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Three minor carotenoids from annatto (*Bixa orellana*) seeds

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Abstract

Three minor carotenoids were isolated from the seed coat of *Bixa orellana* fruits by chromatographic (CC, TLC, HPLC) methods. The structures were established as 6-geranylgeranyl 8′-methyl-6,8′-diapocaroten-6,8′-dioate, 6-geranylgeranyl 6′-methyl (9′*Z*)-6,6′-diapocaroten-6,6′-dioate and 6-geranylgeranyl 6′-methyl-6,6′-diapocaroten-6,6′-dioate by means of UV–visible, ^{1}H- and ^{13}C-NMR spectroscopy and mass spectrometry. This is the first time that geranylgeraniol has been found esterified with a carotenoid carboxylic acid. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Bixa orellana; Bixaceae; Annatto; Carotenoids; 6-Geranylgeranyl 8′-methyl-6,8′-diapocaroten-6,8′-dioate; 6-Geranylgeranyl 6′-methyl (9′*Z*)-6,6′-diapocaroten-6,6′-dioate; 6-Geranylgeranyl 6′-methyl-6,6′-diapocaroten-6,6′-dioate

1. Introduction

In our previous studies (Mercadante, Steck, Rodriguez-Amaya, Pfander, & Britton, 1996; Mercadante, Steck, & Pfander, 1997a; Mercadante, Steck, & Pfander, 1997b) on minor apocarotenoids from annatto (*Bixa orellana* L.) seeds, the occurrence of four apocarotenoids (C_{30} and C_{32}), five diapocarotenoids (C_{19}, C_{22}, C_{24}, C_{25}) and one carotenoid derivative (C_{14}) were reported for the first time. Additionally, two apocarotenoids that have not previously been detected in annatto were also isolated (Mercadante et al., 1997a). In continuation of this work, the isolation and structure elucidation of another three new minor diapocarotenoids (ca. 1% of the carotenoid content) are described.

2. Results and discussion

Three new diapocarotenoids 6-geranylgeranyl 8′-methyl-6,8′-diapocaroten-6,8′-dioate (**1**), 6-geranylgeranyl 6′-methyl-(9′*Z*)-6,6′-diapocaroten-6,6′-dioate (**2**) and 6-geranylgeranyl 6′-methyl-6,6′-diapocaroten-6,6′-dioate (**3**) were isolated from the ethyl acetate–*t*-BuOMe extract of *B. orellana* by CC and HPLC (Mercadante et al., 1997a).

2.1. UV–visible and mass spectra

Carotenoid **1** with 8 conjugated carbon–carbon double bonds (c.d.b.) in the polyene chain conjugated with two carboxylic ester groups showed λ_{max} 441 nm with spectral fine structure (%III/II 91) and a low intensity of the *cis*-peak (%A_B/A_{II} 4). After iodine-catalysed isomerization, a hypsochromic shift of 3 nm in the λ_{max} with a decreased spectral fine structure (%III/II 77) and a higher intensity of the *cis*-peak (%A_B/A_{II} 10) was observed, indicating an $(E) \rightarrow (Z)$-isomerization. The molecular ion of **1** appeared at *m/z* 640 which is compatible with $C_{43}H_{60}O_4$. The fragment ion at *m/z* 367 $[M\text{-}273]^+$, with higher intensity than the molecular ion, indicated the loss of geranylgeranyl; characteristic fragments at *m/z* 335 $[M\text{-}273\text{-}32]^+$ and 307 $[M\text{-}273\text{-}60]^+$ due to losses from both end groups were detected. Elimination of xylene from the polyene chain combined with loss of a geranylgera-

