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C-27 Apocarotenoids in the Flowers of *Boronia megastigma* (Nees)

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Five C-27 apocarotenoids were detected in acetone extracts of the flowers of *Boronia megastigma* (Nees) using HPLC with UV−vis photodiode array and MS detection. Comparison of methylated and unmethylated extracts aided identification when considered with the UV−vis and MS data. The five apocarotenoids identified here were hydroxy-apo-10′-carotenoic acid (**1**), methyl hydroxy-apo-10′-carotenoate (**2**), apo-10′-carotenoic acid (**3**), apo-10′-carotenal (**4**), and methyl apo-10′-carotenoate (**5**). The data obtained was not sufficient to allow the specific isomeric forms to be unequivocally identified. The results further support speculation that the C-13 norisoprenoids found in boronia are derived from C-40 carotenoids. Possible parent molecules of β-ionone, an important component of boronia extract, were identified. An understanding of C-13 norisoprenoid biosynthesis may assist in the selection and postharvest processing of boronia flowers for flavor and fragrance applications.

KEYWORDS: ***Boronia megastigma*** **(Nees); brown boronia; essential oils; carotenoids; apocarotenoids; β-carotene; β-ionone; C-13 norisoprenoids; biosynthesis; 3-hydroxy-β-apo-10′-carotenoic acid; methyl 3-hydroxy-β-apo-10′-carotenoate; β-apo-10′-carotenoic acid; β-apo-10′-carotenal; methyl β-apo-10′-carotenoate**

INTRODUCTION

Boronia megastigma (Nees) is grown commercially in Tasmania to produce a flower extract. The extract, which is also made into an absolute, has a complex range of components including C-13 norisoprenoids. These are regarded as important compounds in many natural and synthetic flavor and fragrance products (*1*, *2*). Extracts from boronia flowers contain more than 20 C-13 norisoprenoids out of 129 components positively identified (*3*−*5*). β-Ionone, first identified in boronia before 1927 (*6*), is a high yielding norisoprenoid ingredient of that extract. Other C-13 norisoprenoids identified in boronia include 3-hydroxy-β-ionone, 4-oxo-β-ionone, various β-ionols, and 7,8-dihydro-β-ionone which has a "fruity, ionone-orris-like smell" (*4*). While substantial progress in improving extract quality has been made in recent years (*7*−*9*), improved understanding of the biochemistry of norisoprenoid production may provide an opportunity to further enhance the quality of boronia extracts and provide opportunities for market differentiation.

It is widely assumed that various volatiles, including C-13 noriosoprenoids, are derived from C-40 carotenoids. This is based on structural comparisons, and various authors have published data demonstrating the combined presence in flowers of molecules which together structurally match the C-40 carotenoid parent molecules. In quince, Lutz and Winterhalter (*10*) argued that both C-13 and C-15 end group volatiles, and corresponding C-10 and C-12 fragments from the central part of the polyene chain, were evidence of biosynthesis from C-40 carotenoids. In starfruit (*11*) the finding of C-15 and C-10 molecules, matching carotenoid end groups and central chain, respectively, was again seen as evidence of volatile derivation from carotenoids. Similarly in saffron, crocetin (C-20) and a variety of C-10 molecules, including the important flavor compound safranal, have been postulated to be derived from the C-40 molecule zeaxanthin (*12*, *13*).

Extracts of rose flowers are known to contain, as does boronia, a wide range of C-13 norisoprenoids. Eugster and Marki-Fischer (*14*), on the basis of the identification of C-27 apocarotenoids and rosafluene (C-14), postulated a two-step biosynthetic process in roses. The first step proposed was cleavage in the 9,10 position to form one C-13 norisoprenoid molecule and a C-27 apo-carotenoid. This latter molecule is further cleaved to form the C-14 rosafluene and a second C-13 norisoprenoid molecule. While many of the C-13 norisoprenoids in boronia correspond to the end-groups of known carotenoids, no matching carotenoid cleavage products have been previously identified. In particular, extensive identification of volatiles in boronia extracts indicates the absence of rosafluene (or other C-14 volatiles) as is found in rose extracts. However, biosynthesis of β-ionone and other C-13 norisoprenoids through cleavage of C-40 carotenoids has long been considered possible. Previous investigation (*15*) yielded evidence in boronia flowers of lutein, neoxanthin, and β-carotene, a possible precursor of

