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Nuclear magnetic resonance analysis of carotenoids from the burgundy plumage of the Pompadour Cotinga (*Xipholena punicea*)

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ABSTRACT

Previous analysis of carotenoids extracted from the burgundy plumage of the Pompadour Cotinga (*Xipholena punicea*) revealed six novel keto-carotenoid pigments with methoxyl groups in the C3-position of one or both β-rings. High performance liquid chromatography (HPLC), mass spectrometry, chemical analysis and, in some instances ^{1}H NMR spectroscopy were employed to determine the structures of the molecules. Further analysis by NMR was precluded due to lack of material. The recent acquisition of multiple feathers from *X. punicea* specimens has made it possible to complete this work using correlated homonuclear spectroscopy (COSY), nuclear overhauser effect spectroscopy (NOESY) and ^{1}H NMR. These new data conclusively confirm the structures of the six methoxy-carotenoids suggested by the earlier work. In addition, the resonance positions of the protons from the novel 3-methoxy-4-keto-β-ring and 2,3-didehydro-3-methoxy-4-keto-β-ring moieties are reported here for the first time.

Introduction

The brightly colored plumages of birds contain a diversity of pigments, including a number of novel structures derived from four common dietary carotenoids, lutein, zeaxanthin, β-carotene and β-cryptoxanthin [1]. In a recent study [2], extraction and analysis of the pigments from the burgundy plumage of the male Pompadour Cotinga, *Xipholena* (*X.*) *punicea* revealed six previously unreported carotenoids with methoxyl groups in the C3 and C3′-positions. These pigments were proposed to be derived via three metabolic reactions, C4-oxygenation, C3(3′)-methylation and C2, C3-dehydrogenation. Whereas C4-oxygenation of dietary carotenoids is reported to be common in birds [1], the other two metabolic transformations are relatively uncommon for naturally-occurring carotenoids. Methoxylation of β-ring structures of carotenoids had previously only been reported in a few rare cases involving sponges [3–5] and more recently, in the human retina [6]. Carotenoids having a double-bond between C2 and C3, such as astacene (3,3′-dihydroxy-2,3,2′,3′-tetradehydro-β,β-carotene-4,4′-dione), phoeniconone (3-hydroxy-2,3-didehydro-β,β-carotene-4,4′-dione), and α-doradecin (3,3′-dihydroxy-2,3-didehydro-β,ε-caroten-4-one) are also relatively uncommon and are thought to be artifacts formed due to exposure to alkali or oxygen during sample processing [7]. However, a recent analysis of crimson and violet feathers from multiple species of Eurasian Broadbills has revealed a similar xanthophyll identified as 2,3-didehydro-papilioerythrinone [8], providing additional evidence that birds are in fact capable of carrying out the reaction that incorporates a double bond between C2 and C3.

Previous identifications of the carotenoids obtained from the solvent extracts of *X. punicea* plumage were accomplished using high performance liquid chromatography ([1]HPLC), mass spectrometry, chemical analysis and limited ^{1}H NMR spectroscopy [2] (The previous work is summarized in the Supplemental information of this article). Insufficient material was available at that time to carry out a detailed NMR structure analysis of the six novel carotenoids. The recent acquisition of multiple feathers from *X. punicea* specimens has made it possible to complete the NMR work.

In a typical ^{1}H NMR spectrum, the chemical shift, multiplet structure, spacings between the resonances, and integration of the peaks in a ^{1}H NMR spectrum can be used to determine the bond status and connectivity of protons in a molecule which can aid in the elucidation of its structure. The 2D-NMR spectroscopic methods of correlated homonuclear spectroscopy (COSY) and nuclear overhauser effect spectroscopy (NOESY) can be used to obtain even more detailed structural information by analyzing the coupling of protons associated with one or more bonds. COSY elucidates spin-couplings between protons that are connected

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[1] *Abbreviations used:* HPLC, high performance liquid chromatography; COSY, correlated homonuclear spectroscopy; NOESY, nuclear overhauser effect spectroscopy; MTBE, mixture of methyl tert-butyl ether; NP, normal phase; RP, reverse phase.

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through a single bond [9]. NOESY examines relationships between protons that are spatially coupled across multiple bonds [10,11]. The present study uses ^{1}H, COSY, and NOESY NMR to provide detailed structural identifications of the novel, methoxy-containing carotenoids from *X. punicea*.

Methods

Extraction of pigments

Two deceased male *X. punicea* specimens (YPM 141968 and 142924) were obtained by the Yale Peabody Museum from the Dallas World Aquarium and Zoological Garden where they had been living in captivity. The burgundy feathers of these specimens were plucked and soaked in 1 L of technical grade ethanol for 30 min, after which time they were transferred to filter paper and patted dry. The feathers obtained from specimen YPM 141968 were placed in 1 L fresh ethanol for an additional 30 min, because the feathers contained a significant amount of dirt and debris. Both sets of feathers were then soaked in 1 L of technical grade hexanes for 30 min and then patted dry in the same fashion. It should be noted that the feathers of specimen YPM 142924 released some pigment upon treatment with hexanes, which was evidenced by a slight orange coloration of the solvent and filter paper.

