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Fluorinated Analogs of the Carotenoprotein, α-Crustacyanin

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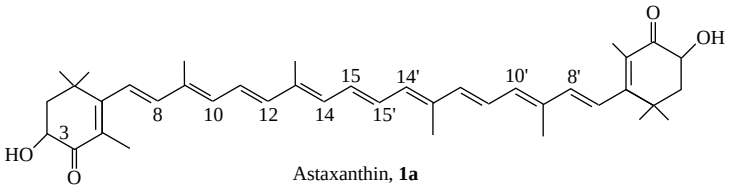
Four analogs of the carotenoprotein α-crustacyanin have been prepared by reconstitution with the all-*trans* isomer of four new carotenoids (10-F, 10,10′-F_2 and 14-F-astaxanthins, and 10′-F-adonirubin). All four blue carotenoproteins exhibit absorption spectra similar to that of the natural α-crustacyanin with λ_{max} in the range of 613–625 nm. Different rates of pigment formation and yields were noted. F NMR spectra of pigments derived from the three 10F carotenoids have been recorded. Four *cis* isomers of 14-F-astaxanthin and one of 10′-F-adonirubin were also isolated.

INTRODUCTION

The vivid red color and other multiple colorful forms that the carotenoid astaxanthin, **1a,** adopts upon interaction with many proteins in Nature make it an important natural and artificial pigment (*1*). In recent years, considerable effort has been devoted toward elucidating the structural properties of the crustacyanins (*2*), i.e., astaxanthin-proteins found in the carapace of crustaceans, and the nature of the nonbonded interaction(s) that leads to the unusually large spectral shift of the absorption maximum from 478 nm (in acetone) for the free carotenoid to 632 nm for α-crustacyanin, the predominant octameric aggregated form of β-crustacyanin. A comprehensive overview (*3*) of important recent developments, specifically in areas of astaxanthin analogs, of the use of resonance Raman spectroscopy (*4*), protein structural analyses (*5*), and ^{13}C NMR techniques (*6*) is available.

The use of α-crustacyanin analogs derived from synthetic astaxanthin (**1a**) analogs has led to much information on specificity of the binding site of the carotenoprotein (*3*). We are now extending this effort to include the use of the fluorine substituent. Formation of four new carotenoprotein analogs from carotenoids **1b–d** (*7*) and **2,** and their spectroscopic characteristics are described in this paper.



Astaxanthin, **1a**

1b, 10-F
1c, 10, 10'-F_2
1d, 14-F

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TABLE 1
Partial H NMR (in $CDCl_3$ 500 MHz) Data, Chemical Shift, and Coupling Constants of the All-*trans* Isomer of Astaxanthin and UV-Vis Absorption Maxima of Fluorinated Astaxanthins

Compounds	H-7,7′	H-8,8′	H-10,10′	H-11,11′	H-12,12′	H-14,14′	H-15,15′
Astaxanthin	6.24	6.44	6.31	6.70	6.46	6.31	6.70
10-fluoro, **1b**	6.22	7.00	−120.26[b]	6.41	6.76	6.41	6.68
	6.21	6.43	6.31	6.68	6.45	6.31	6.68
14-fluoro,	6.26	6.44	6.38	6.75	6.95	−121.69[b]	6.46
1d[a]	6.26	6.44	6.32	6.70	6.47	6.33	6.98
	6.22	7.01	−120.26[b]	6.39	6.77	6.41	6.71
10,10′-F_2, **1c**	6.28	6.92	−121.12[b]	6.52	6.77	6.45	7.03
C_{25}-analog **3**					9.46	6.96	6.72
	6.24	6.96	−121.02[b]	6.40	6.67	6.40	6.67
C_{40}-analog **2**	6.22	6.34	6.30	6.67	6.45	6.30	6.67

