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The Leaves of the Common Box, *Buxus sempervirens* (Buxaceae), Become Red as the Level of a Red Carotenoid, Anhydroeschscholtzxanthin, Increases

Kazuko Ida[1], Kazumori Masamoto[2], Takashi Maoka[3], Yasuhiro Fujiwara[3], Satomi Takeda[1] and Emiko Hasegawa[1]

[1]*Department of Natural Science, Osaka Women's University, Sakai, Osaka, 590 Japan*
[2]*Biological Laboratory, Faculty of Education, Kumamoto University, Kumamoto, 860 Japan*
[3]*Kyoto Pharmaceutical University, Yamashina-ku, Kyoto, 607 Japan*

Carotenoids from the leaves of the common box, *Buxus sempervirens* (Buxaceae), which turn red in late autumn to winter, were analyzed by reversed-phase HPLC. A novel carotenoid, monoanhydroeschscholtzxanthin (3), was isolated from the red-colored leaves. UV-VIS, MS, ^{1}H-NMR and CD spectral data showed that the structure of 3 was (3*S*)-2′, 3′, 4′, 5′-tetradehydro-4, 5′-retro-β, β-caroten-3-ol. As well as anhydroeschscholtzxanthin (2), the major red carotenoid in the leaves, eschscholtzxanthin (4) was identified. Very small amounts of yellow carotenoids (neoxanthin, violaxanthin, lutein and β-carotene), which are major components of green leaves, were present in the red-colored leaves. The amounts of chlorophyll *a* and *b* in the leaves decreased markedly during coloration, even at the early stages, whereas those of the yellow carotenoids decreased gradually. In contrast, the content of 2, a red carotenoid, increased steadily during coloration. The biosynthetic pathway of 2 in *B. sempervirens* was deduced tentatively on the basis of the individual carotenoid contents during autumnal coloration.

Key words: Anhydroeschscholtzxanthin — *Buxus sempervirens* — Carotenoid — Leaf coloration — Monoanhydroeschscholtzxanthin

The autumnal leaf coloration of many angiospermous trees occurs as a result of the disappearance of chloroplast pigments from the mesophyll cells and synthesis of anthocyanin in the epidermal cellular vacuoles (Hayashi and Abe 1955). The chromoplasts of the leaves or needles of many gymnosperms (both evergreen and deciduous species) which turn reddish-brown in the autumn and winter contain substantial amounts of rhodoxanthin (**1**) (Ida 1981, Czeczuga 1986, 1987, Ida *et al.* 1991). The leaves of the common box, *Buxus sempervirens* (Buxaceae), an evergreen angiospermous shrub, undergo a gradual color change from green to red in late autumn to winter, but recover their green color in the next growth season. Electron microscopic observations have confirmed that the color change is due to a reversible change from chromoplasts to chloroplasts in the leaves (Koiwa *et al.* 1986) and this change is similar to that in *Cryptomeria*, a gymnospermous evergreen tree (Toyama and Funazaki 1971).

A red carotenoid was obtained from the red leaves of *B. sempervirens*, and identified as anhydroeschscholtzxanthin (**2**) from its chemical data and visible and infrared absorption spectra (Costes 1969), although its detailed stereostructure was not confirmed. This red carotenoid has not been isolated from any other living materials.

In this study, we analyzed the stereostructure of **2** isolated from the red leaves of *B. sempervirens* by proton-nuclear magnetic resonance (^{1}H-NMR), and examined the seasonal changes in the leaf carotenoid components. We also found a novel red carotenoid, which we named monoanhydroeschscholtzxanthin (**3**). The biosynthetic pathway of the red carotenoids during late autumnal leaf coloration is discussed.

Materials and Methods

Plant materials

Leaves were harvested from *Buxus sempervirens* trees growing in sunny areas of the campus of Osaka Women's University (Sakai City, Osaka, Japan) and in sunny areas in Ono-dai (Sayama City, Osaka, Japan). The leaves started to turn red in December and were completely red by mid-February.

Large-scale extraction and isolation of carotenoids

Carotenoids were extracted with acetone from 1.0 kg fresh leaves (stage 7, Fig. 1). After transfer to *n*-hexane : diethylether (1 : 1 v/v) with addition of H_2O, the extracts

Abbreviations: HPLC, high performance liquid chromatography; UV-VIS, ultraviolet-visible light absorption spectra; MS, mass spectra; ^{1}H-NMR, proton-nuclear magnetic resonance; CD, circular dichroism spectra; EI-MS, electron impact-mass spectra; NOE, nuclear Overhauses effect.