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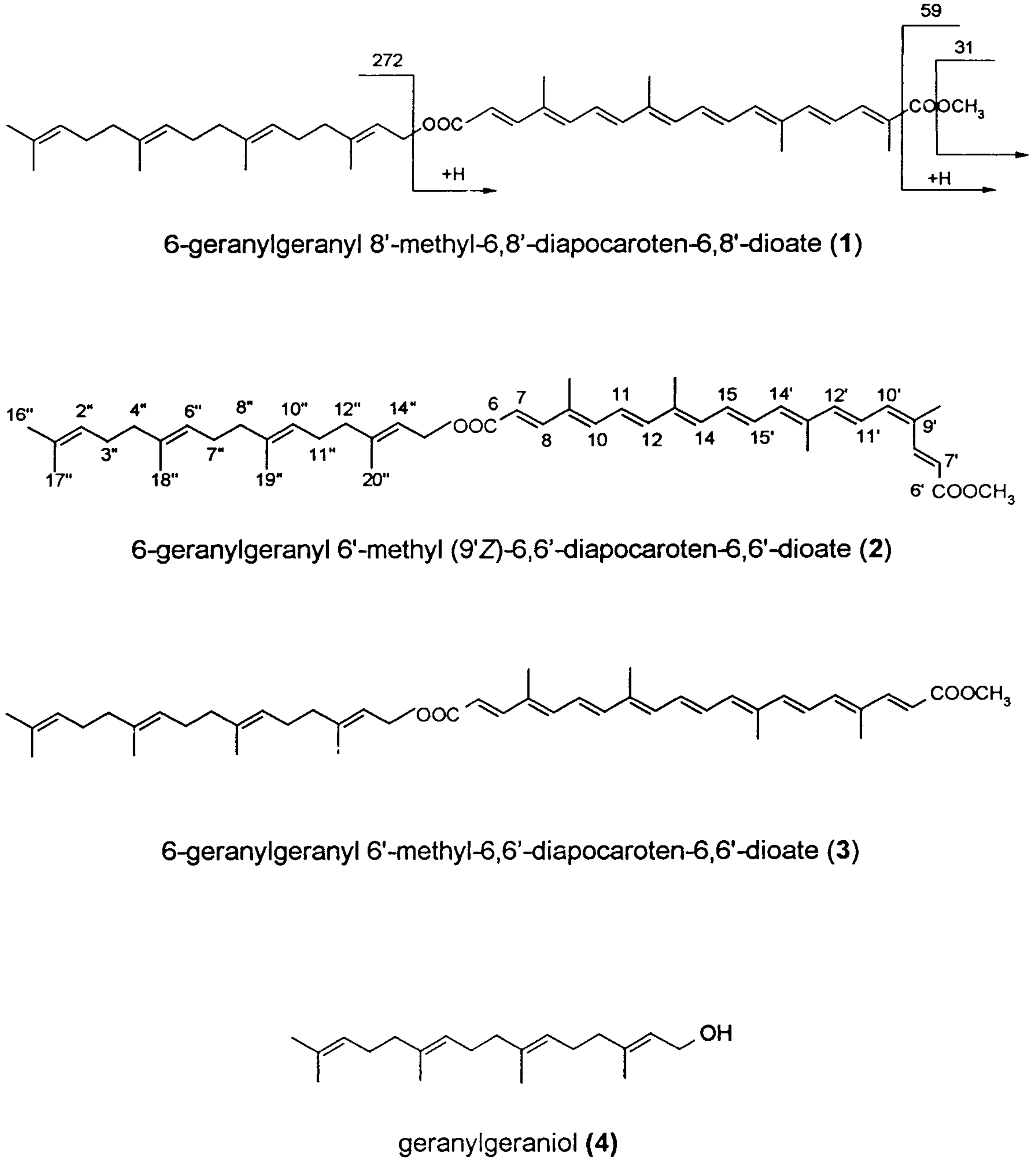

6-geranylgeranyl 8'-methyl-6,8'-diapocaroten-6,8'-dioate (**1**)

6-geranylgeranyl 6'-methyl (9'*Z*)-6,6'-diapocaroten-6,6'-dioate (**2**)

6-geranylgeranyl 6'-methyl-6,6'-diapocaroten-6,6'-dioate (**3**)

geranylgeraniol (**4**)

Scheme 1

nyl moiety (*m*/*z* 262 $[M\text{-}106\text{-}272]^+$) was observed (Scheme 1).

