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# Study of α-Crustacyanin Utilizing Halogenated Canthaxanthins

**Jin Liu,*,† Nicole L. Shelton,† and Robert S. H. Liu‡**

*Department of Chemistry, Murray State University, Murray, Kentucky 42071, and Department of Chemistry, University of Hawaii, Honolulu, Hawaii 96822*

*jin.liu@murraystate.edu*

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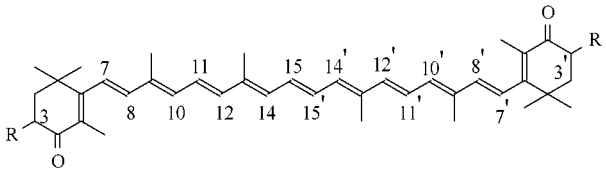
## ABSTRACT

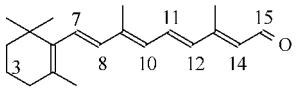


R = OH; Astaxanthin
R = F, Cl, Br, I; Halogenated canthaxanthins

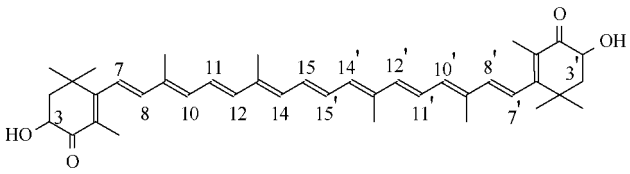
**The preparations and spectroscopic characteristics of five all-trans halogenated canthaxanthins are described in this letter. The air/light-sensitive halogenated canthaxanthins were used to study α-crustacyanin, a blue astaxanthin–protein complex, which is isolated from the carapace of the lobster. Steric and electronegative effects of the halogen substituents on the noncovalent interaction between astaxanthin and the protein in α-crustacyanin were observed.**

Bacteriorhodopsin,[1] a retinal−protein complex, plays several important biological roles. In recent years, considerable effort has been concentrated on the understanding of astaxanthin−protein complexes. The well-known astaxanthin−protein complexes, crustacyanins, are isolated from the carapace of the lobster *Homarus gammarus*.[2]

α-Crustacyanin and γ-crustacyanin are two isolated crustacyanins that have a deep blue color. UV−vis absorptions of α-crustacyanin and γ-crustacyanin are 632 and 625 nm, respectively. The unusual characteristic of α-crustacyanin and γ-crustacyanin is the large bathochromic shift (α-crustacyanin, 5100 $cm^{-1}$; γ-crustacyanin, 5050 $cm^{-1}$) caused by the noncovalent interaction between astaxanthin and the proteins.[3] Because of a lack of the crystal structures of



all-*trans*-Retinal



all-*trans*-Astaxanthin

crustacyanins, some techniques such as solid-state $^{13}C$ NMR,[4] Stark spectroscopy,[5] and $^{19}F$ NMR[6] have been used to study the structure of α-crustacyanin to explain its large bathochromic shift. Recombination studies between the colorless

* Corresponding author. Phone: 270-762-6626. Fax: 270-762-6474.
† Murray State University.
‡ University of Hawaii.

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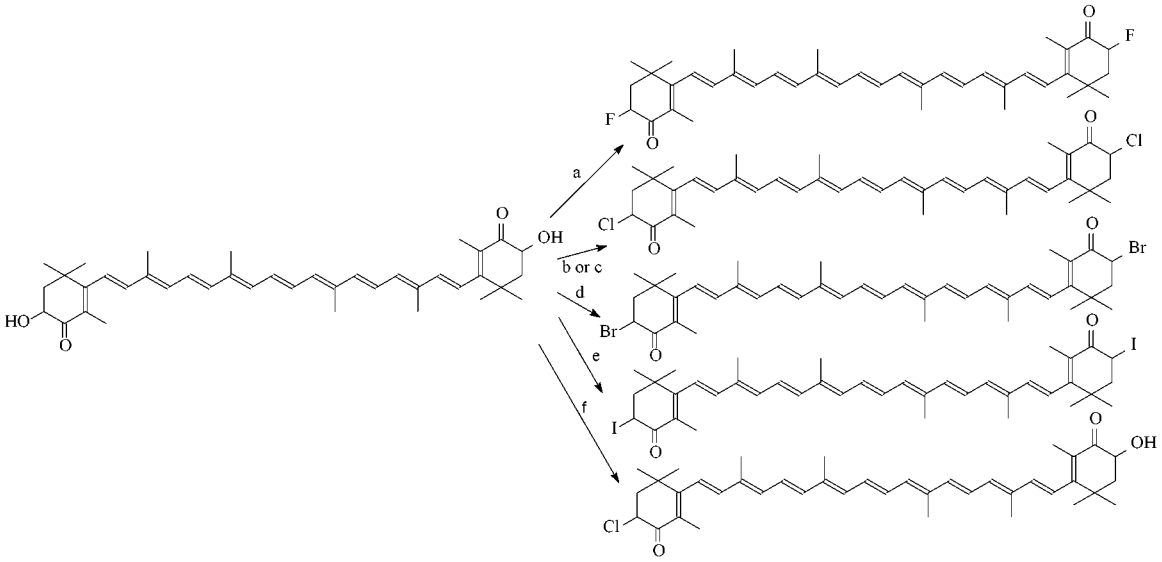
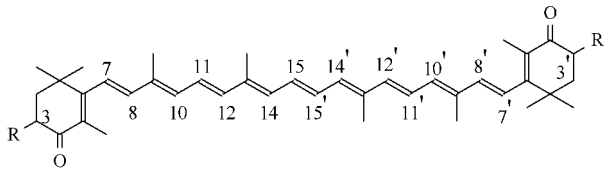
**Scheme 1**[a]