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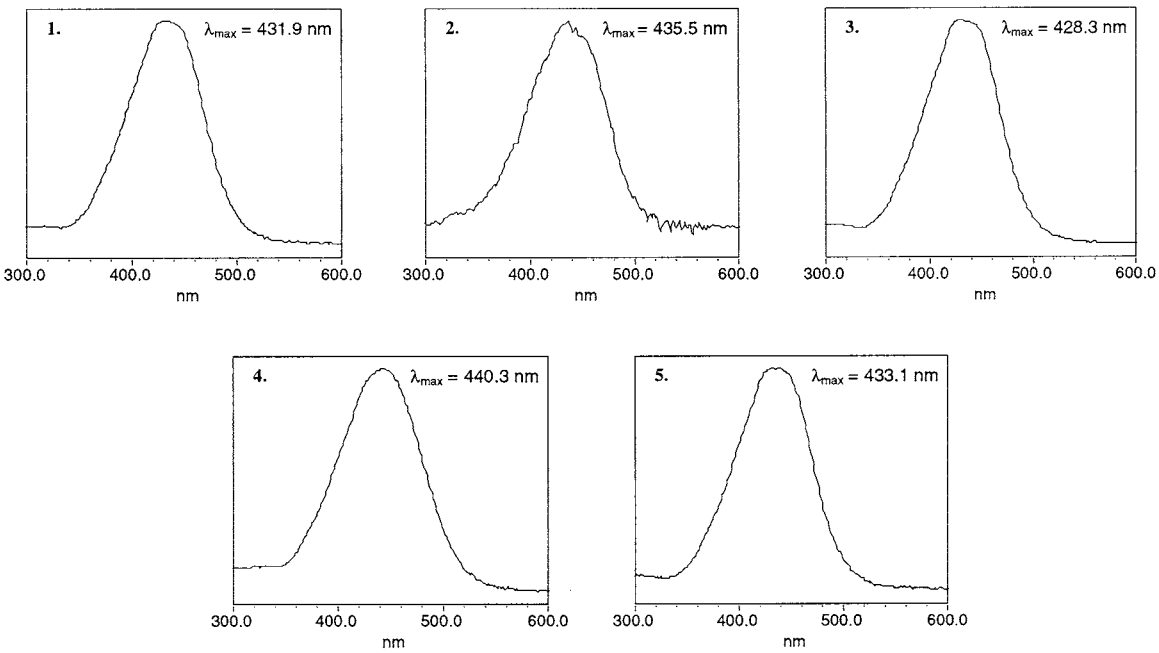

Figure 1. UV–vis spectra for five components of boronia flowers obtained using HPLC program C.

β-ionone, along with a number of unidentified carotenoid peaks in chromatograms. The availability of HPLC linked to photodiode array and atmospheric pressure chemical ionization−mass spectrometry (APCI-MS) detection has allowed more detailed investigation into the carotenoid profile of boronia flowers.

MATERIALS AND METHODS

Materials. *Boronia megastigma* (Nees) clone 3 flowers developed by the University of Tasmania and grown in southern Tasmania were used. All chemicals and solvents were analytical grade.