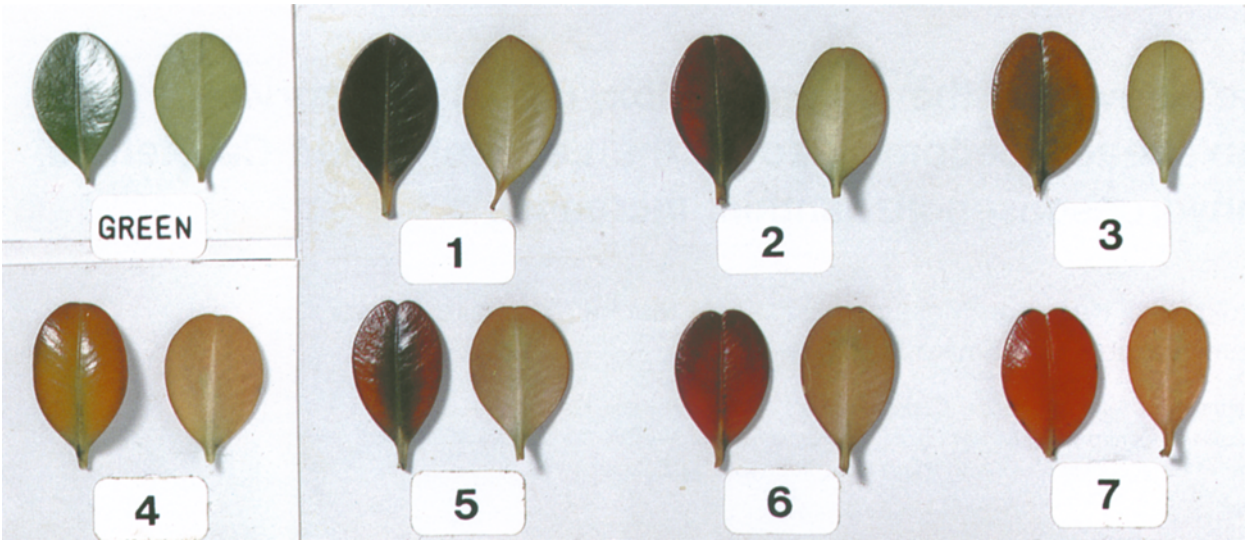


Fig. 1. Color development during the autumnal coloration of leaves of *Buxus sempervirens*. The numerals in the figure represent the intensity (stages 1 to 7) of the red color. The leaf on the right for each pair shows the abaxial side. The harvested leaves were differentiated into 8 stages (including the green leaves), judging from their apparent color.

were evaporated to dryness and saponified with 10% KOH in methanol at 30C for 12hr. The unsaponifiable material was extracted with *n*-hexane : diethylether (1 : 1 v/v) with addition of H_2O. The *n*-hexane-diethylether solution was dried over Na_2SO_4, evaporated to dryness and the residue was subjected to column chromatography on silica gel with *n*-hexane and acetone as the mobile phase. Each fraction was subjected to further purification by preparative high performance liquid chromatography (HPLC) using a LC-6AD with a SPD-6AV detector (Shimadzu Co. Ltd., Kyoto). The samples were separated on a C18 column (Shim-pak PREP-ODS, 20 mm×250 mm, 5 μm) using a solvent of CH_3CN-CH_2Cl_2 (8 : 2 v/v). Ultraviolet-visible light absorption spectra (UV-VIS) of samples in diethylether were recorded with a spectrophotometer (UV-240, Shimadzu). Mass spectra (MS) were recorded using a SX 102A (JEOL, Tokyo) with a direct inlet system at 70 eV, 190-200C. Proton-nuclear magnetic resonance (^{1}H-NMR ; 300 MHz) spectra of samples in $CDCl_3$ were recorded using a XL-300 (Varian Instrument Ltd., Palo Alto, California) with tetramethylsilane as the internal standard at 22-24C. Infrared (IR) spectra of samples in KBr pellets were recorded using a FT-IR 1600 (Perkin Elmer Ltd., Norwalk, Connecticut) and circular dichroism spectra (CD) of samples in diethylether-isopentane-ethanol (5 : 5 : 2 v/v/v) solution were obtained using a J-500C spectropolarimeter (JASCO Ltd., Tokyo) at room temperature.

Analysis of pigments by HPLC

At each coloration stage, the leaf pigments were extracted twice with 90% acetone under dim light at room temperature after measuring the fresh weights of the leaves and homogenizing them with a pestle and mortar. The extracts were centrifuged for 3 min at 4,000×*g* and the resulting supernatants were filtered and subjected to HPLC. All the pigment extraction procedures were completed within 20 min.