Subsequently, the pigmented barbs were trimmed and divided into 8 roughly equal lots. Each lot was placed into a 50 mL screw-cap glass jar, covered with ~30 mL of acidified pyridine and heated in a 90 °C water bath for 90 min, after which time

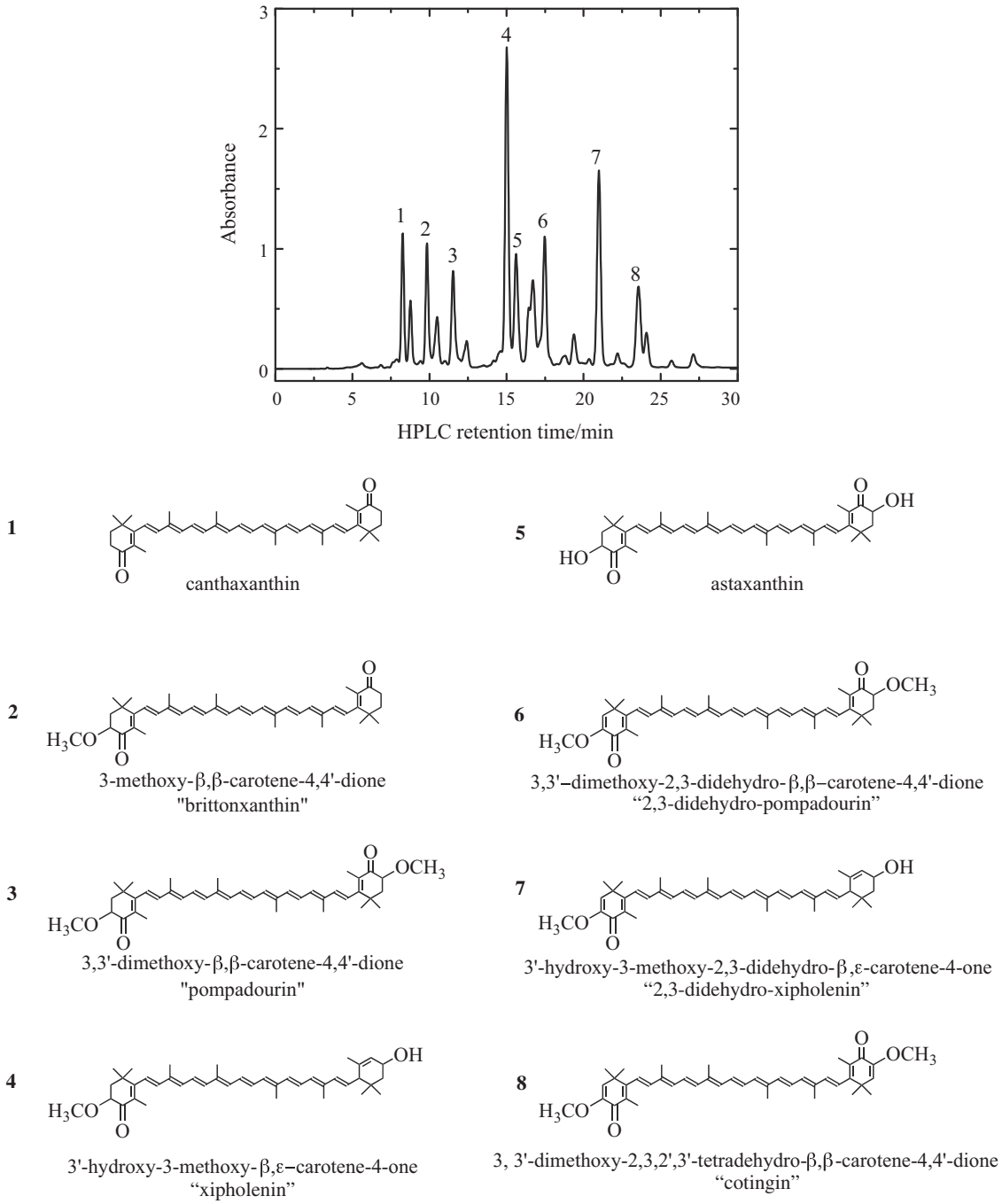


Fig. 1. Preparative NP-HPLC chromatogram of *X. punicea* extract, detected at 450 nm, with eight major peaks denoted numerically. Proposed structures are also given as reported in [2].

extraction appeared to be complete. The use of acidified pyridine and heating for the extraction of carotenoids from feathers has been reported to result in partial isomerization of the carotenoids to cis-conformations, but yields no apparent structural degradation [2,12]. The dark red extract was pipetted out of each jar and into a volumetric flask to which was added ~200 mL of a 3:1 (v/v) mixture of methyl tert-butyl ether (MTBE) and water. The water layer was separated, and the MTBE layer was washed twice with water. The MTBE fractions from each jar were collected, combined, and dried using a rotary evaporator.

High-performance liquid chromatography

The pigment extract was analyzed by HPLC using previously described protocols [2,13]. Eight major bands corresponding to the individual pigments were separated and collected using a normal phase (NP) HPLC protocol, dried under nitrogen gas, and then stored in a −20 °C freezer. Within two days prior to NMR analysis, individual pigments were re-purified using a Waters Atlantis reverse phase (RP) preparative T3 OBD 5 μm (19 × 100) HPLC column, with acetonitrile (100%) as the mobile phase solvent eluting at 7.0 mL/min. This RP-HPLC purification was performed in order to separate isomers and co-eluting pigments, as well as any potential breakdown products. The samples were then dried under nitrogen gas, dissolved in 0.6 mL of deuterated chloroform (Cambridge Isotope Laboratories, Inc.), placed in glass NMR tubes, and stored in a −20 °C freezer until analysis. The concentration of each sample was determined by absorption spectroscopy using a Varian Cary 50 UV/visible spectrophotometer [14].