Carotenoid Extraction from Boronia Flowers. Typically 10−20 g of flower material was extracted using a method adapted from that of Jaren-Galen et al. (*16*). Accordingly, 20 g of boronia flowers or buds, plus 5.0 g of calcium carbonate for acid neutralization (*17*), was homogenized in 100 mL of acetone (4 °C) for 60 s using an Ultra-Turrex (T25 basic, Ika labortechnik, setting 6), fitted with an 18 mm head. The mixture was centrifuged for 5 min at 15000*g* (Beckman J2−21 M/E, rotor 20.0). The pellet was extracted twice more in 50 mL of acetone, and the supernatants were combined in a separating funnel with 100 mL of diethyl ether. The mixture was shaken, and 200 mL of 10% NaCl was added to promote separation of the ether layer. The aqueous layer was discarded and the ether layer washed several times with 100 mL aliquots of 10% NaCl. The ether fraction containing the pigments was dried over sodium sulfate and filtered, and the solvent was removed by rotary evaporation. The pigments were stored at −70 °C prior to analysis. All operations were at 4 °C under reduced or pale yellow light (*18*).

HPLC. A Waters Alliance 2690 HPLC and 996 Photodiode array detector were used for chromatography and identification of carotenoids and apocarotenoids. A Waters Nova-Pak 150 × 3.9 mm i.d. C18 column fitted with an Alltech Econosphere C18 guard cartridge was used to achieve separation. Three different programs were used for analytical purposes.

Program A: Initial conditions were 87% acetonitrile/10% methanol/3% water for 3 min programmed to 85% methanol/15% hexane at 10 min which was then isocratic for 5 min. The flow rate was 1 mL/min. Following the completion of each run, the column was returned to starting conditions over 1 min and equilibrated for 8 min prior to the next run.

Program B: Initial conditions were 50% acetonitrile/50% water for 2 min followed by a linear gradient to 85% acetonitrile/15% methanol at 15 min. A further linear gradient to 85% methanol/15% hexane at 25 min was then held for 10 min. The flow rate was 1 mL/min. The column was reequilibrated to start conditions for 10 min between samples.

Program C: Starting conditions were 50% acetonitrile/50% water for 2 min. This was followed by a linear gradient to 85% acetonitrile/15% methanol at 22 min and a further program to 85% methanol/15% hexane at 32 min which was held for 8 min. The flow rate was 1 mL/min. Reequilibration was to 100% methanol for 3 min and 50% acetonitrile/50% water for 9 min.

Data Processing. Waters Millenium software was used to analyze data and chromatograms were extracted at 430 and 451 nm. Data were recorded from 250 to 700 nm every 1 s at 1.2 nm resolution. Lutein was identified on the basis of published UV−vis data and retention times.

HPLC/MS. Mass spectral data was obtained with a Finnigan LCQ equipped with an atmospheric pressure chemical ionization (APCI) ion source. Settings were sheath gas 60 psi, auxiliary gas 15 psi, vaporizer temperature 450 °C, discharge current 6 μA, capillary temperature 170 °C, capillary voltage 20 V, default MS/MS collision energy 25%. Scanning usually occurred over the *m*/*z* range 100 to 1200. Signals for the apocarotenoids reported here were maximized by shortening the *m*/*z* range to 100−680.

Synthesis of Diazomethane. Diazomethane was prepared in a 250 mL conical flask by adding *N*-methyl-*N*-nitrourea (3.0 g, 22.5 mmol) in small portions to a mixture of aqueous potassium hydroxide (40% w/v, 40 mL) and diethyl ether (50 mL) at 5 °C with constant stirring (magnetic stirrer) for 20−30 min. The ether layer was separated and the aqueous layer further extracted with 25 mL of ether. The combined ether fractions were allowed to stand over some solid potassium hydroxide (30 min minimum) and then transferred to a screw top bottle for storage at −15 °C.

Methylation of Carotenoid Extracts. Aliquots of carotenoid extract (10 mg) were treated with diazomethane (1 or 2 mL of the ether fraction above) for 30 min after which the solvent was evaporated under a stream of nitrogen. The resultant methylated extract was redissolved in 2 mL of acetone and analyzed by HPLC. This process was conducted under low or nil light conditions.