	$J_{7,8}$	$J_{10,11}$	$J_{11,12}$	$J_{14,15}$	$J_{15,15'}$	λ_{max}[d]
Astaxanthin	16.4	11.4	15.0	11.5	14.0	478.4
10-fluoro	16.3	[c]	15.0	—	—	475.2
	15.9		15.0			
14-fluoro	16.0	11.8	14.6	28.6	14.7	473.6
	16.0	11.3	15.1	11.4		
10,10′-F_2	16.2	27.1	15.6	—		472.8
C_{25}-analog **3**	16.3	27.0	15.4	11.6	14.3	417.0
		11.5				
C_{40}-analog **2**	16.4	27.1	15.7	[c]	[c]	469.0
	16.3	[c]	14.8	[c]	[c]	

[a] In CD_2Cl_2.
[b] Chemical shifts for F NMR.
[c] Broad peak, not sufficiently well resolved for measurement.
[d] nm, in acetone.

EXPERIMENTAL DETAILS

Preparation and purification of astaxanthin analogs. Procedure for preparation of 10′-F-3-hydroxycanthaxanthin (10′-F-adonirubin), **2,** is described below as a representative example. Spectral data of the fluorinated astaxanthin analogs **1b–d** along with those of compound **2** are listed in Table 1. Before binding studies, each all-*trans* isomer was purified by preparative hplc using the following conditions: 5μ, 10 mm C_{30} column from Y M Corp.; solvent, $CH_3CN:EtOAc:MeOH = 3:1:1$; flow rate, 1 ml/min, detecting wavelength, 510 nm. For 10-F and 10,10′-F_2-astaxanthins, the all-*trans* isomer was the major isomer in the mixture. For 14-F-astaxanthin, because the middle C10 fragment was prepared as a mixture of isomers, in addition to the all-*trans* isomer, four *cis* isomers were present in sufficient amounts in the synthetic mixture for isolation. Their NMR data (Table 2) are consistent with (in the order of appearance on the chromatogram) those of the 13,13′-*dicis*, 13′-*cis*, 13-*cis*, all-*trans*, and 9′-*cis* isomers.

TABLE 2
Partial H NMR (in $CDCl_3$ 500 MHz) Data, Chemical Shift, and Coupling Constants and UV-Vis Absorption Maxima of Four *cis* Isomer of 14-F-Astaxanthin and 9-*cis*-**2** and **3**

Isomer	H-7,7′	H-8,8′	H-10,10′	H-11,11′	H-12,12′	H-14,14′	H-15,15′
13-*cis* (all*E*)	6.26	6.43	6.37	6.74	7.03	−115.9[b]	6.56
	6.28	6.44	6.34	6.69	6.73	6.18	7.08
9′-*cis* (*9′Z, 13Z*)	6.27	6.44	6.37	6.70	6.95	−121.6[b]	6.48
	6.27	6.44	6.26	6.80	6.39	6.31	6.96
13′-*cis* (*13Z, 13′Z*)	6.27	6.44	6.37	6.75	7.04	−121.6[b]	6.40
	6.28	6.44	6.37	6.69	6.95	6.19	7.13
13,13′-*dicis* (*13′Z*)	6.26	6.43	6.37	6.74	7.03	−115.9[b]	6.56
	6.28	6.44	6.34	6.69	6.73	6.18	7.08
9-*cis*-(all*E*)-**3**[a]	6.28	6.62	−114.8[b]	6.56	6.71	6.43	7.02
					9.47	6.97	6.75
9-*cis*-(*allE*)-**2**[a]	6.23	6.72	−114.5[b]	6.40	6.67	6.40	6.66
	6.21	6.42	6.30	6.66	6.44	6.30	6.66

	$J_{7,8}$	$J_{10,11}$	$J_{11,12}$	$J_{14,15}$	$J_{15,15'}$	λ_{max}[d]
13-*cis*	16.2	10.5	14.6	29.3	14.6	460
	16.2	11.7	15.0	11.7	—	
9′-*cis*	16.4	11.3	15.2	28.8	14.8	470
	16.4	11.3	14.8	11.7	—	
13′-*cis*	16.0	11.3	15.0	28.0	14.6	462
	16.0	11.3	15.0	12.1	—	
13,13′-*dicis*	16.1	11.3	14.7	29.4	14.9	—
	16.4	10.6	14.9	12.2	—	
9-*cis*-**3**[a]	15.9	26.7	15.4	11.8	14.6	401
				11.5		
9-*cis*-**2**[a]	15.8	26.7	14.7	[c]	[c]	458
	16.1	[c]	15.1	[c]		

[a] 300 MHz spectra.
[b] Chemical shift for F NMR.
[c] Not sufficiently well resolved for measurement.
[d] In hexane.