Nuclear magnetic resonance

The pigments corresponding to HPLC peaks 2, 3, 6, 7 and 8 were analyzed on a Bruker AVANCE III 400 MHz NMR instrument using one-dimensional ^{1}H spectroscopy. Typical parameters for 1D acquisition were: delay of 2 s, acquisition time of 4.56 s, pulse width of 5 μs (flip angle of 30°), number of scans of 64, and spectral width of 3.35 kHz. The pigment corresponding to HPLC peak 7 was analyzed additionally by two-dimensional (2D) nuclear overhauser effect spectroscopy (NOESY) using this instrument (same conditions as HPLC peak 4, below).

The ^{1}H NMR analysis of the pigment corresponding to HPLC peak 4 was carried out using one-dimensional, 2D H, H COSY (i.e., correlation spectroscopy), and 2D NOESY experiments. These measurements were done on a 500 MHz Bruker AVANCE instrument. The same parameters for COSY and NOESY were spectral width of 3.97 kHz; and 1024 × 256 data points. COSY was acquired with 1.5 s delay and 4 scans/increment whereas NOESY with 2.0 s delay,

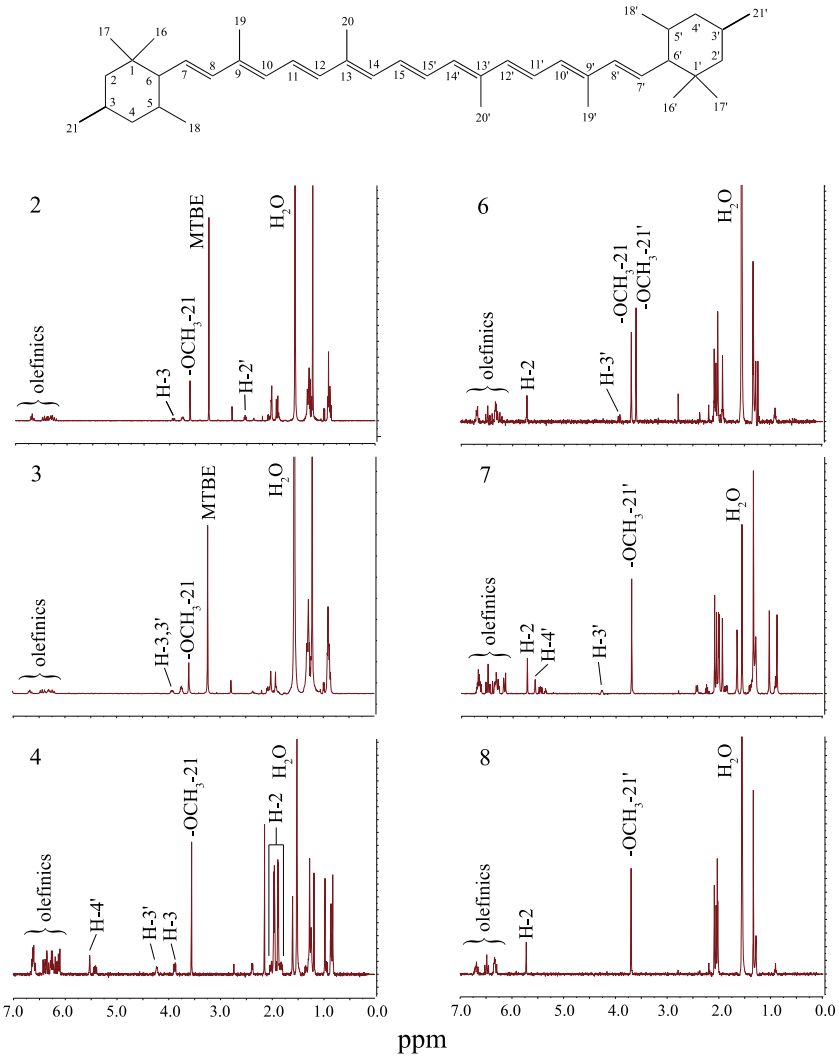


Fig. 2. ^{1}H NMR spectra of pigments corresponding to HPLC peaks 2, 3, 4, 6, 7, and 8. Resonances are labeled numerically according to the carotenoid skeleton shown above [7]. The carbons of the methoxy group are numbered 21 and 21′.