RESULTS AND DISCUSSION

Five C-27 apocarotenoids were identified for the first time in the flowers of *Boronia megastigma* (Nees), using HPLC with diode array detection and HPLC-MS. This identification was

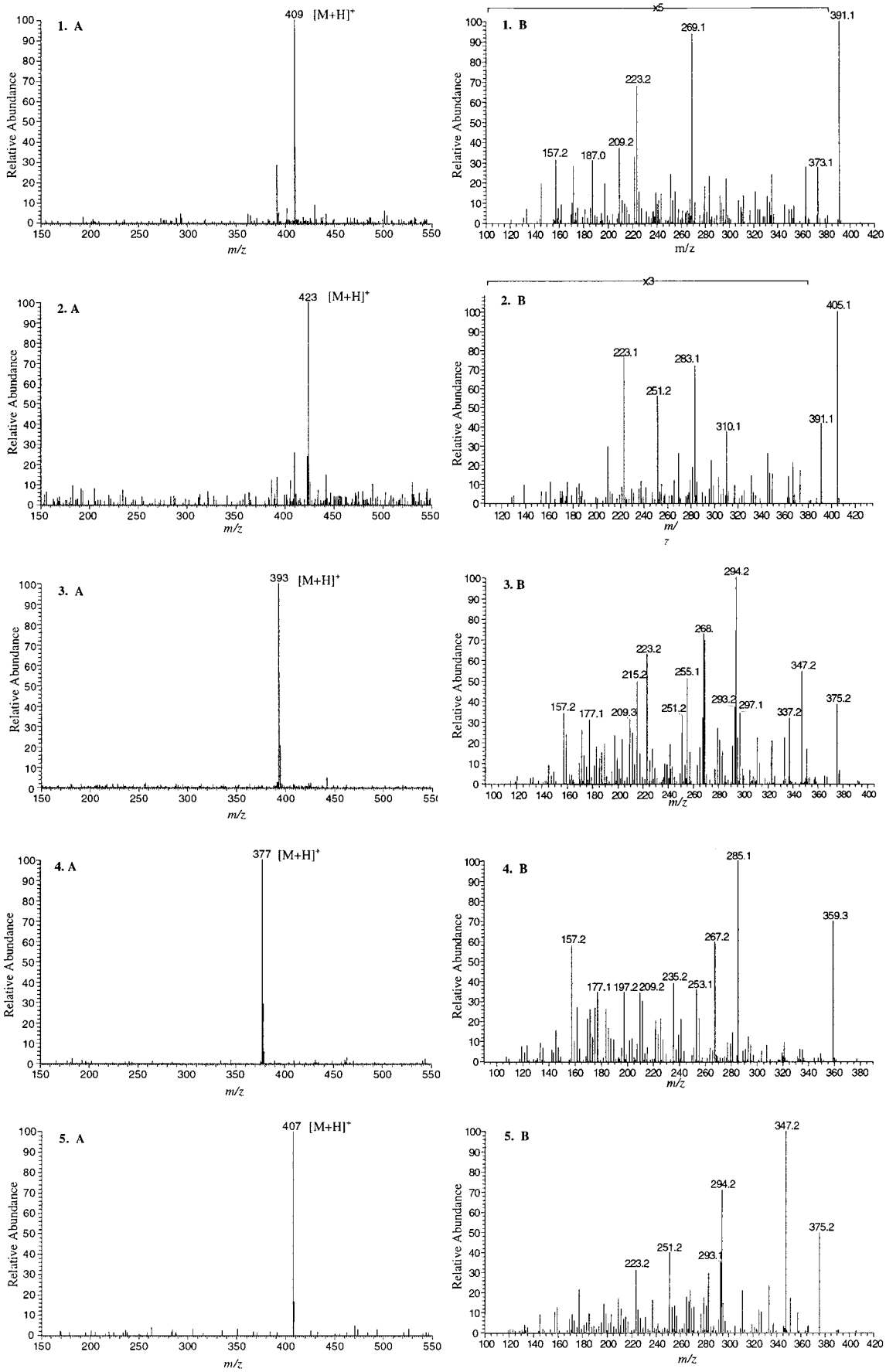

Figure 2. APCI mass spectra (A) and MS/MS spectra (B) for components 1 through 5.

on the basis of published UV−vis spectroscopic data, HPLC-MS data, and changes following methylation.