10-F-12′-apocanthaxanthinal (C_{25}), **3.** To a stirred solution of all-*trans*-4-keto-10F-*β*-ionylideneacetaldehyde, the 4-keto-10-F-C_{15}-CHO, (8 mg, 0.032 mmol), and 7-oxo-2,4,6-octatrien-1-*yl*-triphenylphosphonium bromide, the C_{10}-Wittig salt, (15.75 mg, 0.032 mmol, in 5 ml dichloromethane) at room temperature under an argon atmosphere was added a suspension of 60% sodium hydride (1.56 g, 0.039 mmol) in dry CH_2Cl_2. After being cooled to 0°C, the reaction mixture was quenched by the slow addition of water (5 ml). Isolation of the product by column chromatography over silica gel gave the 10F-canthaxanthinal **3** in the form of red crystals (7 mg, 57% yield). UV and NMR data are listed in Table 1. HR-MS: Calculated for $C_{25}H_{31}FO_2$: 382.2309. Found, 382.2318. Crystals of compound **3** were obtained by the procedure of diffusion crystallization by allowing a concentrated hexane solution of **3** (in a small beaker) to stand under an atmosphere of methanol (in a closed container).

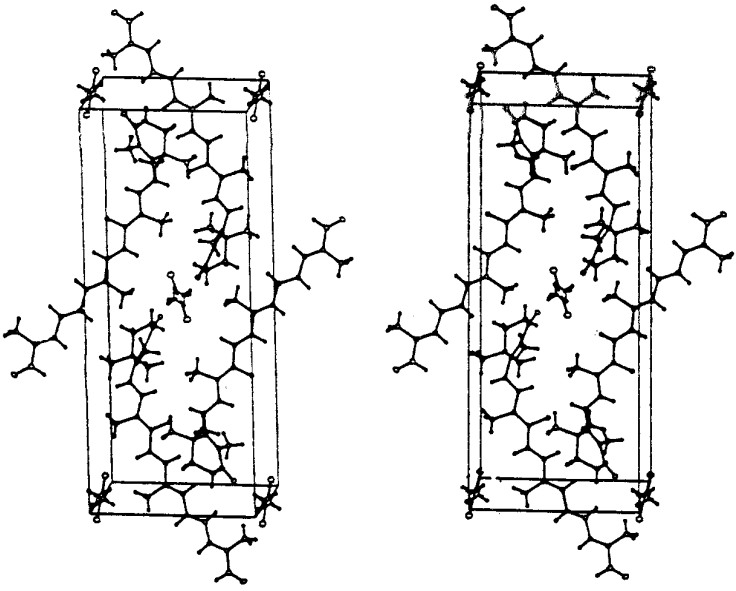

FIG. 1. Crystal packing diagram of 10-fluoro-4-oxo-12′apo-β-caroten-12′-al **3** · $\frac{1}{2}CH_3OH$.

10′-F-Adonirubin **2.** Under dim light, to a solution of apocanthaxanthinal **3** (9 mg, 0.024 mmol) and 3-hydroxy-4-keto-C_{15}-triphenylphosphonium bromide salt (17 mg, 0.03 mmol) in methanol (2 ml) at room temperature under Argon was added a methanol solution of sodium methoxide (0.024 mmol). After complete disappearance of the starting material (by TLC), the reaction was diluted with water, extracted with CH_2Cl_2. Evaporation of the solvent and chromatography over silica gel (30% ethyl acetate/hexane) gave the desired C_{40}-carotenoid **2** (12 mg, 85% yield). Its spectral data are listed in Table 1. HR-MS: Calculated for $C_{40}H_{51}FO_3$: 598.3824. Found: 598.3836.