With 9 c.d.b. in the polyene chain conjugated with two carboxylic ester groups, carotenoids **2** and **3** displayed λ_{max} at 452 and 456 nm, respectively, with the same spectral fine structure (%III/II 76). The (9′*Z*)-isomer (**2**) exhibited, as expected, higher intensity of the *cis*-peak. The molecular ion of **2**, with intensity < 1%, was observed at *m*/*z* 666; however, the mass spectrum of **3** did not show the molecular ion at *m*/*z* 666. Both mass spectra showed the same features observed in the spectrum of **1** (Scheme 1), loss of a geranylgeranyl moiety (peak at *m*/*z* 393 with higher intensity than the molecular ion), fragment ions at *m*/*z* 361 and *m*/*z* 333 compatible with the methyl ester end group and at *m*/*z* 288 due to elimination of xylene plus a geranylgeranyl moiety.

In order to get even more evidence of the presence of geranylgeraniol esterified with **1**, **2** and **3**, free geranylgeraniol (**4**) was obtained by saponification of **3**. As expected for alcohols of relatively high mass, **4** did not show the molecular ion in the mass spectrum. The characteristic fragment at *m*/*z* 272 due to loss of water and ions from the cleavage of the polyene chain at *m*/*z* 257, 203, 136 and 69 were observed.

2.2. NMR spectra

The structures of **1**, **2** and **3** were assigned based on their ^{1}H, COSY, inverse-HMQC and ^{13}C NMR spectra. Although strong signal overlapping even in the 2D spectra, mostly affecting the resonances of the geranylgeranyl moieties, meant that some signal assignments must be regarded as interchangeable, the constitutions of the carotenoids, however, were established without doubt.

In **2**, the connection of the geranylgeranyl moiety to C-6 and of the ester methyl group to C-8′ was proven unequivocally by an inverse gs-HMBC experiment, adapted to $^3J_{\mathrm{HC}}$ long range couplings. Three intensive crosspeaks between the (bold-printed) nuclei $\mathbf{H_3}C(8') \leftrightarrow \mathbf{C}(8')$, $\mathbf{H_2}C(14') \leftrightarrow C(6)$ and $\mathbf{H}C(8) \leftrightarrow C(6)$ showed coupling interactions which confirmed the structure of **2**. Furthermore, these long range interactions served to assign the carbon-13 resonances of both carboxyl centres C-6 and C-8′.

The information mentioned above was used to identify the fragment connections and carboxyl resonances of **1** and **3** by data comparison due to the fact that the ^{13}C chemical shifts of C-6 were identical in all cases, whereas those of C-8′ showed significant deviations. The (9′*Z*) configuration of **2** was assured by the characteristic ^{1}H and ^{13}C isomerization shift values $\Delta\delta = \delta_{9'Z} - \delta_{\text{all-}E}$ obtained previously (Mercadante et al., 1996, 1997a, 1997b) which corresponded to averaged values given by Englert (1995).

The diapocarotenoids **1**–**3** have not been described before and this is the first report of geranylgeraniol esterified with a carotenoid carboxylic acid. Geranylgeranyl diphosphate is well known as an intermediate in the biosynthesis of isoprenoid compounds, including carotenoids, and geranylgeraniol, in the free form, has already been found in annatto (Jondiko, & Pattenden, 1989).