Initial chromatography of acetone extracts of boronia flowers yielded five peaks with UV−Vis spectra that had single

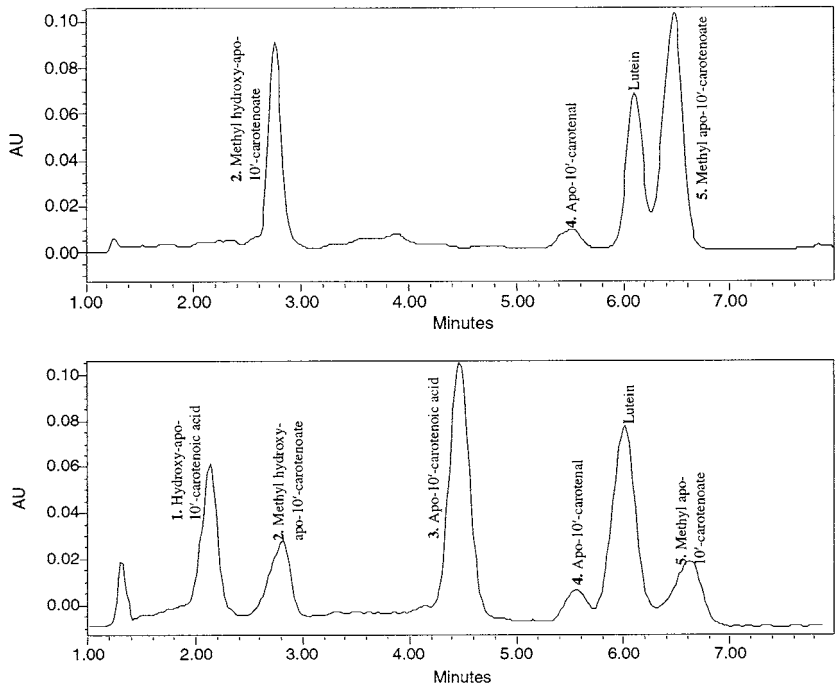

Figure 3. Change in the HPLC (Program A) apocarotenoid profile following methylation with diazomethane at 430 nm. The bottom chromatogram is the unmethylated sample.

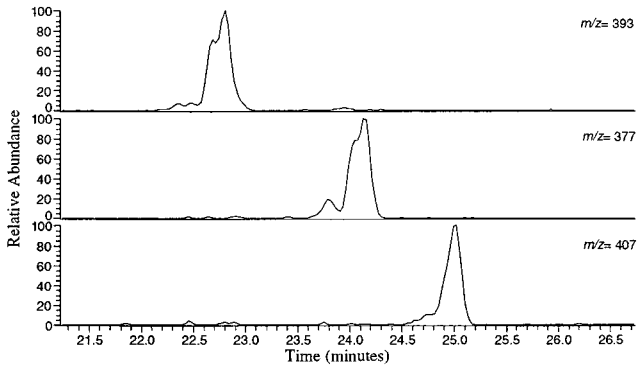

Figure 4. Mass chromatograms, obtained using analytical program C, of apo-10′-carotenoic acid (*m/z* 393), apo-10′-carotenal (*m/z* 377), and methyl apo-10′-carotenoate (*m/z* 407), showing the presence of multiple isomers.

absorption maxima between 428 and 440 nm (**Figure 1**). These UV−vis maxima were consistent with those previously reported for C-27 apocarotenoids. Data reported by Singh et al. (*19*) includes β-apo-10′-carotenoic acid (λ_{max} = 425 nm in light petroleum) and β-apo-10′-carotenal (λ_{max} = 437 nm in light petroleum). Yokoyama and White (*20*) reported 3-hydroxy-β-apo-10′-carotenal as having a similar spectrum to β-apo-10′-carotenal, both consistent with the data published in the aforementioned study. Given the relatively high level of C-13 norisoprenoids and the accepted biosynthetic link between C-40 carotenoids and norisoprenoids in other plants the presence of C-27 apocarotenoids in boronia might be expected.

