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Glycosidic Conjugates of C13 Norisoprenoids, Monoterpenoids, and Cucurbates in *Boronia megastigma* (Nees)

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Supporting Information

ABSTRACT: Analysis of a methanolic extract of marc from *Boronia megastigma* (Nees) using LC-MS (APCI, nominal mass) provided strong evidence for the presence of both glycosides and malonyl glycosides of methyl cucurbates, C13 norisoprenoids including megastigmanes, and monoterpene alcohols. Subsequent fractionation of an extract from the marc using XAD-2 and LH 20 chromatography followed by LC-UV/MS-SPE-NMR and accurate mass LC-MS resulted in the isolation and identification of (1*R*,4*R*,5*R*)-3,3,5-trimethyl-4-[(1*E*)-3-oxobut-1-en-1-yl]cyclohexyl β-D-glucopyranoside (3-hydroxy-5,6-dihydro-β-ionone-β-D-glucopyranoside); 3,7-dimethylocta-1,5-diene-3,7-diol-3-O-β-D-glucopyranoside; and a methyl {(1*R*)-3-(β-D-glucopyranosyloxy)-2-[(2*Z*)-pent-2-en-1-yl]cyclopentyl}acetate stereoisomer (a methyl cucurbate-β-D-glucopyranoside); and provided evidence for 3,7-dimethylocta-1,5-diene-3,7-diol-3-*O*-(6′-*O*-malonyl)-β-D-glucopyranoside in boronia flowers.

KEYWORDS: *Boronia megastigma* (Nees), brown boronia, alangionoside L, glycosides, malonyl glycosides, C13 norisoprenoids, LC-UV/MS-SPE-NMR

INTRODUCTION

Boronia megastigma (Nees) (brown boronia, family *Rutaceae*) is a woody understorey shrub that is endemic to the southwest of Western Australia. The plant produces a highly fragrant flower, and clones of *B. megastigma* (Nees) are grown commercially in Tasmania. A complex range of volatile compounds have been identified in boronia[1−3] including methyl jasmonates, dodecyl acetate and other organoleptically interesting esters, monoterpenols, sesquiterpenes, β-ionone, and a range of other C13 norisoprenoids.

Historically, an extract has been obtained from boronia flowers using a solvent extraction process. In Tasmania, improved large scale extraction technologies developed in the 1980s has established boronia as an intensive horticultural crop. Research and publications that followed from doctoral studies by MacTavish[4] represented important advances in the field of boronia production. A series of studies optimized the solvent extraction process[5] and harvest time technologies.[6] Further work[7,8] established that post harvest incubation led to increases in volatiles and a commercial post harvest incubation process which resulted in increased yields of β-ionone.[9]

The role of metabolic processes in the appearance of C13 norisoprenoids, including β-ionone, was investigated by Cooper et al.[10,11] The authors identified a group of five C27 apocarotenoids in boronia flowers and presented evidence that the appearance of β-ionone was correlated with increases in carotenoids during flower development. This has led to speculation that biosynthesis of hydroxylated C13 norisoprenoids from xanthophylls may occur in boronia. The possibility that those compounds may be present as glycosidic precursors was also considered.

Glycosides of flavor and aroma compounds including glucosides and 6′-*O*-malonyl glucosides of C13 norisoprenoids, monoterpenes, and shikimates are ubiquitous in the plant kingdom, and methods for their isolation and detection are widely documented.[12−19] High pressure liquid chromatography−mass spectrometry (LC-MS) techniques using electrospray ionization (ESI) combined with the use of reference standards were previously used by Withopf et al.[19] and Boss et al.[12] to screen for glycosides in several different types of fruits and leaves. Tandem MS/MS using a triple stage quadrupole analyzer with atmospheric pressure chemical ionization (APCI) has also been reported.[13]

The work presented here was conducted, using hyphenated HPLC, MS, and NMR techniques, in order to investigate the presence of glycosylated flavor and aroma compounds in *Boronia megastigma*.

MATERIALS AND METHODS

Materials. Boronia marc was obtained from flowers grown in southern Tasmania. Chemicals and solvents were either of analytical or of HPLC grade as required. Deuterated acetonitrile (CD_3CN, D 99.96%) was sourced from Cambridge Isotope Laboratories. The XAD-2 was obtained from Supelco, and the Sephadex LH 20 resin was purchased from Sigma-Aldrich.

Extraction of Glycosides. Boronia marc (typically 100 g), which had been extensively extracted with petroleum ether to remove the

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nonpolar compounds, was homogenized with ice-cold methanol (MeOH, 250 mL) in a compressed-air blender for two minutes. The ensuing homogenate was blended (in batches) for a further 1 min (Sorvall Omni-mixer, highest speed setting), shaken for 20 min at 200 cycles/s (Janke and Kunkel Ika-Werk, KS 500, rotary shaker), and filtered through a Büchner funnel (Whatman no. 1). The solids were washed with small volumes of MeOH. The resultant primary MeOH extract was dried by rotary evaporation, shaken with 50 mL of distilled water to form a slurry, and again filtered (Whatman No. 2, 2 layers). The remaining solids were washed with 2 × 50 mL aliquots of distilled water to yield 160 mL of a purple filtrate, which was stored at 4 °C prior to chromatography on XAD-2.

XAD-2 Chromatography. A glycosidic extract was prepared from the purple filtrate using XAD-2 chromatography based on the method of Günata et al.[20] The aqueous extract (typically 320 mL) was poured onto an equilibrated XAD-2 column measuring 40 × 4.5 cm. The column was washed with 4 L of distilled water to remove sugars. The glycosidic fraction was eluted with 4 L of MeOH, and the solvent was reduced by rotary evaporation to yield typically 3.3−3.8 g of a purple solid. The glycosidic MeOH extract was stored at −10 °C prior to further analysis.

