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CAROTENOID SCIENCE



*Proceedings of the 33rd
Annual Meeting on*

Carotenoid Research

*Held in Chiba, Japan
22-23 September 2019*



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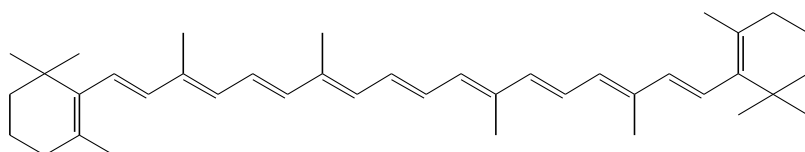
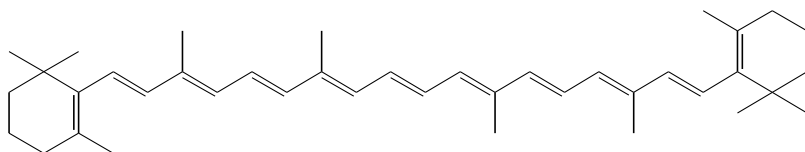
VOLUME 24 (2020)

Japanese Society for Carotenoid Research

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Japanese Society for Carotenoid Research

Preface for the 33rd Annual Meeting

The 33rd Annual Meeting of Japanese Carotenoid Society was held in Nishichiba Campus of Chiba University located on September 22nd-23rd, 2019. The scientific program of this meeting consisted of three invited lectures by Prof. Akimori Wada (Kobe Pharmaceutical University), Prof. Kyoichi Saito (Chiba University), Prof. Kazuki Saito Tsukamoto (Chiba University), 29 oral presentations, 83 participants in total. On the day before (21st), we hosted the Annual meeting of young carotenoid scientists, where we got three tutorial lectures by Dr. Shinichi Takaichi (Tokyo Agricultural University), Dr. Maiko Furubayashi (AIST), and Dr. Takashi Maoka, together with as much as 18 poster presentations by young carotenoid scientists!

The presentations covered a wide variety of research fields on carotenoids and apocarotenoids, including physics, physical chemistry, synthetic and analytical chemistry, molecular biology, genetics, omics, pathway and metabolic engineering, nutrition and medicine of carotenoids. All presentations literally demonstrated the state of the art of the diverse field on carotenoids, symbolizing the rapid growth of carotenoid science, as well as the high standard of Japanese carotenoid scientists.

Hereby, I deeply thank all the participants for delivering valuable presentations, comments, and keeping the good atmosphere all through the meeting. Also greatly appreciated are the panels of sponsors of this wonderful meeting:

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Ever since, this meeting have been playing an extraordinarily important role on promoting the exploration of the uses/values of carotenoids via cross-disciplinary cooperation among Japanese carotenoid researchers both from academic and industrial sectors. And let this meeting stay cool as ever!

We are looking forward to seeing you again in the next 34th Annual Meeting of Japanese Carotenoid Society, which will be held in Kwansei Gakuin University (Osaka Umeda Campus) on September 19th – 20th, 2020.

Daisuke Umeno

Professor, Chiba University

The Meeting Chair of the 33rd Annual Meeting of Japanese Carotenoid Society

Contents

Preface for the 33rd Annual Meeting

Mini Reviews

Enzymatic Reactions with Multilayered Enzymes

Kyoichi Saito and Shiho Asai..... 1

Development of Red-Shifted Channelrhodopsin Using Long Conjugated Retinal Analog as Chromophore

Akimori Wada..... 7

Structures of Carotenoids Determined by Our Research Group During 2009 to 2019

Takashi Maoka..... 12

Regular Papers

Molecular breeding of a biosynthetic pathway to C₆₀ phytoene

Takahiro Seki, Tomoyuki Ichikawa, Takumi Ojima, Shigeko Kawai-Noma and Daisuke Umeno..... 19

Application of DOSY Experiment to Analysis of Astaxanthin and its' Analogues

Chisato Tode, Takashi Maoka and Atsuko Takeuchi..... 25

Proceedings of 33rd Annual Meeting on Carotenoid Research

Reconstitution of carotenoids into the reaction center from the purple photosynthetic bacterium *Rhodobacter sphaeroides* using the chromatophores of the carotenoidless mutant strain R26.1

Mayu Mitarai, Marina Yoshida, Nao Yukihiro, Chiasa Uragami, Kouta Horiuchi, Hiroki Hashimoto, Alastair T. Gardiner, Richard J. Cogdell and Hideki Hashimoto..... 36

Experimental reports

Structural determination of a new minor yellow carotenoid, 5,6-dihydrocrustaxanthin from the prawns *Penaeus japonicus* and *P. vannamei*

Takashi Maoka..... 41

Abstracts

Analysis of methyl 5-glucosyl-5,6-dihydro-apo-4,4'-lycopenoate (C30 carotenoid) biosynthetic pathway, and antioxidant activities of its metabolic intermediates

Chiharu Takagi, Mayuri Aikawa, Kanaho Araki, Miho Takemura, Seon-Kang Choi, Mitsuyasu Itaya, Norihiko Misawa and Kazutoshi Shindo..... 44

Isolation and characterization of myxol biosynthesis genes from marine flavobacteria

Keisuke Nakazawa, Kenjiro Sugiyama, Masaharu Yamada, Susumu Yoshizawa and Shinichi Takaichi..... 46

Esterification of carotenoid in aerial microalgae *Coelastrella* sp. KGU-Y002 under aerial condition with LED light

Takahide Onda, Nobuhiro Aburai and Katsuhiko Fujii 48

Novel biosynthetic precursor of siphonaxanthin found in *Codium fragile* trichomes cultivated under blue-green light

Soichiro Seki, Yumiko Yamano, Kentaro Fujiwara, Ryuichi Kano and Ritsuko Fujii..... 50

In-vitro reconstitution of photosynthetic light-harvesting complex from siphonous green alga *Codium fragile*

Yuki Isaji, Nami Yamano, Masahiko Iha, Tetsuko Nakaniwa, Rei Tohda, Naoko Norioka, Genji Kurisu and Ritsuko Fujii 52

Increased lycopene concentration in egg yolk of hens fed Z-isomer-rich lycopene and development of tomato powder rich in lycopene Z-isomer

Masaki Honda, Hiroto Ishikawa and Yoshiaki Hayashi..... 54

Effects of astaxanthin on cognitive function, physical function, and mood condition in elderly people

Rika Hirashima, Nobuko Hongo, Jiro Okuda, Maki Suzuki, Naoki Miura and Kumi Tominaga..... 56

Synthesis of New Derivatives Similar to Carotenoids and Evaluation of Singlet Oxygen Quenching Ability

Satoshi Kobayashi, Ryoma Shinohara, Ryo Sakata, Kiyotaka Moriizumi, Takashi Fujita, Chiasa Uragami and Hideki Hashimoto..... 58

Pharmacokinetics of Astaxanthin Derivative in Knee Osteoarthritis Models

Katsuhiro Yamano, Yasuhiro Nishida, Makoto Ogawa and Tomihisa Yokoyama..... 60

Investigation of anti-inflammatory mechanism of apo-fucoxanthinoids

Naoki Takatani, Fumiaki Beppu, Takashi Okitsu, Yumiko Yamano, Akimori Wada, Kazuo Miyashita and Masashi Hosokawa..... 62

Synthetic study of ζ -Carotene-Part 1-

Chisato Tode, Takashi Maoka, Kenjiro Sugiyama and Atsuko Takeuchi..... 63

Synthesis of (*R*)- and (*S*)- α -Carotenes and Their HPLC Separation

Yumiko Yamano, Naoto Hayashi, Yuko Hamada and Akimori Wada..... 65

Ultrafast Excitation Energy Transfer Dynamics of Carotenoids bound to Photosynthetic Reaction Centers from Green Sulfur Bacteria *C. tepidum*

Chihiro Azai, Masato Ueno, Shogo Fujimoto, Takumi Inoue, Jiro Harada, Shinji Masuda and Daisuke Kosumi..... 66

Control of an Intramolecular Charge Transfer Character of Artificial Short-Polyene Carotenoid

Daisuke Kosumi, Toshiyuki Kusumoto and Hideki Hashimoto..... 68

Construction of the pathways toward non-natural retinoids

Yohei Saito, Shigeko Kawai-Noma, Kyoichi Saito and Daisuke Umeno..... 70

Yellow xanthophylls in shrimp belonging to *Penaeus* Structures and Metabolism

Takashi Maoka, Futoshi Sunada, Ikuo Kuroda and Yuna Ono..... 71

Carotenoids of Insects belong to Hemiptera, Perspective of their origin, metabolism, chemical ecology

Takashi Maoka, Ritsuo Nishida, Naoki Kawase, Mitutaro Hironaka, Miho Takemura and Norihiko Misawa..... 72

Carotenoid Fatty Acid Esters from Prokaryote: *Chloracidobacterium thermophilum*

Shinichi Takaichi, Jiro Harada, Yoshiki Shirotori, Marcus Tank and Satoshi Hanada..... 73

Dynamics of ICT State in Fucoxanthin Investigated by Multi-Pump Femtosecond Spectroscopy

Masayuki Yoshizawa, Ren Sato, Martin R. Steinel, Nao Yukihiro, Chiasa Uragami and Hideki Hashimoto..... 74

Carotenoid biosynthetic pathway of the filamentous anoxygenic phototrophic bacterium *Chloroflexus aurantiacus*

Jiro Harada, Ken Yamamoto and Shinichi Takaichi..... 75

The construction of a strigolactone production system in cyanobacteria

Yutaka Sakamaki, Shinichi Takaichi, Yuki Kimura, Daisuke Umeno, Shinsaku Ito and Satoru Watanabe..... 76

De novo construction of C60 carotenoid pathways

Takahiro Seki, Takumi Ojima, Yusuke Otani, Takuya Hosoi, Shigeko Kawai-Noma, Li Ling, Takashi Maoka, Maiko Furubayashi, Kyoichi Saito and Daisuke Umeno..... 77

Transformation of *Freesia* × *hybrida* with astaxanthin biosynthesis genes and evaluation of generated transformants

Yohei Uwagaki, Motoyasu Otani, Masako Komaki, Minoru Murahama, Miho Takemura and Norihiko Misawa..... 78

Phytochemical genomics: from Arabidopsis to medicinal plants

Kazuki Saito..... 79

Necessity is the mother of extension? Polymeric adsorbents created using graft polymerization

Kyoichi Saito..... 80

Development of Red-Shifted Channelrhodopsin Using Long Conjugated Retinal Analog as Chromophore

Akimori Wada..... 81

Enzymatic Reactions with Multilayered Enzymes

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Abstract: Enzymes catalyze specific reactions under mild conditions to form useful products. Immobilization of the enzyme to solid surface enables repeated use with stable activity of the enzyme. In this study, the enzymes were immobilized in multilayers by the ionizable polymer chain grafted onto the pore surface of a porous hollow-fiber membrane. The enzymes bound to the graft chain were enzymatically crosslinked with transglutaminase to prevent the enzyme leakage. High activities of ascorbic acid oxidase, urease, and cycloisomaltooligosaccharide glucanotransferase were demonstrated in a permeation mode of their substrate solutions through the enzyme-multilayered porous hollow-fiber membranes.

1. Introduction

Apples in most area of Japan are produced from trees using grafting technique. A branch yielding delicious apples is jointed to a healthy trunk tree at grafting sites and then the tree will produce delicious apples. The grafting is an effective agricultural technique for gardeners and farmers. When a tree that is resistant to severe climate and poor soil bears little fruit on its branches, the gardener forms a graft site by cutting a branch with scissors. Then, he/she grafts a branch capable of producing succulent fruit.

An analogy between grafting performed by a gardener and that by a radiation chemist is illustrated in Figure 1. Chemists produce radicals by irradiating a trunk polymer with gamma rays and electron beams to form grafting sites. Then, a polymer branch with various functionalities is grafted onto these sites of the trunk polymer. Graft polymerization enables us to prepare desirable and useful materials for separation and reaction.

Excitation sources for generating radicals by graft polymerization include radiation, plasma, light, and

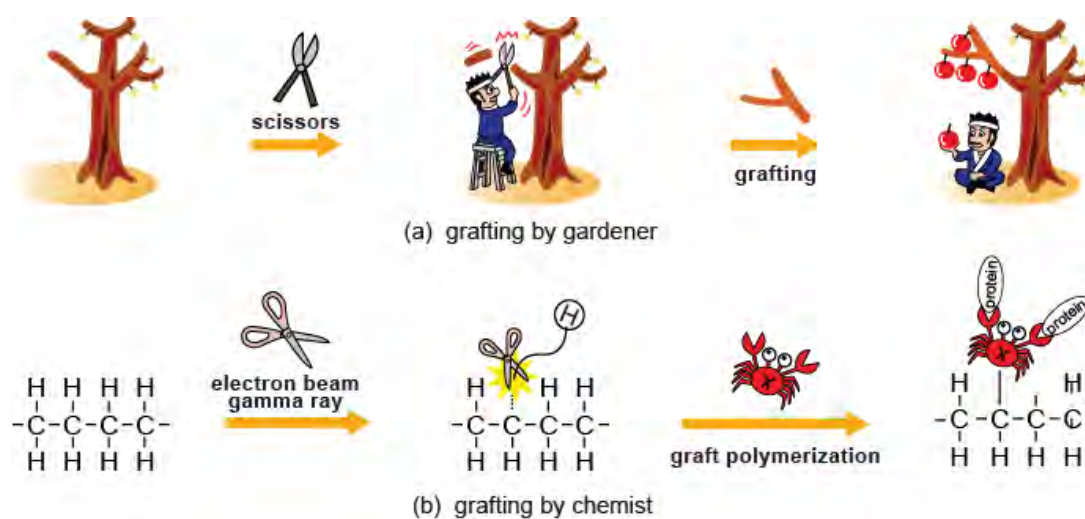


Figure 1. Principle of grafting

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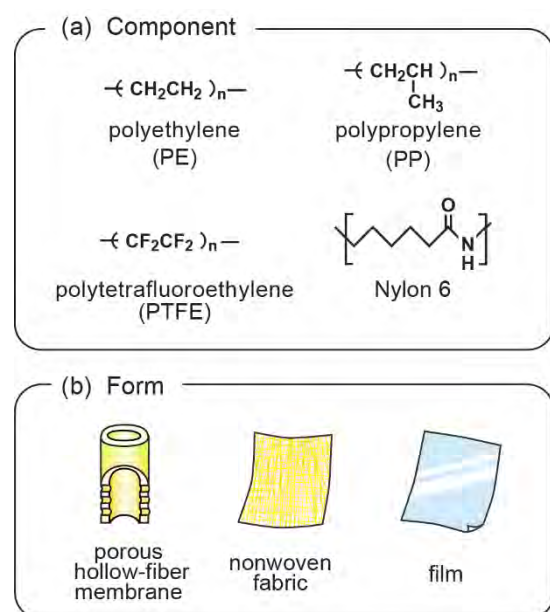


Figure 2. Components and forms of trunk polymer

chemicals. Among graft polymerization procedures using these sources, radiation-induced graft polymerization is advantageous over other grafting techniques because gamma rays or electron beams produce a large number of radicals in polymers of various components and forms. The components of polymer include polyethylene, polypropylene, polytetrafluoroethylene, and nylon. The forms include porous hollow-fiber membranes, nonwoven fabrics, and films (Figure 2).

Some covalent bonds must be destroyed by excitation to form radicals in the trunk polymer as the starting points of graft polymerization. The successive

contacts of vinyl monomers with the radicals in the trunk polymer extend a polymer chain before termination occurs. The resultant polymer chain grafted onto the trunk polymer is referred to as a graft chain [1].

An appropriate combination of component and form will provide us with a new class of adsorbents. For example, an ion-exchange polymer chain grafted onto the pore surface of a porous hollow-fiber membrane enables high-capacity binding of enzymes through multilayer binding of enzymes into the extended graft chain. In addition, high-rate binding of enzymes proceeds because of negligible diffusional mass-transfer resistance of the enzymes to the ion-exchange group, promoted by the permeative flow of an enzyme solution through the pores [2, 3].

The introduction schemes for typical ion-exchange hollow-fiber membranes by radiation-induced graft polymerization are shown in Figure 3. These schemes using glycidyl methacrylate (GMA) as a precursor monomer consist of three steps: (1) irradiation by gamma rays and electron beams to produce radicals on the porous hollow-fiber membrane, (2) graft polymerization of GMA, and (3) conversion of the epoxy group produced into an cation- and anion-exchange groups on the poly-GMA chain grafted onto the porous hollow-fiber membrane.

As illustrated in Figure 4(a), from the term “graft”, the graft chain is depicted as a branch growing from a tree trunk; however, this imagery is not accurate. Figure 4(b) is closer to an actual structure. Let us describe the structure of a grafted polymeric material prepared by pre-irradiation graft polymerization of GMA onto a high-density polyethylene membrane of

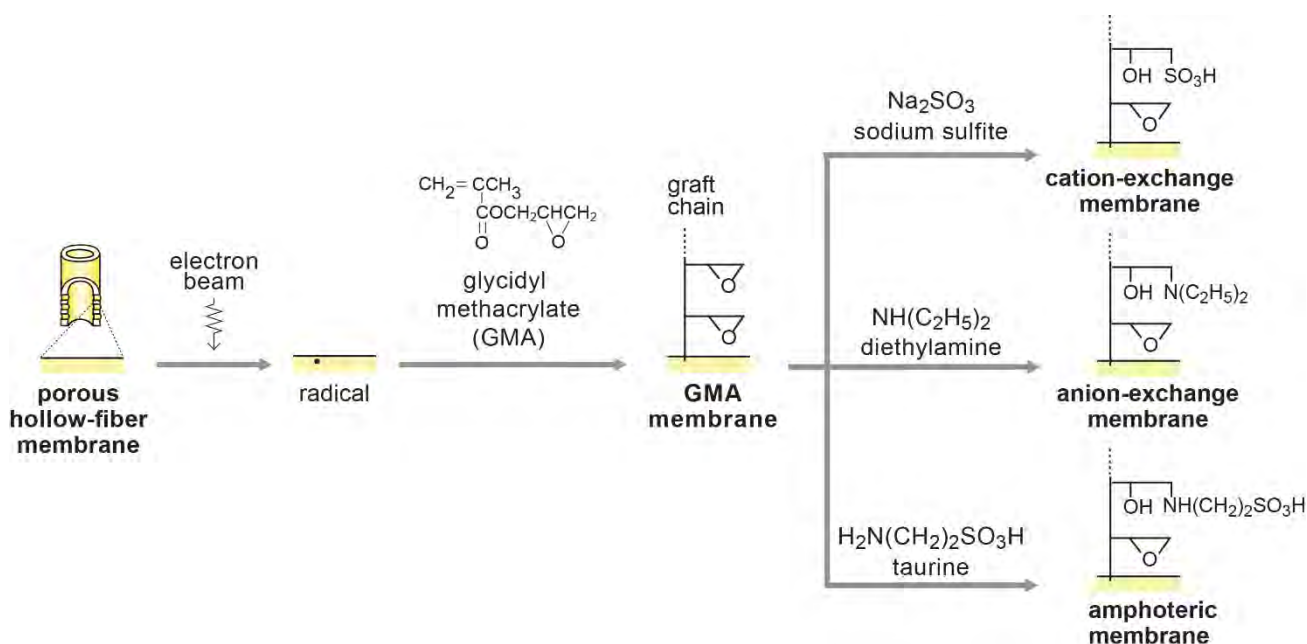


Figure 3. Introduction of ion-exchange groups into graft chains.

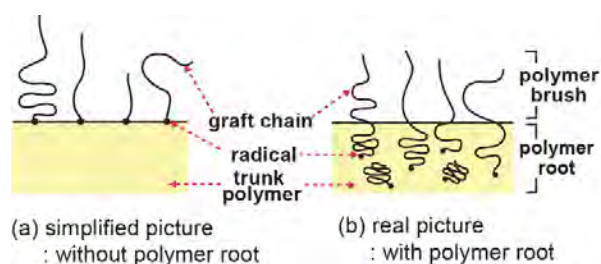


Figure 4. Graft chain consisting of polymer brush and polymer root.

a hollow fiber. High density polyethylene consists of crystalline and amorphous domains. The crystalline domain is an integrated structure of lamella formed by the tightly folded polyethylene chain, whereas the amorphous domain is formed by loose polyethylene chain.

When a porous hollow-fiber membrane with inner and outer diameters of 2 and 3 mm, respectively, is irradiated with electron beams at a dose sufficient for electrons to penetrate the porous polymer, radicals are formed uniformly throughout its entire volume. Polymer chains grafted onto a porous polyethylene hollow fiber can be classified into two categories: polymer chains extending from the pore surface toward the pore interior and polymer chains invading the polymer matrix. The former is referred to as a polymer brush and the latter as a polymer root. The polymer brush and root in porous polymeric adsorbents share respective roles in binding enzymes during their permeation. The polymer brush captures enzymes during the permeation of an enzyme solution through the pores that are rimmed by the polymer brush, whereas the polymer root swells the polymer matrix, which alleviates the pore size reduction caused by the polymer brush.

2. Multilayering of Enzymes

Most enzymes are soluble in aqueous media and catalyze specific reactions under mild conditions, e.g., at ambient temperature and atmospheric pressure. Substrates are captured by enzymes in a homogeneous system and participate in reactions; however, from the viewpoint of the industrial use of enzymes, even after a complete enzymatic reaction, separation of products from enzymes is required to purify the products and reuse the enzymes. To promote the use of enzymatic reaction systems, enzymes have been immobilized on various solid supports by different methods such as physical entrapment, adsorption, and covalent binding.

Ion-exchange or ionizable graft chains hold enzymes in multilayers, as illustrated in Figure 5. An enzyme-multilayered structure can be achieved within

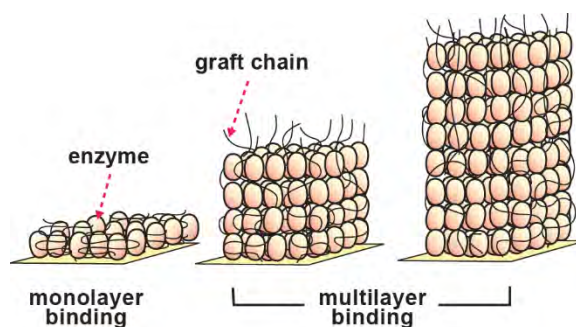


Figure 5. Multilayer binding of enzyme to polymer chains grafted onto pore surface.

the polymer brushes grafted onto porous hollow-fiber membranes. Furthermore, enzymes immobilized on hollow fibers at a high density exhibit high activity because the substrates are fed with a negligible diffusional mass-transfer resistance to the immobilized enzymes. The total activity will be governed by an intrinsic enzymatic reaction or a substrate feed rate.

3. Kinetics of Enzyme-Immobilized Porous Hollow-Fiber Membranes

We discuss the kinetics describing the permeation of a substrate solution through the pores of a porous hollow-fiber membrane with immobilized enzyme. First, the substrate solution is fed into the inner surface of the hollow fiber; second, during the permeation through the pores of the hollow fiber, the substrate reaches the active site of an enzyme immobilized on the polymer brush to accomplish an enzymatic reaction. The overall process consists of three steps: (1) radial diffusion of the substrate toward the graft chain while flowing through the pores driven by the pressure difference, (2) the diffusion of the substrate into the graft chain, and (3) the intrinsic enzyme reaction at the active site of an immobilized enzyme. The observed or overall enzymatic reaction is the combined result of mass transfer and the intrinsic reaction [4].

Ascorbic acid (vitamin C) solutions at concentrations ranging from 0.025 to 0.100 mmol/L were permeated through the pores of a porous hollow-fiber membrane with immobilized ascorbic acid oxidase. The percentage decomposition of ascorbic acid is shown in Figure 6 as a function of space velocity, defined as the ratio of the flow rate of the ascorbic acid solution to the volume of the membrane including the lumen. In this space velocity range, the percentage decomposition was constant at 100%: a quantitative decomposition was achieved [5]. This favorable result can be explained by noting that the time required to pass through steps (1) to (3) is much shorter than the residence time of the substrate solution in the enzyme-immobilized porous hollow-fiber membrane. In other words, the overall reaction is governed by the flow rate

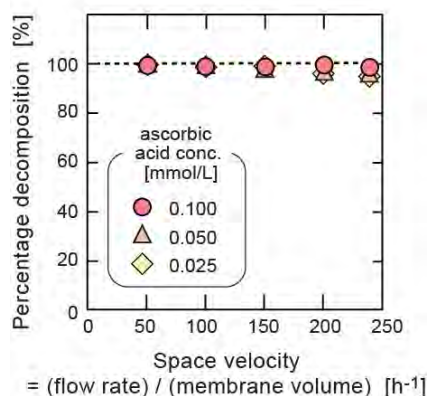


Figure 6. Percentage decomposition of ascorbic acid as a function of space velocity in porous hollow-fiber membrane with immobilized

of the substrate solution and not by diffusion nor intrinsic reaction rate.

4. Examples of Enzymatic Reaction

4.1 Decomposition of urea [6]

The binding of a negatively charged enzyme to an anion-exchange polymer brush is based on an electrostatic interaction, because the pH of the enzyme solution is higher than the isoelectric point of the enzyme. In most cases, the pH suitable for enzyme binding does not coincide with the optimal pH for enzyme activity. To prevent enzyme leakage from the polymer brushes, enzyme molecules were enzymatically crosslinked with transglutaminase.