The extracted pigments were separated by reversed-phase HPLC, as described previously (Masamoto *et al.* 1993). The samples were loaded onto a C18 column (YMC AL-313, 6 mm×250 mm, 5 μm, YMC Ltd., Kyoto) and eluted using 100% methanol for the first 10 min, followed by a 10-min linear gradient of ethanol in methanol to 100% ethanol, elution with which was continued isocratically until the completion of the 30-min separation, at a flow rate of 1.5 ml • min^{-1} at 25C (PU-980, JASCO). The absorptions of the eluates at 450 nm were monitored using a spectrophotometric detector (SPD-6AV, Shimadzu) and analyzed with a Shimadzu CR-6A Chromatopac.

In order to identify the pigments separated by this HPLC system, the absorption spectrum and retention time of each peak were recorded with a photodiode array detector (SPD-M6A, Shimadzu). The authentic pigments used as standards were chlorophyll *a*, β-carotene (β, β-carotene ; Wako Pure Chemical Industries Ltd., Osaka) and (3*R*, 3′*R*)-zeaxanthin ((3*R*, 3′*R*)-β, β-carotene-3, 3′-diol ; Roche Co., Basel, Switzerland).

Chlorophyll *a*, zeaxanthin, β-carotene and the three red carotenoids separated by this HPLC system were quantified by interpolation of calibration curves of the absorptions at 450 nm of the three authentic pigments and the isolated red carotenoids (3*S*, 3′*S*)-eschscholtzxanthin (**4** ; 4′, 5′-didehydro-4, 5′-retro-β, β-carotene-3, 3′-diol), a new carotenoid, monoanhydroeschscholtzxanthin (**3**; (3*S*)-2′, 3′, 4′, 5′-tetradehydro-4, 5′-retro-β, β-caroten-3-ol), and anhydroeschscholtzxanthin (**2** ; 2, 3, 2′, 3′, 4′, 5′-hexadehydro-4, 5′-retro-β, β-carotene), obtained as

described above (*Large-scale extraction and isolation of carotenoids*). The extinction coefficients $E^{1\%}_{1cm}$ used to determine the concentrations of the standard stock solutions were 2620 for β-carotene in ethanol, 2540 for zeaxanthin in ethanol, 3269 for eschscholtzxanthin (**4**) in hexane and 3018 for anhydroeschscholtzxanthin (**2**) in hexane at their absorption maxima (λ_{max}) (Davies 1976). For monoanhydroeschscholtzxanthin (**3**) in hexane the $E^{1\%}_{1cm}$ used was 3215 at its λ_{max} and we used a specific coefficient of 18.42 (g/l) at 450 nm for chlorophyll *a* in 95% ethanol (Lichtenthaler 1987).

We quantified the carotenoids neoxanthin ((3*S*,5*R*, 6*R*, 3′*S*, 5′*R*, 6′*R*)-5′, 6′-epoxy-6, 7-didehydro-5, 6, 5′, 6′-tetrahydro-β, β-carotene-3, 5, 3′-triol), violaxanthin ((3*S*, 5*R*, 6*S*, 3′*S*, 5′*R*, 6′*S*)-5, 6, 5′, 6′-diepoxy-5, 6, 5′, 6′-tetrahydro-β, β-carotene-3, 3′-diol), antheraxanthin ((3*S*, 5*R*, 6*S*, 3′*R*)-5, 6-epoxy-5, 6-dihydro-β, β-carotene-3, 3′-diol), lutein ((3*R*, 3′*R*, 6′*R*)-β, ε-carotene-3, 3′-diol), α-carotene ((6′*R*)-β, ε-carotene) and chlorophyll *b* by estimating their calibration factors at 450 nm in this HPLC system, which were obtained from the ratio of the absorption at 450 nm to that at λ_{max} (obtained from their absorption spectra) of each pigment and the ratios of $E^{1\%}_{1cm}$ at λ_{max} of these carotenoids to that of β-carotene (or zeaxanthin) and of chlorophyll *b* to that of chlorophyll *a* (Davies 1976, Lichtenthaler 1987), which enable the calibration factors for the authentic pigments and red carotenoids, determined as described above, to be used. These estimated calibration factors coincided well with the published relative ratios (Rivas *et al.* 1989).

Results

Absence of anthocyanin in red-colored B. sempervirens *leaves*

In late autumn, the color of *B. sempervirens* leaves starts to change from deep brown (stages 1 and 2, Fig. 1) and progress to bright red (stage 7). Microscopic examination of the mesophyll cells of the red-leaves revealed colorless vacuoles, no green chloroplasts and the presence of red-colored chromoplasts (data not shown). These findings are identical to those reported for gymnosperms (Toyama and Funazaki 1971, Ida 1981, Czeczuga 1986, 1987, Ida *et al.* 1991) and *Buxus* (Koiwa *et al.* 1986). The color changes are due to the appearance of red carotenoids and disappearance of chlorophylls (Costes 1969). The pigments in the leaves during the coloration period were analyzed by reversed-phase HPLC (Fig. 2).