LH 20 Chromatography. This glycosidic MeOH extract was further separated on Sephadex LH 20. Two different LH 20 columns were used (column 1 = 19 × 5.0 cm; column 2 = 80 cm ×3.0 cm). During use, the column was connected to an ÄKTA prime (Amersham Biosciences) pumping and fraction collection system. Samples, usually 1.0 g, were loaded onto the column using an injection loop. The columns were eluted with water, and the fractions (column 1 = 20 mL, $n \approx 10-32$; column 2 = 10 mL, $n \approx 20-65$) were analyzed using LC-MS (LCQ), then pooled to maximize particular glycosidic precursors prior to NMR spectroscopy. Three samples were obtained. Sample 1 was the pooled fractions 13−14 from two column 1 runs. The pooled fraction 22-32 from these two runs was then subjected to further chromatography through column 2 to give sample 2 (pooled fractions 46-47) and sample 3 (pooled fractions 55-57). The columns were washed with ethanol between runs and re-equilibrated with water prior to use. Solvent changes were achieved with a gradient to minimize any disruption to the resin.

LC−MS Analysis. Aqueous LH 20 fractions containing the glycosides were initially analyzed using a Waters 2690 HPLC with a Waters Novapak RP18 3.9 mm × 150 mm column and a Finnigan LCQ detector. Separation was achieved with MeOH (solvent A) and 0.1 M ammonium acetate (solvent B) using LC gradient 1: flow rate mL/min, 30% A/ 70% B to 90% A/10% B over 25 min.

Initial LC-MS analyses were conducted with the HPLC column coupled to a Finnigan LCQ ion trap MS. Typical MS conditions were APCI source; vaporizer, 470 °C; capillary, 175 °C; sheath gas flow, 60 psi; capillary voltage, 46 V, range m/z 150−750. Data dependent and targeted MS^2 and MS^3 experiments were also conducted for many samples. When single ions were targeted or selected in data dependent experiments, an isolation window of at least 3 m/z units was used. In some experiments, related ions 2 m/z units apart were targeted with an isolation window of 6 m/z units around the average of the two values.

Accurate mass analyses were conducted with a Finnigan Surveyor HPLC and a Thermo Orbitrap MS using LC gradient 1. Full scan data was collected in profile mode with 2 ppm mass accuracy. In addition, data dependent MS^2 product ion scans were acquired (resolution = 60,000) followed by 4 data dependent ion trap scans.

LC-MS Coupled with Solid Phase Extraction and Off-Line Nuclear Magnetic Resonance (LC-UV/MS-SPE-NMR) Analysis. The glycosidic samples 1−3 were analyzed by LC-UV/MS-SPE-NMR. For instrument details refer to Motti et al.[21] and Supporting Information. Separation was achieved with a RP18 Gemini 3 μm, 110 Å, 50 × 4.6 mm (Phenomenex) HPLC column using one of the following LC gradients:

LC gradient 2, flow rate 0.5 mL/min, 85% A/15% B to 40% A/60% B over 60 min;

LC gradient 3, flow rate 1 mL/min, 75% A/25% B to 50% A/50% B over 60 min; and

LC gradient 4, flow rate 0.5 mL/min, gradient 65% A/35% B to 40% A/60% B over 35 min.

Detection of compounds was achieved by APCI MS in negative mode. The intensity of the UV response at λ 254 nm was used to define the thresholds to trigger SPE trapping. The loaded SPE cartridges were dried with N_2 and the analytes eluted with CD_3CN directly into a 60 μL active volume 3 mm flow cell and one- and two-dimensional (1D and 2D) NMR spectra acquired referenced to 1.96 ppm (^{1}H) and 118.4 ppm (^{13}C). ^{1}H NMR spectra were recorded using a multiple presaturation 1D nuclear Overhauser effect spectroscopy (NOESY) pulse sequence. ^{1}H−^{1}H correlation spectroscopy (COSY) and heteronuclear single quantum correlation (HSQC) spectra were acquired in phase sensitive mode; heteronuclear multiple bond correlation (HMBC) spectra (optimized for JCH, 7.5 Hz) were acquired with gradient selection. Selective gradient 1D COSY and total correlation spectroscopy (TOCSY) spectra were also acquired.

RESULTS AND DISCUSSION

Preliminary LC-MS Screening for Glycosides. Initially, primary MeOH extracts of boronia were analyzed by full scan APCI MS with alternating data dependent MS^2 spectra on the most intense ion. It had been anticipated that the full scan data would show evidence of glycoside $[M + H]^+$ ions and that the MS^2 spectra of these ions would include the corresponding $[aglycone + H]^+$ ions. While $[M + H]^+$ ions were not selected by the data dependent MS^2 experiments, inspection of the mass spectra revealed the presence of several m/z 371 and m/z 373 ions (putative $[M + H]^+$ ions) for C13 norisoprenoid glycosides, together with the expected aglycone ions ($[aglycone + H]^+$) at m/z 209 and 211 arising from in-source fragmentation, consistent with C13 norisoprenoids. These ions potentially corresponded to a number of C13 norisoprenoids previously identified in boronia including 3-hydroxy-5,6-dihydro-β-ionone and 3-oxo-5,6-dihydro-β-ionol (all MW = 210); and 4-hydroxy-β-ionone, 3-hydroxy-β-ionone, and 4-oxo-β-ionol (all MW = 208).[2,3]

The absence of $[aglycone + H]^+$ ions in data dependent mode was explained through interference of automatically selected ions from the more intense rutin and related flavanone peaks which eluted nearby. Two strategies were employed to overcome the problem of interference by the flavonoids. These were (1) changes to the separation procedure in order to reduce the amount of rutin in the extract and (2) tandem MS experiments on selected protonated molecules in the glycosidic MeOH extract.