Urease with a relatively high mass of 480 kDa decomposes urea to form ammonium ions and carbon dioxide. Urease was bound to a diethylamino-type anion-exchange porous hollow-fiber membrane at an amount of 1.2 g per g of the hollow fiber. Immobilization of urease at a high density will have practical applications.

Urea solutions at high concentrations such as 4 mol/L have been used to denature proteins. Subsequently, a semipermeable dialysis film can be used to refold proteins. If a dialysis-film bag containing proteins dissolved in a highly concentrated urea solution is immersed in a vessel that contains a larger volume of water, urea is transported into the vessel driven by the concentration difference of urea across the film, while water is transported from the vessel to the bag driven by the osmotic pressure difference across the film. This dialysis technique has disadvantages in that a long time is required and the protein concentration is lowered. To overcome these disadvantages, we proposed the application of porous hollow-fiber membranes with immobilized urease to the removal of urea from protein solutions.

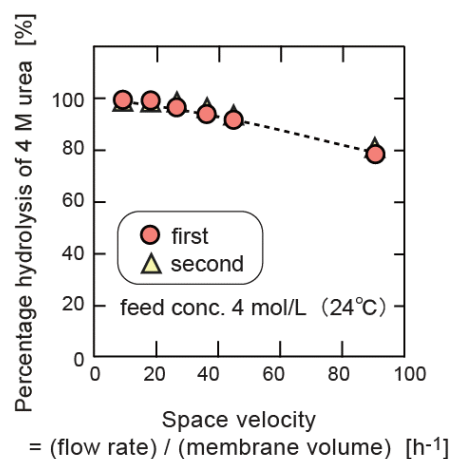


Figure 7. Percentage hydrolysis of 4 M urea as a function of space velocity by porous hollow-fiber membrane with immobilized urease.

A urea solution at a concentration of 4 mol/L was forced to permeate through the pores of a porous hollow-fiber membrane with immobilized urease. The percentage decomposition of urea is shown in Figure 7 as a function of space velocity, defined as the ratio of flow rate to membrane volume. Up to a space velocity of 20 h⁻¹, a quantitative hydrolysis of urea was attained. Urease immobilized on a graft chain and subsequently crosslinked works while being denatured by highly concentrated urea.

4.2 Decomposition of dextran into cycloisomaltooligo-saccharide [7-9]

Cycloisomaltooligosaccharide glucanotransferase (Mr 98 kDa) decomposes dextran, a straight-chain saccharide consisting of glucose monomers, into cycloisomaltooligo-saccharide, a 7- to 9-membered cyclic glucose. Cycloiso-maltooligosaccharide as a product of this enzymatic reaction functions as an inhibitor of glucanotransferase contained in *Streptococcus mutans*. Glucosyltransferase produces glucan with high adhesivity to *S. mutans* in our mouths, increasing the population density of *S. mutans*. Because *S. mutans* produces acid, this increase lowers the pH in the mouth causing cavities to form. Therefore, cycloisomaltooligosaccharide is a promising candidate for the prevention of formation of cavities. In addition, cycloisomaltooligosaccharide may be acceptable to humans because it is tasteless, odorless, and colorless.

Cycloisomaltooligosaccharide glucanotransferase was multilayered in the pore surface of an anion-exchange porous hollow-fiber membrane in the permeation mode. Subsequently, the enzyme molecules were crosslinked with transglutaminase. The conversion of dextran (average molecular mass: 43,000) into cycloisomalto-oligosaccharide increased with an increasing degree of multilayering of cycloisomaltooligosaccharide glucano-transferase.

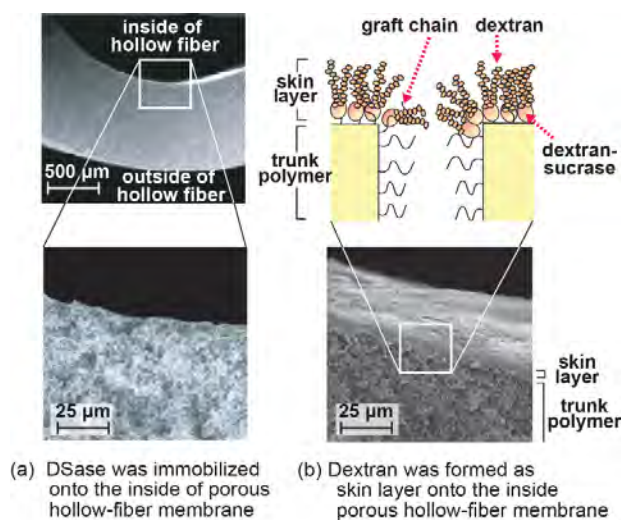


Figure 8. SEM images of skin layer formed on the inside surface of porous hollow-fiber membrane with immobilized DSase.

However, the conversion curve levelled off as the degree of enzyme multilayering increases, because a higher degree of enzyme multilayering into a polymer brush hinders the diffusion of dextran as a high-mass substrate deep into the enzyme-immobilized polymer brush.

4.3 Synthesis of dextran from sucrose [10]

We attempted to produce dextran as a product from sucrose used as a substrate using a dextranase-immobilized porous hollow-fiber membrane. Successive use of dextranase- and cycloisomaltoligosaccharide glucanotransferase-immobilized porous hollow-fiber membranes can produce cycloisomaltoligosaccharide from sucrose. First, an aqueous sucrose solution (0.1 mol/L acetate buffer, pH 5.5) was fed to the inner surface of dextranase-immobilized porous hollow-fiber membranes at a constant flow rate. Initially, the effluent penetrated the outer surface of the hollow fiber without detection of dextran. Then, the pressure required to maintain a constant flow rate increased sharply and the pump stopped because it exceeded the allowable upper limit of applied pressure.

We observed a cross section of a hollow fiber by scanning electron microscopy (SEM). The inner surface of the hollow fiber was coated with a thin white layer (Figure 8). The dextran layer appeared to clog the pores, increasing the permeation pressure. Dextranase continues to capture the product at the active site to polymerize sucrose, as illustrated in Figure 8. This clogging has the potential to influence the pore size under a controlled degree of enzymatic reaction capable of producing the thin layer of dextran. Also, the surface coated with the thin layer of dextran

may have antifouling properties because of the hydrophilicity of dextran.

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Development of Red-Shifted Channelrhodopsin Using Long Conjugated Retinal Analog as Chromophore

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Channelrhodopsins (ChRs, **1**) are a member of microbial rhodopsin, and consist of the chromophore retinal **2a** and a seven-transmembrane helix protein (opsin).¹ Retinal covalently binds with a lysine residue of opsin to form a protonated Schiff base. Upon light stimulation, ChRs exhibit its function (cation channels) accompanying with the conformational changes of retinal and opsin. Recently a new technology, called optogenetics, was developed and used for control of neuronal activity by light.²⁻⁵ This technology has become a very useful tool to clarifying neural circuits and understanding the mechanisms of various neurological diseases.⁶⁻⁷ As ChRs now used in optogenetics are mostly sensitive to blue-green light (430-550 nm), the development of new red-shifted ChRs, which

are activated by the long-wavelength light, are eagerly sought.⁸ In order to develop new type of ChRs having the chromophore responding to the long-wavelength, we prepared new chromophores (**3-5**) having a extended conjugated system than retinal and evaluated their binding experiments with same opsins (Figure 1).

The first approach for new chromophores was to prepare a conjugated double bond in the enamine side of the Schiff base in retinal (A1, **2a**) and 3,4-dehydroretinal (A2, **2b**). Treatment of A1 and A2 with allylamine afforded the corresponding unsaturated imines (**6a, b**) in good yields. Isomerization of double bond was accomplished by potassium *tert*-butoxide in the presence of 18-Crown ether to give the imines (**3a, b**)(Scheme 1).

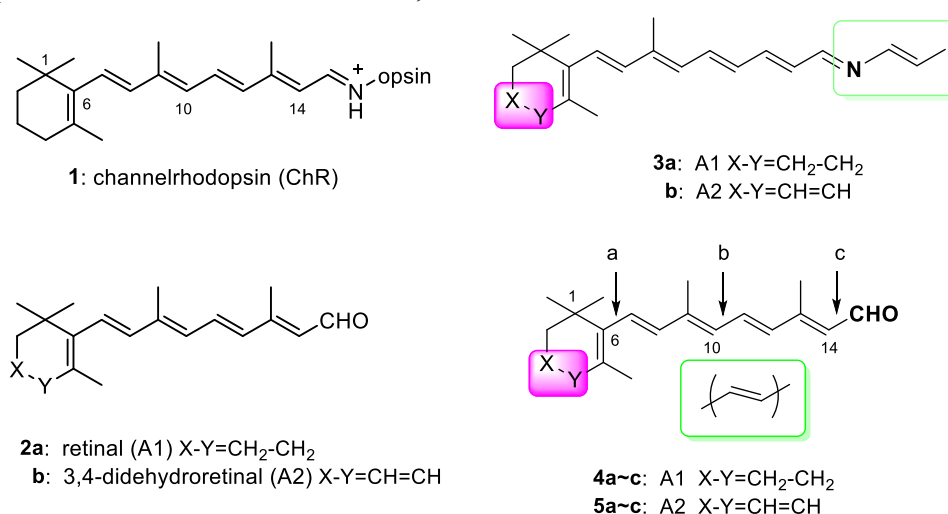
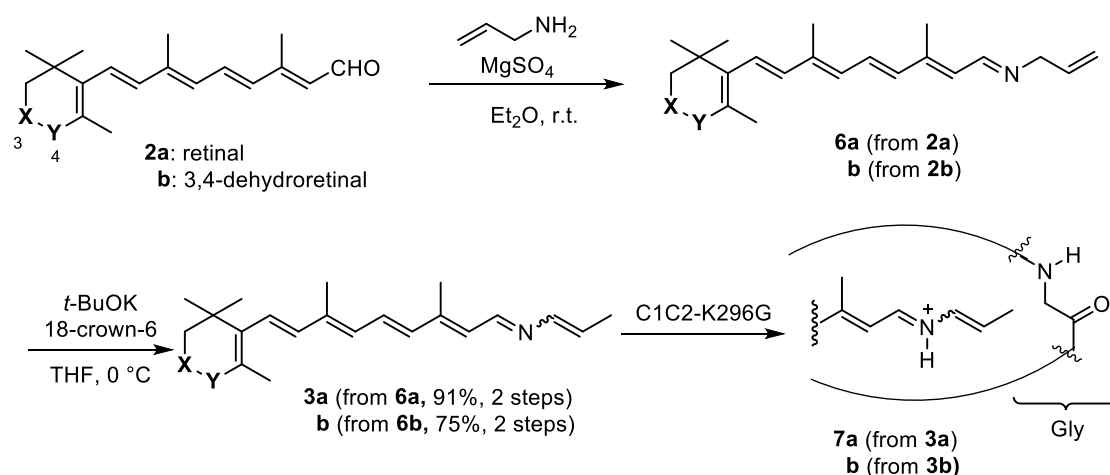


Figure 1



Scheme 1

For the binding experiment, we prepared two C1C2 mutants C1C2-K296A (the lysine at the retinal binding site was replaced by alanine) and C1C2-K296G (the lysine 296 was substituted with glycine). New chromophores (**3a** and **3b**) were only incorporated into C1C2-K296G mutant to afford new ChRs (**7a**, **b**), in which the UV-vis absorption maxima exhibited a 10-30 nm red shift compared with the original C1C2 (Table 1). In the case of C1C2-K296A, the steric repulsion of the two methyl groups (terminal methyl of the chromophore and the mutated alanine residue) seemed to disturb the incorporation of **3** into the opsin.⁹

The second approach was elongation of

side chain of retinal. We prepared six kinds of new chromophores with one double bond inserted into the polyene side chain of A1 or A2 (**4a~c** and **5a~c**). We adopted a 3-step sequence for preparation of these compounds. [(i) elongation of conjugate double bond by Horner-Wadsworth-Emmons reaction using phosphonate **A** or **B**, (ii) reduction of ester to alcohol by DIBAL or LiAlH_4 , (iii) oxidation of allylic alcohol to aldehyde using MnO_2 .] Repeating this 3-step sequence afforded the desired chromophores (**4a~c** and **5a~c**) by changing appropriate phosphonate from β -cyclocitral (**8a**) and safranal (**8b**) as starting materials (Scheme 2).

Table 1. UV-vis Absorption Data of Mutated-C1C2 Analogs.

chromophore	Schiff base ^a	λ_{max} (nm)	
		C1C2-K296G ^b	C1C2-K296A ^b
Retinal (1)	360 ^c	452sh, 476 ^d	452sh, 476 ^d
3a	390	484sh, 508	- ^e
3b	399	488, 520sh	- ^e

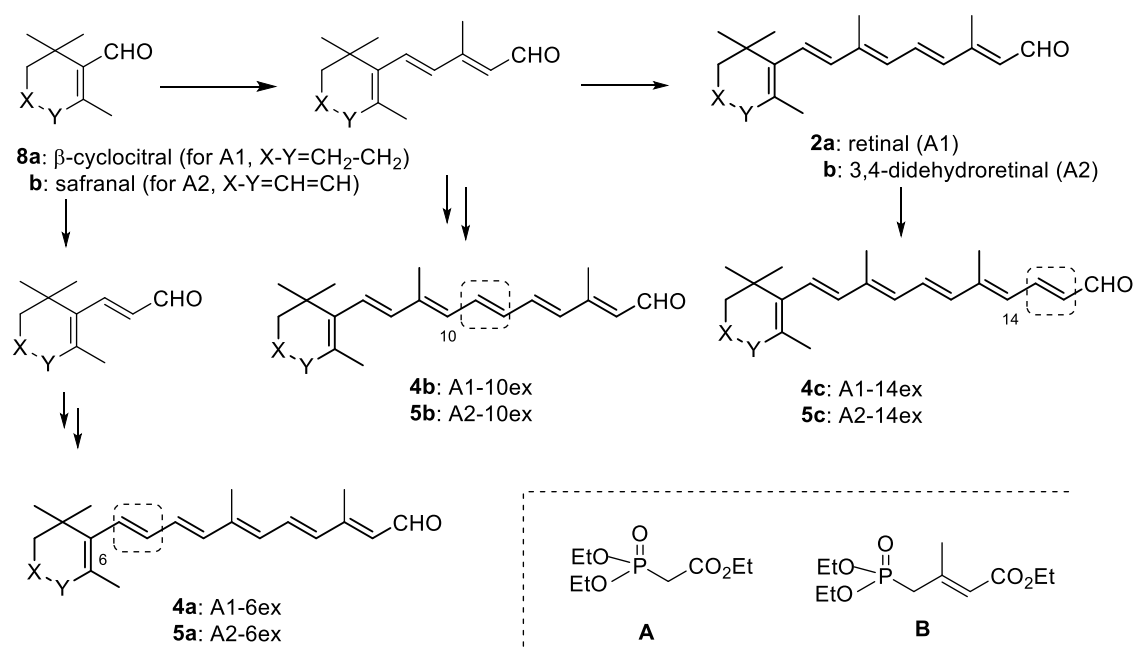
^a In ethanol.

^b In DDM/PM buffer. 0.02% DDM: *n*-dodecyl- β -D-maltoside, PM: 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 140 mM NaCl, 3 mM MgCl_2 , pH 6.0.

^c Prepared from *n*-butylamine.

^d C1C2.

^e No binding.



Reagents and conditions for 3 step sequence: (i) phosphonate **A** or **B**, NaN(TMS)₂ or NaH, THF, 0 °C; (ii) LiAlH₄, Et₂O, 0 °C (for A1 analogs) or DIBAL, THF, -78 °C (for A2 analogs); (iii) MnO₂, Et₂O, r.t.

Scheme 2

We next examined their binding efficiency with two opsins (ReaChR and ChrimsonR). These opsins were selected because their absorption maxima were 61 nm and 110 nm red-shifted, respectively, compared to that of C1C2,¹⁰⁻¹¹ and were expected to afford new red-shifted ChRs. All analogs bound with opsins to afford new ChRs (Table 2 and 3).¹² Among them, A2-10ex (an extra double bond is inserted at the C10-C11 position of A2, **5b**) afforded the novel new ChrimsonR analog, having the

greatest red-shift in the absorption spectrum, with a maximum absorbance at 654 nm (67 nm red-shifted from that of A1-ChrimsonR).

In summary, we designed and prepared new chromophores, with extra one double bond in the enamine of Schiff base part or the polyene side chain of retinal. All chromophores bound with a few opsins to afford the new red-shifted ChRs. These results provide valuable insights toward the generation of a new type of ChR model.

Table2. Absorption characteristics of ReaChR analogs.

	A1-analog				A2-analog			
	A1	4a	4b	4c	A2	5a	5b	5c
$\lambda_{\max}$ (nm) ^a	538	570	569	546	580	607	596	579
shift (Δ nm) ^b	-	32	31	8	42	69	58	41

^a In DDM/PM buffer. 0.02% DDM: *n*-dodecyl- β -D-maltoside, PM: 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 140 mM NaCl, 3 mM MgCl₂, pH 6.0.

^b Shift from A1-ReaChR.

Table 3. Absorption characteristics of ChrimsonR analogs.

	A1-analog				A2-analog			
	A1	4a	4b	4c	A2	5a	5b	5c
$\lambda_{\max}$ (nm) ^a	587	625	636	597	636	651	654	634
shift (Δ nm) ^b	-	38	49	10	49	64	67	47

^a In DDM/PM buffer. 0.02% DDM: *n*-dodecyl- β -D-maltoside, PM: 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 140 mM NaCl, 3 mM MgCl₂, pH 6.0.

^b Shift from A1-ChrimsonR.

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Structures of Carotenoids Determined by Our Research Group During 2009 to 2019

Takashi Maoka*

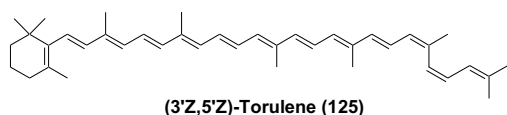
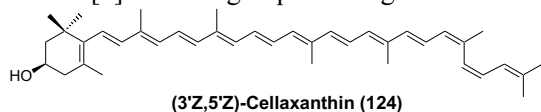
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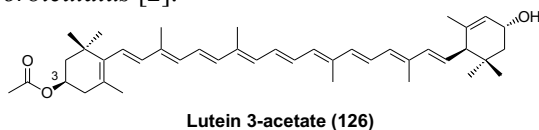
Abstract : Our research group determined 123 structures of carotenoids during 1978 to 2008. Since then, structures of 78 carotenoids were newly determined. They were 43 natural products of carotenoids, 22 reaction products of carotenoids with reactive oxygen species, and 13 carotenoids produced by pathway engineering. In this review, structures of 78 carotenoids determined by our research group during this decade were described.

Key Words: structure, new carotenoids

Since 1978, I have contributed structural studies of various carotenoids. In 2009, I described the list of 123 carotenoids determined by our research group during 1978 to 2008 [1]. The next decade, our research group determined structure of 78 carotenoids, including 43 naturally occurring compounds, 22 reaction products of carotenoids with reactive oxygen species, and 13 carotenoids produced by pathway engineering. In the present review, structures of these 78 carotenoids were described. The compounds numbers are described continuously previous review [1] according to publishing order.

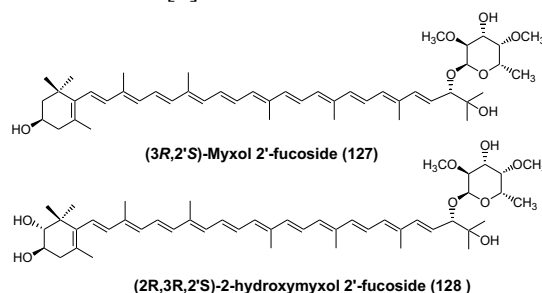


Carotenoids (124) and (125) were isolated from the seeds of oriental bitter sweet *Celastrus orbiculatus* [2].

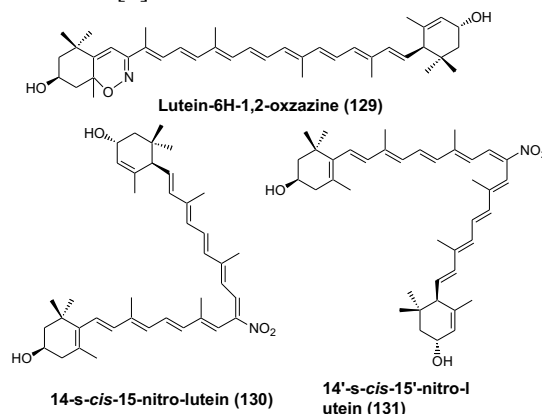


Carotenoid (126) was found in senescent

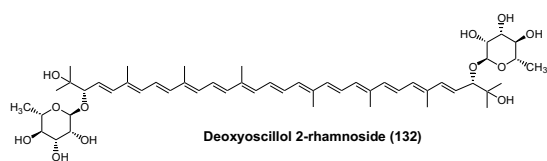
leaves of rice [3].



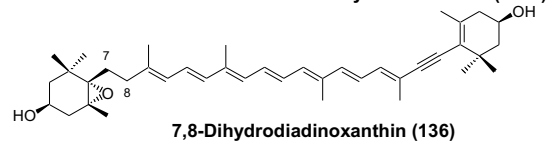
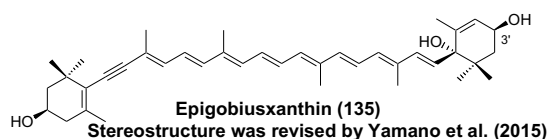
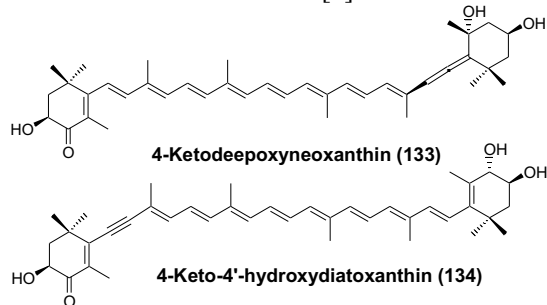
Carotenoids (127) and (128) are isolated from cyanobacterium *Nostoc commune* NIES-24 [4].



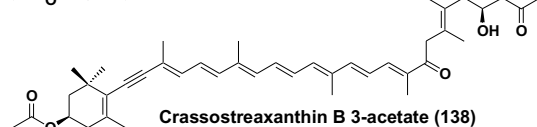
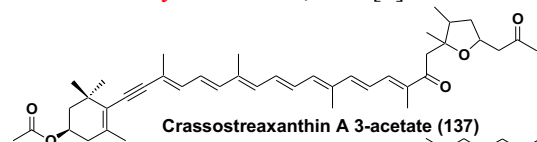
Carotenoids (129) ~ (131) was obtained as reaction products of lutein with peroxyxynitrite [5].



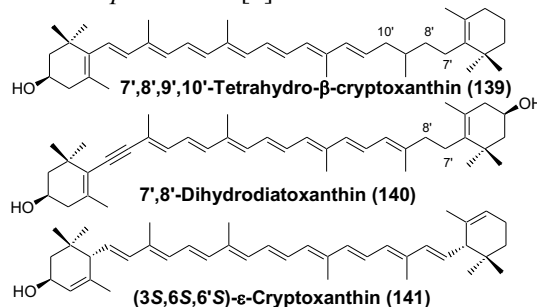
Carotenoid (132) was isolated from *Gemmatimonas aurantiaca* [6].



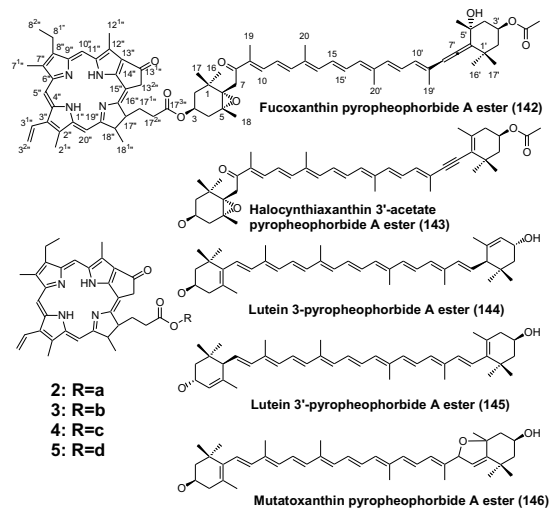
Carotenoids (133) ~ (136) were isolated from the crown-of-thorns starfish *Acanthaster planci* [7]. Stereostructure of epigobiusxanthin (135) was revised by Y. Yamano, et al [8].



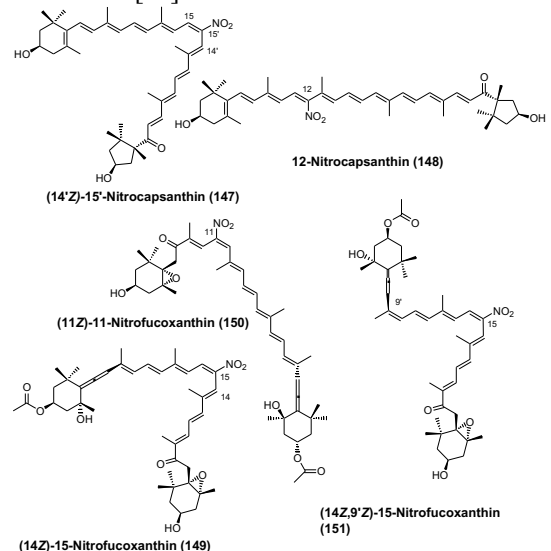
Carotenoids (137) and (138) were isolated from clams, *Ruditapes philippinarum* and *Meretrix petechialis* [9].