[a] Reagents and conditions: (a) $(CF_3SO_2)_2O$, pyridine, $CH_2Cl_2$, then *n*-$Bu_4NF$ in THF, 49% yield; (b) $(CF_3SO_2)_2O$, pyridine, $CH_2Cl_2$, then *n*-$Bu_4NCl$ in THF, 50% yield; (c) *N*,*N*-dimethyl-*N*-1-chloro-2-methylpropenylamine, $CH_2Cl_2$, 95% yield; (d) $(CF_3SO_2)_2O$, pyridine, $CH_2Cl_2$, then NaBr in acetone, 97% yield; (e) $(CF_3SO_2)_2O$, pyridine, $CH_2Cl_2$, then NaI in acetone, 98% yield; (f) (i) $CH_3COCl$, pyridine, $CH_2Cl_2$, (ii) *N*,*N*-dimethyl-*N*-1-chloro-2-methylpropenylamine, $CH_2Cl_2$, (iii) $NaOCH_3$, $CH_3OH$, 61% yield.

apoprotein of crustacyanin and various carotenoids have revealed that the 4,4′-keto groups are essential for reconstituting the blue carotenoprotein.[3] The unique solid-state $^{13}C$ NMR study of α-crustacyanin reported by Lugtenburg and co-workers supported the idea that the 4,4′-carbonyl groups of astaxanthin played a crucial role in the noncovalent interaction between astaxanthin and the protein. Unfortunately, the resonance signals from the 4,4′-$^{13}C$-labeled astaxanthin in the reconstituted α-crustacyanin were weaker than regular $^{13}C$ resonance signals.[7]

Halogenated retinal analogues have been demonstrated to be useful synthetic chromophores in a number of bacteriorhodopsin studies.[8−10] In an effort to understand the important role of the 4,4′-carbonyl groups of astaxanthin in crustacyanins, we decided to introduce four different halogen atoms at the 3- and 3′-positions of astaxanthin and to examine the effects of the size and electronegativity of halogen atoms on the noncovalent interactions between astaxanthin and the proteins.




R = OH, Astaxanthin;
R = H, Canthaxanthin;
R = F, 3,3'-Difluorocanthaxanthin;
R = Cl, 3,3'-Dichlorocanthaxanthin;
R = Br, 3,3'-Dibromocanthaxanthin;
R = I, 3,3'-Diiodocanthaxanthin;
R = Cl and OH, 3-Chloro-3'-hydroxycanthaxanthin.

The strategy for introducing halogen substitutes at the 3- and 3′-positions is shown in Scheme 1. The 3- and 3′-hydroxy groups of all-trans astaxanthin were reacted with triflic anhydride and pyridine in $CH_2Cl_2$ to afford a bis-triflate. Upon treatment with 2 equiv of *n*-$Bu_4NF$ in THF, the bis-triflate was converted into 3,3′-difluorocanthaxanthin. Also, 3,3′-dichlorocanthaxanthin, 3,3′-dibromocanthaxanthin, and 3,3′-diiodocanthaxanthin were prepared by treatment of the bis-triflate with 2 equiv of *n*-$Bu_4NCl$ in THF, 3 equiv of NaBr, and 3 equiv of NaI in acetone, respectively. The yields of the bromination and the iodination were almost quantitative. The yields of the chlorination and fluorination were ∼50%. The low yields were due to the formation of an elimination product.