The structure of red carotenoids in B. sempervirens *leaves*

The red leaves contained three red carotenoids (peak numbers 4, 8 and 10, Fig. 2C), which showed maximal absorption peaks in the flow solvent of 470 (peak 4), 482 (peak 8) and 497 (peak 10) nm. These wavelengths are considerably longer than those of the yellow carotenoid

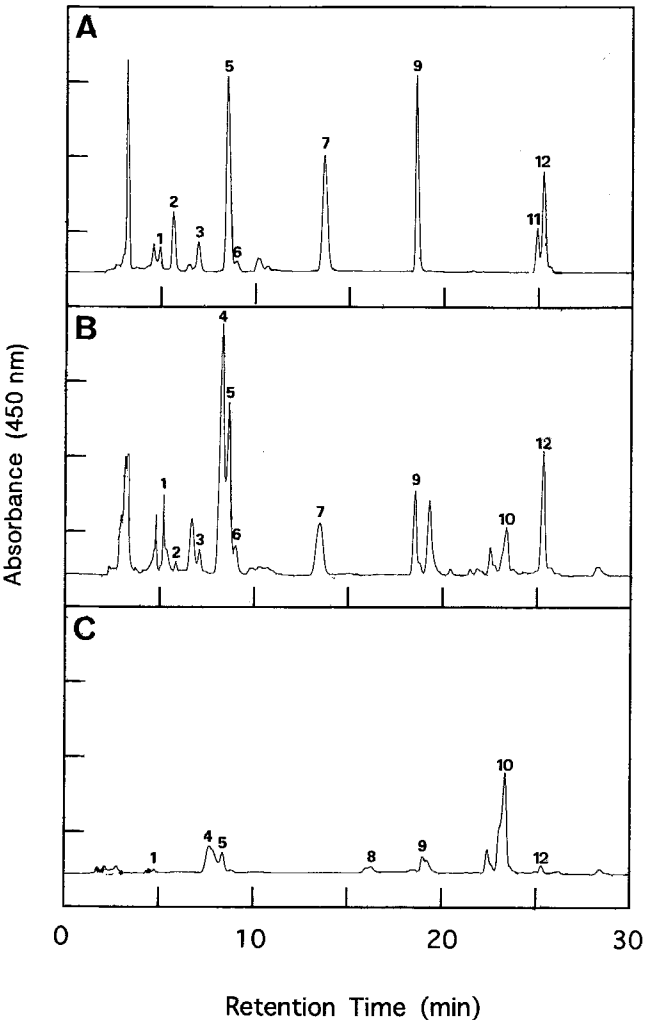


Fig. 2. HPLC profiles of the pigments extracted from the leaves of *B. sempervirens*. The extracted pigments were analyzed by reversed-phase HPLC (see **Materials and Methods**). A; green leaves, B; stage 2 (deep brown leaves), C; stage 7 (red leaves). The numbered peaks are 1; neoxanthin, 2; violaxanthin, 3; antheraxanthin, 4; eschscholtzxanthin, 5; lutein, 6; zeaxanthin, 7; chlorophyll *b*, 8; monoanhydroeschscholtzxanthin, 9; chlorophyll *a*, 10; anhydroeschscholtzxanthin, 11; α-carotene, 12; β-carotene.

maximal absorption peaks (around 450 nm).

The carotenoid from peak 10 (yield 8 mg) in ether showed visible absorption maxima at 500 and 530 nm and IR absorption maxima at 3030 (CH_3), 2961 (CH_3), 2920 (CH_3), 1454 (CH_3), 974 (olefin), 952 (olefin) and 738 cm^{-1} in KBr. Characteristic doublet IR absorption at 974 and 952 cm^{-1} showed the presence of *trans* retro-polyene (Nicoara *et al.* 1966). These spectral data were identical to those of anhydroeschscholtzxanthin (**2**) reported by Costes (1969). Furthermore, high resolution electron impact-mass spectra (EI-MS) yield a molecular ion peak at *m/z* 530.3906, which is compatible with the molecular formula $C_{40}H_{50}$ (calc. 530.3912). The ^{1}H-NMR data for this car-

Table 1. ^{1}H-NMR assignment of anhydroeschscholtzxanthin, monoanhydroeschscholtzxanthin and eschscholtzxanthin in $CDCl_3$