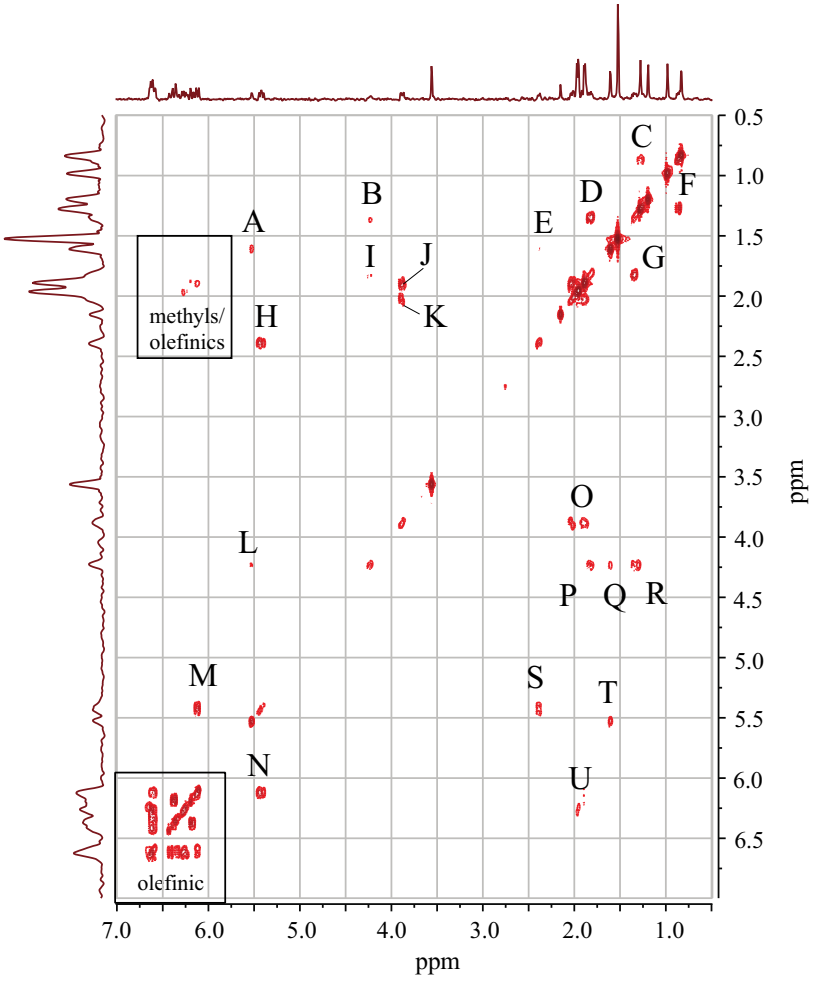
64 scans/increment, and with 1.0 s mixing time. The data were analyzed using the software program MestReNova v8.0.2 (© 2012 Mestrelab Research S. L.). Prior to integrating the resonances the baselines were corrected using Whittaker smoothing.

The pigments corresponding to HPLC peaks 1 and 5 were previously identified as canthaxanthin and astaxanthin based on chemical analysis, mass spectrometry, and co-chromatography with known standards (see Supplemental information). Therefore, no NMR analyses were conducted on these molecules.

Results

The preparative HPLC separation of the pigment extract from the two captive specimens of *X. punicea* revealed the same eight major pigments as two previously analyzed specimens, which included one wild-caught bird [2]. The chromatogram is shown in Fig. 1, along with the structures and IUPAC names proposed in the previous work [2]. In the interest of simplicity, each pigment will be referred to hereafter by the number corresponding to the HPLC peak as denoted in Fig. 1, as follows: pigment 2, 3-methoxy-β,β-carotene-4,4′-dione; pigment 3, 3,3′-dimethoxy-β,β-carotene-4,4′-dione; pigment 4, 3′-hydroxy-3-methoxy-β,ε-carotene-4-one; pigment 6, 3,3′-dimethoxy-2,3-didehydro-β,β-carotene-4,4′-dione; pigment 7, 3′-hydroxy-3-methoxy-2,3-didehydro-β,ε-carotene-4-one; and pigment 8, 3,3′-dimethoxy-2,3,2′,3′-tetradehydro-β,β-carotene-4,4′-dione.

The amount of the six novel pigments in each sample following RP-HPLC purification was as follows: pigment 2, 0.03 mg; pigment 3, 0.01 mg; pigment 4, 0.7 mg; pigment 6, 0.03 mg; pigment 7, 0.2 mg; and pigment 8, 0.03 mg.



A: H-4'/H-18'	H: H-6'/H-7'	O: H-2/H-3
B: H-2'/H-3'	I: H-2'/H-3'	P: H-2'/H-3'
C: H-16/H-17	J: H-2/H-3	Q: H-3'/H-18'
D: H-2'α/H-2'β	K: H-3/H-18	R: H-2'/H-3'
E: H-6'/ H-18'	L: H-3'/H-4'	S: H-6'/H-7'
F: H-16/H-17	M: H-7'/H-8'	T: H-4'/H-18'
G: H-2'α/H-2'β	N: H-7'/H-8'	U: H-7/H-18

Fig. 3. ^{1}H NMR COSY spectrum of pigment 4, 3′-hydroxy-3-methoxy-β,ε-carotene-4-one. Numbers correspond to the location of the proton on a carbon as denoted in Fig. 2.