To improve the yield of the chlorination, astaxanthin was reacted with *N*,*N*-dimethyl-*N*-1-chloro-2-methylpropenylamine in $CH_2Cl_2$ at room temperature to give the desired product in a good yield (>95%).[11] However, treatment of astaxanthin with *N*,*N*-dimethyl-*N*-1-fluoro-2-methylpropenylamine failed to give 3,3′-difluorocanthaxanthin ascribed to the highly electronegative fluorine atom. The starting material, the elimination product, and other side products were recovered after the reaction. Diethylaminosulfurtrifluoride (DAST) has been proved to be a versatile reagent

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**Table 1.** Partial $^1$H NMR Data and UV−vis Absorption Maxima of the Five Halogenated Canthaxanthins

| compounds | H-3,3′[a] | H-7,7′ | H-8,8′ | H-10,10′ | H-11,11′ | H-12,12′ |
|---|---|---|---|---|---|---|
| astaxanthin | 4.31 | 6.21 | 6.43 | 6.30 | 6.66 | 6.45 |
| 3,3′-diF | 5.09 | 6.19 | 6.43 | 6.30 | 6.65 | 6.44 |
| 3,3′-diCl | 4.71 | 6.18 | 6.42 | 6.30 | 6.65 | 6.44 |
| 3,3′-diBr | 4.91 | 6.18 | 6.42 | 6.30 | 6.65 | 6.45 |
| 3,3′-diI | 5.27 | 6.17 | 6.41 | 6.30 | 6.65 | 6.45 |
| 3-Cl-3′-OH | 4.31, 4.71 | 6.18, 6.21 | 6.42 | 6.30 | 6.65 | 6.45 |

| compounds | H-14,14′ | H-15,15′ | 5-Me | 1,1′-Me,Me | $\lambda_{max}$[b] |
|---|---|---|---|---|---|
| astaxanthin | 6.30 | 6.68 | 1.95 | 1.21, 1.32 | 477 |
| 3,3′-diF | 6.30 | 6.65 | 1.93 | 1.25, 1.32 | 477 |
| 3,3′-diCl | 6.30 | 6.65 | 1.95 | 1.23, 1.30 | 477 |
| 3,3′-diBr | 6.30 | 6.65 | 1.95 | 1.23, 1.29 | 477 |
| 3,3′-diI | 6.30 | 6.65 | 1.96 | 1.20, 1.26 | 478 |
| 3-Cl-3′-OH | 6.30 | 6.65 | 1.95 | 1.21, 1.23, 1.30, 1.32 | 477 |

[a] In $CDCl_3$, 200 MHz. [b] Units = nm, in acetone.

in preparation of bioactive molecules with fluorine atoms.[12] Because astaxanthin was decomposed by DAST, it was unsuitable for the preparation of 3,3′-difluorocanthaxanthin.

The first step in the preparation of 3-chloro-3′-hydroxycanthaxanthin was the protection of one hydroxy group of astaxanthin as an acetoxy group using acetyl chloride and pyridine. Treatment of 3-acetoxy-3′-hydroxycanthaxanthin with *N*,*N*-dimethyl-*N*-1-chloro-2-methylpropenylamine, followed by deprotection with sodium methoxide, gave 3-chloro-3′-hydroxycanthaxanthin in 61% overall yield.

All the analogues obtained as deep red solids are air/light-sensitive. The all-trans isomers of the five halogenated canthaxanthins were isolated by HPLC (YMC Carotenoid Column, 5$\mu$, 60:25:15 $CH_3CN/CH_3OH$/EtOAc, flow rate = 1 mL/min) in dim red light. The structures of the analogues were confirmed by their $^1$H and $^{13}$C NMR spectra, HR-MS, and UV−vis data and by comparison with those of astaxanthin and canthaxanthin (Table 1).[13,14] Because the reconstitution of α-crustacyanin with (3*S*,3′*S*)-, (3*R*,3′*R*)-, and (3*R*,3′*S*)-astaxanthins gave the same reconstituted α-crustacyanin, separation of the stereoisomers of the halogenated canthaxanthins was considered unnecessary for the study of α-crustacyanin.[15]

Reconstitutions of the five halogenated canthaxanthins with the apoprotein isolated from natural α-crustacyanin were carried out.[6] 3,3′-Difluorocanthaxanthin was combined with the apoprotein to give a blue α-crustacyanin analogue ($\lambda_{max}$ = 600 nm). The spectra (Figure 1) show that the fluorine atoms at the 3- and 3′-positions cause the blue-shifted UV−vis absorption of the α-crustacyanin analogue. It was suggested that hydrogen-bonding interactions between the carbonyl groups of astaxanthin and hydrogen donors from the protein caused the usual red-shift of the chromophore in α-crustacyanin.[7] The electronegative fluorine atoms at the 3- and 3′-positions should induce weaker hydrogen bonds between the carbonyl groups and hydrogens in the carotenoprotein, thus causing the smaller bathochromic shift of the reconstituted analogue.