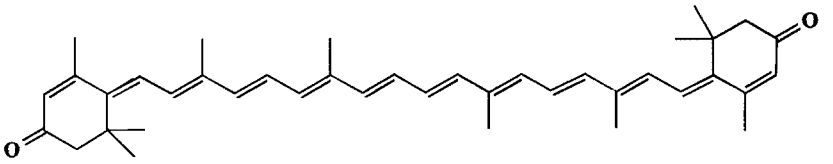
Protons	δ Values in ppm : multiplicity (coupling constants in Hz)					
	Anhydroeschscholtzxanthis (**2**)		Monoanhydroeschscholtzxanthin (**3**)		Eschscholtzxanthis (**4**)	
H-2	5.49 d (9.5)	H-2ax	1.51 d, d (12, 8)	H-2ax	1.51 d, d (12, 8)	
		H-2eq	1.81 d, d (12, 5)	H-2eq	1.81 d, d (12, 5)	
H-3	~5.79 d, m (9.5)		4.35 m	4.35 m*	4.35 m	4.35 m*
H-4	~5.79 m		5.75 br. s	5.67 br. s*	5.75 br. s	5.67 br. s*
H-7	6.60 d (13)		6.48 d (12)		6.48 d (12)	
H-8	6.87 d (13)		6.73 d (12)		6.73 d (12)	
H-10	6.45 d (15)		6.43 d (15)		6.43 d (15)	
H-11	6.64 d, d (15, 11)		6.66 d, d (15, 11)		6.66 d, d (15, 11)	
H-12	6.25 d (11)		6.23 d (11)		6.23 d (11)	
H-14	~6.40 m		~6.40 m		~6.40 m	
H-15	~6.41 m		~6.41 m		~6.41 m	
H_3-16	1.39 s		1.25 s	1.10 s*	1.25 s	1.10 s*
H_3-17	1.39 s		1.45 s	1.25 s*	1.45 s	1.25 s*
H_3-18	1.97 s		1.95 d (1.5)+	2.13 d (1.5)*+	1.97 d (1.5)+	2.13 d (1.5)*+
H_3-19	1.97 s		1.97 s		1.97 s	
H_3-20	1.97 s		1.97 s		1.97 s	
H-2′			5.49 d (9.5)			
H-3′			~5.79 d, m (9.5)			
H-4′			~5.79 m			
H-7′			6.60 d (13)			
H-8′			6.87 d (13)			
H-10′			6.45 d (15)			
H-11′			6.64 d, d (15, 11)			
H-12′			6.25 d (11)			
H-14′			~6.40 m			
H-15′			~6.41 m			
H_3-16′			1.39 s			
H_3-17′			1.39 s			
H_3-18′			1.97 s			
H_3-19′			1.97 s			
H_3-20′			1.97 s			

s, singlet ; d, doublet ; m, multiplet ; br.s, broad singlet.
* : 6 *cis*-isomer.
+ : allyl coupling (Englert and Vecchi 1982).

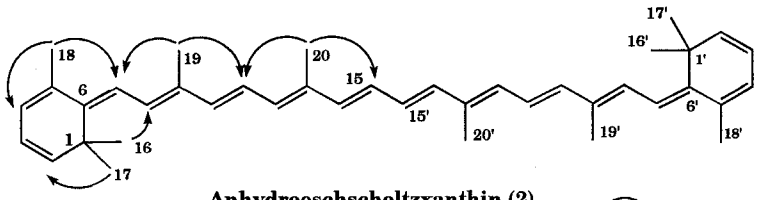
otenoid are shown in Table 1. Assignments were made by carrying out ^{1}H-^{1}H decoupling and ^{1}H-^{1}H nuclear Overhauser effect (NOE) experiments. From these spectral data, the carotenoid from peak 10 was identified as anhydroeschscholtzxanthin (2, 3, 2′, 3′, 4′, 5′-hexadehydro-4, 5′-retro-β, β-carotene), although the stereostructure of this carotenoid, including the configuration of the Δ^6-double bond, has not yet been determined by Costes (1969). In this study, the stereostructure of **2** was determined from the NOE difference spectra shown in Fig. 3 : the differences between H_3-16, 17 and H-8 and between H_3-18 and H-7 indicated that the configuration at the Δ^6 double bond was *trans*.

Peak 4 was identified as eschscholtzxanthin (**4**) using the methods employed to identify the red carotenoid. The UV-VIS, MS, ^{1}H-NMR (Table 1) and CD (Fig. 4) spectral data of this carotenoid isolated from *B. sempervirens* (peak 4) were the same as those of **4** reported by Andrewes *et al.* (1979).