Tandem MS Experiments. The tandem MS screening experiments were assisted by the serendipitous observation that LC-MS of the glycosidic MeOH extract using a column previously eluted with MeOH and an aqueous ammonium acetate buffer resulted in the formation of strong ammonium adducts in APCI. Product ions at m/z 209 and 211 were obtained from MS^2 of the protonated molecules at m/z 457 and 459, and from MS^3 of the ammonium adducts at m/z 474 and 476. Product ions of m/z 209 were also observed to be derived from MS^3 on m/z 492 and from MS^2 on m/z 475. Examination of these mass

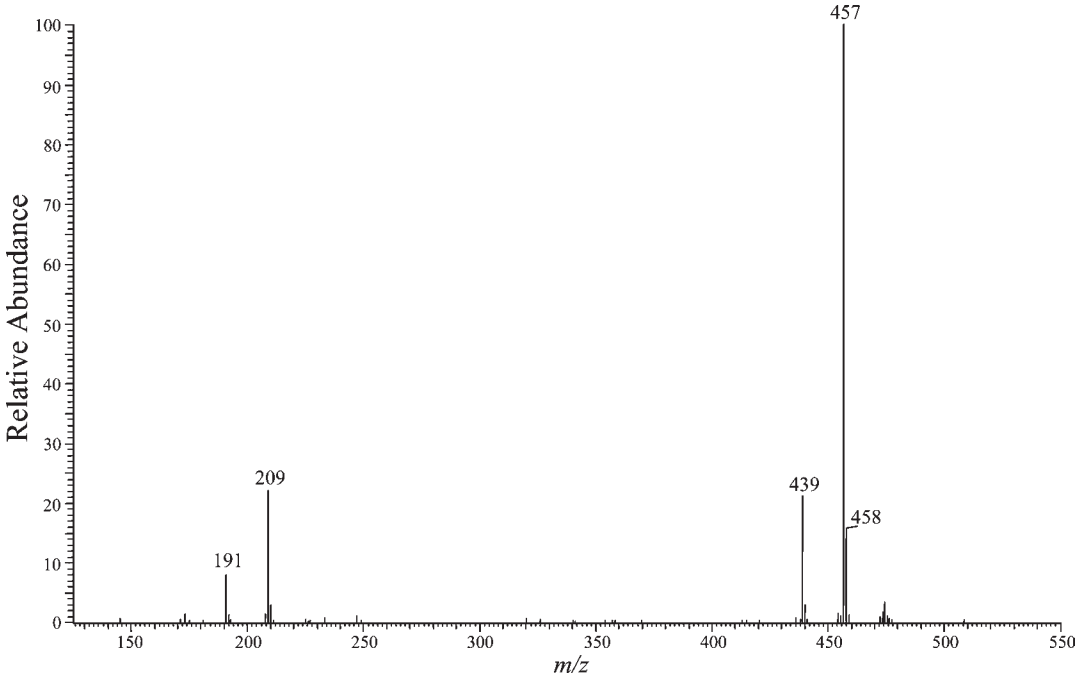

Figure 1. Typical MS^2 spectrum observed for a putative malonyl glycoside of a C13 norisoprenoid from the $[M + NH4]^+$ ion at m/z 474.

differences allowed speculation that malonyl glycosides of C13 norisoprenoids (MW 456 and 458) or methyl cucurbates (MW = 474) were contributing to these outcomes. The methyl cucurbates (MW = 226) were considered here through reasoning that the $[\text{aglycone} + H - H_2O]^+$ ion for the methyl cucurbates was equivalent in mass to the $[\text{aglycone} + H]^+$ ion of C13 norisoprenoids. The presence of the $[\text{aglycone} + H]^+$ ion at m/z 227 was also observed for the putative malonyl glycosides of methyl cucurbates. Figure 1 shows the MS^2 mass spectrum generated from the ammonium adduct of a putative malonyl glycoside of a C13 norisoprenoid.

Consequently LC-MS experiments using 0.1 M ammonium acetate as the polar mobile phase (LC gradient 1) with specific targeting of the parent ions were designed to screen explicitly for malonyl glycosides of C13 norisoprenoids and methyl cucurbates. A similar approach was then used to screen for glycosides of monoterpenes. Results clearly demonstrated that the putative aglycone product ions could be obtained through MS^2 experiments from ions with m/z values equivalent to $[M + H]^+$ or similarly by MS^3 experiments of the ammonium adduct ($[M + NH_4]^+$) for a range of compounds with masses equivalent to C13 norisoprenoids, monoterpenols, and methyl cucurbates. The formation of ammonium adducts was also observed by Withopf et al.[19] when screening for malonylated glycoconjugates in plants using ESI MS.

A more comprehensive analysis of the possible glycosides in the glycosidic MeOH extract was performed using a Thermo Orbitrap MS. In most cases, the molecular formulas of the parent molecules and aglycones were found to be consistent with the proposed glycosides. The range of putative glycosides in boronia was found to be extensive, and Table 1 lists the calculated and measured masses for each diagnostic ion, including spontaneously generated daughter ions. The generation of accurate mass data has been previously used as support for the identification of glycoside content in biological samples. This includes anthocyanins in raspberries,[22] a flavanoid glucoside in artichoke leaf,[23] and monoterpene glycosides in the roots of *Paeonia lactiflora*.[24]

NMR Identification. Three boronia samples, fractionated using XAD-2 and LH 20 chromatography, were investigated by LC-UV/MS-SPE-NMR and glycosides corresponding to the putative identifications made through nominal and accurate mass LC/MS were identified. A summary of the NMR assignments in CD_3CN is presented in Table 2, and the structures for each of the identified compounds are presented in Figure 2.

*3,7-Dimethylocta-1,5-diene-3,7-diol-3-O-β-D-glucopyranoside (**2**).* The 1D and 2D NMR data for the compound with molecular formula $C_{16}H_{28}O_7$ isolated from sample 1 (LC gradient 2, RT = 31−33 min) were in good agreement with those expected for **2** (Figure 2).[25] Three olefinic protons consistent with an isolated double bond were observed at δ_H 5.11 (1H, d, 17.5; H-1a), 5.16 (1H, d, 11.0; H-1b), and 5.99 (1H, dd, 17.5, 11.0; H-2). A second disubstituted double bond, δ_H 5.61 (1H, dt, 15.8, 7.2; H-5) and 5.44 (1H, d, 15.8; H-6), was assigned *E* geometry based on the large coupling constant measured. Three methyl groups were observed as two coincidental singlets at δ_H 1.18 (2 × 3H, s; H-8/9) and a methyl singlet at δ_H 1.25 (3H, s; H-10). COSY and TOCSY correlations confirmed the aglycone moiety to be 3,7-dimethylocta-1,5-diene-3,7-diol (**1**), also referred to in the literature as 7-hydroxy hotrienol.[3]

Selective COSY experiments established correlations from an anomeric proton at δ_H 4.35 (1H, d, 7.8; H-1′) through to the shielded methylene protons H-6a′/6b′ (δ_H 3.53 and 3.68) adjacent to a hydroxyl functionality. Vicinal $^1H-^1H$ coupling constants $J_{1',2'}$ = 7.8, $J_{2',3'}$ = 8.6, and $J_{3',4'}$ = 9.4 confirmed the glycone moiety was β-glucopyranose (**X**, Figure 2).[26] HMBC correlations observed from H10 into C1′ and from H1′ into C3 (weak) indicated the two moieties were joined via the C-1′-O-C-3 ether linkage and confirmed the presence of 3,7-dimethylocta-1,5-diene-3,7-diol-3-*O*-β-D-glucopyranoside (**2**), previously isolated from *Portulaca oleracea*,[25] in boronia.