Starting with 9-*cis*-10-F-C_{15}-aldehyde, 9-*cis*-**3** was also prepared by the similar Wittig coupling reaction. Subsequent reaction with the 3-hydroxy-4-keto-C_{15}-Wittig salt yielded 9-*cis* **2.** Spectral data of these isomers are also listed in Table 2 for comparison.

Crystal structure of **3.** In determining the crystal structure of **3,** (*8*) X-ray diffraction data were collected at 102 K using an Enraf-Nonius CAD4 MACH diffractometer (Mo Kα = 0.7107 Å). A θ-2θ scan mode to $2\theta_{max}$ = 48°; crystal size and shape-sphere, r = 0.42 mm. Of the 3752 unique reflections collected, 2117 had $I > 3.0\ \sigma(I)$. Diffraction intensities were corrected for Lorentz polarization and absorption (Ψ scans, transmission range 0.91–1.0). The structure was determined using the direct method programs in TeXsan and refined by full-matrix least squares procedures. H atom positions were determined by a ΔF synthesis. A stereoview of the crystal structure is shown in Fig. 1.

Formation of α*-crustacyanin pigment analog.* The protein extraction and pigment reconstitution procedures were essentially those described by Zagalsky (*9*). However, in our case, carapace from *Homarus americanus* lobsters was used instead

of *H. gammarus* lobsters. On the average, from six lobsters ~65 mg of α-crustacyanin were obtained. All chromatographic separation and pigment reconstitution procedures were conducted at 2–4°C.

The reconstitution procedure was essentially the same as that described by Zagalsky. Briefly, to 2 mg of α-crustacyanin in 2 ml of 50 mM sodium phosphate buffer, pH 7.0, in a test tube was added rapidly, with stirring, 3 ml of acetone followed by 10 ml of diethyl ether. After quick mixing, the orange layer containing extracted astaxanthin was removed by a pipette. Extraction with ether was repeated until no more carotenoid could be extracted. The remaining volatile solvent was removed by a stream of nitrogen. To this preparation, 25% molar excess of the carotenoid analog in 1.5 ml of acetone was added quickly with stirring, followed by 10 ml of 50 mM sodium phosphate buffer. The solution was dialyzed overnight at 4°C against 5 liters of the same buffer. The product was then collected and excess unbound carotenoid was removed by centrifugation. The reconstituted product was purified and separated on a short DE52 column with a step elution of 0–1 M NaCl in 50 mM sodium phosphate buffer.

For the F NMR sample, ~15 mg of an α-crustacyanin analog was used. The sample was concentrated to a total volume of ~1 ml using stirred Omega cell and filtration centrifugation. The NMR sample contained 50% D_2O and was of approximately 10^{-4} M concentration. A 10 mm Shigemi NMR tube was used, and the spectra were recorded on a GE NMC 500 spectrometer. Some of the spectrometer parameters were: 15-ms pulse width; 1.3-s delay time; 30,000 scans, LB 80-300 Hz; probe temperature, 10°C.

UV and CD spectra were recorded respectively on a P.E. λ-19 and a JASCO-600 spectrometer.

RESULTS AND DISCUSSION

The four fluorinated astaxanthin analogs (optically inactive) were prepared via sequences of reactions parallel to the $C_{15} + C_{10} + C_{15}$ route established for the synthesis of parent astaxanthin (*10*). The specific fluorinated intermediates used in the preparation of 10-F, 14-F, and 10,10′-F_2-astaxanthins (**1b–d**) were described previously in a preliminary paper (*7*) and will not be repeated here. Instead, below is shown, as a representative example, the coupling reactions leading to the new C_{40}-analog **2** (10′-fluoroadonirubin) via the intermediate 10-F-4-oxo-12′-apo-β-carotenal (C_{25}) **3:**