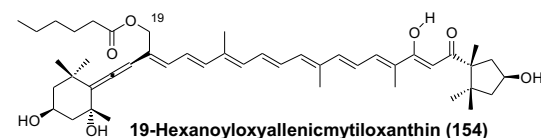
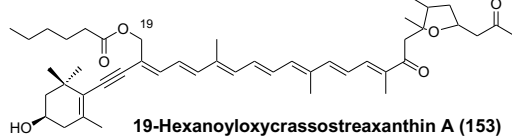
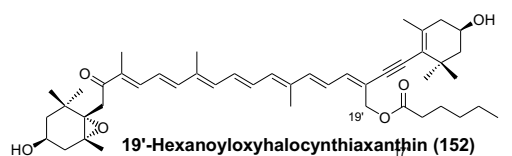
Catotenoids (139) ~ (141) were isolated from the Japanese common catfish *Silurus asotus* [10].



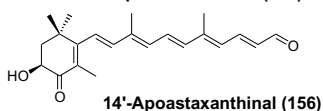
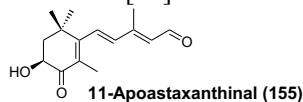
Novel carotenoids and pyropheophorbide A esters (142) ~ (146) were isolated from viscera of abalone [11].



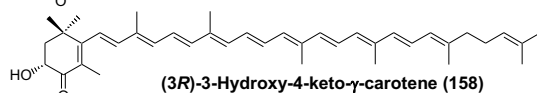
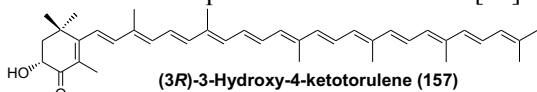
Carotenoids (147) ~ (151) were obtained as products of fucoxanthin with peroxynitrite [12].



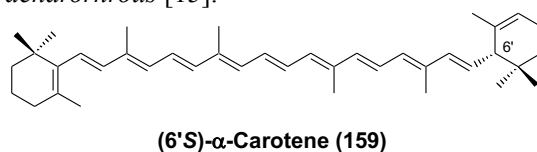
Carotenoids (152) ~ (154) were from the sea mussel *Mytilus galloprovincialis*, grown in the Black Sea [13].



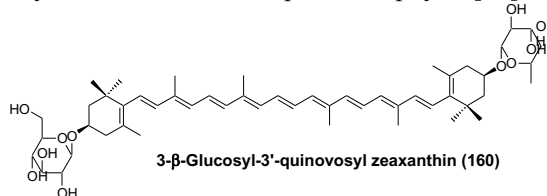
Carotenoids (155) and (156) were obtained as auto-oxidation products of astaxanthin [14].



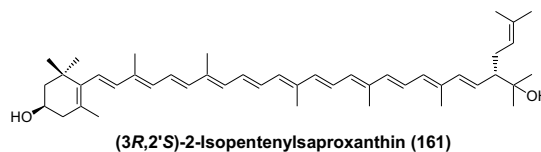
Carotenoids (157) and (158) were isolated from the yeast *Xanthophyllomyces dendrorhous* [15].



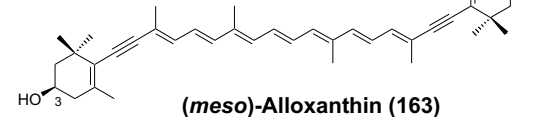
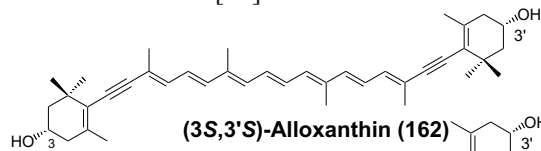
Carotenoid (159) was isolated from unusual Cyanobacteria with unique chlorophylla [16].



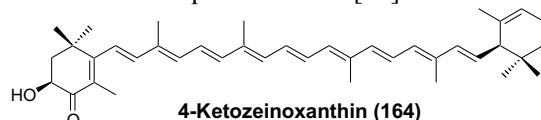
Carotenoid (160) was obtained by *Escherichia coli* cells expressing the *Pantoea ananatis* carotenoid biosynthesis gene cluster [17].



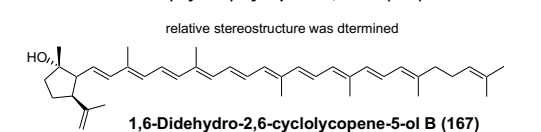
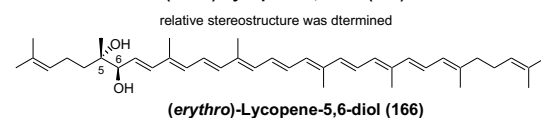
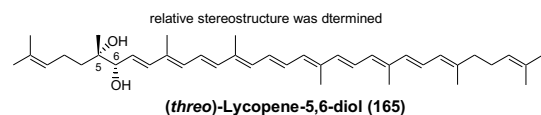
Carotenoid (161) was isolated from yellow-orange bacterium *Jejuria pallidilutea* strain 11shimoA1 [18].

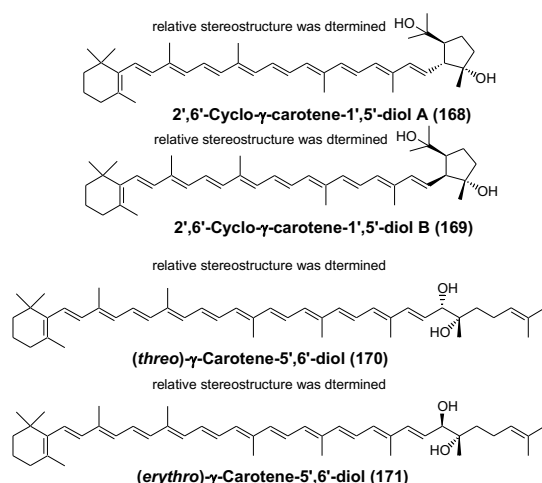


Alloxanthin optical isomers (162) and (163) were found in aquatic animals [19].

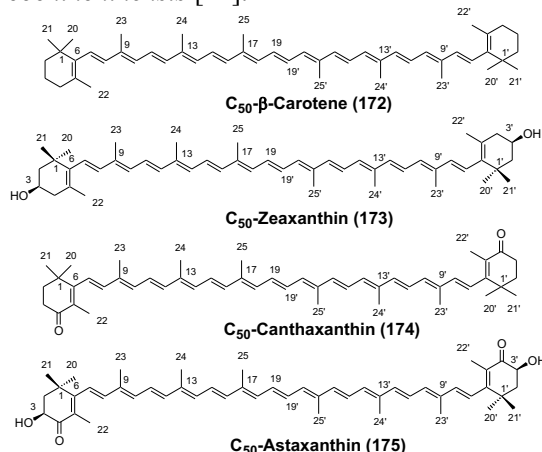


Carotenoid (164) was produced in *Escherichia coli* through metabolic engineering using carotenogenic genes of bacterium and liverwort [20].

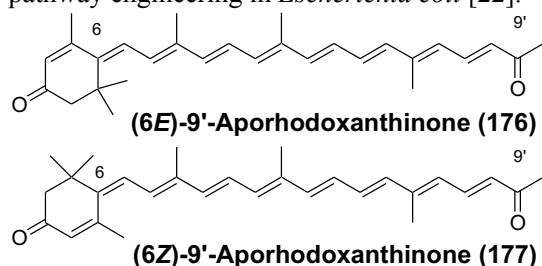




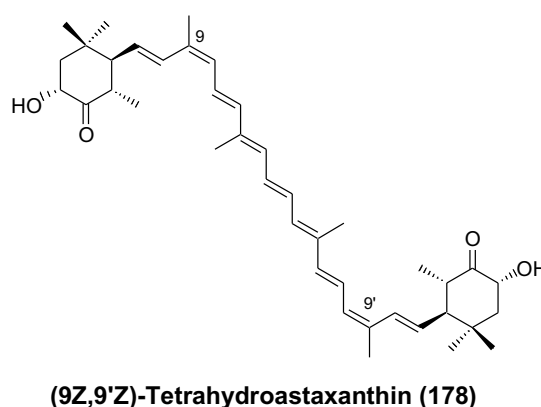
Carotenoids (165) to (171) were isolated from the fruits of gac *Momordica cochinchinensis* [21].



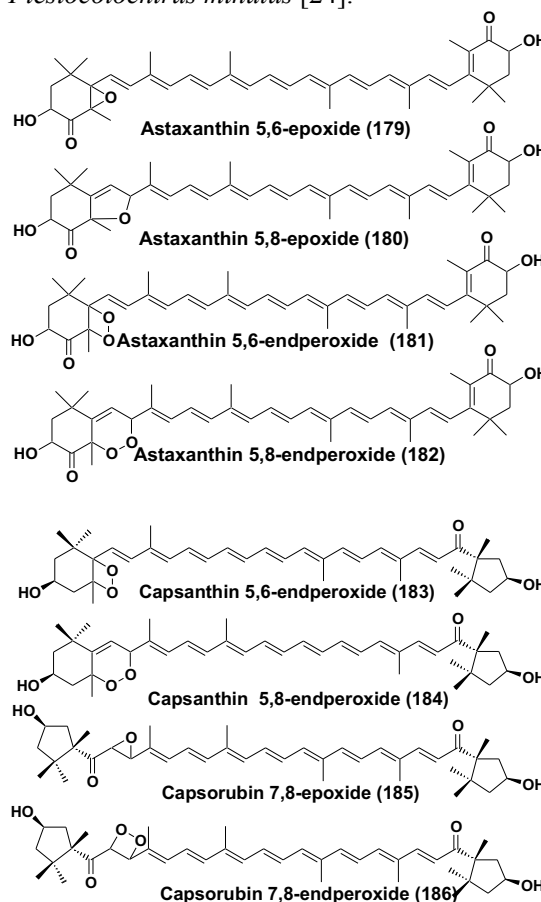
Carotenoids (172) ~ (175) produced by pathway engineering in *Escherichia coli* [22].



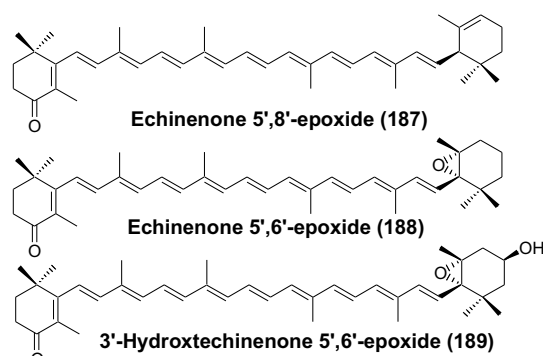
Carotenoids (176) and (177) were obtained in zeaxanthin-synthesizing *Escherichia coli* by redox stress [23].



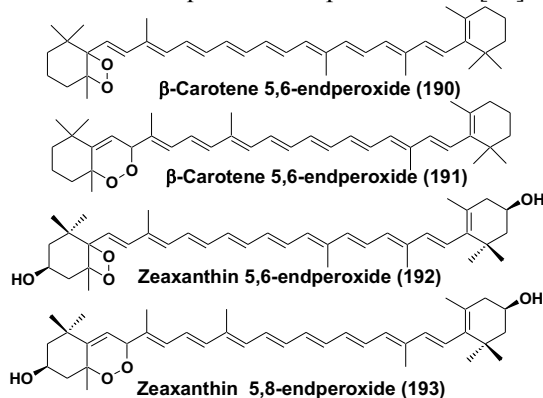
Carotenoid (178) from the sea cucumber *Plesiocolochirus minutus* [24].



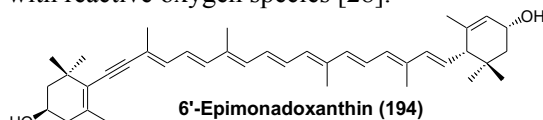
Carotenoids (179) ~ (184) was obtained as reaction products of astaxanthin with reactive oxygen species [25]. Carotenoids (185) and (186) were obtained as capsanthin and capsorubin with reactive oxygen species [26].



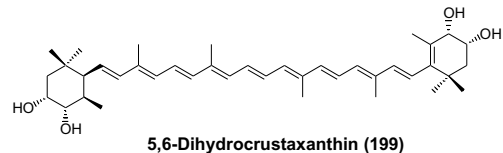
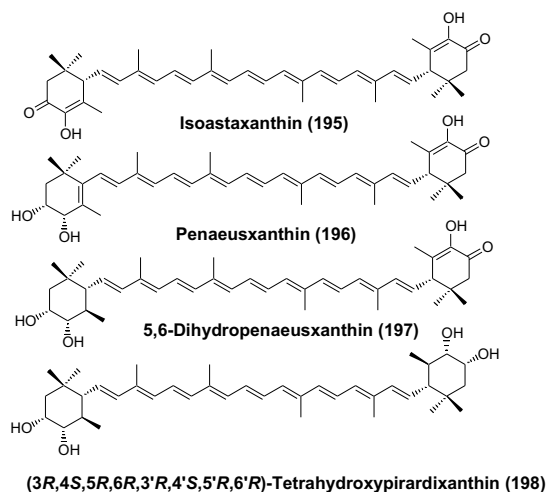
Carotenoids (187) ~ (189) were obtained by the interaction of the foreign carotenoid ketolase CrtW and endogenous epoxy carotenoids unique to sweet potato tubers [27].



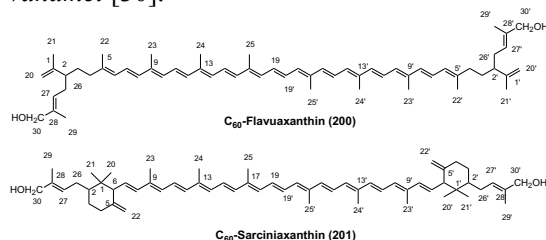
Carotenoids (190) ~ (193) were obtained as reaction products of β -Carotene and zeaxanthin with reactive oxygen species [28].



Carotenoid (194) was isolated from the rosary goby *Gymnogobius castaneus* [29].



Carotenoids (195) ~ (199) were isolated from the prawn *Penaeus japonicus* and *Penaeus vanamei* [30].



Carotenoids (200) and (201) were obtained genetically engineering biosynthetic product in *Escherichia coli* [31].

Improvements of analytical instruments such as NMR, MS, and HPLC have made it possible to perform the structural elucidation of very minor (141, 199) or complex structures of carotenoids (142~146) in nature. Determination of complex stereo-structure of carotenoids (165~171) is needed chemical synthetic approach. Progress of LC/MS, LC/MS/MS methods can be determined unstable compounds such as reaction products of carotenoids with reactive oxygen species (179~184 and 190~193). These results indicated that carotenoids scavenged not only singlet oxygen but also hydroxyl radical and superoxide anion radical through chemical reactions. Furthermore, carotenoids are able to capture peroxynitrite and nitrogen dioxide

radicals from their molecules to form nitro-carotenoids (**129~131** and **147~151**). Pathway engineering produced several interesting structural carotenoids, which are not found in nature.

In conclusion, I determined total 201 carotenoids during for decade as described above. I acknowledged all of co-authors of my publications.

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Molecular breeding of a biosynthetic pathway to C₆₀ phytoene

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Abstract: Creation of carotenoids with non-natural large backbones is probably the most straightforward strategy for expanding the biological and non-biological functions of carotenoids. To establish biosynthetic pathways that can accommodate larger number of conjugated double bonds (CDBs), we have been evolving natural carotenoid biosynthetic genes into those capable of making C₅₀, C₆₀ and even larger carotenoids. In the present study, we report our latest efforts to improve >C₆₀ carotenoid pathways we have previously constructed. By mutating HexPS and C₆₀-producing CrtM variants, we have succeeded in increasing the efficiency of C₆₀ phytoene biosynthesis in *Escherichia coli* by 50%. We also examined, for the first time, the possible competition between C₆₀ carotenoid pathways and endogenous quinone biosynthetic pathways.

1. Introduction

Carotenoids are characterized for the long polyene chains stretching along their backbones. The size of polyene (the number of conjugated double bonds (CDB)) is determinant factor for the photochemical properties of the carotenoid. In general, increase in CDB results in the red-shifted absorption spectrum [1], and in the elevation of antioxidant properties [2]. Besides these established properties, carotenoids are gathering growing interest as field-effect transistors (FETs)[3-5] and/or photovoltaic /photonic devices [6-8]. Here again, the CDB is one of the most important determinant factors of carotenoids' device specification as nanodevices.

One of the most straightforward way to expand the functional scope of carotenoids is to make larger carotenoids that can accommodate larger CDBs. Fully conjugated acyclic natural carotenoids diapolycopene (C₃₀) and 3,4,3',4'-tetrahydrolycopene (C₄₀), can harbor 11 and 15 CDBs, respectively. On the other hand, C₅₀, C₆₀, and C₇₀ carotenoids can theoretically accommodate 19, 23, and 27 CDBs when they could be fully desaturated. In the last decade, we have successfully established the biosynthetic pathways toward C₅₀- [9,10], C₆₀- [11] and C₆₅-phytoenes [11]. After multiple rounds of directed evolution [9,10, 12-14], the efficiency and selectivity of C₅₀ carotenoid pathways have grown to the level comparable with or even superior to natural ones, and this allowed us to create diverse set of carotenoids with various functionalities [15-17]. In contrast, we have had only a limited success in improving C₆₀ carotenoid pathways so far, and the amount of C₆₅-phytoene we can make remains minimal [11].

In order to improve the production level of C₆₅-phytoene, we introduced size-shifting mutations to *Micrococcus luteus* HexPS [18]. Unfortunately, none of them were decent suppliers of C₃₅PP and C₃₀PP, but we found one mutant HexPS_{V76A} showed unique feature: the expression of this gene resulted in the accumulation of

C₂₀PP, C₂₅PP, C₃₀PP, and C₄₀PP with similar level. We also discovered that the introduction of T153A mutation shifted the size specificity of the best known C₆₀-synthesizing mutant of *Staphylococcus aureus* CrtM [11], namely CrtM_{4AG}, without compromising its foldability. Using thus obtained new CrtM mutant CrtM_{5AG}, together with HexPS_{WT} we could have established the C₆₀-phytoene pathway that is 1.5 time more efficient than the best pathway previously constructed. In addition, we found the direct proof of the competition between C₆₀-phytoene pathways and endogenous quinone biosynthetic pathways for their shared precursor C₃₀PP. According to the result we obtained, the C₆₀-phytoene pathway we created already outmatches the endogenous quinone pathways.

2. Materials and Methods.

2.1 Strains and Reagents.

E. coli XL-10-Gold was used for cloning, and *E. coli* XL-1-Blue was used for carotenoids production. All enzymes used in DNA works were purchased from New England Biolabs. The chemicals used were Lennox-LB Broth Base (Life Technologies), Bacto Yeast Extract and Bacto Tryptone (BD Biosciences). All other chemicals and reagents used were purchased from Nacalai Tesque (Kyoto, Japan). The antibiotics used were carbenicillin (50 µg/mL) and chloramphenicol (30 µg/mL).

2.2 Plasmid Construction.

pUC-CrtM, pUC-CrtM_{3A} pUC-CrtM_{4AG} and pAC-HexPS2 were prepared in our previous paper [11]. pAC-HexPS_{V76G} was newly constructed by inverse PCR with pAC-HexPS2 as template and with the primer set, 5'-CAGCAGTATCTCTGGAGCTCggtCATATGGCAAGTCTCGTTCATGATG-3' and 5'-GAGCTCCAGAGATACTGCTGTACGAATCAAG-3' (bases with small letter correspond to the codon introduced), followed by the Gibson assembly [19] (underlined denotes the overlapped

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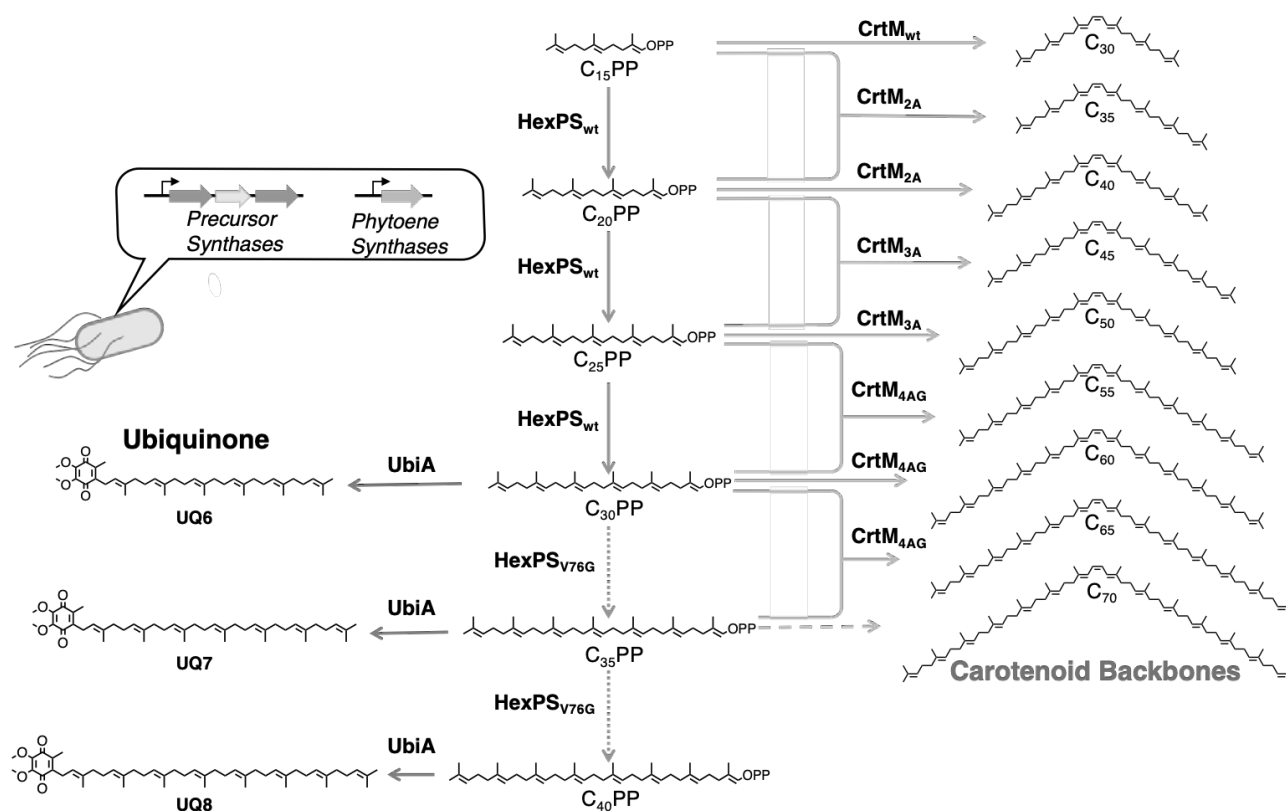


Figure 1. *Escherichia coli* production of C₆₀- and C₆₅-phytoene.

region necessary for assembly). pAC-HexPS_{V76A} was constructed also Gibson assembly of the product of inverse PCR with pAC-HexPS2 as template and the primer set, 5'-CAGCAGTATCTCTGGAGCTCgctCATATGGCAAGTCTCGTTTCATGATG-3' and 5'-GAGCTCCAGAGATACTGCTGTACGAATCAAG-3'. pUC-CrtM_{5AG} was constructed by the Gibson assembly of the product of inverse PCR, which was prepared using pUC-CrtM_{4AG} as template and the primer set, 5'-CATGAAACCCACCAGgctTATGACGTCGCGCGTC-GTCTGGGC-3' and 5'-CTGGTGGGTTTCATGGTCGGACG-3'.

2.3 Bioproduction and extraction of carotenoids.

Single colonies were inoculated into 2 mL of LB media with antibiotics in culture tubes and shaken at 37°C for overnight. The pre-culture was diluted 100-fold into 40 mL of fresh Terrific Broth in a 200-mL flask and shaken at 30°C and 200 rpm for 48 h. Each cell culture was centrifuged at $3,270 \times g$ and 4°C for 15 min. The cell pellets were washed with 10 mL of 0.9% (w/v) NaCl_{aq} and re-pelleted by centrifugation. Products were extracted by adding 10 mL of acetone, followed by vigorous vortexing. Hexane (1 mL) and 1% (w/v) NaCl_{aq} (35 mL) were added, and the samples were centrifuged at $3,250 \times g$ for 15 min. The product-containing hexane phase was collected. Hexane was evaporated by a vacuum concentrator. Then, the extracts were dissolved in 50 µL of THF:methanol (6:4) for further analysis.

2.4 HPLC-MS analysis.

A 5 µL aliquot of the final extract was analyzed using the Shimadzu Prominence HPLC-PDA-MS (APCI) system. The column was a Waters Spherisorb ODS2 Analytical Column (4.6 x 250 mm, 5 µm, PSS831915). The mobile phase used was methanol/isopropanol (6:4) at 0.25 mL/min. The detectors used were a photodiode array (200-700 nm) and APCI-MS. Mass scans were measured from m/z 10 up to m/z 1000 at a 300°C interface temperature, 300°C DL temperature, ± 4500 V interface voltage, and neutral DL/Qarray with N₂ as the nebulizing gas.