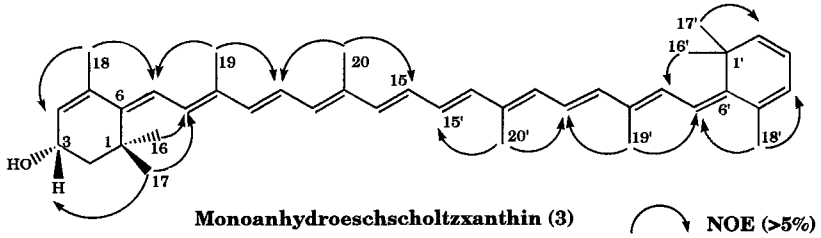
The new carotenoid from peak 8 (yield 0.8 mg) in ether showed UV-VIS absorption maxima at 458, 482 and 515 nm. High resolution EI-MS yield a molecular ion peak at *m/z* 548.4046, which is compatible with the formula $C_{40}H_{52}O$ (calc. 548.4018). Acetylation of this new carotenoid in dry pyridine with acetic anhydride at room temperature produced a monoacetate with a molecular ion peak at 590 ($C_{42}H_{54}O_2$), which demonstrated it possessed one primary or secondary hydroxyl group. The



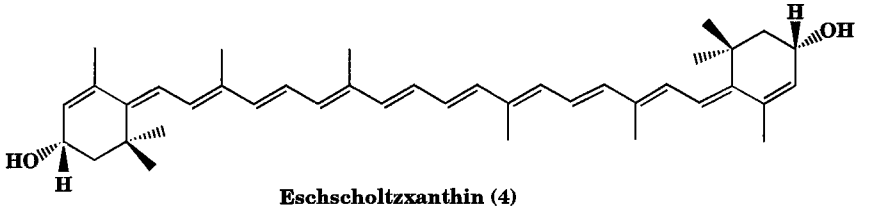
Rhodoxanthin (1)



Anhydroeschscholtzxanthin (2) NOE (>5%)



Monoanhydroeschscholtzxanthin (3) NOE (>5%)



Eschscholtzxanthin (4)

Fig. 3. Structures of red carotenoids.

^{1}H-NMR data for this new carotenoid are presented in Table 1. Assignments were also accomplished by ^{1}H-^{1}H decoupling and ^{1}H-^{1}H NOE experiments and by comparing these data with those of eschscholtzxanthin (**4**) and anhydroeschscholtzxanthin (**2**) described above. The ^{1}H-NMR data for this new carotenoid indicated that one half of the molecule (H-2 to H_3-20) was identical to that of **4** and the other (H-2′ to H_3-20′) was identical to that of **2**. The proton-proton connectivities of the new carotenoid were confirmed by ^{1}H-^{1}H decoupling, long range decoupling (Englert and Vecchi 1982) and NOE (Fig. 3) experiments. Furthermore, dehydroxylation of the new carotenoid with dry hydrogen chloride in chloroform yielded **2**, which was identified by UV-VIS, EI-MS and ^{1}H-NMR data. Therefore, the structure of the new carotenoid was concluded to be 2′, 3′, 4′, 5′-tetradehydro-4, 5′-retro-β, β-caroten-3-ol and it was named monoanhydroeschscholtzxanthin (**3** ; Fig. 3). The CD spectrum of **3** showed almost the same Cotton effect as that of (3*S*, 3′*S*)-eschscholtzxanthin (**4**) reported by Andrewes *et al.* (1979), except the wavelength shift was 15 nm longer than that of **4**, which is attributable to the presence of one more double bond at 2′ in **3**. Therefore, the chirality at C-3 of **3** was postulated tentatively to be *S*. Furthermore, the presence of a minor inseparable 6-*cis* isomer of **3**, which was also reported for **4** by Andrewes *et al.* (1979) was confirmed by the ^{1}H-NMR data. The ratio of 6-*trans* to 6-*cis* (88 : 12) isomers of **3** was determined by measuring the ^{1}H-NMR signal areas of the corresponding 6-*trans* and 6-*cis* (88 : 12) isomers.

Identification of pigments from B. sempervirens *leaves*

In order to identify chlorophyll *a*, zeaxanthin, β-carotene and the three red carotenoids (eschscholtzxanthin

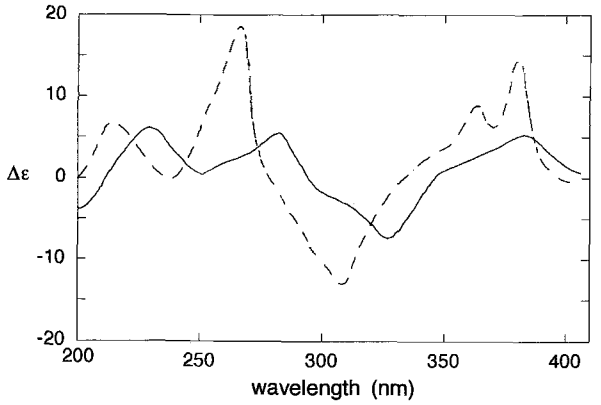


Fig. 4. CD spectra of monoanhydroeschscholtzxanthin (**3**) (solid line) and (3*S*, 3′*S*)-eschscholtzxanthin (**4**) (broken line) in ether-isopentane-ethanol (5 : 5 : 2) solution.