3. Experimental

3.1. Spectroscopy

UV–vis spectra: *tert*-butyl methyl ether (*t*-BuMeO). Spectral fine structure is expressed as %III/II and relative intensity of the *cis*-peak as $\%A_B/A_{II}$ (Ke, Imsgard, Kjosen, & Liaaen-Jensen, 1970; Britton, 1995). I_2-catalysed isomerization reactions were performed in *t*-BuMeO (Davies, 1976). MS: AE MS 9 instrument with a VG console and a Finnigan MAT SS300 data system. The samples were introduced with a direct inlet system at 70 eV, 190–210°C. The ^{1}H, COSY, inverse-HMQC (heteronuclear multiple-quantum correlation) and ^{13}C NMR experiments were recorded on a Bruker DRX-400 spectrometer (^{1}H: 400.13 MHz, ^{13}C: 100.25 MHz) with a broadband inverse probe head. The inverse gradient-selective HMBC (heteronuclear multiple-bond correlation) experiment was performed on a Bruker DRX-500 instrument (^{1}H: 500.13 MHz, ^{13}C: 125.77 MHz) equipped with a triple-resonance broadband inverse probe head with *z*-gradients. All spectra were recorded at 23°C in $CDCl_3$ (99.95%) purified over Al_2O_3 before use. Chemical shifts were related to the residual solvent signals and only relevant coupling constant values are given.

3.2. HPLC and GC/MS

HPLC separations were carried out on a rev. phase Nucleosil-5RP-18 column (250 × 10 mm) at a flow rate of 3 or 3.5 ml/min. The GC/MS was operated with injector at 250°C, detector ion source (EI-70 eV) at 230°C, split 2:5. A capillary DB-5 column (30 m, 0.25 μm film thickness) was used with the temp. programmed 40°C for 1 min to 280°C at 6°C min^{-1}.

3.3. Plant material and isolation

Carotenoid extraction and two subsequent flash CC steps were performed according to Mercadante et al. (1997). The subject of this communication is the fifth band (fraction V) eluted from the silica gel column with EtOAc–hexane (3:2). Fraction V was applied to a column packed with MgO–celite (1:2), the fraction eluted with Me_2CO–hexane (9:1) was discarded and the fractions eluted with Me_2CO–H_2O (9:1) (fr. 1) and H_2O (fr. 2) were collected.

In order to partially remove the oil, fr. 1 was submitted to flash CC on silica gel. The mixture of pigments eluted with 50 to 70% EtOAc–hexane (0.77 g) was subjected to MgO–kieselguhr TLC with Me_2CO–hexane (1:1) as mobile phase. This gave carotenoid **1** (R_f 0.1), the final purification of which was achieved by HPLC (MeOH–*iso*-PrOH, 85:15) (R_t 16.2 min), yielding ca. 1 mg.

Fr. 2 was submitted to TLC on silica gel with EtOAc–hexane (1:4) as mobile phase. The major band (R_f 0.58) was scraped off and subjected to TLC on MgO–kieselguhr, developed with Me_2CO–hexane (7:3) (R_f 0.2). When analysed by HPLC, this band was found to be composed of at least two pigments and a further separation was carried out by alumina TLC (*t*-BuMeO–hexane, (3:7)), to give carotenoids **2** (R_f 0.25) and **3** (R_f 0.18). The final purification was achieved by HPLC with MeOH–*t*-BuMeO as mobile phase: 49:1

for carotenoid **2** (R_t 18.6 min) and 9:1 for **3** (R_t 10.9 min).

3.4. 6-Geranylgeranyl 8′-methyl-6,8′-diapocaroten-6,8′-dioate (*1*)