Table 1. Summary of LC-MS Spectral Data for 30 Proposed Boronia Glycosides and Malonyl Glycosides[a]

assigned identity	type of glycoside	molecular formulas	retention time (min)	$[M + NH_4]^+$	$[M + NH_4\text{-}CO_2]^+$	$[M + H]^+$	$[M + H-H_2O]^+$	$[Aglycone + H]^+$	$[Aglycone + H-H_2O]^+$
C13 norisoprenoids	glucoside	$C_{19}H_{30}O_7$ (MW = 370)		**388.2335**		**371.2070**	**353.1964**	**209.1542**	**191.1436**
(aglycone MW = 208)			5.15	388.2339		371.2073	353.1967	209.1539	191.1433
			6.54	388.2336		371.2071		209.1538	191.1432
			10.48	388.2332		371.2064	353.1965	209.1536	191.1430
	malonyl glucoside	$C_{22}H_{32}O_{10}$ (MW = 456)		**474.2339**	**430.2441**	**457.2074**	**439.1968**	**209.1542**	**191.1436**
			5.12	474.2340	430.2445	457.2076		209.1539	191.1433
			6.62	474.2337	430.2439	457.2066		209.1537	191.1431
			8.18	474.2336	430.2442			209.1539	191.1432
C13 norisoprenoids	glucoside	$C_{19}H_{32}O_7$ (MW = 372)		**390.2491**		**373.2226**	**355.2120**	**211.1698**	**193.1592**
(aglycone MW = 210)			5.52	390.2491		373.2217	355.2114		193.1587
			6.94	390.2492		373.2226	355.2119	211.1694	193.1588
			8.88	390.2491		373.2229	355.2113	211.1693	193.1588
	malonyl glucoside	$C_{22}H_{34}O_{10}$ (MW = 458)		**476.2495**	**432.2597**	**459.2230**	**441.2124**	**211.1698**	**193.1592**
			4.49	476.2494	432.2597	459.2230		211.1694	193.1588
			5.52	476.2493	432.2595		441.2105	211.1693	193.1587
			7.31	476.2496	432.2598	459.2229	441.2123	211.1695	193.1589
monoterpenols	glucoside	$C_{16}H_{28}O_6$ (MW = 316)		**334.2229**		**317.1964**	**299.1858**	**155.1436**	**137.1330**
(aglycone MW = 154)			7.67	334.2228		317.1962	299.1855	155.1428	137.1323
			11.16	334.2229		317.1965	299.1856		137.1325
			13.87	334.2225		317.1956	299.1850		137.1323
	malonyl glucoside	$C_{19}H_{30}O_9$ (MW = 402)		**420.2233**	**376.2335**	**403.1968**	**385.1862**	**155.1436**	**137.1330**
			11.20	420.2233	376.2333				137.1324
monoterpenediols	glucoside	$C_{16}H_{28}O_7$ (MW = 332)		**350.2178**		**333.1913**	**315.1807**	**171.1385**	**153.1279**
(aglycone MW = 170)			4.49	350.2171		333.1914	315.1805	171.1376	153.1277
			5.72	350.2178		333.1915	315.1808	171.1379	153.1275
			6.31	350.2176		333.1908	315.1800		153.1272
			6.66	350.2178		333.1912	315.1805		153.1273
	malonyl glucoside	$C_{19}H_{30}O_{10}$ (MW = 418)		**436.2182**	**392.2284**	**419.1917**	**401.1811**	**171.1385**	**153.1279**
			2.82	436.2180	392.2280		401.1816	171.1374	153.1272
			3.95	436.2180	392.2280			171.1377	153.1274
			4.49	436.2182	392.2285			171.1376	153.1273
			4.62	436.2178	392.2281			171.1380	153.1273
methyl cucurbates	glucoside	$C_{19}H_{32}O_8$ (MW = 388)		**406.2440**		**389.2175**	**371.2069**	**227.1647**	**209.1541**
(aglycone MW = 226)			13.03	406.2433		389.2167	371.2057	227.1639	209.1535
			13.27	406.2434		389.2169	371.2063	227.1640	209.1535
			14.01	406.2443		389.2177	371.2059	227.1645	209.1538
	malonyl glucoside	$C_{22}H_{34}O_{11}$ (MW = 474)		**492.2444**	**448.2546**	**475.2179**	**457.2073**	**227.1647**	**209.1541**
			10.48	492.2437	448.2542	475.2173		227.1642	209.1536
			10.99	492.2443	448.2548			227.1642	209.1538
			11.81	492.2441	448.2545	475.2169	457.2071	227.1642	209.1537

[a] The numbers in bold are the calculated values, and the other numbers refer to values obtained from peaks at distinct HPLC elution times. Measurements were within 2 ppm of these in most instances, and all were within 5 ppm.