Table 2. Contents of each carotenoid in green and red leaves of *Buxus sempervirens*

Carotenoid component (mol %)	Color of Leaves	
	Green	Red (Stage 7)
Yellow carotenoid		
Neoxanthin	12	1
Violaxanthin	13	trace
Antheraxanthin	2	trace
Lutein	44	7
Zeaxanthin	5	trace
α-Carotene	3	trace
β-Carotene	20	2
Red carotenoid		
Eschscholtzxanthin (**4**)	0	13
Monoanhydroeschscholtzxanthin (**3**)	0	4
Anhydroeschscholtzxanthin (**2**)	0	58

(**4**), monoanhydroeschscholtzxanthin (**3**) and anhydroeschscholtzxanthin (**2**)) using this HPLC system, authentic samples of the former three and the isolated red carotenoids (see *Large-scale extraction and isolation of carotenoids*) were used to compare the retention times and spectra. Other peaks were identified similarly by comparing them with those obtained from spinach (neoxanthin, violaxanthin, antheraxanthin, lutein and chlorophyll *b*) and from carrot roots and the leaves of woody plants (α-carotene) using this HPLC system, with reference to the data of Rivas *et al.* (1989), Lichtenthaler (1987) and Thayer and Björkman (1990). In addition, β-carotene, lutein, violaxanthin and neoxanthin were identified by MS, CD and ^{1}H-NMR.

The leaf pigment changes during the autumnal coloration process

Pigments from green leaves of *B. sempervirens* (Fig. 2A) contained carotenoid components typical of higher plants (Ida 1981, Ida *et al.* 1991, Rivas *et al.* 1989, Demmig-Adams 1990, Thayer and Björkman 1990). The carotenoid compositions of the green and the red leaves of *B. sempervirens* are summarized in Table 2. Lutein (44%) and β-carotene (20%) were the major components of the green leaves, in which no red carotenoids were detected. The amounts of yellow carotenoids (Fig. 5B) decreased depending on the coloration (472 and 28 nmol/g fr-wt in green and red (stage 7) leaves, respectively). No α-carotene was detected in the leaves at any stage of coloration. At stage 1, the amount of violaxanthin had decreased markedly and that of antheraxanthin had increased in comparison with the amount present in green leaves (Fig. 5B). The molar ratio of members of the xanthophyll cycle (violaxanthin, antheraxanthin and zeaxanthin) to chlorophyll ($a+b$) in the green leaves was 8.1%, which is slightly lower than that for typical sun-growing plants (Thayer and Björkman 1990). These carotenoid components were detected in the red leaves, but only in very small amounts (Table 2).

The pigment composition of red leaves (stage 7 ; Fig. 2C and Table 2), differed considerably from that of green ones. The yellow carotenoids were minor components of the former, whereas the red carotenoids (**3** and **2**) and a related one (**4**) became the major ones, accounting for 75% of the total carotenoid content. These major components were not detected in green leaves, but were present in all colored ones (Figs. 2 and 5C). The content of **3** increased up to stage **3** and only increased very slightly thereafter. In contrast, the content of **2** increased in parallel with the color change (Fig. 5C).

In order to check that the red carotenoid rhodoxanthin (**1**), was not present in the red leaves of *B. sempervirens*, we analyzed the HPLC profiles of the extracts from metasequoia, which contains 6, 6′-di*cis*, 6-*cis* and all *trans* rhodoxanthins (Englert and Vecchi 1982, Ida *et al.* 1991). These rhodoxanthins appeared at retention times of around 15 min (data not shown), whereas no such peaks were detected in *B. sempervirens* extracts (Fig. 2).

The contents of chlorophylls in *B. sempervirens* leaves decreased as coloration progressed (Fig. 5A). The content of the major pigments the leaves contained, chlorophylls, decreased to one third when the leaves turned deep brown (stage 1) and were virtually absent from red leaves (stage 7 ; Fig. 5A). At stage 7, a trace amount of chlorophyll *a* was present, but no chlorophyll *b* was detected (Fig. 2C). The chlorophyll *a*/*b* ratio was almost constant (about 3.0) throughout the leaf color change process, except at stage 7.

