles is dominated by 4-hydroxybenzyl alcohol, 4-hydroxybenzylmethyl ether, benzyl alcohol, and 2-phenylethanol.

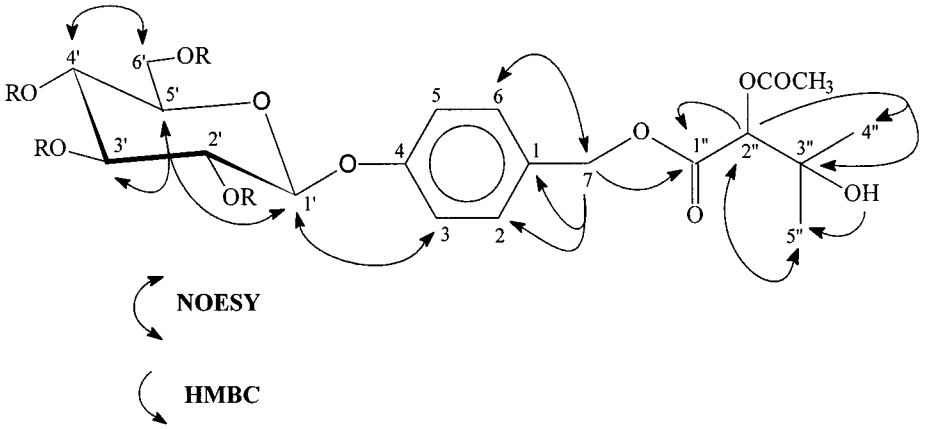
The aroma evaluation of each free volatile separated from free and bound volatiles extract by HRGC did not reveal any compound which could be considered as the character impact compound in *Sicana odorifera* fruit. However, (*Z*,*E*)-2,6-nonadienal and (*Z*)-6-nonenol seem to be important contributors to the watery aroma of this fruit.

Isolation and Characterization of Glucoconjugates. To isolate the intact glycoconjugates, the glycosidic fraction was prefractionated by MLCCC. The subfractions thus obtained were submitted to RP-HPLC and subsequently acetylated and purified by liquid column chromatography as described in the Materials and Methods section. In this way, two glucosidic compounds were obtained in free (**1** and **2**) and peracetylated form (**1a** and **2a**).

Compound **1** showed a UV absorption maximum at 272 nm characteristic of 4-hydroxybenzyl alcoholic derivatives (Taguchi et al., 1981). ^{1}H and ^{13}C NMR data corresponded with data published for 4-(β-D-glucopyranosyloxy)benzyl alcohol by Taguchi et al. (1981). The peracetylated compound **1a** showed UV absorption maximum at 270 nm, and its FAB−MS yielded a pseudo-molecular ion at *m*/*z* 497 $[M+H]^+$ and diagnostic ions *m*/*z* 331, 271, and 169, indicating a monosaccharide (hexose) as sugar moiety (Osorio et al., 1999). From comparison of the ^{1}H and ^{13}C NMR data (Taguchi et al., 1981), it was evident that compound **1a** was the per-*O*-acetylated 4-(β-D-glucopyranosyloxy)benzyl alcohol. HRGC and HRGC−MS analyses of the enzymatic hydrolysis products of compound **1** indicated that this glucoside is a natural precursor in melón de olor of



1 R=H 1a R=Ac



2 R=H 2a R=Ac

Figure 1. Structures of glucoconjugates **1** and **2** isolated from melón de olor (*Sicana odorifera*).