Also, 3,3′-dichlorocanthaxanthin, 3,3′-dibromocanthaxanthin, and 3,3′-diiodocanthaxanthin were combined with the apoprotein to form red canthaxanthin−protein complexes ($\lambda_{max} \approx$ 500 nm) within 1 min of mixing. The halogenated canthaxanthins were completely dissociated from the protein after 12 h at 4 °C. The results show that if the size of a substituent is larger than that of a fluorine atom, the substituents at the 3,3′-positions of astaxanthin affected the interactions between astaxanthin and the protein. This is probably due to the rigid portion of the protein host around the 3,3′-OH groups of astaxanthin. The protein surrounding for the six-membered rings of astaxanthin in α-crustacyanin

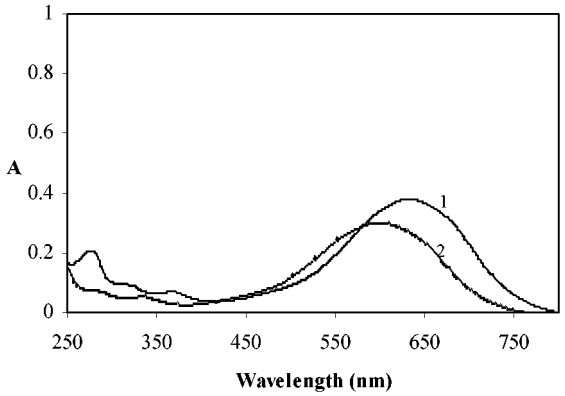


**Figure 1.** Absorption spectra of α-crustacyanin (**1**) and the α-crustacyanin analogue reconstituted with 3,3′-difluorocanthaxanthin (**2**) in 50 mM sodium phosphate buffer.

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seems to be very different from the relatively flexible protein pocket for the six-membered ring of retinal in bacteriorhodopsin.[16]

Moreover, 3-chloro-3′-hydroxycanthaxanthin was reconstituted with the apoprotein to give a similar canthaxanthin−protein complex initially. The synthetic chromophore was released from the protein complex after 12 h (Figure 2) due

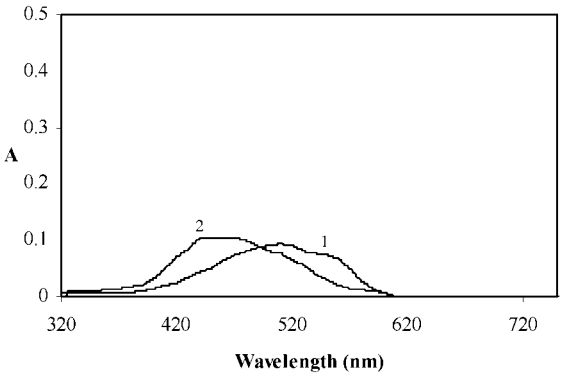


**Figure 2.** Absorption spectra of the 3-chloro-3′-hydroxycanthaxanthin−protein complex (**1**) in 50 mM sodium phosphate buffer within 1 min of mixing and the dissociated complex (**2**) in the same buffer after 12 h at 4 °C.

to the steric limitation. However, UV−vis absorption of the 3-chloro-3′-hydroxycanthaxanthin−protein complex ($\lambda_{max}$ = 510 and 562 nm) was more red-shifted than those of the other red complexes. This was attributed to the hydroxy group of 3-chloro-3′-hydroxycanthaxanthin, which made it possible for a half of the synthetic chromophore to be fitted correctly to the apoprotein. On the basis of our results and the previous reconstitution studies of α-crustacyanin,[3] we conclude that the large bathochromic shift of α-crustacyanin can only be achieved when the astaxanthin chromophore snugly fits into the apoprotein of α-crustacyanin.

In this letter, we demonstrate that the air/light-sensitive halogenated canthaxanthin analogues can be prepared from all-trans astaxanthin by convenient methods. These analogues are useful for the study of α-crustacyanin. Recently, we found an ideal method for the reconstitution of $\gamma$-crustacyanin. Detailed studies of $\gamma$-crustacyanin using these halogenated canthaxanthins and $^{19}$F NMR study of the reconstituted crustacyanin analogues are in progress.

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**Supporting Information Available:** Experimental procedures for preparations of the five halogenated canthaxanthins and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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