Discussion

In red leaves of *B. sempervirens*, a carotenoid, which was not present in its green leaves, was detected by TLC and identified, from its chemical and spectral data, as anhydroeschscholtzxanthin (**2**) by Costes (1969). In this study, we confirmed the presence of this red carotenoid

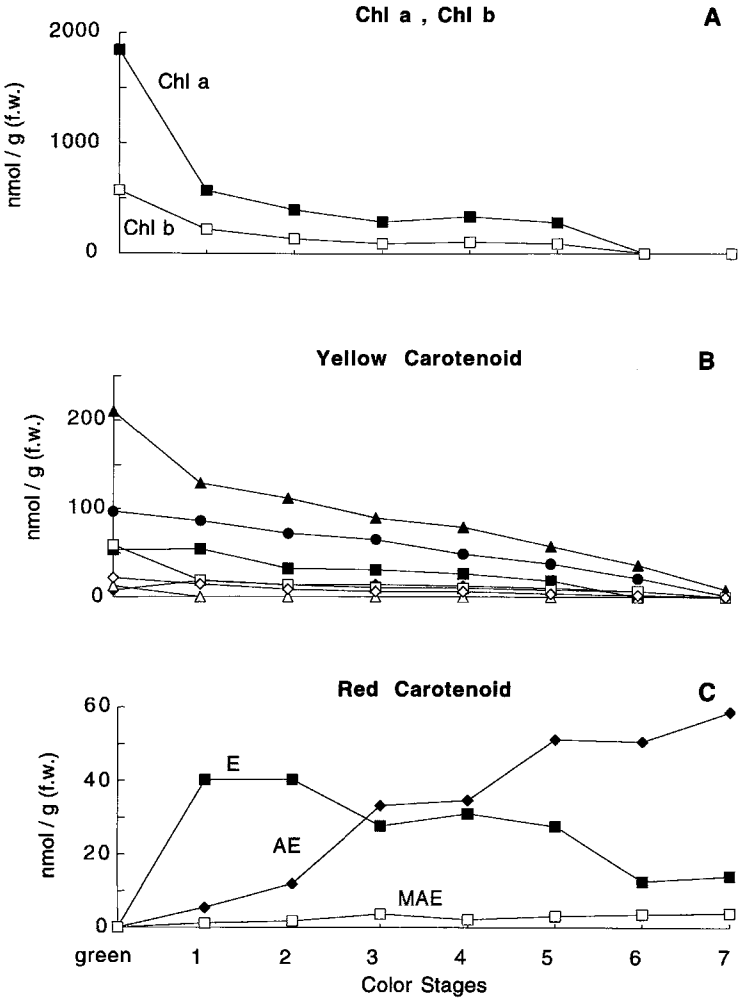


Fig. 5. Changes in the contents of chlorophylls (A), yellow carotenoids (B) and red carotenoids (C) in *B. sempervirens* during autumnal coloration. The symbols in (B) are neoxanthin (■), violaxanthin (□), antheraxanthin (◆), lutein (▲), zeaxanthin (◇), α-carotene (△), β-carotene (●). In C, the abbreviations for the red carotenoids are E (eschscholtzxanthin (**4**)), MAE (monoanhydroeschscholtzxanthin (**3**)) and AE (anhydroeschscholtzxanthin (**2**)).

(**2**) and determined its stereostructure. Eschscholtzxanthin (**4**) was first isolated from *Eschscholtzia california* petals (Strain 1938) and its configuration was determined by Andrewes *et al.* (1979). So far, two types of biosynthetic pathway for **2** have been postulated. Costes (1969) proposed a pathway of biosynthesis of **4** from **2** *via* rhodoxanthin (**1**), a diketo-carotenoid, and a pathway for retro-carotenoids (**1**, **4** and related carotenoids) *via* an epoxy-carotenoid, antheraxanthin, was proposed by Williams *et al.* (1966). Karrer and Leumann (1951) demonstrated the chemical synthesis of **2** from **4** by a dehydroxylation reaction involving the removal of two water molecules.

During the early coloration stage (stages 1 and 2 in Fig. 1), the leaf content of **4** was relatively high, but declined during the later stages, whereas that of **2** increased continuously during the coloration process. Carotenoid **3** appeared to have intermediate characteristics in view of its chemical structure determined in this study and its contents during coloration (Fig. 5C). We were unable to detect **1** during the process of leaf coloration. Unlike other yellow carotenoids, the antheraxanthin content increased during coloration, although only slightly, and that of violaxanthin decreased to one-third of the green leaf content and was nearly same as that of **4**, at stage 1. In the light of these findings, the suggested biosynthetic route to **4** from zeaxanthin and antheraxanthin (Williams *et al.* 1966, Andrewes *et al.* 1979) and the chemical synthesis of **2** from **4** (Karrer and Leumann 1951), we propose that the biosynthetic pathway for the red carotenoids in *B. sempervirens* leaves during the coloration process is:

xanthophyll cycle components → eschscholtzxanthin (**4**) → monoanhydroeschscholtzxanthin (**3**) → anhydroeschscholtzxanthin (**2**).

However, the actual biosynthetic reactions have not been

established and further studies to discover the biosynthetic enzymes involved are needed to substantiate this proposed pathway.

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