Table 2. ^{13}C and ^{1}H NMR Spectral Data of Compound 2a ($CDCl_3$, 500 MHz, δ Relative to TMS)

position[a]	δ_C	δ_H
1	130.05	
2, 6	130.00	7.30 (2H, *d*, $J = 8.5$)
3, 5	116.98	6.98 (2H, *d*, $J = 8.5$)
4	156.90	
7	66.72	5.14 (1H, *d*, $J = 12.0$)
		5.19 (1H, *d*, $J = 12.0$)
1'	98.88	5.09 (1H, *d*, $J = 7.2$)
2'	71.10	5.28 (1H, *dd*, $J = 8.6, 7.2$)
3'	72.64	5.31 (1H, *dd*, $J = 9.1, 8.6$)
4'	68.17	5.18 (1H, *dd*, $J = 10.0, 9.1$)
5'	72.06	3.87 (1H, *ddd*, $J = 10.0, 5.4, 2.4$)
6'	61.89	4.17 (1H, *dd*, $J = 12.0, 2.4$)
		4.30 (1H, *dd*, $J = 12.0, 5.4$)
acetates	20.6−20.8 (4×)	2.0−2.1 (12H, 4*s*)
	169.3−170.7 (4×)	
1″	168.55	
2″	78.45	4.84 (1H, *s*)
3″	71.07	
4″, 5″	25.89, 26.00	1.27 (3Hx2, *s*)
2″-Ac	20.67	2.16 (3H, *s*)
	170.20	
3″-OH		2.46 (1H, *s*)

[a] Assigments were based on $^{1}H-^{1}H$ COSY, HMQC, HMBC, and NOESY.

4-hydroxybenzyl alcohol, the major volatile generated by hydrolysis of the glycosidic fraction (Table 1). Glucoside **1** as natural compound has been isolated previously from *Vanda parishii* (Dahmén and Leander, 1976) and *Anoectochilus koshunensis* (Ito et al., 1993), plants belonging to the *Orchidaceae* family. As far as we know, this is the first time that 4-(β-D-glucopyranosyloxy)-benzyl alcohol has been found in fruits. It has also been reported that both 4-hydroxybenzyl alcohol and 4-(β-D-glucopyranosyloxy)benzyl alcohol **1** facilitate memory consolidation and retrieval in rats (Hsieh et al., 1997).

The structural elucidation of **2** was determined from its acetate derivative by UV and FAB−MS, as well as by ^{1}H and ^{13}C NMR one and two-dimensional spectroscopy. Compound **2a** showed a UV absorption maximum at 270 nm, also suggesting a structure containing 4-hydroxybenzyl alcohol too. HRFAB−MS displayed a pseudo-molecular ion at *m/z* 613.2139 corresponding to a molecular formula of $C_{28}H_{37}O_{15}$, and FAB−MS showed signals at *m/z* 635 $[M+Na]^+$ and 613 $[M+H]^+$, together with the diagnostic ions at *m/z* 331, 271, 211, and 169 characteristic of a peracetylated hexose unit (Osorio et al., 1999). From the ^{1}H and ^{13}C NMR data the presence of a β-D-glucopyranoside unit was confirmed. NMR spectra also displayed a set of signals which showed that the structure of compound **1a** is a part of compound **2a**, except for the absence of the methylene carbinol ($-CH_2OH$) singlet signal at δ 5.05, which in the spectrum of **2a** appeared as two doublets corresponding to non-equivalent carbinol protons at δ 5.14 and 5.19. Furthermore, the ^{1}H and ^{13}C NMR spectra of **2a** showed additional signals for one methine (δ 4.84; 78.45), two methyl (δ 1.27; 25.89 and 26.00), one acetate (δ 2.16; 20.67 and 170.20), one carbinol (δ 2.46 exchangeable; 71.07), and one carboxylic acid (δ 168.55) group. Additional $^{1}H-^{1}H$ COSY, HMQC, HMBC, and NOESY (two-dimensional NMR experiments) (cf. Table 2 and Figure 1) permitted the unequivocal connection of these functionalities, leading to the formula **2a** for the peracetylated derivative of **2**. Thus, compound **2** was determined to be [4-(β-D-glucopyranosyloxy)benzyl] 2,3-dihydroxy-3-methylbutanoate. To our knowledge, this is the first time that glucoconjugate **2** has been isolated from natural sources. HRGC and HRGC−MS of the enzymatic products of compound **2** revealed this compound as another natural precursor of 4-hydroxybenzyl alcohol in melón de olor fruit.

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