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Table 2. ^{1}H and ^{13}C NMR Data (600 and 150 MHz) for Boronia Glycosides 2, 3, 5, and 7 in CD_3CN

compound **2**					compound **3**				compound **5**						compound **7**				
								selective					selective						
no.	δ_C[a]	δ_H (J, m)[a,b]	COSY	HMBC[c]		δ_H (J, m) [a,b]	COSY	TOCSY		δ_C[a]	δ_H (J, m) [a,b]	COSY	TOCSY	HMBC[c]	No.	δ_C[a]	δ_H (J, m) [a,b]	COSY	HMBC[c]
1a	115.3	5.11 (17.5, d)	2	C2, C3	**1a**	5.13 (18.0, d)	2	2	**1**	34.5					**1″a**	37.1	2.32 (m)	1″b	C2″, C2
1b		5.16 (11.0, d)	2		**1b**	5.17 (10.8, d)	2	2							**1″b**		2.41 (m)	1″a	C2″, C2
2	144.7	5.99 (17.5, 11.0, dd)	1a, 1b		**2**	5.95 (18.0, 10.8, dd)	1a, 1b	1a, 1b	**2a**	46.0	1.43 (14.8, 2.8, dd)	2b, 4	2b, 3, 4, 5, 13	C1, C11, C12	**2″**	175.1			
									2b		1.77 (14.8, 2.8, dd)	2a, 4	2a, 3, 4, 5, 13	C1, C3, C4, C6					
3	80.2				**3**				**3**	74.2	4.03 (2.5, brt)	2a, 2b, 4	2a, 2b, 4, 5, 13	C1	**3″**	52.2	3.58 (s)		C2″
4	44.2	2.35 (m)	5		**4a**	2.22 (m)		5/6	**4**	38.5	1.09 (12.9, 3.2, dd)	3, 5		C1, C2, C5, C6	**1**	38.8	2.40 (m)	1″a, 1″b	C4
					4b	2.30 (m)		5/6							**2**	48.1	1.93 (7.4, 1.9, dd)	3	C1″
5	126.8	5.61 (15.8, 7.2, dt)	4, 6		**5**	5.60 (15.2, br t)	4a, 4b, 6	4a, 4b	**5**	26.8	1.98 (2.4, brt)	4, 6, 13			**3**	80.2	4.21 (br)	4, 2	C5, C1′
6	139.9	5.44 (15.8, d)	5		**6**	5.60 (15.2, br t)	5	4a, 4b	**6**	58.8	1.64 (11.0, 10.3, dd)	7		C1, C7, C8, C11, C12	**4**	30.7	1.77 (m)	5a, 3	
7	75.5				**7**				**7**	150.5	6.66 (16.2, 10.3, dd)	6, 8	8, 6, 5, 13	C5, C6, C9, C11	**5a**	29.9	1.49 (m)	4	
8	26.4	1.18 (s)		C6, C7, C9	**8**	1.19 (s)			**8**	134.1	6.01 (16.2, d)	7	7, 6, 13	C5, C6, C9	**5b**		1.32 (m)	4	
9	26.4	1.18 (s)		C6, C7, C8	**9**	1.19 (s)			**9**	199.4					**1‴a**	23.9	2.12 (8.1)	2‴	C3, C2, C2‴, C3‴
10	24.1	1.25 (s)		C2, C3, C4, C1′	**10**	1.21 (s)			**10**	26.8	2.20 (m)			C7, C8, C9	**1‴b**		2.16 (7.4)	2‴	C1″, C3, C2, C2‴, C3‴
									11	23.6	1.05 (s)			C1, C2, C6, C11	**2‴**	129.8	5.41 (10.5, 8.1, dd)	1‴, 3‴	C4‴
									12	32.2	0.82 (s)			C1, C2, C6, C11	**3‴**	132.7	5.34 (10.5, 7.5, dd)	2‴, 4‴	C1‴
									13	21.4	0.79 (6.4, d)	5	2a, 2b, 3, 4, 5, 6, 7, 8	C4, C5, C6	**4‴**	21.6	2.06 (7.5, q)	3‴, 5‴	C2‴, C3‴
															5‴	14.9	0.94 (7.5, t)	4‴	C3‴, C4‴
1′	99.0	4.35 (7.8, d)	2′	C3	**1′**	4.30 (7.8, d)	2′	2′, 3′, 6a′, 6b′	**1′**	101.9	4.27 (8.2, d)	2	2, 3, 4/5, 6a, 6b	C3	**1′**	101.1	4.22 (8.0, br d)	2′	C3
2′	75.1	3.03 (8.6, brt)	1′, 3′	C3′	**2′**	3.05 (10.0, 7.8, dd)	1′, 3′		**2′**	74.6	3.05 (9.5, 8.2, dd)	1, 3	4/5, 3, 1	C1′, C3′	**2′**	74.8	3.03 (10.1, 8.0, dd)	1′, 3′	
3′	78.1	3.26 (m)	2′		**3′**	3.26 (m)	2′		**3′**	77.5	3.27 (m)	2, 4	2, 4/5, 6a, 6b	C5′	**3′**	78.0	3.26 (10.7, 7.2, dd)	2′, 4′	C5′

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Table 2. Continued

	compound 2				compound 3				compound 5				compound 7			
no.	δ_C[a]	δ_H (J, m)[a,b]	COSY	HMBC[c]		δ_H (J, m)[a,b]	COSY	selective TOCSY		δ_C[a]	δ_H (J, m)[a,b]	COSY	selective TOCSY	HMBC[c]	No.	δ_C[a]	δ_H (J, m)[a,b]	COSY	HMBC[c]
4′	72.1	3.19 (9.4, brt)	3′, 4′	C3′	4′	3.19 (m)			4′	76.9	3.21 (m)	3, 5	6a, 6b, 3, 2, 1	C1′	4′	77.3	3.19 (m)	3′	
5′	77.1	3.14 (m)	4′, 6b′		5′	3.32 (m)	6b′		5′	71.5	3.23 (m)	4, 6a	6a, 6b, 3, 2, 1		5′	72.0	3.19 (m)	6a′	
6a′	63.2	3.53 (12.0, 4.9, dd)	5′, 6b′		6a′	4.16 (11.7, d)	6b′		6a′	63.0	3.59 (11.9, 6.2, dd)	5, 6b	6b, 4′/5, 3, 2, 1	C4′	6a′	63.2	3.55 (10.7, 7.7 dd)	6b′	C4′
6b′		3.68 (12.0, 2.3, dd)	5′, 6a′		6b′	4.27 (11.7, 4.8, dd)	5′, 6a′	3′, 5′, 6b′	6b′		3.74 (11.9, 6.2, dd)	5, 6a	6a, 4′/5, 3, 2, 1		6b′		3.71 (10.7, 7.7 dd)	6a′	C4′
					7′														
					8′	3.60 (s)													
					9′														

[a] ppm. [b] J = coupling constant in Hz, m = multiplicity. [c] HMBC correlations from H to C.