APCI mass spectrometric data were then obtained from each of the five HPLC peaks (assigned as peaks **1** through to **5**) and used to propose identities for these apocarotenoids. **Figure 2** gives the MS data (**A**) and MS/MS data (**B**), respectively. The molecular weights obtained from the APCI mass spectra of peaks **3** and **4** corresponded to known C-27 apocarotenoids (*21*). The APCI mass spectrum for peak **3** was consistent with both β-apo-10′-carotenoic acid and a hydroxy-β-apo-10′-carotenal ($C_{27}H_{36}O_2/[M + H]^+ = 393$). The spectrum for peak **4** was consistent with β-apo-10′-carotenal ($C_{27}H_{36}O/[M + H]^+ = 377$). For further clarification, flower extract was methylated with diazomethane and analyzed in comparison to an unmethylated extract using HPLC program A (**Figure 3**). Peaks **1** and **3** were removed by methylation indicating the presence of a carboxylic acid functional group. The resulting methyl esters appeared at the same retention time and with the same APCI MS data as components **2** and **5**, augmenting the existing peak areas relative to that of lutein. Peak **4** was not affected by methylation with diazomethane, consistent when the UV−vis and molecular weight data was considered, with an apo-10′-carotenal. The methylation experiment demonstrated peak **3** to be a carboxylic acid which, when considered in conjunction with UV−vis and molecular weight data was consistent with an apo-10′-carotenoic acid. Peak **5**, based on its later retention time and its exact match in HPLC, UV, and MS data to the product of methylation of peak **3**, was proposed as methyl apo-10′-carotenoate. Similarly, peaks **1** and **2** were consistent with a hydroxy-apo-10′-carotenoic acid and a methyl hydroxy-apo-10′-carotenoate, respectively.

The MS/MS data detailed in **Figure 2** further supported the proposed identities. The five peaks gave the relatively complex product ion profiles typical of carotenoids (*22*). These included characteristic ions such as the loss of toluene (e.g., the base peak for compound **4**). In addition, specific functional group losses from the apocarotenoids support the structures proposed. Hydroxy-apo-10′-carotenoic acid (**1. B**, **Figure 2**, $[M + H]^+$ at *m/z* 409) shows successive loss of water from the hydroxyl and carboxyl groups respectively (peaks at *m/z* 391 and *m/z* 373). The peak at *m/z* 363 ($[M + H - 46]^+$) is consistent with the loss of formic acid, which is typical of carboxylic acids. Methyl hydroxy-apo-10′-carotenoate (**2. B**, **Figure 2**, $[M + H]^+$ at *m/z* 423) shows losses of 18, 32, 50, and 60 Da which are consistent with losses of water, methanol, methanol plus water, and methyl formate, respectively. The MS/MS spectrum for apo-10′-carotenoic acid (**3. B**, **Figure 2**, $[M + H]^+$ at *m/z* 393) shows peaks for losses of water at *m/z* 375 and formic acid at *m/z* 347 from the hydroxyl and carboxyl groups, respectively. The apo-10′-carotenal MS/MS spectrum (**4. B**, **Figure 2**, $[M + H]^+$ at *m/z* 377) shows loss of only one water at *m/z* 359 as would be