3. Results and Discussion.

3.1 Testing HexPS mutants as feeders for C₃₅PP and C₄₀PP

Hexaprenyl diphosphate (C₃₀PP) synthase is highly specific C₃₀PP synthase, but several mutations are known to change its products to those with larger sizes (C₃₅PP, C₄₀PP, C₄₅PP) [18, 20, 21]. Replacement of Valine 76 shift the size specificity of this enzyme, and the in vitro feeding of excess amount of substrates (C₁₅PP and the elongation unit isopentenyl diphosphate) to the purified HexPS_{V76G} resulted in the accumulation of C₄₀PP [18]. We expected the moderate expression of this mutant, using medium copy plasmid, could result in the intercellular accumulation of

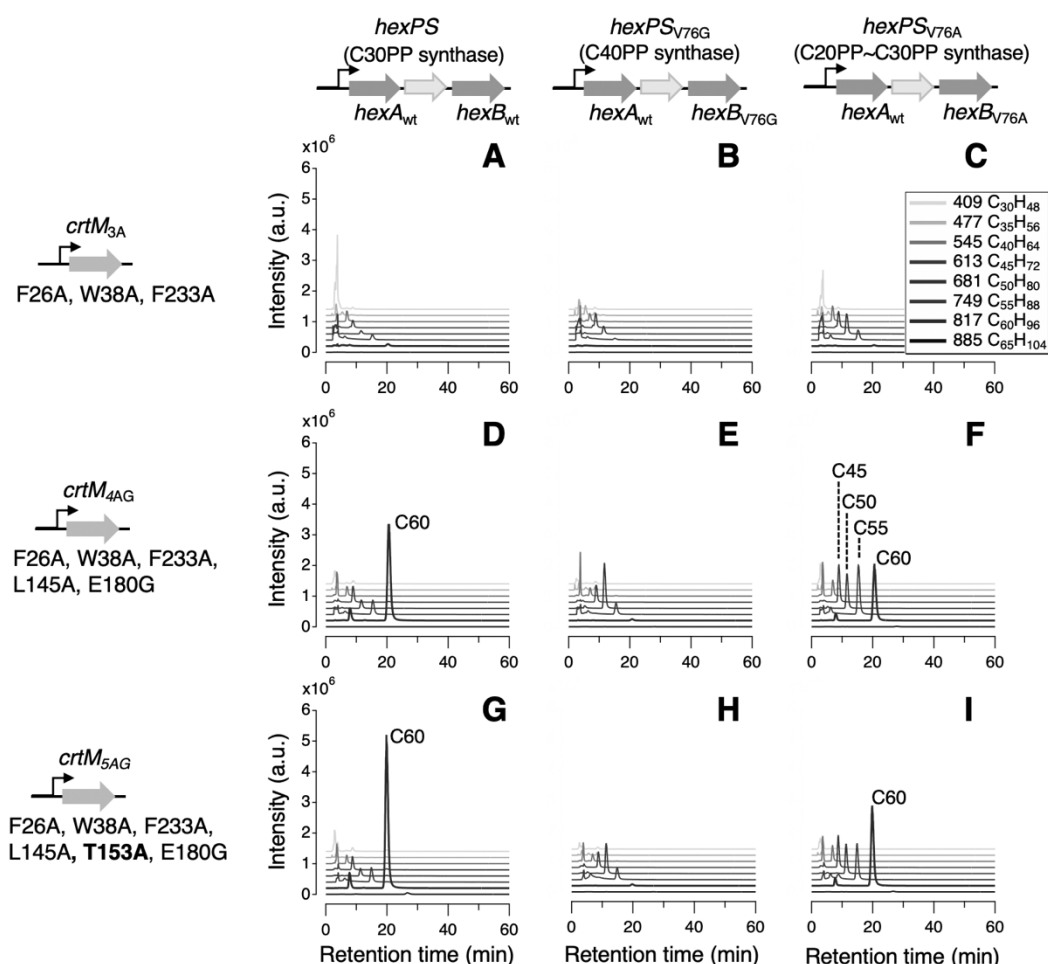


Figure 2. HPLC analysis of the carotenoid fractions of the *Escherichia coli* cells harboring pathways for C₆₀-phytoene. All of the chromatograms (A-I) were taken from the *E. coli* cell harboring indicated pairs of HexPS variants (shown upper) and CrtM variants (shown left).

C₄₀PP, C₃₅PP, in addition to the cognate product of HexPS C₃₀PP.

CrtM_{3A} is the size shifted mutant of CrtM known as efficient C₅₀-phytoene synthase [10]. CrtM_{4AG} was created by adding two mutations (L145A and E180G) to CrtM_{3A} and it is known as the best available the C₆₀-phytoene synthase [11]. Either by co-expressing with wild-type HexPS (HexPS_{WT}) or HexPS_{V76G}, CrtM_{3A} accumulated almost no carotenoids (**Fig. 2A** and **Fig. 2B**), indicating that CrtM_{3A} is the poor C₃₀PP consumer. In sharp contrast, expression of CrtM_{4AG} with HexPS_{WT} resulted in the accumulation of C₆₀ phytoene (**Fig. 2D**). When CrtM_{4AG} was co-expressed with HexPS_{V76G}, however, host cell accumulated virtually no carotenoid products (**Fig. 2E**). This indicates HexPS_{V76G} is highly active in heterologous host (*Escherichia coli*) and behave as C₄₀PP synthase. It is known that CrtM_{4AG} cannot accept C₄₀PP as substrate [11].

Given the results above, we decided to place alanine at the residue 76 instead. Alanine is slightly larger than glycine but smaller than the original amino acid valine. Co-expression of CrtM_{4AG} with HexPS_{V76A} accumulated the C₆₀-phytoene accompanied by several other phytoenes with smaller sizes (C₅₅, C₅₀, C₄₅). We do not know exactly how

these minor carotenoids showed up. Probably, V76A somehow decreased the affinity (1/K_M) of HexPS to its reaction intermediates (C₂₀PP, C₂₅PP), thereby allowing these intermediates to be intercepted by the promiscuous, multi-functional synthase CrtM_{4AG}. As a consequence, this pathway accumulated number of <C₆₀-phytoenes and the amount of C₆₀-phytoene was rather decreased. We did not observe appearance of C₆₅-phytoene (**Fig. 2F**).

3.2 Consumption of C₃₀PP and C₃₅PP by ubiquinone pathways.

Living cells are extremely ‘dirty’ reaction tube contaminated with thousands of metabolites and enzymes (catalysts). When a new artificial pathway is installed into the cell, one must consider the possible cross-reactions with or intervention from endogenous metabolic networks. Because C₃₀PP and C₃₅PP are structurally very close to the C₄₀PP, the native size chain of quinones in *E. coli*, they could be consumed by the endogenous quinone pathways. Indeed, HPLC analysis revealed the molecular size of ubiquinone drastically change upon installing pathways for large carotenoids (**Fig. 3**).

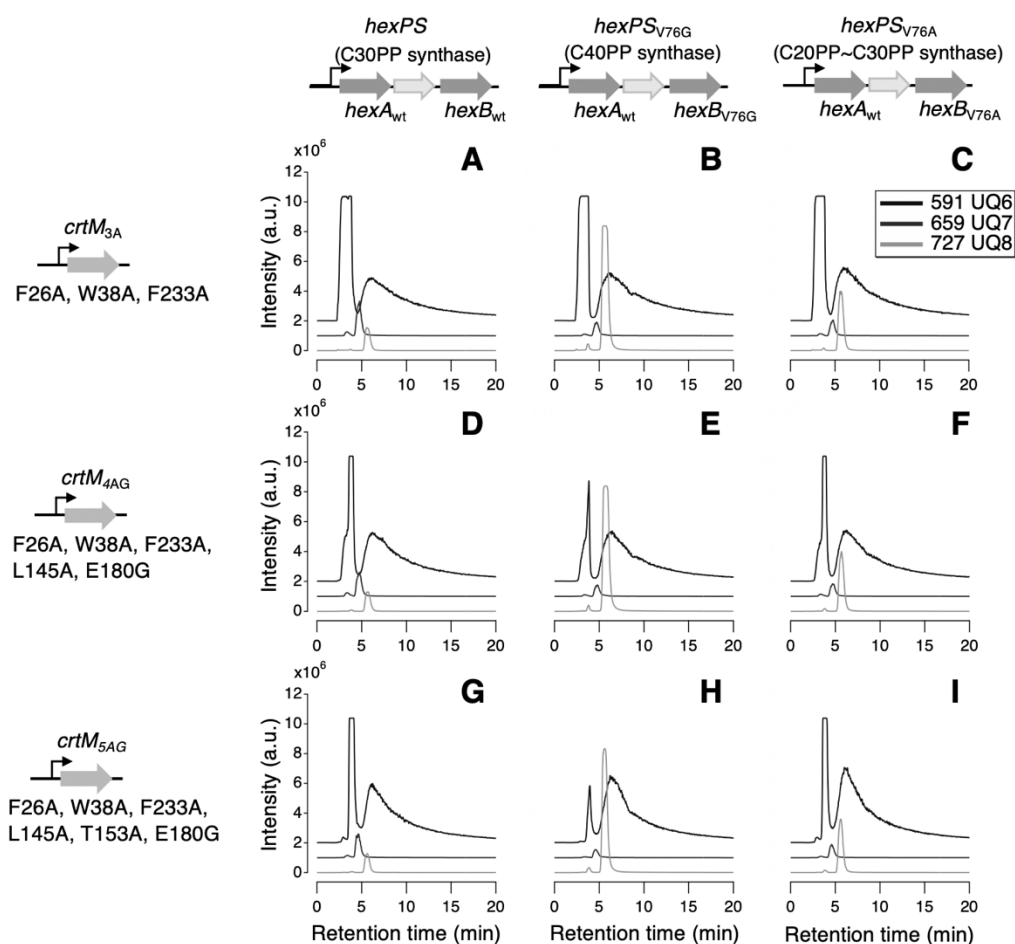


Figure 3. Size distribution of ubiquinones in the cells harboring pairs of the variant of HexPSs and CrtMs. All of the chromatograms (A-I) were taken from the *E. coli* cell harboring indicated pairs of HexPS variants (shown upper) and CrtM variants (shown left).

Because CrtM_{3A} does not consume prenyl diphosphate larger than C₃₀PP, cells harboring CrtM_{3A} directly represents size distribution of C₃₀PP, C₃₅PP, and C₄₀PP available to ubiquinone pathways. Cellular ubiquinones (UQs) were found to be either forms of UQ6 (UQ with C₃₀ side chain), UQ7 (C₃₅), or UQ8 (C₄₀), irrespective to the HexPS variants expressed in the cell (**Fig. 3A-C**). The natural or cognate *E. coli* UQ is UQ8, but when HexPS is expressed, UQ6 becomes dominant. None of the three tested HexPSs led to the accumulation of UQ7 with significant amount, suggesting we were not successful in establishing feeding system of C₃₅PP (**Fig. 2E** and **Fig. 2F**). Thus, we failed to improve the production level of C₆₅-(C₃₀+ C₃₅) phytoene, trace amount of which was previously shown to be accumulated by CrtM_{4AG} [11].

It should be noted that the cell expressing CrtM_{4AG} accumulated markedly decreased UQ6 (**Fig. 3D-F**) compared with those expressing CrtM_{3A} (**Fig. 3A-C**). This indicates that heterologous the C₆₀-phytoene pathway surpass the promiscuous activity of endogenous quinone prenyl transferase (UbiA) on C₃₀PP consumption. In contrast, the peak size of UQ8 remained same between the cells expressing CrtM_{3A} and those expressing CrtM_{4AG}: this

is in good agreement with the fact that neither of them consumes C₄₀PP [11].

3.3 Evolving C₆₀ phytoene synthase activity of CrtM variant by T153A.

Currently, the most size-shifted mutant of CrtM ever created is CrtM_{4AG} [11]. This mutant efficiently takes up two molecules of C₃₀PP to as substrates and condense them into the C₆₀-phytoene. It also accepts at least one molecule of C₃₅PP, yielding trace amount of C₆₅-phytoene. This mutant has four size-specificity mutations (F26A, W38A, FL145A, F233A) together with one stabilizing mutation (E180G). There are many other bulky residues inside the pockets, and calculation indicated that alanine substitution to these residues indeed enlarge the volume of its reaction pocket. However, the CrtM_{4AG} is already very unstable (according to the Fold-X calculation [22, 23], CrtM_{4AG} is 12.3 kcal/mol more unstable than CrtM_{WT}). Thus, our previous attempt to add possible size-shifting mutations (F22, L29, V37, Y41, C133, L141, L160, and I241) failed to produce better C₆₀/C₆₅-phytoene synthase [11].

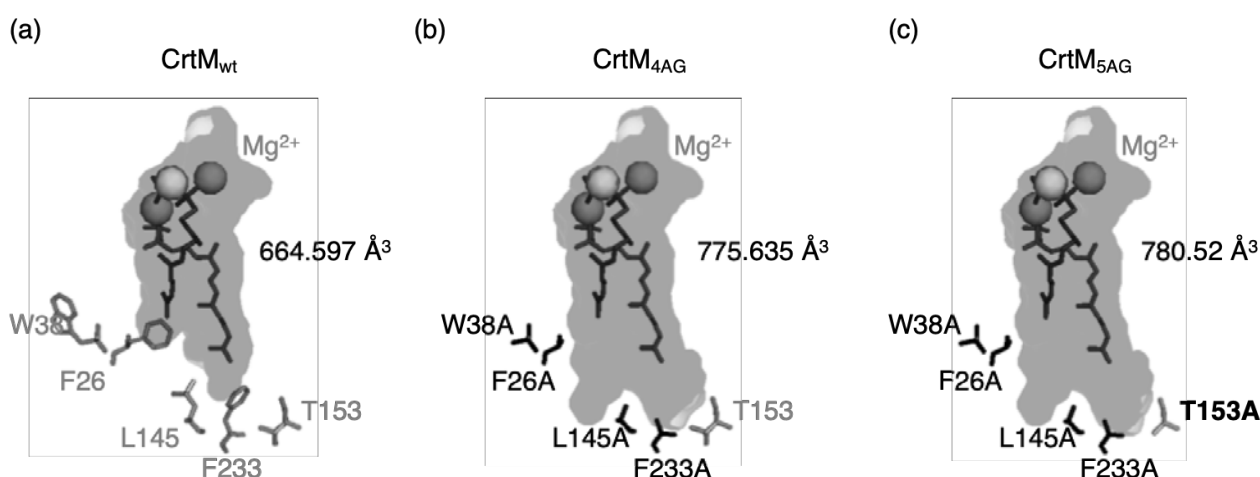


Figure 4. Structural mapping of T153A mutants. The structure of (a) CrtM_{WT} (PDB 3W7F), modeled structure of, (b) CrtM_{4AG}, and (c) CrtM_{5AG}. Shaded in gray are the calculated shape of substrate pockets of the respective variants.

In this work, we turned our attention to possibly more subtle size-shifting, but less destabilizing mutations. Among the many residues sitting in the substrate pockets, one residue T153 caught our attention (**Fig. 4**). Fold-X calculation indicated a positive but almost negligible score ($\Delta\Delta G_{\text{fold}}$ of +0.66 kcal/mol). It resides at the very bottom of the reaction pocket of CrtM_{4AG}, and alanine substitution of this residue slightly increased the calculated pocket volume of CrtM_{4AG} from 775.635 Å³ to 780.52 Å³. Interestingly, this mutation alone did not change the cavity size of the CrtM_{WT} (data not shown).

We conducted site-directed mutagenesis to introduce T153A into CrtM_{4AG} to make new mutant CrtM_{5AG}. This mutant was co-expressed with HexPS and its variants (**Fig. 2G-I**) in hope for observing improved yielding in >C₆₀-phytoenes. Indeed, we observed ~50% increase in the C₆₀-phytoene yield. Unfortunately, however, the production of C₆₅-phytoene remained trace even with this mutation.

4. Conclusions.

Carotenoid with larger backbones have a promise as superior antioxidant [2], novel pigment with new, red-shifted color, as well as the unique and extended properties as molecular photonic/photovoltaic devices.

We attempted to further evolve the efficiency of >C₆₀ carotenoid pathways, by creating mutating HexPS and CrtM variants. Although our attempt to shift size specificity of HexPS failed to increase either of C₆₀ and C₆₅ carotenoids, alanine substitution of CrtM_{4AG} resulted in as much as 50% increase in the yield of the C₆₀-phytoene (**Fig. 2G**). As of today, this is the best available the C₆₀-phytoene biosynthetic pathway.

Interestingly, the expression of HexPS could result in the domination of UQ6 (**Fig. 3A-C**). Thus, a part of the precursor for the C₆₀-phytoene is possibly drained out into quinone biosynthetic pathways. Luckily, our CrtM variants (CrtM_{4AG} and CrtM_{5AG}) seem already efficient enough so that their expression drastically decrease in UQ6 (**Fig. 3D-**

I), indicating our prototype C₆₀-phytoene pathway is already outcompeting the quinone pathways. As far as we know, this is the first examination of the possible competition between carotenoid and quinone pathways.

As of today, the apparent bottleneck lies in the shortage of backbone synthase (CrtM variants) in making C₆₅, C₇₀, or even larger carotenoids. To further increase the efficiency of making C₆₀ carotenoids and to make meaningful amount of C₆₅ or C₇₀ carotenoids, extensive engineering effort on CrtM will be required. The first step needs to discover mutations stabilizing the C₆₀-phytoene synthase, this time CrtM_{5AG}, without altering its size specificity.

Acknowledgements

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Application of DOSY Experiment to Analysis of Astaxanthin and its' Analogues

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Abstract: Diffusion-ordered spectroscopy (DOSY) is a relatively new method of separating the NMR signals of different species in a mixture according to their diffusion coefficients. Although a few applications of this attractive method to limited compounds have been presented, to date there have been no reports of its application to the field of carotenoids. This work reports the application of DOSY to the mixture of a variety of astaxanthin, its analogues and other compounds.

1. Introduction

Diffusion-ordered spectroscopy (DOSY) is a relatively new method of separating the NMR signals of different species in a mixture with characterization based on their diffusion coefficients [1]. The DOSY spectrum is a kind of

2D spectrum that adds the axis of diffusion to ¹H-1D spectrum. Additionally, we can measure 3D spectrum, for example such as DOSY-COSY, by adding the axis of diffusion to the original 2D spectrum of COSY or HSQC, etc. This is a very useful method in which separate signals of the

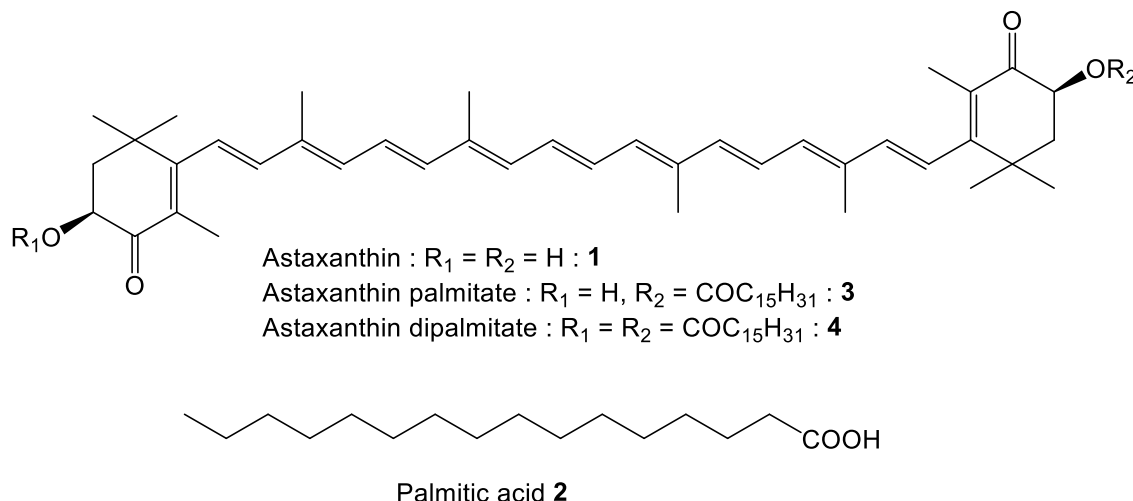


Figure 1 Chemical structures of carotenoid and aliphatic acid

particular compound are observed from the overlapping signals of the other components and/or solvent.

Although this attractive method has been applied to mixed samples in fields such as organic chemistry [2], natural products research [3], and food chemistry [4] so far there have been no reports of applications in the field of carotenoids. In this field, it is sometimes difficult to separate each carotenoid component from natural mixed extracts, because the extracts occasionally contain aliphatic acids and many lipids. We chose astaxanthin from many kinds of carotenoids. Astaxanthin **1**, one of the dominant carotenoids in marine animals, has become a focus of attention due to its extremely strong antioxidant activities [5]. Astaxanthin **1** has two hydroxyl groups in the molecule as shown in Figure 1, therefore, it is existed monoester and/or diester of **1** and aliphatic acids as well in nature. In the first present work, DOSY experiments were applied to a mixture of astaxanthin **1** and palmitic acid **2** (Figure 1) as a model of carotenoid extracts (*Experiment 1*). In the next works, we tried some changed the measurement parameters in DOSY experiments (*Experiment 2*) for the optimum conditions using the mixture of more similar to natural mixed extracts, were **1**, **2** and synthesized astaxanthin esters **3**, **4** (Figure 1). In the last work, we applied to DOSY experiment (*Experiment 3*) for the extracted lipid containing astaxanthin esters [3] from Spiny Lobster (*Panulirus japonicus*). The

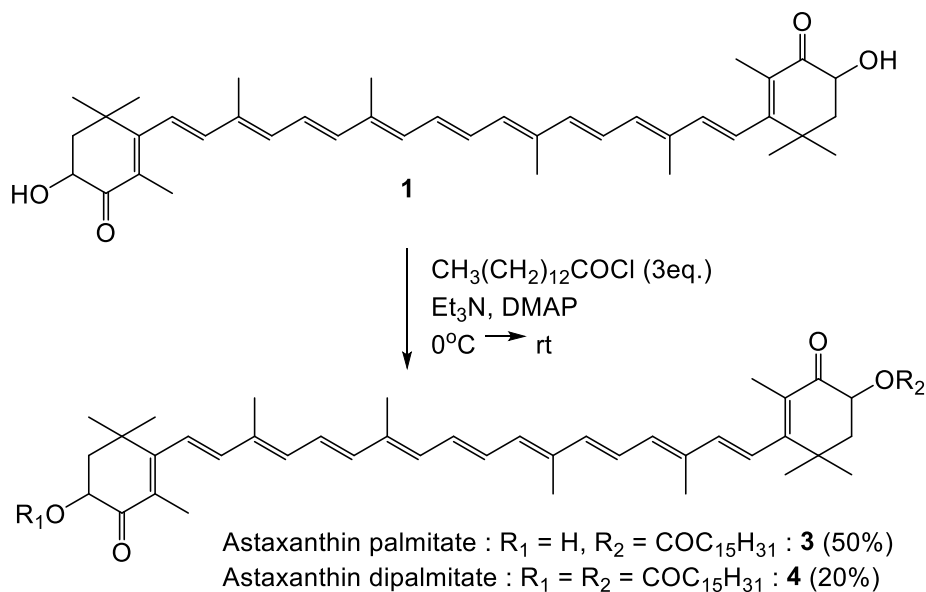
purpose of this work is to examine the usefulness of this method in the field of carotenoids.

2. Experimental

2.1 Instruments. The UV-VIS spectra were recorded on a JASCO Ubest-55 instrument for ethanol solutions. IR spectra were measured on a Perkin Elmer FT-IR spectrometer, model Paragon 1000, for chloroform solutions. ESI/MS data were obtained using an EXACTIVE (Thermo Fisher Scientific Inc., Bremen, Germany). ¹H-NMR spectra were measured on a Varian NMR system 500 spectrometer (¹H : 500 MHz), respectively, for CDCl₃ solutions (tetramethyl silane as internal reference).

2.2 Synthesis of astaxanthin esters.

Palmytoyl chloride (0.05 mL, 0.15 mmol) was added to a stirred solution of astaxanthin (30 mg, 0.05 mmol), distilled Et₃N (0.04 mL, 0.25 mmol) and DMAP (24 mg, 0.2 mmol) in dry CH₂Cl₂ (10 mL) at 0°C. The mixture was stirred at 0 °C for 30 min and at rt for 2h. Then, the reaction mixture was poured into ice-water and extracted with Et₂O. The extracts were washed successively with 5% aq. HCl, saturated aq. NaHCO₃ and brine. Evaporation of the dried solution followed by Column Chromatography (acetone-Hex, 1~3 : 9~7) afforded the astaxanthin palmitate **3** (21 mg, 50%)[6] as a red solid and astaxanthin dipalmitate **4** (10 mg, 20 %)[7] as a red solid (Scheme 1).



Scheme 1

2.3 Extraction of lipid containing astaxanthin from Spiny Lobster

(*Panulirus japonicus*) [8] Acetone (300 mL)

was added to two fractured Spiny Lobster's shell, and mixture was filtered through filter paper. The filtered solution was concentrated to one-third of original volume. This solution was then partitioned with Et_2O /Hex (1 : 1) and water. The organic layer was dried over Na_2SO_4 and allowed to evaporate. The resulting reddish residue was dissolved Hex and loaded onto silica gel column chromatography. The fraction eluted with Hex (fr.1), Et_2O -Hex (1 : 4) (fr.2 : ca 4 mg), Et_2O -Hex (1 : 1) (fr.3 : ca 3 mg), Et_2O (fr.4 : ca 3 mg), and acetone (fr.5). Their absorption maxima were indicated in about 480 nm. Consequently, it was suggested that they contained carotenoids. It proved by ^1H NMR measurement that fr.1 was contained β -carotene and fr.5 was contained

astaxanthin. And, fr2-4 were contained various compounds.