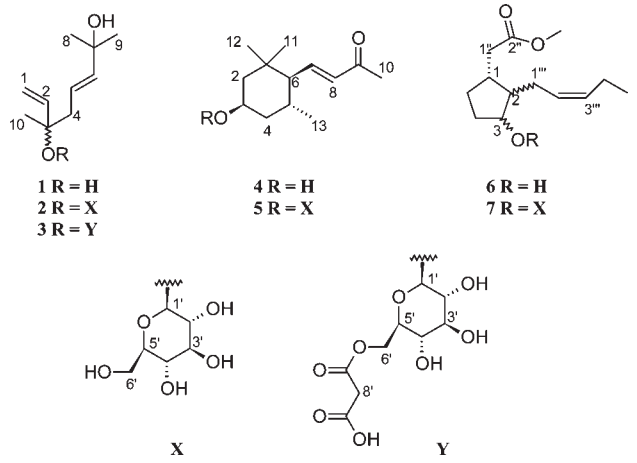

Figure 2. Structures of aglycones and the glycosides found in *Boronia megastigma* (Nees) confirmed by NMR spectroscopy. Twenty-six other related partially characterized glycosides are also listed in Table 1. 1 = 3,7-dimethylocta-1,5-diene-3,7-diol; 2 = 3,7-dimethylocta-1,5-diene-3,7-diol-3-*O*-β-D-glucopyranoside; 3 = 3,7-dimethylocta-1,5-diene-3,7-diol-3-*O*-(6′-*O*-malonyl)-β-D-glucopyranoside; 4 = (1*R*,4*R*,5*R*)-3,3,5-trimethyl-4-[(1*E*)-3-oxobut-1-en-1-yl]cyclohexanol (3-hydroxy-5,6-dihydro-β-ionone); 5 = (1*R*,4*R*,5*R*)-3,3,5-trimethyl-4-[(1*E*)-3-oxobut-1-en-1-yl]cyclohexyl-β-D-glucopyranoside (3-hydroxy-5,6-dihydro-β-ionone-β-D-glucopyranoside); 6 = methyl {(1*R*)-3-hydroxy-2-[(2*Z*)-pent-2-en-1-yl]cyclopentyl}acetate (stereoisomers present in boronia include (2*S*,3*S*) (methyl cucurbate), (2*S*,3*R*) (methyl 3-epicucurbate), and (2*R*,3*R*) (methyl 2,3-diepicucurbate); 7 = a methyl {(1*R*)-3-(β-D-glucopyranosyloxy)-2-[(2*Z*)-pent-2-en-1-yl]cyclopentyl}acetate stereoisomer (a methyl cucurbate β-D-glucopyranoside stereoisomer); X = β-D-glucoside; and Y = (6′-*O*-malonyl)-β-D-glucoside.

*3,7-Dimethylocta-1,5-diene-3,7-diol-3-O-(6′-O-malonyl)-β-D-glucopyranoside (**3**).* Spectral data for the second compound isolated from sample 1 at RT = 20 min were similar to those obtained for **2**, with one isolated double bond (δ_H 5.13 [1H, d, 10.8; H-1a], 5.17 [1H, d, 18.0; H-1b], and 5.95 [1H, dd, 18.0, 10.8; H-2]) and one *E*-disubstituted double bond (δ_H 5.60, 2 × 1H, br t, 15.2; H-5/6). A methyl singlet at δ_H 1.21 (3H, s; H-10) and two coincidental singlets at δ_H 1.19 (2 × 3H, s; H-8/9) were also observed. This established 3,7-dimethylocta-1,5-diene-3,7-diol as the aglycone moiety (Figure 2).

The ^{1}H, COSY and TOCSY spectra confirmed correlations for a spin system from an anomeric proton at δ_H 4.30 (1H, d, 7.8; H-1′) with a β-glycosidic linkage, to methylene protons at δ_H 4.16 (1H, d, 11.7; H-6a′) and 4.27 (1H, dd, 11.7, 4.8; H-6b′), similar to the glycone moiety in **2**. The presence of an additional signal at δ_H 3.60, the deshielded methylene protons at δ_H 4.16 and 4.27, similar to that found for a malonyl glycoside by Withopf et al.,[19] and the molecular formula $C_{19}H_{30}O_{10}$, provided evidence of a malonyl side chain on the glycone moiety (**Y**, Figure 2). The low amount of the glycoside isolated was not sufficient to fully elucidate its stereochemistry. The data suggested the presence of 3,7-dimethylocta-1,5-diene-3,7-diol-3-*O*-(6′-*O*-malonyl)-β-D-glycopyranoside (**3**) in boronia. This compound has not been previously reported in the literature as a natural product.

*(1R,4R,5R)-3,3,5-Trimethyl-4-[(1E)-3-oxobut-1-en-1-yl]cyclohexyl β-D-glucopyranoside (3-hydroxy-5,6-dihydro-β-ionone-β-D-glucopyranoside) (**5**).* Sample 2, separated using LC gradient 3, yielded a C13 norisoprenoid glycoside with formula $C_{19}H_{32}O_7$

at RT = 21.8 min. Two olefinic protons observed at δ_H 6.01 (1H, d, 16.2; H-8) and 6.66 (1H, dd, 10.3, 16.2; H-7) were consistent with an *E*-disubstituted double bond. COSY correlations were observed from H-7 to H-8 and to a methine proton at δ_H 1.64 (1H, dd, 10.3, 11.0; H-6). Three methyl groups were observed as two methyl singlets (δ_H 1.05, 3H, s; H-11 and 0.82, 3H, s; H12), and a methyl doublet (δ_H 0.79, 3H, d, 6.4; H-13) with a COSY correlation to a methine at δ_H 1.98 (1H, m; H-5). Selective TOCSY experiments established the spin system based on correlations from H-8 through to H-2a/2b and to H-13 (Table 2) and established the presence of the aglycone moiety 3-hydroxy-5,6-dihydro-β-ionone (**4**, Figure 2).