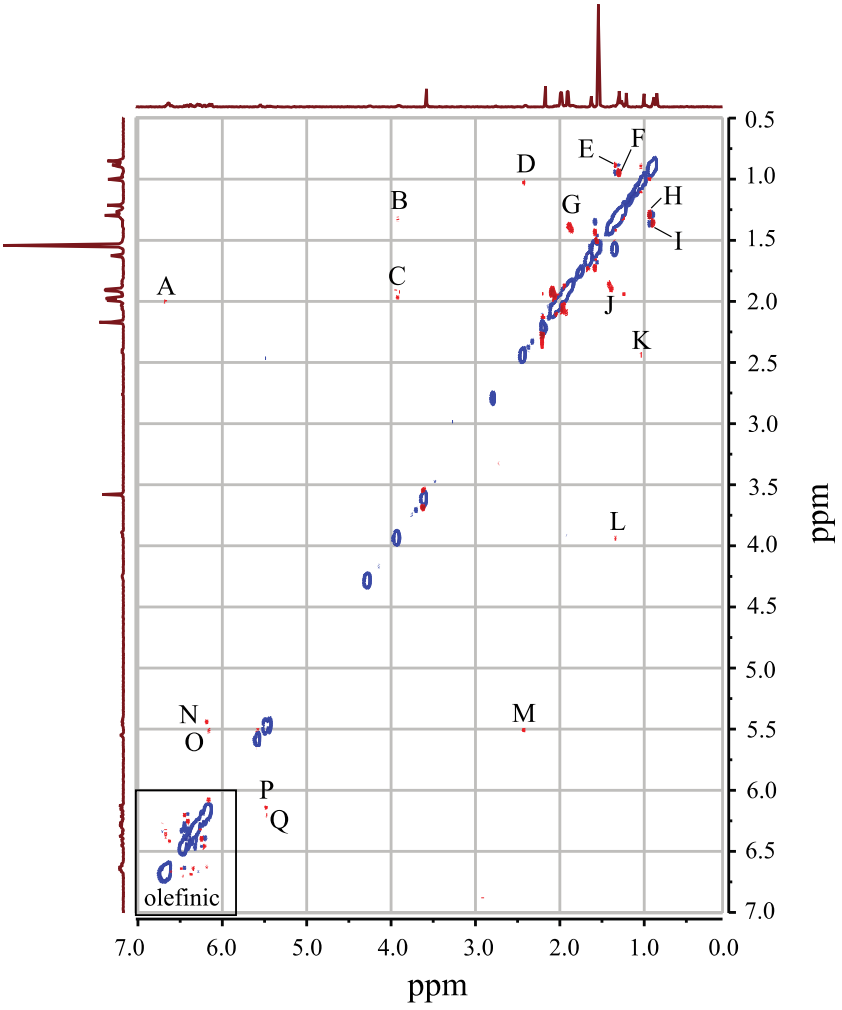
^{1}H NMR spectroscopy

The ^{1}H NMR spectra of the six pigments revealed several characteristic features of carotenoid spectra (Fig. 2). Note also that Fig. 2 displays a carotenoid skeleton numbered according to IUPAC nomenclature guidelines, with the carbons in the methoxyl groups attached to C3 and C3′ labeled as C21 and C21′, respectively. These numbers are used throughout the manuscript to reference the location of protons associated with specific chemical shifts.

The spectra of all six pigments revealed a number of resonances in the region of 6.0–7.0 ppm, which represent different olefinic protons along the π-electron conjugated chain. The integrated area of this region corresponds to 12 protons, and all six pigments integrated to within 20% of this amount (See Table S1 in Supplemental information).

The resonances corresponding to the functional groups in the rings are of particular interest, because these are associated with the novel structural features of these carotenoids; viz. the C3 (C3′) methoxyl group(s) appearing in pigments 2, 3, 4, 6, 7 and 8 (Fig. 1) and C2, C3 (C2′, C3′) double bonds appearing in pigments 6, 7 and 8 (Fig. 1). Resonances that are significant to the identification of these functional groups are labeled in Fig. 3. Resonances of the solvents used for the samples were observed at 1.56 ppm (water) and 3.22 ppm (MTBE) [15].

The resonances of the protons associated with the methoxyl groups are observed at 3.6 ppm in the spectra of pigments 2, 3, 4 and 6, and at 3.7 ppm in the spectra of pigments 6, 7 and 8 (Fig. 2). Pigments 2, 3, 4 and 6 were proposed to contain at least one 3-methoxy-4-keto-β-end-group, while pigments 6, 7, and 8 each contain at least one 2,3-dehydro-3-methoxyl-4-keto-β-end-group. Integration of these resonances (See Table S1 in Supplemental information) shows that that pigments 2, 4 and 7 each contain only a single methoxyl group, whereas pigments 3 and 8 contain two equivalent methoxyl groups. Pigment 6 contains two inequivalent methoxyl groups.



A: H-11/H-20
B: H-3/H-16,17
C: H-3/H-18
D: H-6'/H-16'
E: H2'β/17'
F: H-16/H-17
G: H-2'α/H-2'β
H: H-16/H-17
I: H2'α/H-17'
J: H-2'α/H-3'
K: H-6'/H-16'
L: H-3/16,17
M: H-4'/H-6'
N: H-7'/H-8'
O: H-4'/H-8'
P: H-4'/H-8'
Q: H-7'/H-8'

Fig. 4. NOESY spectrum of pigment 4, 3′-hydroxy-3-methoxy-β,ε-carotene-4-one. Positive correlations are shown in red, and negative correlations are shown in blue. Numbers correspond to the location of the proton on a carbon as denoted in Fig. 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