2.4 DOSY measurement.

DOSY spectra were measured on a Varian UNITY INOVA 500 or Varian NMR system 500 spectrometer (^1H : 500 MHz), respectively, using the pulse sequence of BPPSTE (DOSY bipolar pulse pair stimulated echo experiment), BPPSTE-cc (bipolar pulse pair stimulated echo experiment with convection compensation) and GCSTESL (gradient compensated stimulated echo with spin lock). J -Values are given in Hz. The gradient amplitude (g) was changed from 13 to 60 Gcm^{-1} in 20 steps for the DMSO solution and from 4 to 20 Gcm^{-1} in 20 steps for the CDCl_3 solution. The field gradient pulse width was 1 ms and the actual diffusion delay was 50 ms. For DOSY-COSY (GCSTE-COSY : gradient compensated stimulated echo COSY), the gradient amplitude (g) was changed from 1 to 40

Gcm⁻¹ in 10 steps and the number of increments was 512. All samples included TMS (tetramethylsilane) as the internal reference.

Experiment 1. Commercially available astaxanthin **1** and palmitic acid **2** were used. To compare the influence of convection currents, two kinds of solvents were used, DMSO-d₆ and CDCl₃. Sample A was prepared with **1** (ca 3 mg) and **2** (ca 2 mg) in DMSO-d₆ (0.75 mL). Sample B was CDCl₃ solution containing **1** and **2** (4 mg each in 0.75 mL). A 3 mm inside diameter (i.d.) NMR sample tube was used to prevent the effect of convection currents.

Experiment 2. Sample C was prepared with **1** (ca 6 mg, 0.01mmol), **2** (ca 3mg, 0.01mmol), **3** (ca 8mg, 0.01mmol) and **4** (ca 10 mg, 0.01mmol) in CDCl₃ (0.75mL), and this solution was applied into 5 mm inner diameter (i.d.) NMR sample tube. We performed DOSY experiments for the above solution was applied into 3 mm i.d. NMR sample tube (sample C'), too.

Experiment 3. We subjected DOSY experiments to fr.4 (sample D) were dissolved with CDCl₃ (0.75mL) into 3 mm i.d. NMR sample tube. Sample E was prepared with fr.3 in CDCl₃ (0.75 mL). Fr.2 was dissolved with CDCl₃ (0.75mL) by same method and sample F was prepared.

3. Results and Discussions

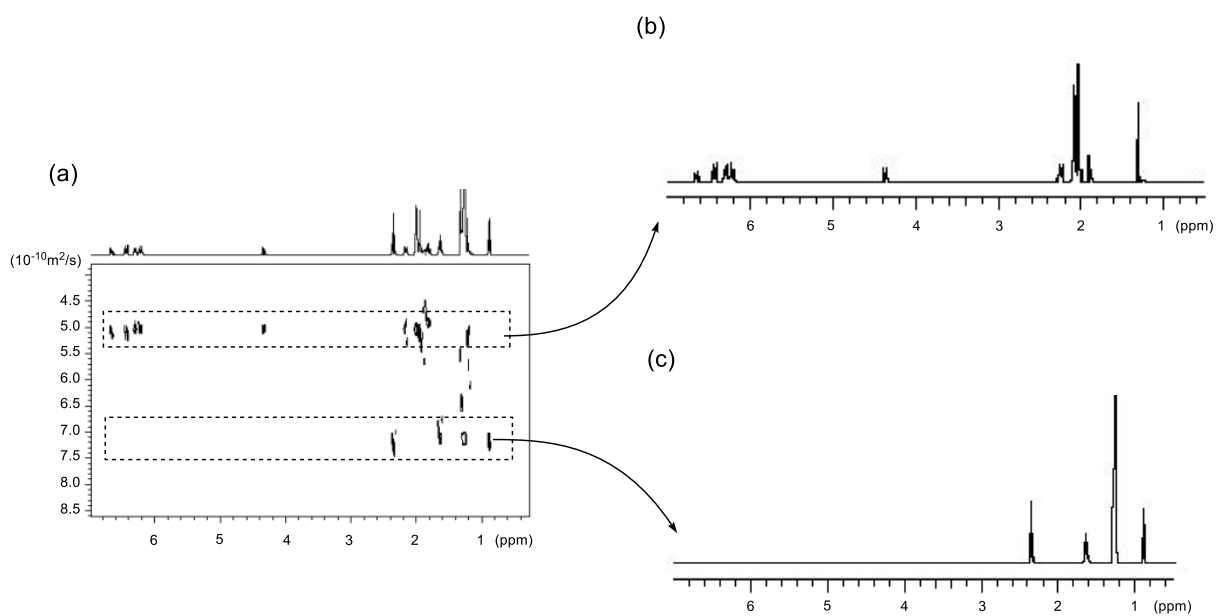
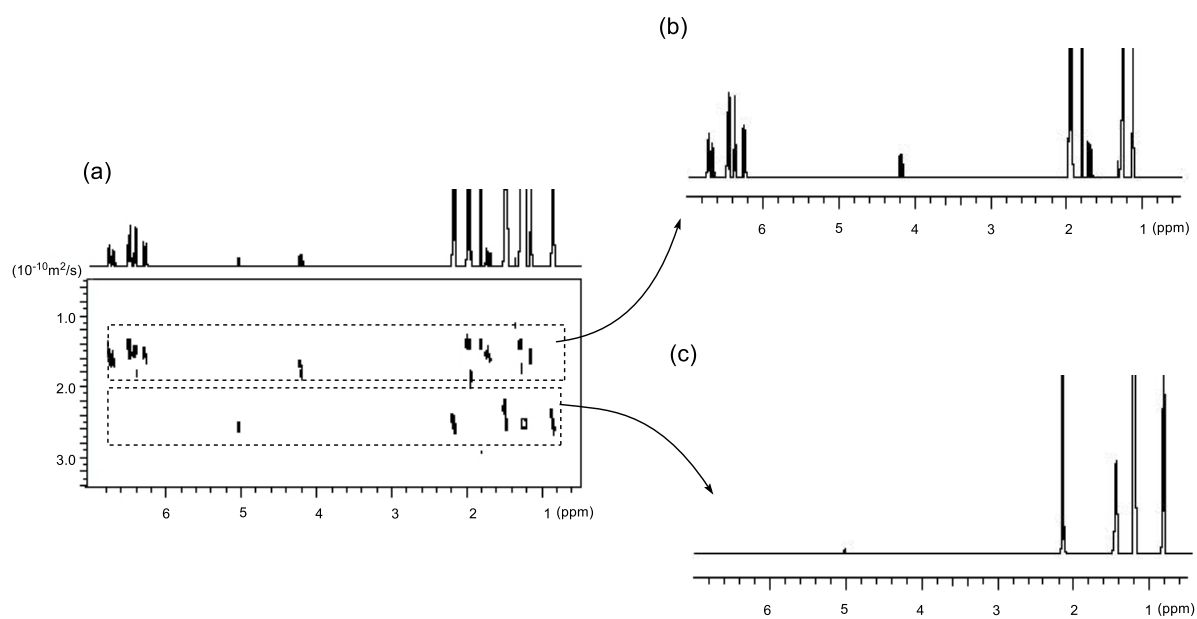
3.1 Experiment 1 : Separation of the mixture (astaxanthin 1 and palmitic acid 2),

and comparison of solvents (DMSO-d₆ and CDCl₃).

For two samples of different solvents, DMSO-d₆ (sample A) and CDCl₃(sample B), DOSY experiments have been carry out with 3mm i.d. sample tube using the BPPSTE pulse sequence, sequence commonly used for DOSY measurement. As shown in Figure 2 and 3, clear separations have been observed for both samples. Sample A (DMSO-d₆ solution) gave the diffusion coefficients of $1.2\text{-}2.0 \times 10^{-10} \text{ m}^2/\text{s}$ for **1** and $2.1\text{-}3.0 \times 10^{-10} \text{ m}^2/\text{s}$ for **2**, and in CDCl₃ solution those of $4.8\text{-}5.2 \times 10^{-10} \text{ m}^2/\text{s}$ for **1** and $6.7\text{-}7.4 \times 10^{-10} \text{ m}^2/\text{s}$ for **2** were obtained. Furthermore, the projected spectrum of each region showed the proper pattern (shown in Figure 2(b) (c) and 3 (b) (c)).

DOSY-COSY, 3D spectrum added the diffusion axis to the original COSY, were also measured for Sample A, in which two COSY spectra corresponding to **1** and **2**, respectively, were observed (Figure 4). This experiment verified the usefulness of 3D DOSY for the structural analysis of carotenoids in a mixture of natural extracts.

In generally, in the DOSY experiment, it is preferable to use a solvent having a high viscosity in order to suppress the convection effect. However, in these experiments, CDCl₃ solution shows the larger separation, so this solvent which is the most general for NMR experiment has been selected to use in our experiments of this field.



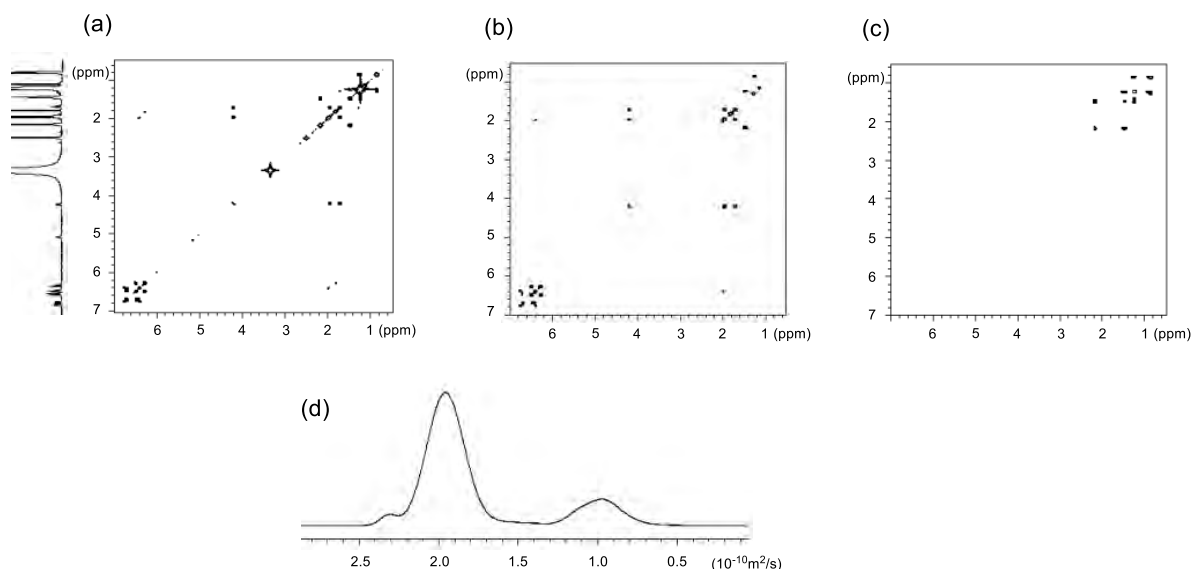


Figure 4. (a) DOSY-COSY spectrum of sample A (b) Slice spectrum of **1** (c) Slice spectrum of **2** (d) Diffusion coefficient of sample A

3.2 Experiment 2 : Separation of the mixture sample mimicked natural mixed extracts and examinations of sample tube's i.d. and pulse sequence.

In this section, the mimetic mixture closer to natural mixed extract were used as the sample, in which astaxanthin **1**, its mono- **3** and di- **4** palmitate and palmitic acid **2** were contained, since **1** having two hydroxyl groups was usually extracted as the mixture with its esters of higher aliphatic acid from natural products.

For this sample (Sample C and C' in CDCl₃), the influence of sample tube's inner diameter (i.d.), 5 mm and 3 mm, was examined. Comparison between Figure 5a and 5b indicates that the thinner (3 mm i.d.) sample tube is better. The thicker inner diameter should induce convection more.

However, the separation of each components on the spectrum of Figure 5b using conventional BPPSTE pulse sequence was unsatisfactory, though dispersion of magnetization suppressed partly.

Two more sophisticated pulse sequence were tried to use for measuring this sample. BPPSTE-cc (Bipolar Pulse Pair Stimulated Echo with convection compensation) [9] is the modified BPPSTE sequence to inhibit convection effect and GCSTESL (Gradient Compensated Stimulated Echo with Spin Lock) [10] is the sequence adding a spin lock purge pulse to GCSTE [11] suppressing the spread of diffusion values. The resulting spectra were shown in Figure 6, in which fairly improvements were observed with both sequences.

Although, in Figure 6(a) with BPPSTE-cc sequence, each diffusion coefficients were estimated as $5.4\text{--}6.2 \times 10^{-10} \text{ m}^2/\text{s}$ for **1**, $7.3\text{--}8.6 \times 10^{-10} \text{ m}^2/\text{s}$ for **2**, $4.7\text{--}5.7 \times 10^{-10} \text{ m}^2/\text{s}$ for **3** and $4.3\text{--}5.1 \times 10^{-10} \text{ m}^2/\text{s}$ for **4**, the fully separations were not observed, especially among **1**, **3** and **4**. On the other hand, with GCSTESL sequence, slightly well separation obtained, resulting diffusion coefficients of $2.8\text{--}3.4 \times 10^{-10} \text{ m}^2/\text{s}$ for **1**, $3.7\text{--}4.4 \times 10^{-10} \text{ m}^2/\text{s}$ for **2**, $2.4\text{--}2.9 \times 10^{-10} \text{ m}^2/\text{s}$ for **3** and $1.9\text{--}2.6 \times 10^{-10} \text{ m}^2/\text{s}$ for **4**.

for **4**.

These experiments showed that GCSTESL is the proper sequence for the measurements of this field, but the results were not completely satisfied,

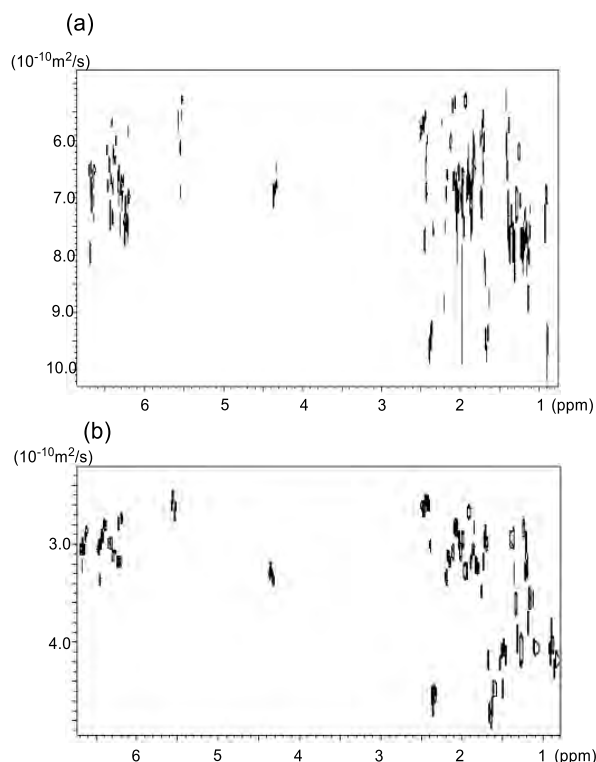


Figure 5 (a) DOSY spectrum of sample C
(b) DOSY spectrum of sample C'

3.3 Experiment 3 : Application to the mixtures extracted from Spiny Lobster (*Panulirus japonicus*).

The crude extracted sample from Spiny Lobster was expected to contain many substances, so loaded on to silica gel column chromatograph to separate roughly. Several fractions were selected to measure DOSY spectra, Sample D, E, F. The broad contents of each sample were estimate from the 1D-NMR spectra (7a, 8a and 9a), sample D include astaxanthin monoesters and aliphatic acids

yet, **1** and its analogs may be not suitable subjects for measurement of DOSY because these molecular shapes are anisotropic resulting not to fit with the Einstein-Stokes equation.

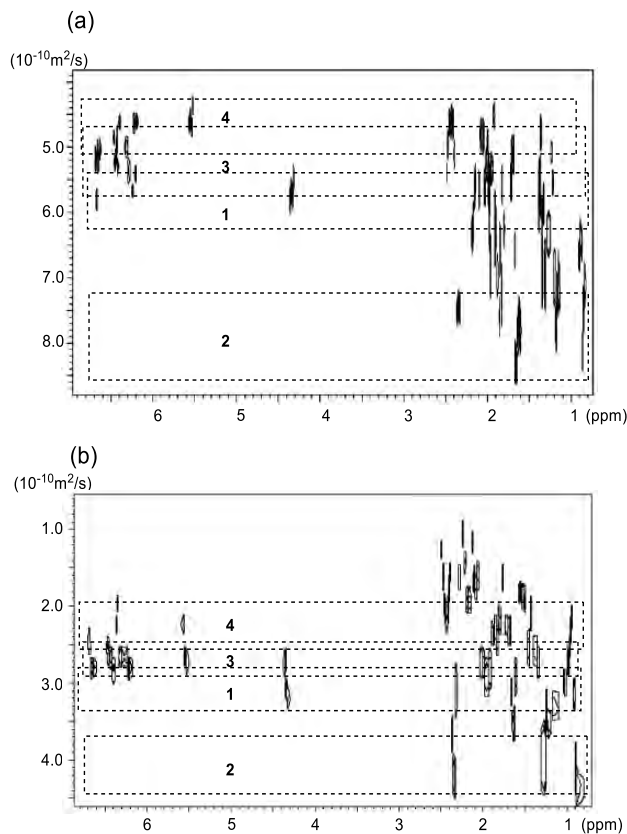


Figure 6 (a) DOSY spectrum of C' using BPPSTE-cc experiment (b) DOSY spectrum of sample C' using GCSTESL experiment

sample E included astaxanthin monoesters, aliphatic acids and steroids, and sample F included astaxanthin diesters, aliphatic acids and many other compounds. The content of carotenoid analogs is the highest for sample D and lowest for sample F. DOSY spectra with BPPSTE sequence were measured for these 3 samples.

In the spectra in Figure 7, the projected spectrum in the region observed olefinic protons (at the diffusion coefficient of $3.2\text{--}4.0 \times 10^{-10}$) was comparable with the spectrum of isolated monoester,

confirming its existence.

For sample E and F, since a lot of signals were observed in higher field region and the contents of carotenoids were low as can be seen in Figure 8a and 9a, DOSY measurements in the all regions were expected to be unsuccessful.

So we tried to measure in the low field region (3-8 ppm), as shown in Figure 8b and 9b. In Figure 8b, the projection of the region of the diffusion coefficient of $3.6\text{--}4.8 \times 10^{-10} \text{ m}^2/\text{s}$ is comparable with the spectrum of isolated monoester.

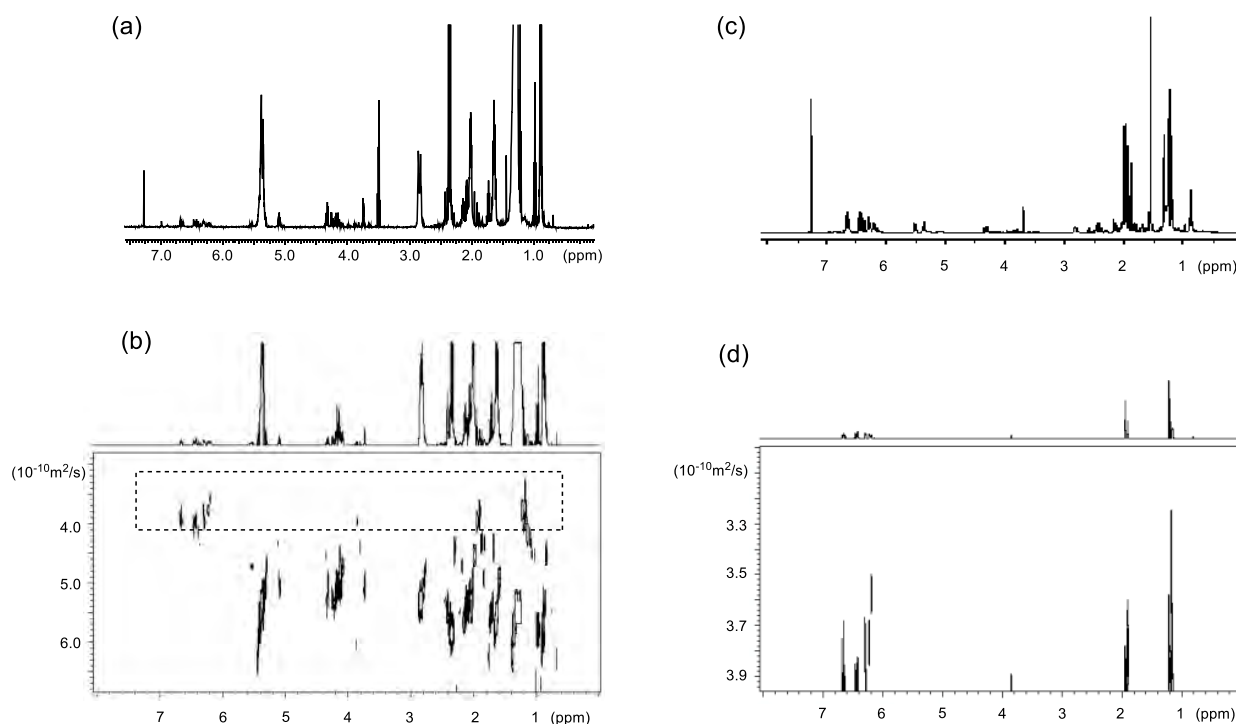


Figure 7 (a) ^1H -NMR spectrum of sample D (b) DOSY spectrum and its projected spectrum of sample D (c) ^1H -NMR spectrum of isolated monoester (d) Slice spectrum and its projected spectrum of sample D

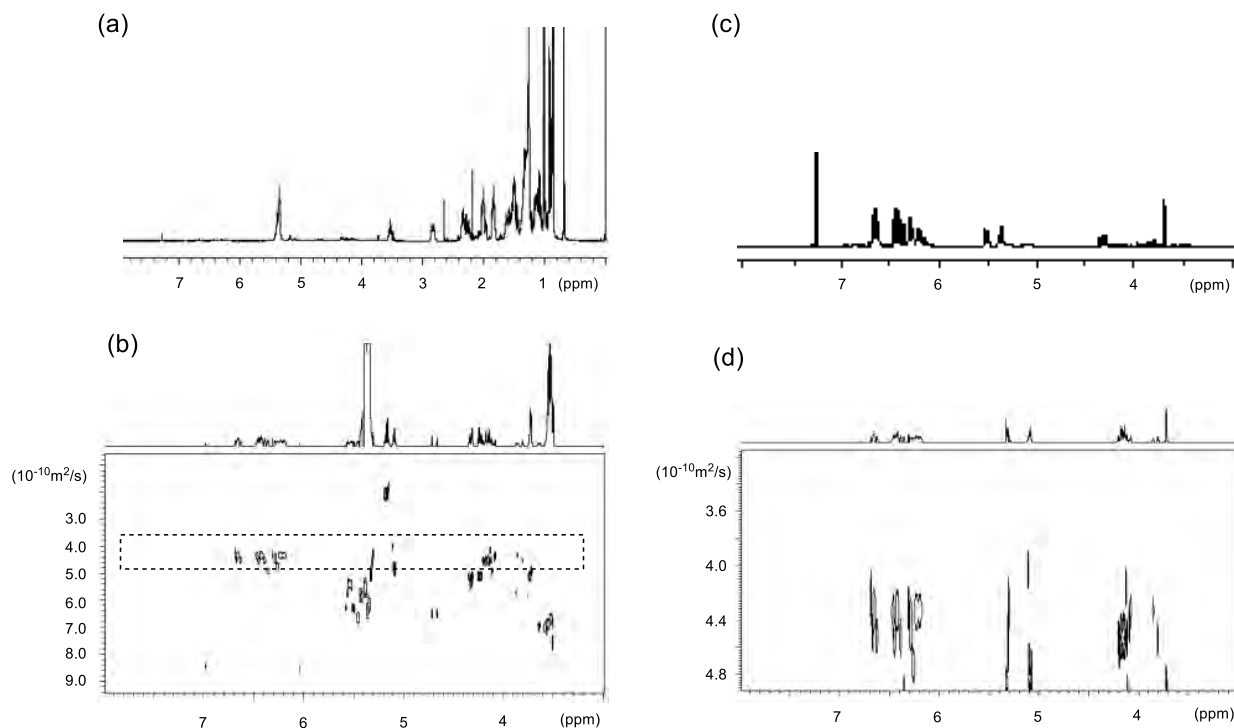


Figure 8 (a) ^1H -NMR spectrum of sample E (b) DOSY spectrum and it's projected spectrum of sample E (c) ^1H -NMR spectrum of isolated monoester (d) Slice spectrum and it's projected spectrum of sample E

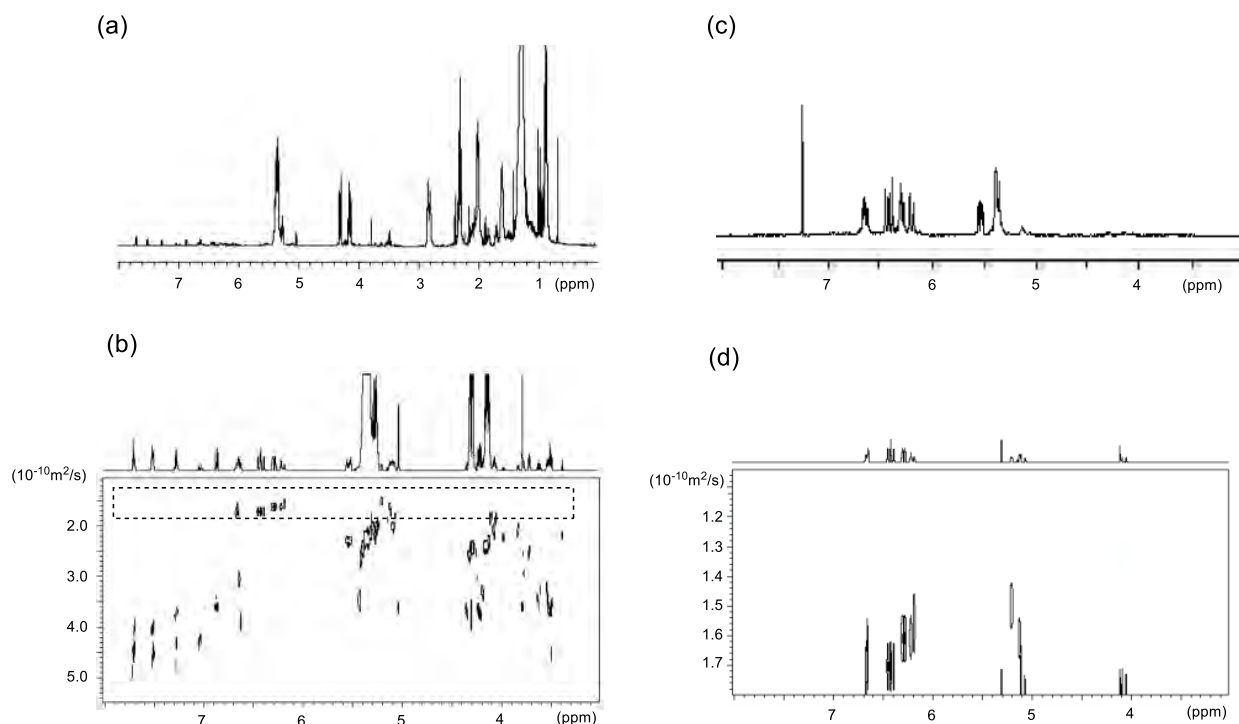


Figure 9 (a) ^1H -NMR spectrum of sample F (b) DOSY spectrum and it's projected spectrum of sample F (c) ^1H -NMR spectrum of isolated monoester (d) Slice spectrum and it's projected spectrum of sample F

On the other hand, for sample F the projected spectrum in the region of olefinic

proton, the diffusion coefficient of $1.2\text{--}1.8 \times 10^{-10} \text{ m}^2/\text{s}$, was partly agreement with the

spectrum of isolated diester.