Selective TOCSY experiments identified a spin system from an anomeric proton at δ_H 4.27 (1H, d, 8.2; H-1′) through to methylene protons at δ_H 3.59 (1H, dd, 11.9, 6.2; H-6a′) and δ_H 3.74 (1H, dd, 11.9, 6.2; H-6b′) indicative of a glycone with a β-glycosidic linkage. The aglycone methine carbon at δ_C 74.2 (C-3) showed a HMBC correlation to the glycone proton H-1′, revealing the two subunits were linked via the ether linkage C-1′-O-C-3. These data were in good agreement with 3-hydroxy-5,6-dihydro-β-ionone- β-D-glucopyranoside (**5**), for which the 1R,4S,5R stereoisomer (Alangionoside L) has previously been isolated from *Alangium premnifolium*[27]. The main aglycone stereoisomer in boronia is 1R,4R,5R (2).

*Methyl {(1R)-3-(β-D-glucopyranosyloxy)-2-[(2Z)-pent-2-en-1-yl]cyclopentyl} Acetate Stereoisomer (a Methyl Cucurbate β-D-Glucopyranoside Stereoisomer) (**7**).* A compound (RT = 26−28 min) corresponding to formula $C_{19}H_{32}O_8$ was isolated from sample 3 using LC gradient 4. The aglycone moiety was identified as a methyl cucurbate (**6**, Figure 2). Two olefinic protons were observed with a smaller coupling constant of J = 10.5 Hz (δ_H 5.34, 1H, dd, 10.5, 7.5; H-3‴, and 5.41, 1H, dd, 10.5, 8.1; H-2‴) characteristic of a *Z*-disubstituted double bond. Two methyl groups were identified as a methoxy at δ_H 3.58 (3H, s; H-3″) and a triplet at δ_H 0.94 (3H, t, 7.5; H-5‴). The COSY data established two spin systems based on correlations from the methyl triplet H-5‴ through to H-1‴; and from H-2 through to H-5 (Table 2), which were shown to be joined by HMBC correlations from the methylene protons at H-1‴ to C-2 and C-3. HMBC correlations were also observed from the carbonyl C-2″ to the methoxy singlet H-3″, and to the methylene protons C-1″a/1″b. The methine carbon at δ_C 80.2 (C-3) showed a HMBC correlation to H-1‴, the C-1‴ correlated to H-2, and C-2 correlated to H-1″ linking the two side chains to the ring as shown in Figure 2.

The glycone (**X**, Figure 2) was established on the basis of a spin system from an anomeric proton d_H 4.22 (1H, br d, 8.0; H-1′) through to δ_H 3.55 (1H, dd, 10.7, 7.7; H-6a′), and 3.71 (1H, dd, 10.7, 7.7; H-6b′). The large coupling constant of the anomeric proton, J = 8.0 Hz, was indicative of a β-glycosidic linkage, while the $^1H-^1H$ coupling constants $J_{1',2'}$ = 8.0, $J_{2',3'}$ = 10.1, and $J_{3',4'}$ = 7.2 provided evidence that the glycone moiety was β-glucopyranose. Furthermore, HMBC correlations between C-3 of the aglycone and H-1′ of the glycone confirmed the two subunits were linked via the ether linkage C-1′-O-C-3 and confirmed the presence of a methyl cucurbate-β-D-glucopyranoside (**7**)[2] in boronia.

Overall, the evidence obtained from LC-MS analyses of the glycosidic MeOH extract of boronia marc supported the presence of both glycosides and malonyl glycosides of three methyl cucurbates, several C13 norisoprenoids including megastigmanes, and several monoterpene alcohols. Subsequent fractionation of the glycosidic MeOH extract using XAD-2 and LH 20 chromatography followed by accurate mass LC-MS and LC-UV/MS-SPE-NMR analysis allowed for the formal identification of three known glycoconjugates, 3,7-dimethylocta-1,5-diene-3,7-diol-3-*O*-β-D-glucopyranoside (**2**), (1*R*,4*R*,5*R*)-3,3,5-trimethyl-4-[(1*E*)-3-oxobut-1-en-1-yl]cyclohexyl β-D-glucopyranoside (3-hydroxy-5,6-dihydro-β-ionone-β-D-glucopyranoside) (**5**), and a methyl {(1*R*)-3-(β-D-glucopyranosyloxy)-2-[(2*Z*)-pent-2-en-1-yl]cyclopentyl}acetate stereoisomer (methyl cucurbate β-D-glucopyranoside stereoisomer) (**7**), as well as the previously unreported 3,7-dimethylocta-1,5-diene-3,7-diol-3-*O*-(6′-*O*-malonyl)-β-D-glycopyranoside (**3**) .

■ ASSOCIATED CONTENT

Supporting Information. Principle of operation of the HPLC-UV/MS-SPE-NMR instrument. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

MeOH, methanol; XAD-2, chromatography resin; LH-20, chromatography resin; LC, liquid chromatography; RP, reverse-phase; RT, retention time; UV, ultraviolet; MW, molecular weight; MS, mass spectrometry; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; m/z, mass to charge ratio; NMR, nuclear magnetic resonance; SPE, solid phase extraction; CD_3CN, deuterated acetonitrile; δ_H, proton chemical shift; δ_C, carbon chemical shift; J, coupling constant; m, multiplicity; COSY, $^1H-^1H$ Correlation spectroscopy experiment; HSQC, heteronuclear single quantum correlation experiment; HMBC, heteronuclear multiple bond correlation experiment; TOCSY, total correlation spectroscopy experiment; NOESY, multiple presaturation 1D nuclear Overhauser effect spectroscopy.

■ REFERENCES

(1) Davies, N. W.; Menary, R. C. Volatile consituents of *Boronia megastigma* flowers. *Perfum. Flavor.* **1983**, *8*, 3–8.

(2) Weyerstahl, P.; Marschall, H.; Bork, W.-R.; Rilk, R. Megastigmanes and other constituents of the absolute of *Boronia megastigma* from Tasmania. *Liebigs Ann. Chem.* **1994**, 1043–1047.

(3) Weyerstahl, P.; Marschall, H.; Bork, W.-R.; Rilk, R.; Schneider, S.; Wahlburg, H.-C., Constituents of the absolute of *Boronia megastigma* Nees. from Tasmania. *Flavour Fragrance J.* **1995**.

(4) MacTavish, H. Factors Affecting Yield and Compostion of Floral Extract from *Boronia megastigma* Nees. Ph.D. Thesis, University of Tasmania, Hobart, 1995.