Ca. 1 mg. UV–vis λ_{max} nm: 330, 416, 441, 469 (%III/II 91, %A_B/A_{II} 4), after I_2: 330, (414), 438, 466 (%III/II 77, %A_B/A_{II} 10); EI-MS *m/z* (rel. int.): 640 $[M]^+$ (4), 396 (2), 367 $[M-273]^+$ (19), 335 $[M-273-32]^+$ (13), 317 $[M-273-44]^+$ (5), 307 $[M-273-60]^+$ (4), 289 (6), 270 (10), 262 $[M-272-106]^+$ (8), 145 (26), 119 (36), 91 (39), 81 (60), 69 (100); ^{1}H NMR (400 MHz, $CDCl_3$): carotenoid part: δ 1.94 (3H, s, H-19), 1.99 (6H, s, H-20 and H-20′), 2.00 (3H, d, *J* = 1.3 Hz, H-19′), 5.90 (1H, d, *J* = 15.6 Hz, H-7), ~6.35 (1H, N part of ABMN, H-14), ~6.37 (1H, M part of ABMN, H-14′), 6.49 (1 H, d, *J* = 11.2 Hz, H-10), 6.50 (1H, d, *J* = 14.7 Hz, H-12), 6.54 (1H, dd, *J* = 10.8 and 15.0 Hz, H-11′), 6.62 (1H, d, *J* = 15.0 Hz, H-12′), 6.63 (1H, dd, *J* = 11.2 and 14.7 Hz, H-11), ~6.69 (1H, B part of ABMN, H-15′), ~6.70 (1H, A part of ABMN, H-15), 7.29 (1H, dq, *J* = 10.8 and 1.3 Hz, H-10′), 7.38 (1H, d, *J* = 15.6 Hz, H-8); alcohol part: δ 1.61 (3H, dq, H-17″), 1.61 (6H, d, H-18″ and H-19″), 1.68 (3H, dq, *J* = 1.1 Hz, H-16″), 1.73 (3H, d, *J* = 1.3 Hz, H-20″), 1.99 (2H, m, H-8″), 2.02 (2H, m, H-7″), 2.07 (2H, m, H-12″), 2.10 (2H, m, H-11″), 2.11 (4H, m, H-3″ and H-4″), 3.77 (3H, s, 8-$COOCH_3$), 4.69 (2H, d, *J* = 7.1 Hz, H-15″), 5.09 (1H, m, H-2″), 5.10 (1H, m, H-10″), 5.11 (1H, m, H-6″), 5.40 (1H, tq, *J* = 7.1 and 1.3 Hz, H-14″); ^{13}C NMR (100 MHz, $CDCl_3$): carotenoid part: δ 12.67 (C-19), 12.77 (C-20)[a], 12.80 (C-20′)[a], 12.89 (C-19′), 116.42 (C-7), 123.66 (C-11′), 124.60 (C-11), 126.27 (C-9′), 130.86 (C-15′), 131.54 (C-15), 133.71 (C-9), 134.56 (C-14′), 135.50 (C-14)[b], 136.43 (C-13′), 137.13 (C-13), 138.93 (C-10′), 139.24 (C-10), 141.50 (C-12), 143.82 (C-12′), 148.84 (C-8), 167.51 (C-6), 168.95 (C-8′), alcohol part: δ 16.01 (C-18″)[c], 16.04 (C-19″)[c], 16.53 (C-20″), 17.69 (C-17″), 25.70 (C-16″), 26.62 (C-11″), 26.76 (C-7″), 26.22 (C-3″), 39.57 (C-12″)[d], 39.69 (C-4″)[d], 39.72 (C-8″)[d], 51.82 (Me of C-6), 61.22 (C-15″), 118.46 (C-14″), 123.64 (C-6″), 124.17 (C-10″), 124.38 (C-2″), 131.28 (C-1″), 134.96 (C-13″), 135.48 (C-15″)[b], 142.20 (C-9″) ([a,b,c,d]assignment may be interchanged).

3.5. 6-Geranylgeranyl 6′-methyl-(9′*Z*)-6,6′-diapocaroten-6,6′-dioate (*2*)