For sample D containing the relatively higher carotenoids, DOSY-COSY experiment was tried. As shown in Figure 10, two different COSY spectra were obtained corresponding to monoester (at diffusion coefficient of 3.8-4.0)(Figure 10b) and aliphatic acid (at diffusion coefficient of

4.8-5.0)(Figure 10c), which must be provide the information about structure analyses. Particularly, in Figure 10b, cross peaks of long range coupling were observed between methyl proton at ca 2.0 ppm and olefinic proton at ca 6.2 ppm which is characteristic of carotenoids.

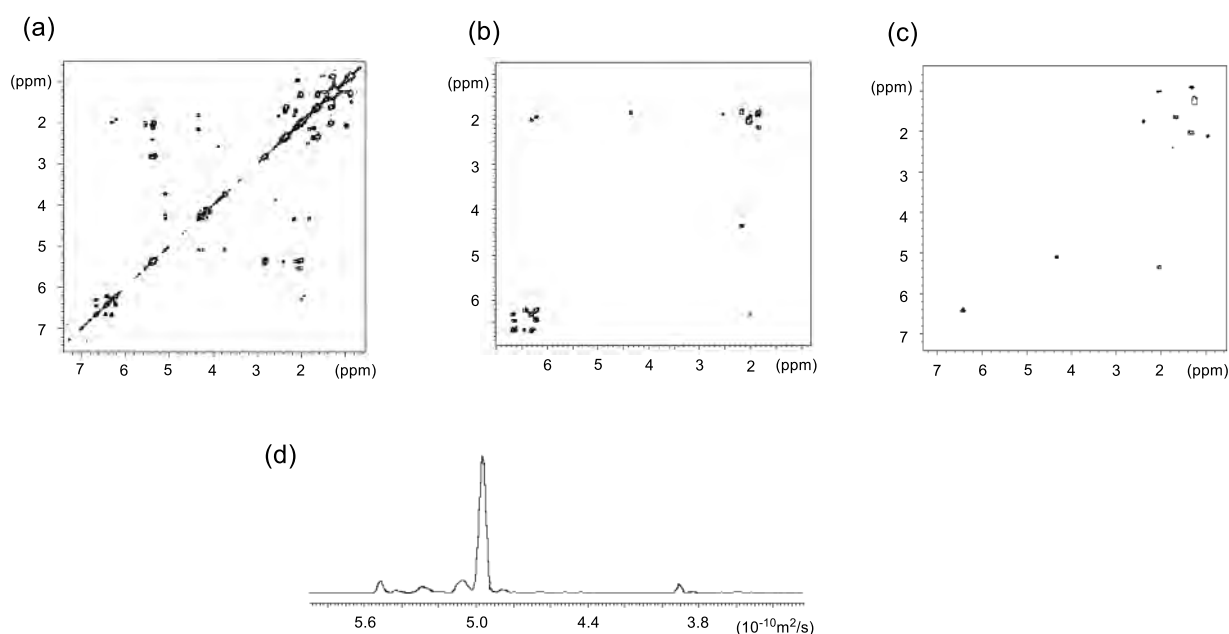


Figure 10 (a) DOSY-COSY spectrum of sample D (b) Slice spectrum of astaxanthin monoester (c) Slice spectrum of aliphatic acid (d) Diffusion coefficient of sample D

4. Conclusion

The present results suggest that the DOSY method enables not only the identification but also

the structural analysis of certain carotenoids in mixed extracts.

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[6] **Astaxanthin palmitate** ; $\lambda_{\max/\text{nm}}$ 479 ; $\nu_{\max/\text{cm}^{-1}}$ 3506 (OH), 1706 (CO₂R), 1664 (conj.C=O) ; δ_{H} 0.88 (3H, t, $J=7$ Hz, 16''-H₃), 1.21, 1.22, 1.32, 1.35 (each 3H, s, gem-MeX₂), 1.26 (24H, s, 4''~15''H₂), ca.1.66 (2H, m, 3''-H₂), 1.81 (1H, t, $J=13$ Hz, 2'ax-H), 1.90 (3H, s, 5-Me), 1.94 (3H, s, 5'-Me), 1.99, 2.00 (each 6H, s, 9,9',13,13'-Me), ca.2.01 (1H, m, 2'eq-H), 2.07 (1H, t, $J=13.5$ Hz, 2ax-H), 2.15 (1H, dd, $J=13$, 6 Hz, 2'eq-H), 2.34 (2H, t, $J=7.5$ Hz, 2''-H₂), 4.32 (1H, dd, $J=13, 6$ Hz, 3'-H), 5.53 (1H, dd, $J=13.5$, 5.5 Hz, 3-H), 6.20 (1H, d, $J=16$ Hz, 7-H), 6.21 (1H, d, $J=16$ Hz, 7'-H), 6.29 (2H, d, $J=12$ Hz, 10,10'-H), ca.6.31 (2H, m, 14,14'-H), 6.40 (1H, d, $J=15.5$ Hz, 8-H), 6.43 (1H, d, $J=15.5$ Hz, 8'-H), 6.44 (2H, d, $J=15$ Hz, 12,12'-H), 6.65 (2H, dd, $J=15$, 12 Hz, 11,11'-H), ca. 6.67 (2H, m, 15,15'-H) ; ESI-HRMS (m/z). Calcd for C₅₆H₈₃O₅⁺ [M+H]⁺, 835.6235; found, 835.6240.

[7] **Astaxanthin dipalmitate** ; $\lambda_{\max/\text{nm}}$ 481 ; $\nu_{\max/\text{cm}^{-1}}$ 1733 (CO₂R), 1675 (conj.C=O) ; δ_{H} 0.88 (6H, t, $J=7$ Hz, 16''-H₃ x 2), 1.22, 1.35 (each 6H, s, gem-Me x 2), 1.26 (48H, s, 4''~15''-H₂ x 2), 1.70 (4H, m, 3''-H₂ x 2),

1.90 (6H, br s, 5, 5'-Me), 1.99, 2.00 (each 6H, br s, 9,9',13,13'-Me), 2.00 (2H, m, 2, 2'eq-H), 2.07 (2H, t, $J=13.5$ Hz, 2,2'ax-H), 2.43 (4H, m, 2''-H₂ x 2), 5.53 (2H, d, $J=11$ Hz, 10,10'-H), 6.31 (2H, m, 14,14'-H), 6.41 (2H, d, $J=16$ Hz, 8,8'-H), 6.45 (2H, d, $J=15$ Hz, 12,12'-H), 6.65 (2H, dd, $J=15$, 11 Hz, 11,11'-H), 6.67 (2H, dd, $J=8.5$, 3 Hz, 15,15'-H) ; ESI-HRMS (m/z). Calcd for C₇₂H₁₁₃O₆⁺ [M+H]⁺, 1073.8532; found, 1073.8548.

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Reconstitution of carotenoids into the reaction center from the purple photosynthetic bacterium *Rhodobacter sphaeroides* using the chromatophores of the carotenoidless mutant strain R26.1

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Abstract: Antenna function in the light-harvesting pigment-protein complexes in photosynthesis is well studied, however, it is not very clear in the reaction center (RC). This is because carotenoids are believed to mainly function as a photoprotector in the RC. In order to understand the detailed functions of carotenoids in the RC, one of the promising methods is to reconstitute any type of carotenoids into the reaction center that is prepared from carotenoidless mutant strain of purple photosynthetic bacteria. In this present study, we have found that spheroidene was successfully reconstituted into the RC with the 15-*cis* configuration when all-*trans* spheroidene was incorporated into the chromatophores from *Rhodobacter (Rba.) sphaeroides* R26.1. In this latest study, we have investigated the excitation energy-transfer between carotenoid and bacteriochlorophyll in the RC using femtosecond time-resolved absorption spectroscopy.

1. Introduction

Carotenoids (Cars) have important roles such as light-harvesting and photoprotection in the primary process of photosynthesis [1, 2]. The photoprotective function of carotenoids has been well studied in the photosynthetic reaction center (RC) from purple photosynthetic bacteria, and it is reported that carotenoids prevent the generation of harmful singlet oxygen by quenching the triplet bacteriochlorophylls (Bchls) [1]. However, the light harvesting function of Cars in RC has not yet been clarified. This is because Car is believed to mainly function as a photoprotector in the RC. In order to make this issue clearer, we have focused on to investigate the light harvesting function of Cars in RCs.

As illustrated in **Fig. 1** [1], in the RC from purple photosynthetic bacteria, a special pair Bchl *a* (P), two molecules of monomeric Bchl *a* (B_A and B_B), two molecules of bacteriopheophytin (H_A and H_B), and two molecules of quinone (Q_A and Q_B) are arranged with pseudo two-fold symmetry. Electron generated at P is transferred to Q_A and then Q_B through a single pathway denoted with the subscript

A (A-branch). The pathway with subscript B (B-branch) is inactive for electron transfer. One carotenoid molecule is bound to the B-branch side with 15-*cis* configuration.

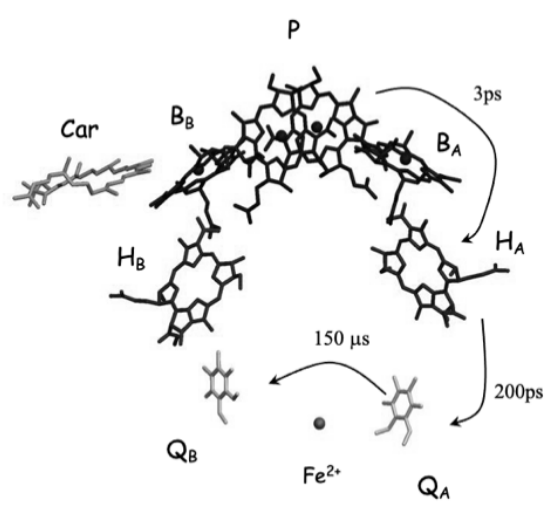


Fig. 1. Arrangement of the pigment molecules in the RC from the purple photosynthetic bacterium *Rba. sphaeroides* 2.4.1. [1]

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Reconstitution of carotenoids into the RC from the carotenoidless mutant of purple photosynthetic

bacteria is one of the promising methods to study the functions of carotenoids [3]. If this method were established, it is possible to extend the studies to the wider range of carotenoids. Moreover, we will be able to expand the studies to non-naturally occurring carotenoids in the photosynthetic pigment-protein complexes.

In the previous study, carotenoids have been directly incorporated into the RC from the carotenoidless mutant of the purple photosynthetic bacterium *Rba. sphaeroides* R26.1 [2]. In this presented study, we were successful to incorporate spheroidene (see Fig. 1 for its chemical structure) into the chromatophores from *Rba. sphaeroides* R26.1 and could isolate the spheroidene-bound RC from there. Spheroidene is a carotenoid that is biosynthesized in the wild type strain of the purple photosynthetic bacterium *Rba. sphaeroides* 2.4.1. We have investigated the EET from spheroidene to Bchls in the spheroidene reconstituted RC using femtosecond time-resolved absorption spectroscopy.

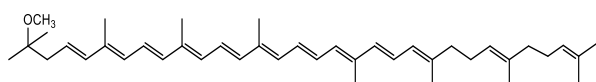


Fig. 2. Chemical structure of all-*trans*-spheroidene ($n=10$)

2. Experimental

2.1 Preparation of chromatophores from *Rba. sphaeroides* R26.1

Photosynthetically grown cells of *Rba. sphaeroides* R26.1 under anaerobic condition were harvested and washed with MES/KCl buffer (20mM MES, 100mM KCl pH6.8) [4]. The cells were suspended in 20 mM Tris-HCl (pH8.0) before the preparation of chromatophores. A small amount of DNase and $MgCl_2$ were added, and the cell walls were broken with French Pressure cell. Chromatophores were obtained by centrifugation ($9,075 \times g$, 10 min, 4 °C) that is followed by ultra-centrifugation ($183,000 \times g$, 90 min, 4 °C). The chromatophores were freeze-dried and stored at $-30^\circ C$ until use.

2.2 Preparation of spheroidene

Cars and Bchl *a* were extracted from the wild type cells of *Rba. sphaeroides* 2.4.1 with a mixed

solvent of acetone and methanol with 50/50 ratio (v/v) for 3 times. The pigment extract was partitioned against *n*-hexane after repeated washing with saturated NaCl aqueous solution. The mixture of methanol and water (95/5 (v/v) ratio) was added to this *n*-hexane solution in order to remove Bchl *a* from the *n*-hexane layer. The Cars including *n*-hexane solution was dried with Na_2SO_4 and evaporated until dryness. The Cars were then dissolved in a small amount of *n*-hexane. All-*trans* spheroidene was purified by silica-gel column chromatography using diethyl ether/*n*-hexane (10 / 90 (v/v)) as an eluent. The purified all-*trans* spheroidene was evaporated and dissolved in petroleum ether.

2.3 Incorporation of all-*trans* spheroidene into the chromatophores from *Rba. sphaeroides* R26.1

We have developed a new method to incorporate Cars directly into carotenoidless chromatophores from *Rba. sphaeroides* R26.1 by referring to the method described in a previous report [3]. The petroleum ether solution of all-*trans* spheroidene ($OD=30$ at λ_{max}) was added to the freeze-dried chromatophores from *Rba. sphaeroides* R26.1. The solution was sonicated for 20-30 minutes to dissolve the aggregate of chromatophores. Then the petroleum ether solution was evaporated until dryness using a rotary evaporator. The chromatophores were resuspended in 20 mM Tris-HCl buffer (pH = 8.0) and pelleted by ultra-centrifugation ($183,000 \times g$, 60 min, 4 °C). During these protocols spheroidene was successfully incorporated into the chromatophores.

2.4 Isolation and purification of the RC from the spheroidene incorporated chromatophores

The spheroidene incorporated chromatophores were homogenized with 20 mM Tris-HCl buffer (pH = 8.0) including 150 mM NaCl, and OD_{850} was adjusted to be equal to 50. The LDAO solution was added so as the detergent concentration to be 0.3 %. The chromatophores were solubilized for 60 min at 26 °C under darkness, and then ultra-centrifugation ($183,000 \times g$, 60 min, 4 °C) was performed. The RC component was extracted to the supernatant. Finally, the RC was purified by ion-exchange column chromatography [5].

2.4 Spectroscopic Methods

Steady-state absorption spectra were recorded using a JASCO V670 spectrophotometer at room

temperature. Resonance Raman spectra were recorded using 532 nm excitation laser light at room temperature. A home-built set-up composed of a spectrometer (Horiba iHR550) equipped with a liquid nitrogen cooled CCD detector (Princeton 7527-0001) was used for the Raman measurements.

Femtosecond time-resolved absorption spectroscopic measurements were performed at room temperature as described previously [6]. The 510 nm excitation laser pulses were generated by an optical parametric amplifier (Spectra Physics, OPA-800CF). The excitation energy was set to be 11 nJ/pulse, and the pulse width was approximately 130 fs. The probe pulse was the white-light continuum that spreads in the wavelength region between 436 to 738 nm with ~100 fs pulse duration. Optical density of the sample solution was set to be 2.3 – 2.8 at the absorption maxima. The sample solution was placed in a quartz cuvette with 2.0 mm optical path-length. During the measurements, the sample solution was continuously stirred using a magnetic micro-stirrer bar to avoid the degradation of the samples.

3. Results and Discussion

3.1 Steady-state absorption spectra

The steady-state absorption spectra of three types of RC; the carotenoidless RC from *Rba. sphaeroides* R26.1, the spheroidene reconstituted RC, the RC from *Rba. sphaeroides* 2.4.1 are shown in Fig. 3. The absorption spectrum of the spheroidene reconstituted RC shows the clear vibronic band ascribable to the RC bound spheroidene in 420 – 530 nm wavelength region. In addition, the intensity of the absorption of the reconstituted spheroidene is comparable to that of the native RC from strain 2.4.1. Therefore, we can conclude that the reconstitution of spheroidene is successful. This finding is quite amazing since we have incorporated all-*trans* spheroidene into carotenoidless chromatophores. Nevertheless, it is very clear that spheroidene can be reconstituted not only into the light-harvesting complexes (both LH2 and LH1) but also into the RC that is surrounded by LH1. This method of reconstitution should be quite useful when we expand the studies to other species of Cars.

3.2 Resonance Raman spectra

Fig. 4. shows the resonance Raman spectra of the spheroidene reconstituted RC and the RC from

Rba. sphaeroides 2.4.1. A key Raman line of the 15-*cis* isomer can be seen at 1239 cm^{-1} in either case of the

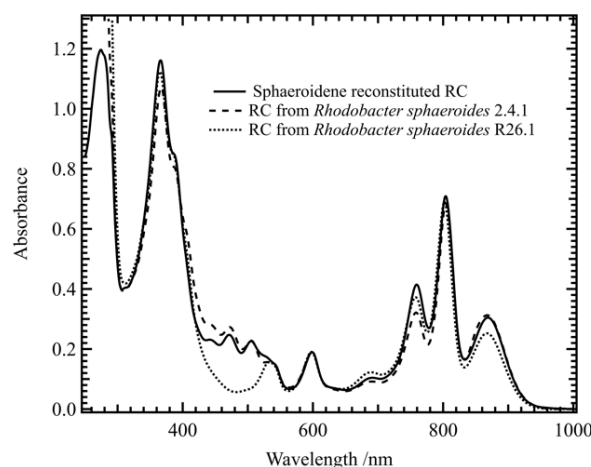


Fig. 3. Comparison of the steady-state absorption spectra of the RC from *Rba. sphaeroides* R26.1 (···), the spheroidene reconstituted RC (—), and the RC from *Rba. sphaeroides* 2.4.1 (---).

RC [7]. Overall spectral features are also similar to each other. Therefore, we can safely conclude that spheroidene is reconstituted with the 15-*cis* configuration in the RC. This is another amazing finding because we have incorporated all-*trans* spheroidene to the carotenoidless chromatophores. Therefore, this finding suggests that all-*trans* spheroidene is somehow isomerized and then successfully reconstituted into the RC in the chromatophores without the interference of LH1 that surrounds the RC.

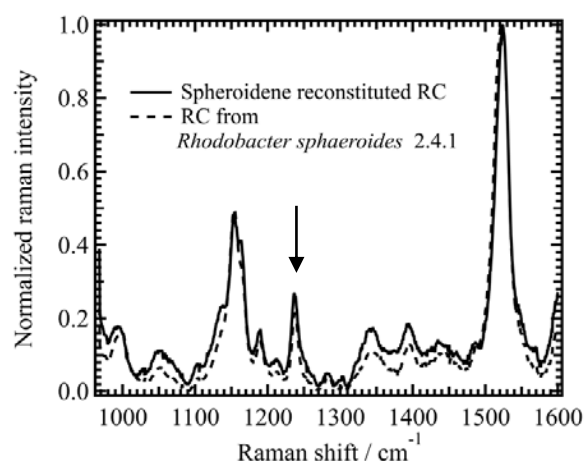


Fig. 4. Resonance Raman spectra of the RC from *Rba. sphaeroides* 2.4.1 (---) and the spheroidene reconstituted RC (—). The arrow points the key Raman line of the 15-*cis* isomer.

3.3 Femtosecond Time-resolved absorption spectra

Fig. 5. shows TA spectra taken at various delay times after the excitation. The ground state bleaching (GSB) of spheroidene is seen as early as 0.4 ps after excitation. This is naturally expected since we have excited the RC at 510 nm, which directly excites the spheroidene. Interestingly, the GSB of the Q_x absorption band of Bchl *a* at 600 nm is also seen and this GSB signal lasts as long as 45.68 ps after excitation (see shadow in Fig. 5(B)). The issue is whether the excitation energy-transfer (EET) from the S_2 excited state of spheroidene to Bchl *a* can take place or not. This will be clarified by the global analysis as shown below. It should be pointed out that the GSB of bacteriopheophytin (Bphe) around 540 nm and the excited state absorption (ESA) band associated with the BPhe around 660-670 nm are seen at 6.18 ps after excitation (see shadows in Fig. 5(B)), which suggests the possibility of EET from Bchl *a* to Bphe takes place.

More details of the EET dynamics can be revealed with global analysis against the whole observed dataset of TA spectra as shown in Fig. 6. The global analysis was performed supposing a sequential model to generate evolution associated difference spectra (EADS). As a result, 5 components were obtained, and these EADS can be identified by comparison with previous study. The EADS with a lifetime of 49.2 fs represents coherent artifact/ S_2 state of spheroidene. The 2nd EADS represents *hot* S_1 state of spheroidene together with the ESA contribution of Bchl *a*. This is a good indication that the EET takes place from the S_2 state spheroidene to Bchl *a*. The 3rd EADS corresponds to the S_1 state of spheroidene that has 1.36 ps lifetime. The 4th EADS (6.40 ps component) is associated with the ESA of Bchls together with the transient absorption of S^* or T_1 state spheroidene, and the 5th EADS (335 ps component) can be associated with the formation of P^+ and H_A^- . From these perspectives, we will be able to estimate the exact pathways and efficiencies of the EET between spheroidene and P via B_B , and those of the electron transfer from P to H_A via B_A .

Conclusion

Spheroidene was successfully incorporated into the RC when it was reconstituted into the chromatophore. In addition, we found that the EET from spheroidene to Bchl takes place. However, the

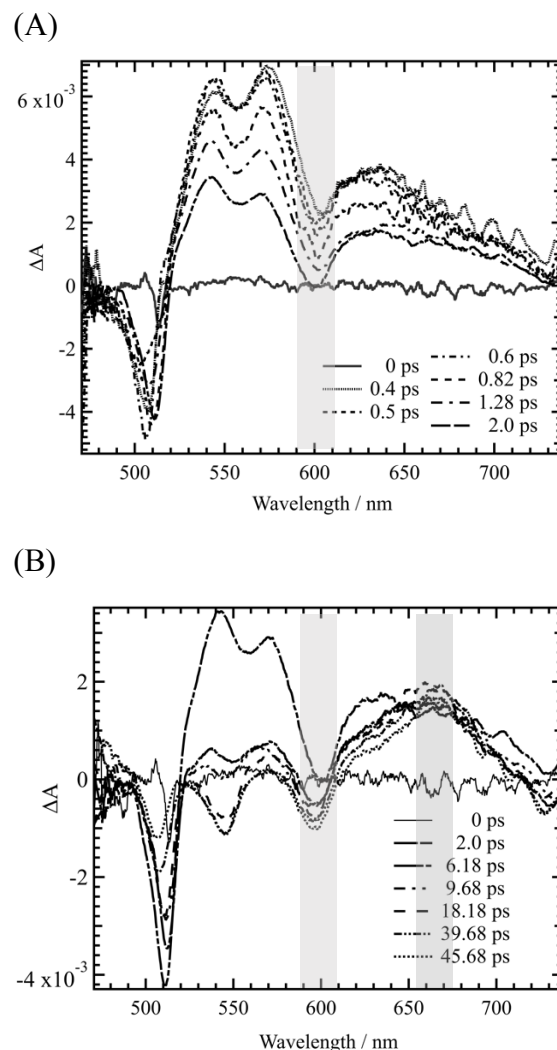


Fig. 5. TA spectra of spheroidene reconstituted RC taken at various time (A) from 0 ps to 2.0 ps and (B) from 1.28 ps to 45.68 ps. The excitation wavelength is 510 nm, which excites the (0-0) vibronic band of spheroidene.