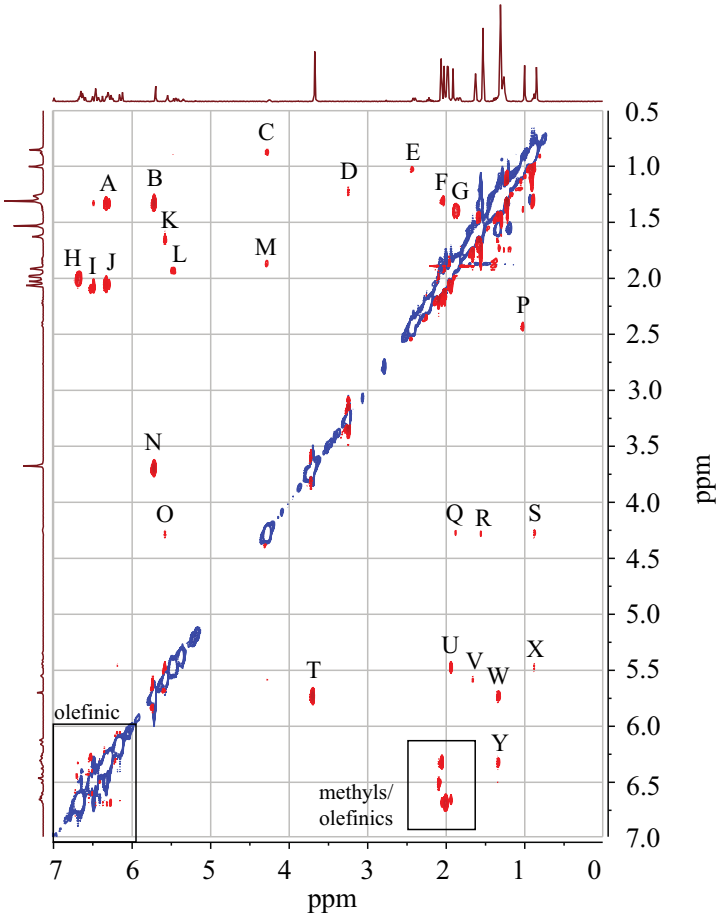
The ^{1}H NMR spectra of pigments 2, 3, 4, and 6 all display doublets of doublets at 3.93 ppm with a J-coupling frequency of 13 Hz. These features are consistent with the proton adjacent to a methoxyl group on C3 in pigments 2, 4, and 6, and on C3′ in pigments 3 and 6. The resonance signal at 3.93 ppm is not observed in the NMR spectrum recorded for pigments 7 and 8, consistent with dehydrogenation of C2 (and C2′ in peak 8). The ^{1}H NMR spectra of pigments 6, 7 and 8 all display a singlet at 5.72 ppm which is consistent with an olefinic proton on C2 for pigments 6 and 7, and the equivalent C2′-position for HPLC peak 8. In addition, the spectrum of pigment 4 displays two doublets of doublets at 1.83 and 2.03 ppm with a J-coupling frequency of 13 Hz, which is consistent with the chemical shifts of the protons on carbon C2 reported for astaxanthin [9]. This was also the value predicted for the proposed structure of pigment 4 using MestreNova software.

One half of the proposed structure for pigment 2 is identical to the well-studied symmetric carotenoid, canthaxanthin. Indeed, the spectrum of pigment 2 shows a triplet at 2.53 ppm which is not present in the NMR spectrum of any of the other pigments. This is consistent with two equivalent protons on carbon C3′, as reported in the ^{1}H NMR spectrum of canthaxanthin in the same solvent [9].

The previously proposed structures for pigments 4 and 7 also contain a 3-hydroxy-ε-ring end-group common to many naturally-occurring carotenoids including lutein. Several of the resonances observed in the ^{1}H NMR spectra of pigments 4 and 7 are consistent with those reported for lutein [9]. These include: 1.63 ppm, which is consistent with the methyl group at C18′; 1.37 and 1.85 ppm, which are consistent with the α- and β-protons on C3′; 4.25 ppm, which is consistent with the proton on C3′ adjacent to the hydroxyl group; and 5.55 ppm, which is associated with the proton on carbon C4′.

Correlated two-dimensional spectroscopy (COSY)

The COSY spectrum for pigment 4 is shown in Fig. 3 and supports the proposed structure of this molecule shown in Fig. 1. Most notably, the spectrum shown in Fig. 3 reveals a 3-methoxy-4-keto-β-ring from cross peaks between H-2α and H-2β with H-3 (J and O), and coupling between the protons associated with the two methyl groups, C16 and C17 (C and F). The spectrum also supports the structure of the 3-hydroxy-ε-ring, with cross peaks between: H-2′α-and H-2′β (D and G); the H-2′ protons with H-3′ (B and I); H-3′ with H-4′ (L) and H-3′ with H-18′ (Q); H-4′ with H-18′



A: H-7/H-17	F: H-16/H-19	K: H-4'/H-18'	P: H-6'/H-17'	U: H-7'/H-19'
B: H-2/H-17	G: H-2'α/H-2'β	L: H-7'/H-19'	Q: H-2'α/H-3'	V: H-4'/H-18'
C: H-3'/H-16'	H: H-11/H-20	M: H-2'/H-3'	R: H-3'/H-18'	W: H-2/H-16
D: MTBE	I: H-12/H-20	N: H-2/H-21	S: H-3'/H-16'	X: H-7'/H-16'
E: H-6'/H-17'	J: H-7/H-19	O: H-3'/H-4'	T: H-2/H-21	Y: H-7/H-16

Fig. 5. NOESY spectrum of pigment 7, 3′-hydroxy-3-methoxy-2,3-didehydro-β,ε-carotene-4-one. Positive correlations are shown in red, and negative correlations are shown in blue. Numbers correspond to the location of the proton on a carbon as denoted in Fig. 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(A and T); H-6′ with H-7′ (H and S); H-6′ with the methyl group, H-18′ (E); and the olefinic proton on H-7′ with the olefinic proton on H-8′ (M and N).

Nuclear overhauser effect spectroscopy (NOESY)

The NOESY spectra of pigments 4 and 7 are shown in Figs. 4 and 5, respectively. Both spectra show a number of spatial correlations supporting the proposed structures given in Fig. 1.

The spectrum of HPLC peak 4 (Fig. 4) shows correlations between: H-3 with the protons of the methyl groups attached to the β-ring, C16 and C17 (B and L); and H-3 and the protons on the methyl group C18 (C). Several NOE correlations were observed in the ε-ring, including those between: H-2′α and H-2′β (G); H-2′ and H-17′ (E and I); H-2′α and H-3′ (J); H-4′ and H-6′ (M); H-6′ and H-16′ (D and K); and H-7′ and H-8′ (N and Q).