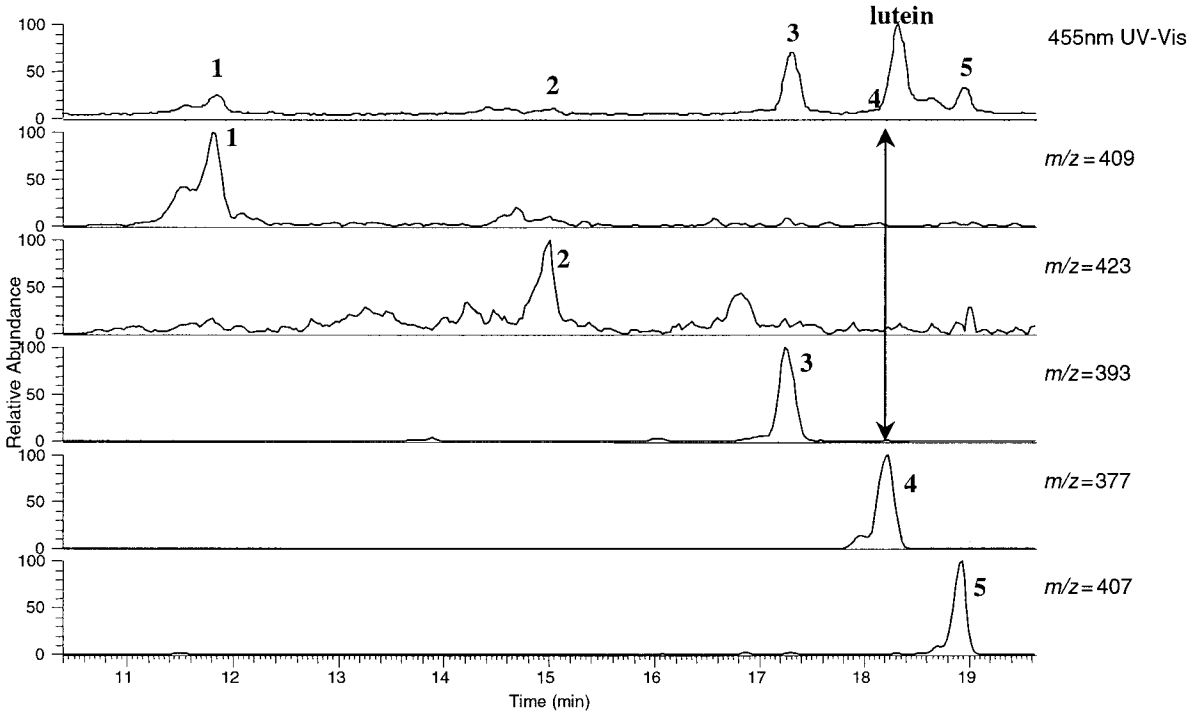

Figure 5. Individual mass chromatograms for each of the apocarotenoids showing alignment with their corresponding HPLC chromatogram positions. Component **4** (apo-10′-carotenal) is an unresolved shoulder slightly to the left of lutein. Separation was achieved using the HPLC program B.

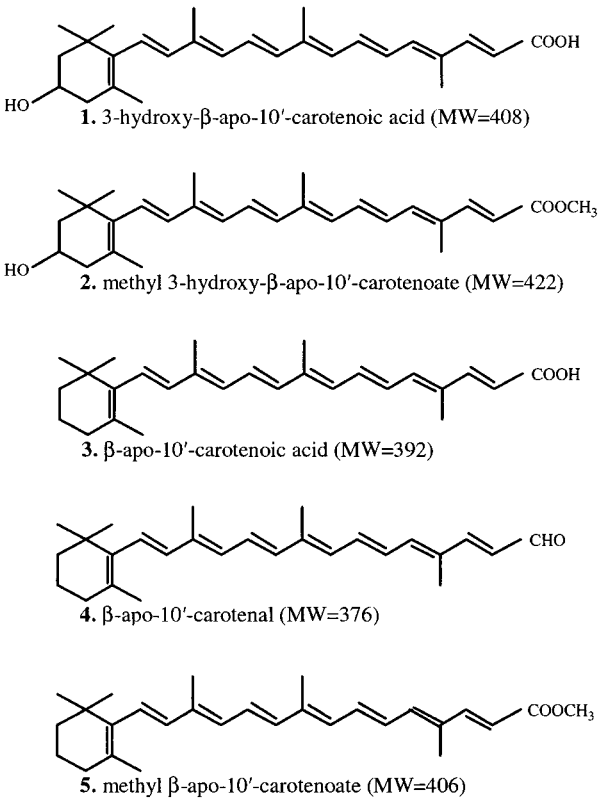

Figure 6. Possible structural formulas of the five apocarotenoids identified in boronia flowers.

expected from an aldehyde. Peaks for losses of methanol at *m/z* 375 and methyl formate at *m/z* 347 are apparent in the MS/MS spectrum for methyl apo-10′-carotenoate (**5. B**, **Figure 2**, $[M + H]^+$ at *m/z* 407).