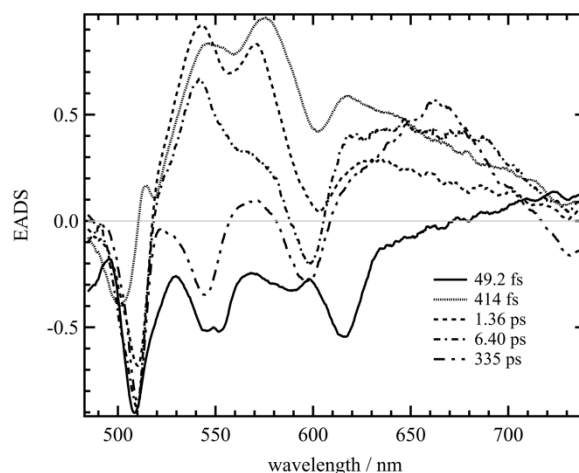


Fig. 6. EADS obtained with the application of a sequential decay model.

detailed mechanisms of the EET between spheroidene and Bchl is under investigation. As the future perspective, for sample preparation, we are planning to incorporate the other species of carotenoids into RC using the same method. In addition, we continue to investigate the detailed mechanisms of the EET between carotenoid and Bchls.

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Structural determination of a new minor yellow carotenoid, 5,6-dihydrocrustaxanthin from the prawns *Penaeus japonicus* and *P. vannamei*

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Abstract: A new minor yellow carotenoid, named 5,6-dihydrocrustaxanthin (**6**), was isolated from the prawns *Penaeus japonicus* and *P. vannamei* with very small amount (about 20 μ g). This structure was determined to be 5,6-dihydro- β,β -carotene-3,4,3',4'-tetrol with 3,4-*cis*-, 3',4'-*cis*-configuration by UV-VIS, MS, and ^1H -NMR spectral data. (3*R*,4*S*,5*R*,6*R*,3'*R*,4'*S*) configuration was proposed from CD spectral data.

Keywords: prawn, *Penaeus japonicus* and *P. vannamei*, 5,6-dihydrocrustaxanthin

1. Introduction

The prawns *Penaeus japonicus* and *P. vannamei* are economically important species of shrimps in Japan. These prawns contain not only astaxanthin but also several yellow xanthophylls. I previously reported the presence of a series of yellow xanthophylls, isoastaxanthin (**1**), 5,6-dihydropenaeusxanthin (**2**), penaeusxanthin (**3**), tetrahydroxypirardixanthin (**4**), and crustaxanthin (**5**), as metabolites of astaxanthin in these prawns [1] (Fig. 1). Recently, in addition to these yellow xanthophylls (**1-5**), a new yellow carotenoid, named 5,6-dihydrocrustaxanthin (**6**) (Fig. 2), was isolated with very small amount (about 20 μ g) from *P. japonicus* and *P. vannamei*. In the present report, I described the isolation and structural elucidation of a new carotenoid **6** from these prawns.

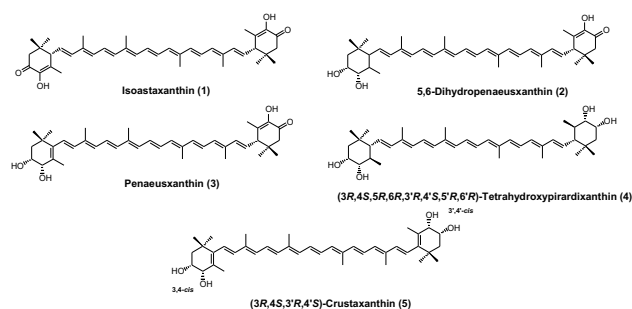


Fig. 1. Yellow xanthophylls (**1-5**) in the prawns *Penaeus japonicus* and *P. vannamei*.

2. Experimentals

The carotenoids were extracted from the carapace of prawns with acetone. They were then transferred to *n*-hexane: Et₂O (1:1, v/v) by addition of water. The *n*-hexane: Et₂O phase was washed with water and dehydrated on anhydrous sodium sulphate. Yellow xanthophylls fraction obtained from silica gel column chromatography eluted with *n*-hexane: Et₂O (8:2, v/v) was saponified with 5 % KOH/MeOH at room

temperature for 3 hrs, as usual manner [2]. They were separated by silica gel HPLC [Cosmosil 5SL-II with acetone: *n*-hexane (4:6, v/v) as a solvent at a flow rate of 1.0 mL/min] and afforded six yellow xanthophylls, isoastaxanthin (retention time 8.3 min), 5,6-dihydropenaeusxanthin (retention time 13.0 min), penaeusxanthin (retention time 16.8 min), a new carotenoid 5,6-dihydrocrustaxanthin (retention time 18.8 min), tetrahydroxypirardixanthin (retention time 20.3 min), and crustaxanthin (retention time 22.3 min). In order to remove lipid impurity, they were re-purified by with ODS HPLC [Wakosil 5C18 with chloroform: methanol (2:8)].

The UV-VIS spectra were recorded with a Hitachi U-2001 spectrophotometer (Hitach Field Navigator, Tokyo, Japan) in Et₂O. The ESI MS was obtained a Waters Xevo G2S Q TOF mass spectrometer (Waters Corporation, Milford, CT, USA). The electro-spray ionization (ESI) time-of-flight (TOF) MS spectra were acquired by scanning from *m/z* 100 to 1,500 with a capillary voltage of 3.2 kV, cone voltage of 40 eV, and source temperature of 120°C. Nitrogen was used as a nebulizing gas at a flow rate of 30 L/h. MS/MS spectra were measured with a quadrupole-TOF MS/MS instrument with argon as a collision gas at a collision energy of 30 V. The ^1H NMR (500 MHz) spectrum was measured with a Varian UNITY INOVA 500 spectrometer (Varian Corporation, Palo Alto, California USA) in CDCl₃ with TMS as an internal standard, using a SHIGEMI micro-tube (sample solution volume 200 μ L). The CD spectrum was recorded in Et₂O at room temperature with a Jasco J-500C spectropolarimeter (JASCO Corporation, Hachioji, Tokyo, Japan). Preparative HPLC was performed with a Hitachi L-6000 intelligent pump and an L-4250 UV-VIS detector (Hitach Field Navigator, Tokyo, Japan) set at 450 nm.

5,6-Dihydrocrustaxanthin (**6**): Yield 20 μ g from 10g carapace, UV-VIS (420), 445, 473 nm, HR-ESI TOF MS *m/z* 602.4317 C₄₀H₅₆O, Calcd for 602.4335, *m/z*

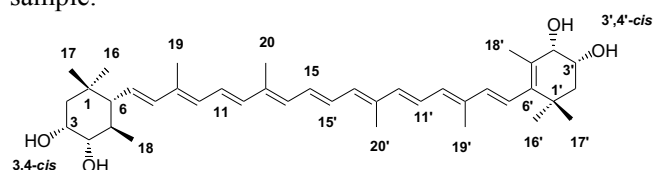
625.4181 $C_{40}H_{58}O_4Na$ Calcd for 625.4233. ESI TOF MS/MS (Fig. 1), 1H -NMR (Table 1), CD λ nm ($\Delta\epsilon$) 224 (+3.0), 230 (0), 245 (-6.0), 260 (0), 284 (+9.0), 320 (0), 330 (-3.5).

3. Results and Discussion

3.1. Structure of a new carotenoid (6)

A new yellow carotenoid (**6**) was obtained as minor carotenoid (20 μ g from 10g carapace, about 4~5% of total carotenoids) from the carapace of *P. japonicus* and *P. vannamei* along with astaxanthin, isoastaxanthin (**1**), 5,6-dihydro-penaeusxanthin (**2**), penaeusxanthin (**3**), tetrahydroxypirardixanthin (**4**), and crustaxanthin (**5**). In order to remove lipid impurity from a new carotenoid (**6**), HPLCs using silica gel and ODS column were carefully repeated. This carotenoid (**6**) showed UV-VIS absorption maxima at (420), 445, 473 nm. The molecular formula of this compound was determined as $C_{40}H_{58}O_4$ by HR ESI TOF MS. The characteristic products ion at m/z 510 $[M-92]^+$ (elimination of toluene moiety from polyene chain) was observed in MS/MS spectrum [2]. 1H NMR of this compound was measured in SHIGEMI micro-tube (sample solution volume 200 μ L) for 12 hrs. 1H NMR signals of this compound (Table 1) showed the presence of a 3,4-*cis*-3,4-dihydroxy-5,6-dihydro- β -end group, a 3',4'-*cis*-3',4'-dihydroxy- β -end group, and an all-*trans*-polyene chain [1,3,4]. This structure was also confirmed by 1H - 1H COSY experiment. The 1H - 1H

COSY spectrum was accuired for 24 hrs. From these spectral data, the structures of this carotenoid was determined as 5,6-dihydro- β,β -carotene-3,4,3',4'-tetrol with 3,4-*cis*- and 3',4'-*cis*-configuration [1-4]. This structure was corresponded to 5,6-dihydro derivative of crustaxanthin. Therefore, this carotenoid was named as 5,6-dihydrocrustaxanthin. The CD spectrum of this compound was almost similar to the additive CD spectrum of (3*R*,4*S*,5*R*,6*R*,3'*R*,4'*S*,5'*R*,6'*R*)-tetrahydroxypirardixanthin (**4**) and (3*R*,4*S*,3'*R*,4'*S*)-crustaxanthin (**5**). Therefore, the absolute configuration of **6** was proposed to be (3*R*,4*S*,5*R*,6*R*,3'*R*,4'*S*) [1,4,5] as shown in Fig. 2. In my experience, this structural elucidation was accomplished with the smallest amount sample.



5,6-Dihydrocrustaxanthin (6)

Fig. 2. Structure of a new carotenoid (**6**).

As well as yellow xanthophylls **1**~**5**, 5,6-dihydrocrustaxanthin (**6**) was increased by astaxanthin administration with dose dependency. Therefore, 5,6-dihydrocrustaxanthin (**6**) was also assumed to be a metabolite of astaxanthin to in prawn [1,6,7].

Table 1. 1H -NMR (in $CDCl_3$) of 5,6-dihydrocrustaxanthin (**6**).

Position	δ	mult. (J in Hz)	Position	δ	mult. (J in Hz)
H-2 ax	n.a.		H-2' ax	n.a.	
H-2 eq	n.a.		H-2' eq	n.a.	
H-3	4.01	m	H-3'	3.84	m
H-4	3.17	dd (10, 3.5)	H-4'	3.96	d (3.5)
H-5	n.a.				
H-6	n.a.				
H-7	5.45	dd (15.5, 9.5)	H-7'	6.07	d (15)
H-8	6.05	d (15.5)	H-8'	6.17	d (15)
H-10	6.11	d (10)	H-10'	6.17	d (10)
H-11	6.62	dd (15, 11)	H-11'	6.62	dd (15, 11)
H-12	6.35	d (15)	H-12'	6.38	d (15)
H-14	6.24	br. d	H-14'	6.27	br. d
H-15	6.65	m	H-15'	6.65	m
H ₃ -16	0.81	s	H ₃ -16'	1.07	s
H ₃ -17	1.06	s	H ₃ -17'	1.09	s
H ₃ -18	0.93	d (6.5)	H ₃ -18'	1.90	s
H ₃ -19	1.93	s	H ₃ -19'	1.97	s
H ₃ -20	1.97	s	H ₃ -20'	1.97	s

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Analysis of methyl 5-glucosyl-5,6-dihydro-apo-4,4'-lycopenoate

(C30 carotenoid) biosynthetic pathway,

and antioxidant activities of its metabolic intermediates

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Abstract: The biosynthetic pathway of a new C30 carotenoid, methyl 5-glucosyl-5,6-dihydro-apo-4,4'-lycopenoate that *Planococcus maritimus* strain iso-3 produced, was investigated by using recombinant *Escherichia coli* that expressed its carotenoid biosynthetic gene candidate(s), followed by analyzing the structures of generated carotenoids. Up to the present, we identified five carotenoid biosynthetic genes and determined the structures of five carotenoids as the metabolic intermediates (two of them were new carotenoids). Furthermore, we examined ¹O₂ suppression activities of the five carotenoids and found that more downstream intermediates in the biosynthetic pathway possessed superior ¹O₂ suppression activity.

1. Introduction

A large number of research works on biosynthetic pathways of C40 carotenoids have been conducted, while biosynthetic pathways of C30 carotenoids that some bacteria produced is still unclear. *Planococcus maritimus* strain iso-3 is a marine bacterium that was isolated from an intertidal sediment sample from the shoreline of the Clyde estuary in the UK, which was heavily industrialized. Our group reported that this strain produced a new C30 carotenoid methyl 5-glucosyl-5,6-dihydro-apo-4,4'-lycopenoate (**1**).¹⁾ In the present study, we aimed to identify genes corresponding to the biosynthesis of **1**, and evaluate ¹O₂ suppression activities of metabolic intermediates of **1**. We report here biosynthesis genes and generated carotenoids in the pathway that is drawn in solid line of Fig. 1.

2. Experimental

A genome library of *P. maritimus* iso-3 was constructed in *Bacillus subtilis* with pGETS103 vector using *Bam*HI that produced relatively large DNA fragments, since we assumed that biosynthesis genes of **1** existed as a cluster on the genome. Consequently, one yellow colony appeared, which contained a 7.3 kb *Bam*HI fragment. Next, we searched sequences homologous to carotenoids biosynthesis genes from other organisms. In order to analyze the function of resultant carotenoid biosynthetic gene candidate(s), we used recombinant *E. coli* that expressed the gene candidate(s).

Each recombinant *E. coli* was cultured in TB medium for 48 to 72 hours at 20°C. The cells are collected by centrifugation and extracted by CH₂Cl₂-MeOH (1:1) with sonication. The extract was concentrated to small volume, and a produced

carotenoid was purified by two-phase separation → silica gel column chromatography → preparative ODS HPLC. The structures of the isolated carotenoids were determined by HRESI-MS, and 1D (^1H and ^{13}C) and 2D (^1H - ^1H DQF COSY, HSQC, and HMBC) NMR analyses.

Antioxidant activities (lipid peroxidation inhibitory activity and $^1\text{O}_2$ suppression activity) were examined on each intermediate carotenoid.

3. Results and Discussions

Through the gene cloning experiments, we found a carotenoid biosynthetic gene cluster that contained seven candidate genes (*orf1-orf7*) on the genome of the iso-3 strain. *Orf1*, *orf2*, *orf4* showed homology to genes encoding Phytoene desaturase, *orf3* to Phytoene synthase, *orf5* to Glycosyl transferase, *orf6* to Acyltransferase, and *orf7* to 1'-hydroxylase. Functional analysis experiments on each gene using the *E. coli* expression system revealed that *orf3* codes for Diapophytoene synthase (CrtM), *orf1* for Diapophytoene desaturase (CrtN), *orf7* for 1'-Hydroxylase (CruF), *orf4* for Hydroxydiaponeurosporene desaturase (CrtNb), and *orf5* for Glucosyl transferase (GT).

As metabolic intermediates of **1**, we isolated five carotenoids (15 *cis* apo-4,4'-phytoene (**2**), apo-4,4'-neurosporene (**3**), 5-hydroxy-5,6-dihydro-apo-4,4'-neurosporene (**4**), 5-hydroxy-5,6-dihydro-apo-4,4'-lycopene (**5**), and ucosyl-5,6-dihydro-apo-4,4'-lycopene (**6**)) (Fig. 1). Compounds **4** and **6** were new carotenoids (**5** was reported as the biosynthetic intermediate of **1** in our previous study). Thus, we clarified biosynthetic pathway from FPP to **6**, by identifying the functions of the five enzymes, CrtM, CrtN, CruF, CrtNb and GT, involved in this pathway, as shown in solid line of Fig. 1. We also elucidated that **2** (15 *cis*) to **3** (15 *trans*) transformation occurs even in C30 carotenoids, same to the case of C40 carotenoids.

We examined the $^1\text{O}_2$ suppression activities of **1** – **6**. The IC_{50} values were 5.1 μM (**1**), >100 μM (**2**), 45 μM (**3**), 56 μM (**4**), 30 μM (**5**), 30 μM (**6**), respectively. These results strongly suggested that more downstream intermediates in the biosynthetic

pathway possess superior $^1\text{O}_2$ suppression activity. Therefore, *P. maritimus* iso-3 may biosynthesize **1** to protect itself from $^1\text{O}_2$ damage. Only compound **4** showed lipid peroxidation inhibitory activity (IC_{50} of 1.8 μM).

4. Conclusion

We identified the genes corresponding to the biosynthetic pathway, and determined the chemical structures of the five carotenoids (**2** – **6**) as the biosynthetic intermediates of **1**. We also examined lipid peroxidation inhibitory activity and $^1\text{O}_2$ suppression activity of **1** – **6**. In the near future, we will complete this study by clarifying the remaining pathway (dotted line in Fig. 1).

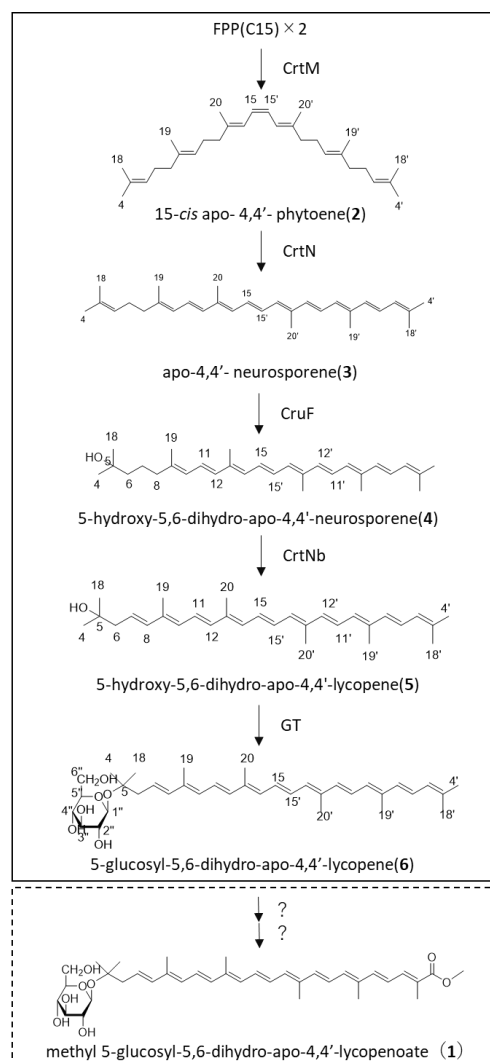


Fig. 1. Biosynthetic pathway of methyl 5-glucosyl-5,6-dihydro-apo-4,4'-lycopenoate

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Isolation and characterization of myxol biosynthesis genes from marine flavobacteria

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Myxol is a monocyclic carotenoid and has been isolated from several microorganisms including cyanobacteria and marine flavobacteria [1, 2]. We identified the myxol as a main carotenoid in marine flavobacteria *Nonlabens spongiae* JCM 13191^T. We isolated a carotenoid biosynthesis gene cluster for myxol production from this flavobacteria. A sequence analysis revealed that the cluster contains 8 open reading frames, including a MerR family transcriptional regulator gene homolog (*merR*) and carotenoid biosynthetic gene homologs, phytoene desaturase (*crtI*), phytoene synthase (*crtB*), β -carotene hydroxylase (*crtZ*), 1-hydroxycarotenoid 3,4-desaturase (*crtD*), 1'-hydroxylase (*cruF*), lycopene β -monocyclase (*crtYm*), and 2-hydroxylase (*crtA-OH*), within a 9.3-kb region (Fig. 1). The *merR*, *crtI*, *crtB*, *crtZ*, *crtD*, *cruF*, and *crtYm* were oriented in the same direction, whereas the *crtA-OH* was oriented in the opposite direction.

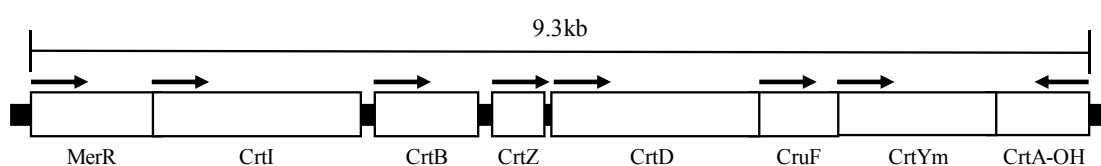


Fig. 1 Organization of the myxol biosynthesis gene cluster from marine flavobacteria *Nonlabens spongiae* JCM 13191^T.

To test the catalytic activity, the cluster of *N. spongiae* was transformed into *Escherichia coli* strains that produced mainly geranylgeranyl diphosphate (GGPP). In the resulting *E. coli* transformants, myxol was accumulated predominantly. This result indicates that the enzymes encoded by the gene cluster of *N. spongiae* function normally in *E. coli*, and suggests that the gene cluster is responsible for the production of myxol in *N. spongiae*. We also identified GGPP synthase gene (*crtE*) of *N. spongiae* by functional complementation in *E. coli*. We propose a biosynthetic pathway for the myxol in *N. spongiae* (Fig. 2).

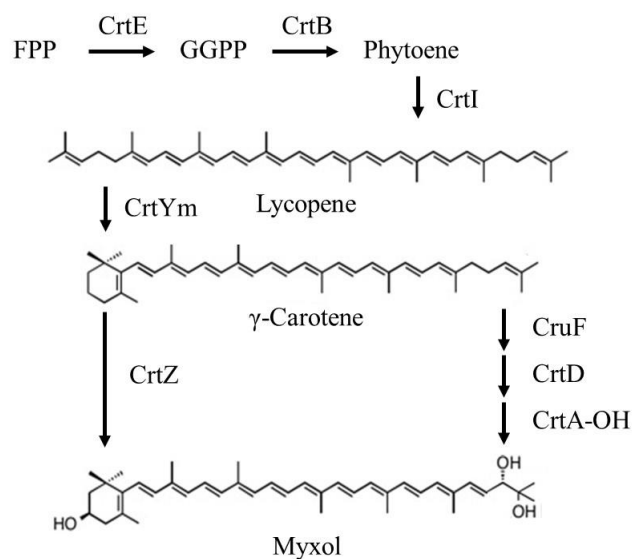


Fig. 2 Proposed biosynthetic pathway of the myxol in marine flavobacteria *Nonlabens spongiae* JCM 13191^T.

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Esterification of carotenoid in aerial microalgae *Coelastrella* sp. KGU-Y002 under aerial condition with LED light

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Abstract: Carotenoids have high-antioxidant effect to protect organism from reactive oxygen species. Esterified carotenoids may have higher physiological activity than the free-types. Aerial microalga, *Coelastrella* sp. KGU-Y002 can accumulate free and esterified carotenoids under aerial condition. In this study, we examined carotenoid esterification with different light wavelength by light emitting diode (LEDs) under aerial condition. Red light was promoted carotenoid esterification of the algal cells. Blue and green light were promoted algal cells division.

1. Introduction

Esterified carotenoids play an important role to physiological activity in humans. Aerial microalga, *Coelastrella* sp. KGU-Y002 isolated from a rock surface in Japan can accumulate large amounts of free and esterified carotenoids [1-3]. *Coelastrella* sp. synthesize xanthophylls including astaxanthin and adonixanthin under aerial condition. We expect that specific light wavelength by LEDs light is promoted the carotenoid esterification in cells. The aim of this study was to examine carotenoid esterification in aerial microalgae *Coelastrella* sp. KGU-Y002 under aerial condition with LED light.

2. Experimental

2.1 preculture

Aerial microalga *Coelastrella* sp. KGU-Y002 was precultured in BB medium for 21 days at 25°C and 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in 500 mL erlenmeyer flask while bubbling with air.

2.2 culture under aerial condition

To prepare biofilms, precultured cell suspension were put on the membrane filter. Biofilms were cultured for 7 days at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with red (625 nm), green (525 nm), blue LEDs (467 nm) or, fluorescent lamp on the membrane filter which is placed on wet cotton including pure water in a petri dish.

2.3 Carotenoid extraction and analysis

Carotenoids were extracted by methanol and dichloromethane and analyzed by HPLC.