Ca. 1 mg. UV–vis λ_{max}: 350, 426, 452, 482 (%III/II 77, %A_B/A_{II}, 11), after I_2: 349, (428), 453, 484 (%III/II 398, %A_B/A_{II} 12); EI-MS *m/z* (rel. int): 666 $[M]^+$ (< 1), 393 $[M-273]^+$ (7), 313 (2), 288 $[M-272-106]^+$ (4), 256 (8), 210 (23), 145 (21), 133 (28), 121 (60), 107 (68), 91 (64), 69 (100); ^{1}H NMR (400 MHz, $CDCl_3$): carotenoid part: δ 1.934 (3H, s, H-19), 1.958 (3H, s, H-19′), 1.982 (6H, s, H-20′), 2.004 (3H, s, H-20), 5.89 (1H, d, *J* = 15.5 Hz, H-7), 5.91 (1H, d, *J* = 15.3 Hz, H-7′), 6.32 (1H, N part of ABMN, H-14), 6.35 (1H, M part of ABMN, H-14′), 6.37 (1H, d, *J* = 11.6 Hz, H-10′), 6.41 (1H, d, *J* = 14.8 Hz, H-12′), 6.49 (1H, d, *J* = 11.6 Hz, H-10), 6.51 (1H, d, *J* = 14.3 Hz, H-12), 6.62 (1H, dd, *J* = 11.6 and 14.3 Hz, H-11), 6.66 (1H, B part of ABMN, H-15′), 6.70 (1H, A part of ABMN, H-15), 6.86 (1H, dd, *J* = 11.6 and 14.8 Hz, H-11′), 7.39 (1H, d, *J* = 15.5 Hz, H-8), 7.96 (1H, d, *J* = 15.3 Hz, H-8′), alcohol part: δ 1.60 (3H, dq, H-17″), 1.60 (6H, d, H-18″ and H-19″), 1.68 (3H, dq, *J* = 1.0 Hz, H-16″), 1.73 (3H, d, *J* = 1.1 Hz, H-20″), 1.99 (2H, m, H-8″), 2.02 (2H, m, H-7″), 2.07 (2H, m, H-12″), 2.10 (2H, m, H-11″), 2.11 (4H, m, H-3″ and H-4″), 3.79 (3H, s, 6′-$COOCH_3$), 4.68 (2H, d, *J* = 7.1 Hz, H-15″), 5.09 (1H, m, H-2″), 5.10 (1H, m, H-10″), 5.11 (1H, m, H-6″), 5.40 (1H, tq, *J* = 7.1 and 1.1 Hz, H-14″); ^{13}C NMR (100 MHz, $CDCl_3$): carotenoid part: δ 12.66 (C-19), 12.77 (C-20′), 13.00 (C-20), 20.29 (C-19′), 116.30 (C-7), 117.47 (C-7′), 123.26 (C-11′), 124.38 (C-11), 130.77 (C-15′), 131.51 (C-15), 133.51 (C-9 and C-9′), 134.25 (C-14), 134.77 (C-14′), 136.63 (C-13), 136.95 (C-13′), 137.95 (C-10′), 139.33 (C-10), 140.41 (C-12′), 140.46 (C-8′), 141.63 (C-12), 148.88 (C-8), 167.53 (C-6), 167.98 (C-6′), alcohol part: δ 16.00 (C-18″)[a], 16.04 (C-19″)[a], 16.52 (C-20″), 17.69 (C-17″), 25.70 (C-16″), 26.62 (C-11″), 26.23 (C-3″), 26.76 (C-7″), 39.57 (C-12″)[b], 39.69 (C-4″)[b], 39.74 (C-8″)[b], 51.61 (Me of C-6′), 61.21 (C-15″), 118.48 (C-14″), 123.66 (C-6″), 124.18 (C-10″), 124.34 (C-2″), 131.27 (C-1″), 134.96 (C-13″)[c], 135.47 (C-5″)[c], 142.19 (C-9″) ([a,b,c]assignment may be interchanged).