It should be noted that while the published data refers to the β isomers of these apocarotenoids, and there is no published data referring to an α isomer, the data obtained in this study cannot readily distinguish between α- and β-isomers. However, the presence of an ion at $[M + H - 56]^+$ in compounds **3**, **4**, and **5** could be interpreted as characteristic of retro Diels−Alder fragmentation from an ϵ carotenoid ring (*22*). Similarly, our data does not allow the cis/trans geometry or the position of the hydroxyl moiety to be established with any certainty. Furthermore, close scrutiny of the mass chromatograms for apo-10′-carotenoic acid, apo-10′-carotenal, and methyl apo-10′-carotenoate (**Figure 4**) clearly shows evidence of two or more unresolved isomers of each compound. However, the protonated molecules, $[M + H]^+$, obtained in each mass spectrum and the MS/MS spectra for the five apocarotenoids, are in agreement with the proposed identities. In addition, the extracted mass chromatograms align with the corresponding HPLC positions (**Figure 5**). Structures of possible 'all trans', β, and 3-hydroxy isomers of the apocarotenoids are presented in **Figure 6**.

Both β-apo-10′-carotenoic acid (**3**, **Figure 6**) and β-apo-10′-carotenal (**4, Figure 6**) have been reported in the literature. The former has been isolated only from animal sources (*23*, *24*) while the latter has been previously identified in other plant species. These reports include identification of β-apo-10′-carotenal in three varieties of *Citrus senensis* (*25*), lichens from Kenya (*26*), and the Brazilian fruit *Mammea americana* (*27*). The related compound 3-hydroxy-β-apo-10′-carotenal, not identified in this study, has been found in the flavedo of sinton citrangequat (*20*). In this study one or more isomers of the four C-27 apocarotenoids, hydroxy-apo-10′-carotenoic acid, methyl hydroxy-apo-10′-carotenoate, apo-10′-carotenoic acid, and methyl apo-10′-carotenoic acid, are reported here as being derived from plant sources for the first time.

The C-27 apocarotenoids detected here and the C-13 norisoprenoids previously characterized (*5*) are matched in both chain length and structure to known longer chain carotenoids. The structural matching of these compounds is evidence that C-13 norisoprenoids are derived from C-40 carotenoids in boronia. Speculation as to a possible C-40 carotenoid parent molecule for β-ionone is important because of its relevance to the aroma

profile of boronia extracts. On the basis of the structures of the five C-27 compounds, five known carotenoids as published in Straub (*21*) are possible parent compounds. They are β-carotene (β,β-carotene), α-carotene (β,ϵ-carotene), isocryptoxanthin (β,β-caroten-4-ol), β-cryptoxanthin (β,β-caroten-3-ol), and β,β-caroten-2-ol. Additionally, selection of clones with different carotenoid profiles may lead to the production of extracts with varying aroma characteristics, which may in turn increase market differentiation for Tasmanian boronia. These results should serve to provide the basis for future studies on the possible mechanism of C-13 norisoprenoid biosynthesis.

Safety. Caution: Diazomethane is a highly toxic, yellow carcinogenic gas that must be handled in a fume cupboard. The gas is explosive but may be used safely as a solution in diethyl ether. Nevertheless, it should be treated with great caution. Due to its potential carcinogenic nature and high potency as a skin irritant, all necessary safety protocols for handling dangerous compounds should be strictly followed.

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