The spectrum of pigment 7 (Fig. 5) shows NOE correlations between: the lone proton in the C2-position with those of the protons on the methyl groups on C16 and C17 (B and W). Most notably, a strong correlation exists between H-2 and the protons of the methoxyl group, C21 (N and T). Numerous correlations are observed between the protons of the ε-ring, including those between the H-2′α and H-2′β with one another (G) and also with the H-3′ (M and Q); between H-3′ and H-4′ (O), H-3′ and H-16′ (C and S), and H-3′ and H-18′ (R); H-4′ and H-18′ (K and V); H-6′ and H-17′ (E and P); and H-7′ with H-16′ (X) and H-19′ (L).

Discussion

It is a fortunate circumstance that the male Pompadour Cotinga deposits a number of metabolic products in its plumage, as this has afforded an opportunity to examine systematically the complex metabolic transformations that dietary carotenoids undergo in this species. Previous assignments of the structures of these carotenoids, including several novel ones, were based on HPLC, mass spectrometry, chemical analysis and limited NMR characterization [2]. In the present work, much more convincing support for the proposed structural identifications lies in the observation of chemical shifts associated with the methoxyl group, the proton on C3 adjacent to the methoxyl group, and the proton(s) on the neighboring carbon, C2.

The present NMR data show that the chemical shift of the protons associated with the methoxyl group on carbons C3 (and also C3′ in pigment 3) is observed at 3.6 ppm in the spectra of pigments 2, 3, and 4, which are proposed to have at least one methoxyl group adjacent to the C2, C3 single bond. In pigments 7 and 8, which are both proposed to contain at least one methoxyl group adjacent to the C2, C3 double bond, a chemical shift is observed at 3.7 ppm. HPLC peak 6 contains two inequivalent methoxyl groups, one adjacent to a C2, C3 single bond and the other adjacent to a C2, C3 double bond, and accordingly shows resonance at both 3.6 and 3.7 ppm. This result suggests that didehydrogenation of the C2, C3 bond causes a slight change in the chemical shift associated with the protons of the methoxyl group. Integration of these shifts using the MestreNova software asserts that pigments 2, 4 and 7 each contain a single methoxyl group, pigments 3 and 8 contain two equivalent methoxyl groups (i.e. both methoxyls are adjacent to the same type of bond), and pigment 6 contains two inequivalent methoxyl groups (i.e. each methoxyl is adjacent to a different type of bond).

The differences in the chemical shift of the proton associated with C3 and C3′ also support the proposed identifications. The assignment of the 3.93 ppm resonance to the proton in the C3 position for pigments 2, 3, 4 and 6 is confirmed by the COSY interactions with H-2α and H-2β (J and O in Fig. 3, Fig. 6C). In the COSY spectrum of pigment 2, the 3.93 ppm shift integrates to ~1, indicating that only one β-ring contains a methoxyl group in the C3 position. The 4-keto-β-ring, which does not contain a methoxyl group, displays a chemical shift at 2.53 ppm, and this is consistent with previous ^{1}H NMR data for canthaxanthin measured in the same solvent (Fig. 6A) [9]. In the spectrum of pigment 3 the chemical shift is observed at 3.93 ppm with an integration of ~2, indicating that both β-rings are functionalized with methoxyl groups in the C3 and C3′ positions. The structure for pigment 6 can be derived from the structure of pigment 3 by a C2, C3 didehydrogenation on one side of the molecule, and the structure for pigment 8 can be further derived by the same transformation occurring on the other side of the molecule. Consistent with this proposal, the chemical shift at 3.93 ppm is observed in the spectrum of pigment 6 with an integration value of ~1, indicating that one of the β-rings has undergone this transformation. The resonance signal disappears completely in the spectrum of pigment 8 indicating that both rings have been transformed. Similarly, the spectrum of pigment 4 contains a singlet at 3.93 ppm with an integration of ~1. This signal disappears in the spectrum of pigment 7 indicating that the sole β-ring has undergone C2, C3 didehydrogenation.

The chemical shifts associated with the protons on C2 are interdependent with those on C3. The spectra of the pigments having a saturated bond between C2 and C3 show two separate resonances at 1.88 and 2.05 ppm, indicating the α- and β-protons (Fig. 6C). The

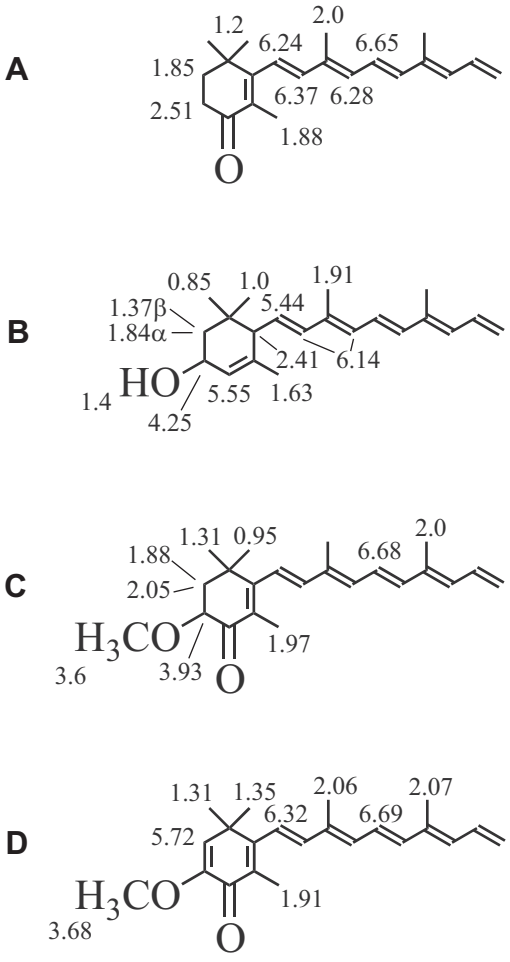


Fig. 6. Chemical structures of the four end groups that make up the six novel chemical structures: (A) 4-keto-β-ring, as reported in [9]; (B) 3-hydroxy-ε-ring, as reported in [9]; (C) 3-methoxy-4-keto-β-ring; (D) 2,3-didehydro-3-methoxy-4-keto-β-ring.