3.6. 6-Geranylgeranyl 6′-methyl-6,6′-diapocaroten-6,6′-dioate (*3*)

Ca. 1.5 mg. UV–vis λ_{max} nm: 350, 432, 456, 489 (%III/II 76, %A_B/A_{II}); EI-MS *m/z* and (rel. int): 393 $[M-273]^+$ (3), 361 $[M-273-32]+$ (< 1), 333 $[M-273-601]^+$ (< 1), 288 $[M-272-106]^+$ (1), 256 (2), 145 (24), 119 (29), 106 (28), 91 (53), 81 (54), 69 (100), ^{1}H NMR (400 MHz, $CDCl_3$): carotenoid part: δ 1.936 (3H, s, H-19), 1.945 (3H, s, H-19′), 1.988 (6H, s, H-20 and H-20′), 5.88 (1H, d, *J* = 15.5 Hz, H-7), 5.90 (1H, d, *J* = 15.7 Hz, H-7), 6.35 (2H, M and M′ part of pseudo-AA′MM′, H-14 and H-14′), 6.49 (1H, d, *J* = 11.7 Hz, H-10), 6.50 (1H, d, *J* = 11.7 Hz, H-10), 6.50 (1H, d, *J* = 14.3 Hz, H-12′), 6.51 (1H, d, *J* = 14.3 Hz, H-12), 6.63 (2H, dd, *J* = 11.7 and 14.3 Hz, H-11 and H-11′), 6.69 (2H, A and A′ part of AA′MM′, H-15 and H-15′), 7.38 (1H, d, *J* = 15.7 Hz, H-8′), 7.39 (1H, d, *J* = 15.5 Hz, H-8); alcohol part: δ 1.60 (3H, dq, H-17″), 1.60 (6H, d, H-18″ and H-19″), 1.68 (3H, dq, *J* = 1.1 Hz, H-16″), 1.73 (3H, d, *J* = 1.3

Hz, H-20″), 1.99 (2H, m, H-8″), 2.02 (2H, m, H-7″), 2.07 (2H, m, H-12″), 2.10 (2H, m, H-11″), 2.11 (4H, m, H-3″ and H-4″), 3.76 (3H, s, 6′-$COOCH_3$), 4.68 (2H, d, $J = 7.2$ Hz, H-15″), 5.09 (1H, m, H-2″), 5.10 (1H, m, H-10″), 5.11 (1H, m, H-6″), 5.40 (1H, tq, $J = 7.2$ and 1.3 Hz, H-14″); ^{13}C NMR (100 MHz, $CDCl_3$): carotenoid part: δ 12.66 (C-19 and C-19′), 12.80 (C-20 and C-20′), 115.86 (C-7′), 116.36 (C-7), 124.38 (C-11 and C-11′), 131.07 (C-15′)^a, 131.14 (C-15)^a, 133.62 (C-9)^b, 133.49 (C-9′)^b, 134.71 (C-14), 134.80 (C-14′), 136.82 (C-13), 136.89 (C-13′), 139.29 (C-10), 139.47 (C-10′), 141.57 (C-12′), 141.70 (C-12), 148.85 (C-8), 149.06 (C-8′), 167.53 (C-6), 167.94 (C-6′); alcohol part: δ 16.00 (C-18″)^c, 16.04 (C-19″)^c, 16.52 (C-20″), 16.69 (C-17″), 25.70 (C-16″), 26.63 (C-11″), 26.76 (C-7″), 26.23 (C-3″), 39.58 (C-12″)^d, 39.69 (C-4″)^d, 39.72 (C-8″)^d, 51.50 (Me of C-6′), 61.22 (C-15″), 118.47 (C-14″), 123.66 (C-6″), 124.18 (C-10″), 124.48 (C-2″), 131.27 (C-1″), 134.96 (C-13″)^e, 135.48 (C-5″)^e, 142.19 (C-9″) (^{a,b,c,d,e}assignment may be interchanged).

3.7. Geranylgeraniol

Recovered from the MeOH–H_2O phase with *t*-BuMeO after saponification of **3** with 10% KOH–MeOH overnight at room temp. (purity 86% determined by GC/FID). GC/MS: 272 $[M-18]^+$ (2.1), 257 $[M-18-15]^+$ (< 1), 203 (1.8), 136 (4.5), 121 (9.2), 107 (14.7), 93 (31.9), 81 (39.7), 69 (100), 55 (19.1), 41 (52.1), 29 (7.7).

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