(5) MacTavish, H.; Menary, R. C. Optimizing solvent extraction of *Boronia megastigma* (Nees) flowers. *J. Essent. Oil Res.* **1998**, *10*, 31–37.

(6) MacTavish, H.; Menary, R. C. The effect of flower maturity and harvest timing on floral extract from *Boronia megastigma* (Nees). *Ann. Bot.* **1997**, *80*, 299–303.

(7) MacTavish, H.; Menary, R. C. Production of volatiles in brown boronia flowers after harvest. I: Effect of clonal type and incubation temperature. *J. Hortic. Sci. Biotechnol.* **1999a**, *74*, 436–439.

(8) MacTavish, H.; Menary, R. C. Production of volatiles in brown boronia flowers after harvest.II: Effect of oxygen consumption. *J. Hortic. Sci. Biotechnol.* **1999b**, *74*, 440–442.

(9) MacTavish, H.; Menary, R. C. Production of volatiles in brown boronia flowers after harvest: Pilot-scale research. *J. Hortic. Sci. Biotechnol.* **2000**, *75*, 455–458.

(10) Cooper, C. M.; Davies, N. W.; Menary, R. C. C-27 Apocarotenoids in the flowers of *Boronia megastigma* (Nees). *J. Agric. Food Chem.* **2003**, *51*, 2384–2389.

(11) Cooper, C. M.; Davies, N. W.; Menary, R. C. Changes in some carotenoids and apocarotenoids during flower development in *Boronia megastigma* (Nees). *J. Agric. Food Chem.* **2009**, *57*, 1513–1520.

(12) Boss, B.; Richling, E.; Schreier, P., HPLC-ESI-MS/MS Analysis of 6′-O-Malonylated β-D-glucosides in Plants. In *Natural Products Analysis. Chromatography, Spectroscopy, Biological Testing*; Schreier, P., Herderich, M., Humpf, H.-U., Schwab, W., Eds.; Wieweg: Wiesbaden, Germany, 1998; pp 187−191.

(13) Roscher, R.; Herderich, M.; Steppen, J.-P.; Schreier, P.; Schwab, W. 2,5-Dimethyl-4-hydroxy-3(2H)-furanone 6′O-malonyl-β-D-glucopyranoside in strawberry fruits. *Phytochemistry* **1996**, *43*, 155–159.

(14) Sarry, J.-E.; Gunata, Z. Plant and microbial glycoside hydrolases: Volatile release from glycosidic aroma precursors. *Food Chem.* **2004**, *87*, 509–521.

(15) Schliemann, W.; Ammer, C.; Strack, D. Metabolite profiling of mycorrhizal roots of *Medicago truncatula*. *Phytochemistry* **2008**, *69*, 112–146.

(16) Stahl-Biskup, E.; Intert, F.; Holthuijzen, J.; Stengele, M.; Schulz, G. Glycosidically bound volatiles - A review 1986 - 1991. *Flavour Fragrance J.* **1993**, *8*, 61–80.

(17) Winterhalter, P.; Schreier, P. C-13 Norisoprenoid glycosides in plant tissues: an overview on their occurence, composition and role as flavour precursors. *Flavour Fragrance J.* **1994**, *9*, 281–287.

(18) Winterhalter, P.; Skouroumounis, G. K. Glycoconjugated Aroma Compounds: Occurrence, Role and Biotechnological Transformation. In *Biotechnology of Aroma Compounds*; Berger, R. G., Ed.; Springer-Verlag: Berlin, Germany, 1997; Vol. 55, pp 73−106.

(19) Withopf, B.; Richling, E.; Roscher, R.; Schwab, W.; Schreier, P. Sensitive and selective screening for 6′-O-malonylated glucoconjugates in plants. *J. Agric. Food Chem.* **1997**, *45*, 907–911.

(20) Gunata, Y. Z.; Bayonove, C. L.; Baumes, R. L.; Cordonnier, R. E. The aroma of grapes. 1. Extraction and determination of free and glycosidically bound fractions of some grape aroma compounds. *J. Chromatogr.* **1985**, *331*, 83–90.

(21) Motti, C. A.; Freckelton, M. L.; Tapiolas, D. M.; Willis, R. H. FTICR-MS and LC-UV/MS-SPE-NMR applications for the rapid dereplication of a crude extract from the sponge *Ianthella flabelliformis*. *J. Nat. Prod.* **2009**, *72*, 290–294.

(22) Beekwilder, J.; Jonker, H.; Meesters, P.; Hall, R. D.; Van der Meer, I. M.; de Vos, C. H. R. Antioxidants in raspberry: On-line analysis links antioxidant activity to a diversity of individual metabolites. *J. Agric. Food Chem.* **2005**, *53*, 3313–3320.

(23) Moglia, A.; Lanteri, S.; Comino, C.; Acquadro, A.; de Vos, R.; Beekwilder, J. , Stress-induced biosynthesis of dicaffeoylquinic acids in globe artichoke. *J. Agric. Food Chem.* **2008**, *56*, 8641–8649.

(24) Li, S.-L.; Song, J.-Z.; Choi, F. F. K.; Qiao, C.-F.; Zhou, Y. Chemical profiling of Radix Paeoniae evaulated by ultra-performance liquid chromatography/photo-diode-array/quadropole time-of-flight mass spectrometry. *J. Pharm. Biomed. Anal.* **2009**, *49*, 253–266.

(25) Seo, Y.; Shin, J.; Cha, H. J.; Kim, Y.-A.; Ahn, J.-W.; Lee, B.-J.; Lee, D. S. A new monoterpene glucoside from *Portulaca oleracea*. *Bull. Korean Chem. Soc.* **2003**, *24*, 1475–1477.

(26) Yamamori, A.; Fukushi, E.; Onodera, E.; Kawabata, S.; Shiomi, N. NMR analysis of mono- and difructosyllactosucrose synthesised synthesized by 1′-fructosyltransferase purified from roots of asparagus (*Asparagus officinalis* L.). *Magn. Reson. Chem.* **2002**, *40*, 541–544.

(27) Otsuka, H.; Yao, M.; Kamada, K.; Takeda, Y. Alangionosides G-M: glycosides of megastigmane derivatives from the leaves of *Alangium premnifolium*. *Chem. Pharm. Bull.* **1995**, *43*, 754–759.