Table 1
Chemical shifts associated with specific carbons in the ^{1}H NMR spectra. Multiplet status and J-coupling frequencies are noted parenthetically when applicable. Olefinic protons and those associated with methyl groups for which shifts could not conclusively be determined simply noted as either "olefin" or "methyl".

Carbon No.	Pigment 2	Pigment 3	Pigment 4	Pigment 6	Pigment 7	Pigment 8
1						
2	2.07 (dd, 13)	2.07 (dd, 12.6)	1.88, 2.05	5.72	5.70	5.72
3	3.93 (dd, 13)	3.93 (dd, 13)	3.90 (dd, 13)			
4						
5						
6						
7	Olefin	Olefin	Olefin	Olefin	6.32	Olefin
8	Olefin	Olefin	Olefin	Olefin	Olefin	Olefin
9						
10	Olefin	Olefin	Olefin	Olefin	Olefin	Olefin
11	Olefin	Olefin	Olefin	Olefin	Olefin	Olefin
12	Olefin	Olefin	Olefin	Olefin	Olefin	Olefin
13						
14	Olefin	Olefin	Olefin	Olefin	Olefin	Olefin
15	Olefin	Olefin	Olefin	Olefin	Olefin	Olefin
16	Methyl	Methyl	1.31	Methyl	1.31	Methyl
17	Methyl	Methyl	0.95	Methyl	1.35	Methyl
18	1.92	1.92	1.97	Methyl	1.91	Methyl
19	Methyl	Methyl	Methyl	Methyl	Methyl	Methyl
20	Methyl	Methyl	Methyl	Methyl	Methyl	Methyl
21	3.6	3.6	3.6	3.7	3.68	3.7
1′						
2′	1.87 (d,6)	2.07	1.37 (dd, 13)	2.07	1.85 (dd, 13)	5.72
3′	2.53 (t,7)	3.93 (dd, 13)	4.25	3.93 (dd, 13)	4.25	
4′			5.55		5.55	
5′						
6′			2.41 (d, 9)		2.41 (d, 9)	
7′	Olefin	Olefin	5.44 (dd, 16)	Olefin	5.46	Olefin
8′	Olefin	Olefin	6.19	Olefin	6.19	Olefin
9′						
10′	Olefin	Olefin	Olefin	Olefin	Olefin	Olefin
11′	Olefin	Olefin	Olefin	Olefin	Olefin	Olefin
12′	Olefin	Olefin	Olefin	Olefin	Olefin	Olefin
13′						
14′	Olefin	Olefin	Olefin	Olefin	Olefin	Olefin
15′	Olefin	Olefin	Olefin	Olefin	Olefin	Olefin
16′	1.2	Methyl	0.85	Methyl	0.87	Methyl
17′	1.2	Methyl	1.0	Methyl	1.0	Methyl
18′	1.90	1.92	1.63	1.92	1.63	1.91
19′	Methyl	Methyl	2.17	Methyl	1.91	Methyl
20′	Methyl	Methyl	Methyl	Methyl	Methyl	Methyl
21′		3.6 (OCH_3)	1.4 (OH)	3.6 (OCH_3)	1.4 (OH)	3.7 (OCH_3)

assignment of these shifts were made based on interactions observed in the COSY spectrum (Fig. 3) and NOESY spectrum (Fig. 4) of pigments 4. In pigments 2 and 3, the H-2 (2′) signal is observed at 2.07 ppm as a doublet of doublets with a J-coupling frequency of 13 Hz and an integration of ~2. While these protons would be expected to produce two separate signals consistent with those observed in pigment 4, it is likely that the 400 MHz instrument was unable to resolve the signals due to the substantially lower concentration of these two samples. The shift of the C2 proton in the structures having a double bond between C2 and C3, which include pigments 6, 7 and 8, was found to be 5.72 ppm based on NOESY data obtained for pigment 7 (Fig. 5). This chemical shift was indeed observed in these three pigments, and the integration values confirm that pigments 6 and 7 each have one proton consistent with a 2,3-didehydro-3-methoxy feature on the ring. Pigment 8 has two such protons because the same feature exists symmetrically on both rings of that molecule.

Conclusions

The present work confirms the chemical structures of the six novel methoxy-carotenoids proposed previously [2]. The four different end groups needed to construct these six structures are shown in Fig. 6 along with their characteristic chemical shifts. The resonance positions of the protons from the 4-keto-β-ring (Fig. 6A) and 3-hydroxy-ε-ring (Fig. 6B) have been reported previously [9], but those for the 3-methoxy-4-keto-β-ring (Fig. 6C) and 2,3-didehydro-3-methoxy-4-keto-β-ring (Fig. 6D) are reported here for the first time. The six novel pigments are formed by combining two of these end groups as follows: pigment 2, A and C; pigment 3, C and C; pigment 4, B and C; pigment 6, C and D; pigment 7, B and D; and pigment 8, D and D. A summary of the chemical shifts, multiplet status and J-coupling frequencies obtained from the spectra is presented in Table 1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.abb.2013.08.012.

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