3. Results and Discussions

The algal biomass increased to 2.5 ~ 3.0-fold of initial level under aerial condition (Fig. 1). Aerial microalga *Coelastrella* sp. can photosynthesize under aerial condition. Cell numbers increased to 3.2, 1.3, 2.0, and 2.6-fold of initial level by fluorescent, red, green, and blue lights (Fig. 2). Cell division was caused by irradiating green and blue wavelength. Ester-type carotenoids in the cells accumulated to 0.84, 1.20, 0.10, and 0.06 mg g⁻¹ dry weight cells under light source by fluorescent lamp, red, green, and blue LEDs (Fig. 3). Esterification xanthophylls (astaxanthin and adonixanthin) in the algal cell was promoted by irradiating red wavelength.

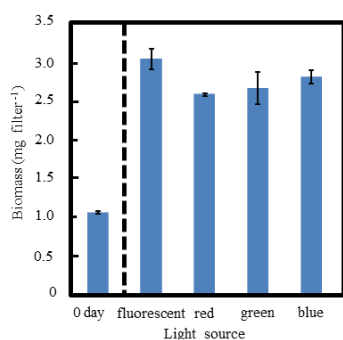


Fig.1 Effect of various light sources on the algal biomass under aerial condition after 7 days

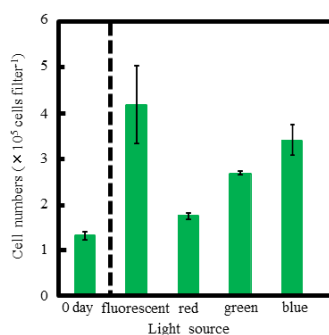


Fig.2 Effect of various light sources on the cell numbers under aerial condition after 7 days

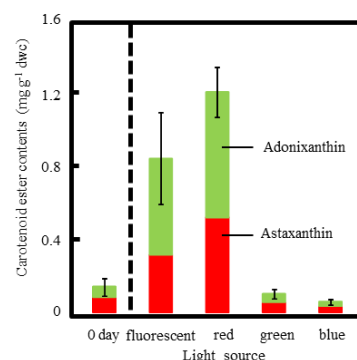


Fig.3 Effect of various light sources on the contents of ester-type carotenoid in the algal cells under aerial condition after 7 days

4. Conclusion

Different wavelength by LEDs affected biomass, cell number, carotenoid esterification in aerial microalgae *Coelastrella* sp. KGU-Y002 under aerial condition. Blue and Green lights were promoted algal cells division. Red light promoted carotenoids esterification. We will identify physiologically active compounds to acyltransferase enzyme activity for elucidating esterification metabolism.

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Novel biosynthetic precursor of siphonaxanthin found in *Codium fragile* trichomes cultivated under blue-green light

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Abstract: A siphonous green alga, *Codium* (*C.*) *fragile*, possess siphonaxanthin and its ester that absorbs blue-green light when bound to the photosynthetic light-harvesting complexes. Influence on pigmentation of five different light regimes in photosynthetic active radiation range (400-700 nm, including blue, green, red, far-red, and white) at four intensities (50, 100, 200, and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) was assessed for *C. fragile* trichomes for 7 days. In addition, a novel carotenoid was clearly accumulated under blue and green LED at higher irradiance. The structure was determined to be 19-deoxysiphonaxanthin ((3S,5R,6S,3'R,6'R)-3,3'-dihydroxy-7,8-dihydro- β,ϵ -caroten-8-one) by comparison with spectroscopic data of organic-synthesized one. A plausible biosynthetic pathway of siphonaxanthin from lutein via 19-deoxysiphonaxanthin was proposed.

1. Introduction

Several species of green algae, known as siphonous green algae, possess siphonaxanthin (Sx) in their light-harvesting complex (LHC) to utilize blue-green light for photosynthesis [1]. Sx has been proposed to biosynthesize from lutein (Lu) via loroxanthin (Lo) in relation to the evolutionary flow from sea to land [2,3]. However, no concrete evidence of this pathway has been reported yet. Recently our group reported that the accumulation of all-*trans* neoxanthin (tNx) in *Codium* (*C.*) *intricatum* at only high light conditions [4]. Chloroplast has been known to accumulate neoxanthin only in 9'-*cis* conformation [5], however accumulation of tNx in chloroplast were reported in Prasinophyceae [6] and Bryopsidales [7]. Interestingly, both species contains Sx as major antenna carotenoids.

In this work, we precisely investigated the effect of light regimes on the pigment compositions of *C. fragile* trichomes cultivated in floating form. As the results, we found a remarkable accumulation of a novel carotenoid observed only with intense blue-green light, and determine the structure as 19-deoxysiphonaxanthin.

2. Experimental

C. fragile (KU-065, KU-MACC, Kobe, Japan) were cultivated in trichomes in PES enriched artificial sea water (Marine Art SF-1, Tomita Pharmaceutical Co., Ltd., Tokushima, Japan).

Wet cultures (~0.5 g) were placed in a glass vial (32 mm i.d.) containing 5 ml of the medium. Each sample bottle was placed in a compartment on the LED and irradiated from the bottom at 20 °C under an irradiation of 50, 100, 200, 300 PPFD (photosynthetic photon flux density, $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) white, red, green, blue and far-red LED (TSLM-series, CCS Inc. Kyoto, Japan).

Pigment compositions were determined by HPLC basically as described previously [4] with some modifications.

UID was purified from 40 g wet weight of *C. fragile* trichomes cultivated under blue LED at 300 PPFD for 7 days. ¹H-NMR (600 MHz cryoprobe NMR, Bruker, Germany), HRMS (FTICRMS, SolariX, Bruker), UV-VIS (UV-1800, Shimadzu), and CD (J720W, JASCO, Tokyo, Japan) were performed.

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3. Results and Discussions

The change in pigment composition under different light conditions was more pronounced for carotenoids compared to chlorophylls. Under blue, green, red and white LED, the higher the light intensities, the higher the accumulation level of both tNx and violaxanthin (Vx). In far-red LED, no remarkable changes in pigment compositions were observed. Under both blue and green LED at highest irradiation, an unidentified peak (UID) appeared just after Vx in HPLC elution profile as shown in Figure 2. The daily increase in UID with the decrease in Sx (Sx + Sn) is very consistent, indicating that UID is a precursor to Sx.

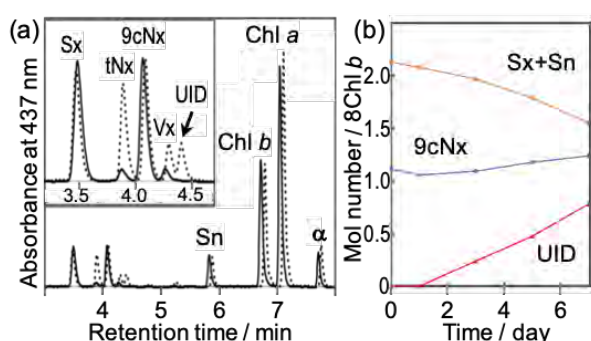


Fig 1. (a) HPLC profiles of *C. fragile* trichomes cultivated under blue LED at 300 PPFD for a week (broken line) and control (solid line). (b) Changes in carotenoids accumulation by 300 PPFD blue LED exposure within a week.

We isolated UID and obtained a set of optical spectroscopic data, i.e., UV-VIS, CD, HRMS, and 1D ^1H -NMR spectra. All the spectral data of UID were in good agreement with the organic synthesized one, so the structure of UID was unequivocally determined to be 19-deoxysiphonaxanthin (dSx, Fig. 2). It was for the first time to isolate dSx from biological source.

Due to its chemical structure and concomitant accumulation behavior of Sx, dSx can be considered as the precursor for Sx in this species. We could not identify Lo throughout this work. To the best of the authors' knowledge, no species containing both Sx and Lo have been reported [3, 8]. Thus, the major precursor to Sx may be not Lo but dSx as proposed in Fig. 2.

Accumulation level of dSx strongly depends on the color: green > blue >> white. Therefore, the biosynthesis from dSx to Sx is thought to be down regulated by strong green light. This may be related to

the natural light environment under the sea [3].

Since an epoxidation of dSx results in preprasinoxanthin, a proposed precursor of prasinoxanthin [2]. Thus, dSx may be a key precursor that serves as a branching point for the biosynthesis of prasinoxanthin and Sx.

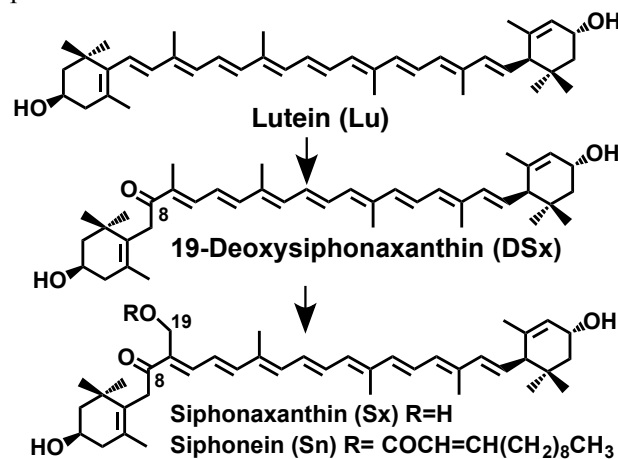


Fig. 2. Proposed biosynthetic pathway of siphonaxanthin

4. Conclusion

In *C. fragile* trichomes, remarkable accumulation of tNx and Vx under blue, green, red, and white LED with increasing intensity was observed. Under both blue and green LED with high intensity, accumulation of a novel carotenoid dSx was clearly observed. The structure was determined by comparison with a chemically synthesized standard.

5. Acknowledge

NMR and HRMS were performed at Analysis Division, Research Center for Artificial Photosynthesis, Osaka City University.

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In-vitro reconstitution of photosynthetic light-harvesting complex from siphonous green alga *Codium fragile*

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Abstract: A siphonous green alga, *Codium* (*C.*) *fragile* has a unique light-harvesting complexes, siphonaxanthin-chlorophyll *a/b*-binding protein (SCP) in which siphonaxanthin (Sx) absorbs green region of light. Recently we obtained SCP cDNAs from *C. fragile* and expressed in *E. coli*. In this work, in vitro reconstitution of SCP was performed with the recombinant protein and natural pigments. After several trials we successfully prepared the trimeric reconstituted SCP. Further improvement of yield and reproducibility will be necessary.

1. Introduction

Photosynthetic light-harvesting complexes (LHC) play the crucial role of absorbing sunlight and transferring the excitation energy to photoreaction center [1]. A siphonous green alga, *C. fragile* has a unique LHC called SCP to utilize blue-green region of light for photosynthesis [2]. The molecular mechanism, however, to capture blue-green light by Sx has not been clarified yet. Fig. 1 shows the chemical structures of carotenoids bound to SCP.

We sequenced several SCP cDNAs of *C. fragile*, which revealed high homology to the higher plant major LHC (LHCII), to which lutein (Lu) is bound instead of Sx [3]. Like LHCII, SCP exists as a trimer. The structural preference of carotenoids to their binding sites were extensively studied for LHCII by using in vitro reconstitution technique [1,4,5]. There are, however, no reports which describes the successful in vitro reconstitution with other-type LHCII. In this work, we aim to establish the in vitro reconstitution of SCP to investigate the energetic perturbations that Sx receives from the protein environment.

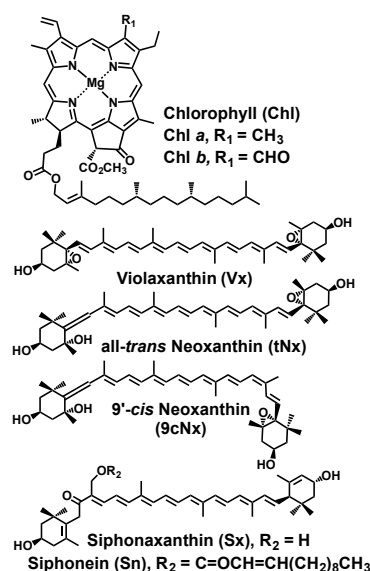


Fig. 1. Chemical structures of pigments bound to SCP

2. Experimental

SCP cDNAs was cloned from cultivated *C. fragile* trichomes (KU-065, KU-MACC, Kobe, Japan), sequenced and expressed in *E. coli*. [3].

Two sets of pigments were extracted from *C. fragile* or *C. intricatum* thalli collected from the sea as follows. “Total extracts” referred to the whole methanol-acetone (1:1) extracts of those algae after worked up with aqueous dichloromethane and dried up. “Carotenoid mixture” referred to the chlorophyll (Chl)-free “total extracts”: Chls were chelated by adding 5ml dioxane with 50 ml water to 100 ml of “total extracts” in acetone and removed by filtration [6]. The filtrate was then purified using an aluminum column with *n*-hexane as an eluent and dried up.

In-vitro reconstitution was performed by mixing the SCP protein expressed as inclusion body in *E. coli* with both “total extracts” and “carotenoid mixture” as described [4] with some modifications.

Pigment compositions were determined by HPLC as described in [7] with a few modifications. Blue-Native (BN) PAGE was performed (Novex Bis-Tris gel, 4-16%, Invitrogen) according to the provided protocol.

3. Results and Discussions

Fig. 2 shows HPLC profiles of the “total extracts” and “carotenoid mixture”. No degradation of Chls was observed in “total extracts”. “Carotenoid mixture” was almost free from Chls, but all the carotenoids except α -carotene were preserved their natural composition.

After invitro reconstitution, the reconstituted SCP (rSCP) was purified by a DE52 short column. BN-PAGE revealed that fractions 2-4 all contain SCP in both monomeric (M) and trimeric (T) assembly (Fig. 3 left). Major absorption maxima due to Chls (440, 480, 650 and 670 nm) were comparable between rSCP and native trimeric SCP (ntSCP), whereas 520-560 nm band due to bound Sx was smaller in rSCP. Since the rSCP (fraction 3) was a mixture of monomeric and trimeric SCP, this may be explained that the spectral shift of Sx is achieved only in trimeric SCP.

In vitro trimerization of LHCII required active addition of phosphatidyl glycerol [8]. In this work, we detected trimeric SCP without active addition of lipid(s). This may indicate SCP does not require lipids for trimerization or the key lipid was supplied from “total extracts”. Further research is needed.

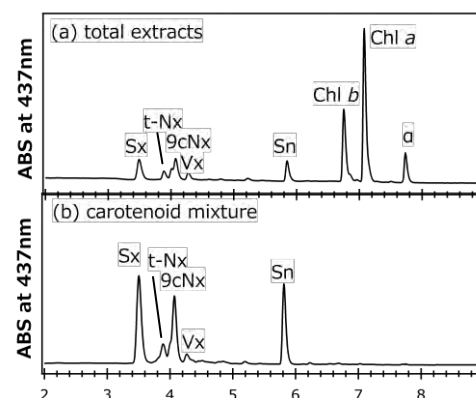


Fig. 2. HPLC elution profiles of “total extracts” (a) and “carotenoid mixture” (b).

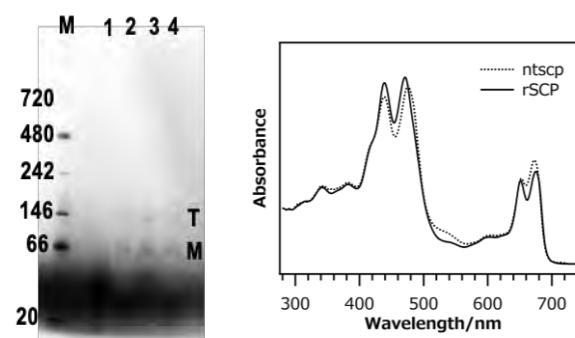


Fig. 3. (left) BN-PAGE of rSCP, (right) Absorption Spectra of rSCP (frac. 3) and ntSCP. Spectra were normalized at Chl *b* (650 nm).

4. Conclusion

We have successfully reconstituted the trimeric SCP without active adding of lipid. Green absorptive band of bound Sx at 540 nm was reproduced in the reconstituted SCP for the first time. Further improvement of yield and reproducibility is necessary.

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Increased lycopene concentration in egg yolk of hens fed *Z*-isomer-rich lycopene and development of tomato powder rich in lycopene *Z*-isomer

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Abstract: This study aimed to investigate the effect of feeding lycopene isomers to laying hens on lycopene concentration in the yolk. As the lycopene *Z*-isomer content in the diet increased, the lycopene concentration in the egg yolk increased. For example, when fed a diet rich in *Z*-isomers (61.3%), the lycopene concentration in the egg yolk was approximately three times higher than when fed the (all-*E*)-lycopene-rich diet for 21 days. We also developed tomato powder rich in lycopene *Z*-isomers by thermal treatment above melting point of lycopene. The resulting tomato powder rich in the *Z*-isomers would be useful for feed having high egg yolk color improving property.

1. Introduction

The egg yolk of hens contains carotenoids in considerable quantity, which are derived from the diet. Several studies reported that when tomatoes were fed to hens, lycopene was accumulated in egg yolk [1]. In nature, most carotenoids, including lycopene in tomatoes, exist in the all-*E* configuration. On the other hand, several studies demonstrated that *Z*-isomers of lycopene exhibit higher bioavailability than the all-*E*-isomers [2]. Hence, this study aims to investigate the effects of feeding lycopene having high *Z*-isomer content to laying hens on lycopene concentration in egg yolk. Moreover, we also developed tomato powder rich in lycopene *Z*-isomers for using animal feed.

2. Experimental

2.1 preparation of lycopene *Z*-isomers

Lycopene containing different *Z*-isomer contents was prepared by thermal treatment of oil-containing tomato oleoresin under different conditions: 130 or 140 °C for 1 h. Although the total *Z*-isomer content in the untreated oleoresin was only 5.5%, the total

Z-isomer contents after the *Z*-isomerization treatments increased to 35.1 and 61.3%, respectively. The prepared tomato oleoresins having different lycopene *Z*-isomer contents were added to the basal diet so that the lycopene content in the diet became a defined value (200 mg/kg of diet), and their diets were used for the test of the animal husbandry as described below (see “2.2 animal husbandry” section).

2.2 animal husbandry

Boris Brown hens were employed in this study to investigate the effect of all-*E*-isomer-rich and *Z*-isomer-rich lycopene-supplemented feeding on lycopene concentration in egg yolk.

2.3 *Z*-isomerization of (all-*E*)-lycopene in tomato powder

Z-Isomerization of lycopene in tomato powder was performed by hot air heating using a cooking oven (AX-XW600, Sharp Corporation, Osaka, Japan). The sample was treated by hot air at 140–250 °C for 5 min. After the treatment, lycopene isomers were immediately extracted for HPLC analysis.

2.4 HPLC analysis

To analyze lycopene isomers, normal-phase HPLC analysis with three Nucleosil 300-5 columns connected in tandem (3 × 250 mm length, 4.6 mm inner diameter, 5 µm particle size; GL Sciences Inc.,

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Tokyo, Japan) was carried out according to the method described previously [3].

3. Results and Discussions

3.1 effect of Z-isomer-rich lycopene supplementation on hen's egg quality

As shown in Fig. 1, although the lycopene content in the diet was the same (200 mg/kg of diet), an increase in Z-isomer contents induced an obvious increase in egg yolk lycopene concentration [4]. For example, when hens were fed diets containing 61.3% of lycopene Z-isomer, the egg yolk lycopene concentration was approximately three times higher than when fed all-*E*-isomer-rich lycopene-containing diet. These results strongly support the suggestion that lycopene Z-isomers have higher bioavailability and/or higher transfer efficiency to the egg yolk than the all-*E*-isomer in Boris Brown hens.

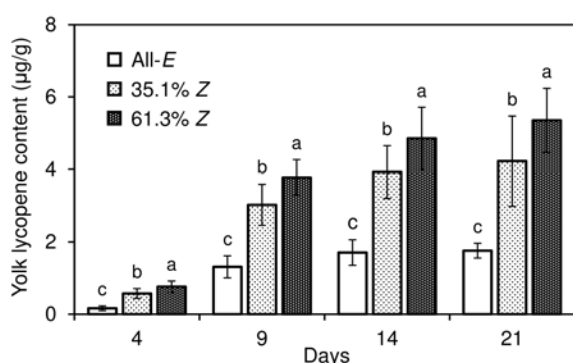


Fig. 1. Effects of lycopene isomer supplementation for 21 days on lycopene concentration of egg yolk. Error bars show standard deviation ($n = 10$). Means with different letters in each day are significantly different ($p < 0.05$), whereas means with similar letters are not different from each other [4].

3.2. effects of heating temperature on Z-isomerization of lycopene in tomato powder

When the tomato powder was heated below the melting point of lycopene (approximately 175 °C), the Z-isomerization reaction hardly progressed (Fig. 2). On the other hand, the heating over 180 °C markedly promoted the Z-isomerization of lycopene; as the heating temperature increased, the total Z-isomer content raised proportionally. On heating at 250 °C, the total Z-isomer content reached approximately 80% within a short time (5 min). On the other hand, when tomato powder was heated above 240 °C, lycopene in tomato powder was significantly decomposed.

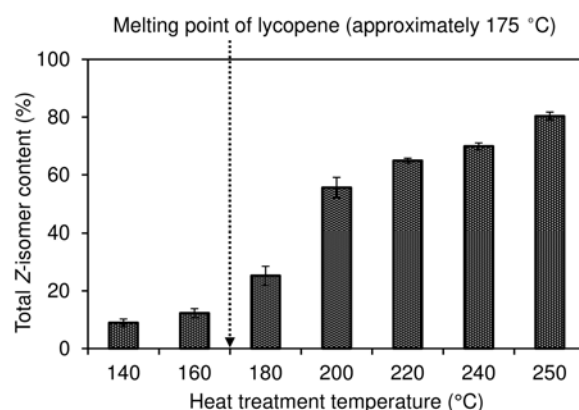


Fig. 2. Effect of heat treatment temperature of tomato powder for 5 min on Z-isomerization of lycopene. Error bars show standard deviation ($n = 3$).

4. Conclusion

Lycopene Z-isomers had higher accumulation efficiency in the egg yolk of Boris Brown hens than the all-*E*-isomer. This result clearly demonstrated that lycopene Z-isomers have higher bioavailability and/or higher transfer efficiency to the egg yolk than the all-*E*-isomer. Moreover, this study clearly showed that heating above the melting point of lycopene promoted thermal Z-isomerization of the all-*E*-isomer in tomato powder. This result indicated that the Z-isomerization of lycopene might be enhanced in the molten state.

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Effects of astaxanthin on cognitive function, physical function, and mood condition in elderly people

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Abstract: We conducted this clinical study to investigate the effects of the intake of astaxanthin on cognitive function, physical function, and mood condition in elderly people and showed that astaxanthin shortened the reaction time in 2-back working-memory tasks compared to a placebo, and that POMS negative mood scores were improved versus pre-intervention levels only in the astaxanthin group. Astaxanthin supplementation also improved judgment for unmemorized faces in elderly people with greater adaptation in endurance. Additional analysis showed that increased plasma astaxanthin significantly correlated with the decreased POMS “Fatigue-Inertia” score in the astaxanthin group. Astaxanthin may contribute to cognitive ability and emotional health in elderly people.

1. Introduction

Cognitive or physical decline in aging is directly associated with changes in the quality of life (QOL). A decline or a subjective experience of decline in cognitive or physical function leads to a reduction in daily activities and social relationships, which in turn results in deterioration in mood condition such as lowered motivation and depression. A complex approach to the preservation of cognitive function, physical function, and mood condition is important to maintain the health of elderly people for a long period. We therefore conducted this clinical study to investigate the efficacy of astaxanthin on cognitive function, physical function, and mood condition [1]. We here report our further analysis on the relationships between plasma astaxanthin

concentration and the effects of astaxanthin intake on the cognitive function and mood condition of the elderly people.

2. Experimental

A randomized, double-blind, placebo-controlled, parallel-group, comparative study was performed. Healthy men and women aged 60–79 years, who exhibited subjective decline in cognitive function or physical strength in their daily lives were randomly allocated to either the astaxanthin group, which consumed 2 capsules daily containing 6 mg astaxanthin, 5 mg tocotrienol, 3 mg zinc, and 300 IU vitamin D for 12 weeks, or the control group, which daily received 2 placebo capsules containing the aforementioned substances except astaxanthin for 12 weeks. The following evaluations were performed to investigate the cognitive function, physical function

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and mood condition for the subjects on week 0 and week 12. The 2-back working-memory task and recognition memory task were used for the evaluation of cognitive function, and the short physical performance battery, 6-minute walking test (6MWT), and 10 meter incremental shuttle walking test (ISWT) were used for the evaluation of physical function. Moreover, the subjects evaluated their own cognitive ability, physical condition, and mood condition using the visual analogue scale (VAS). The profile of mood states (POMS) was also used to evaluate their mood condition. Plasma samples were prepared from blood samples taken on week 0 and week 12. The astaxanthin concentrations were determined by reversed-phase HPLC according to the procedure we previously reported [2].

All data are expressed as mean $\pm$ standard error (mean $\pm$ SE). For comparison between the groups, after the normality and homogeneity of variance were confirmed, Student's *t*-test was performed. Intra-group comparison was performed by paired *t*-test. Pearson correlation coefficient was used for correlation analysis.

3. Results and Discussions

Astaxanthin was detected in the plasma samples from only two subjects on week 0 (1.6 ng/mL and 0.7 ng/mL respectively). After intervention of 12 weeks, the plasma astaxanthin concentrations in the astaxanthin group was significantly increased to 85.7 ± 14.0 ng/mL compared with that on week 0 and significantly higher than that in the control group. The plasma astaxanthin concentration in the control group remained low at 0.9 ± 0.6 ng/mL on week 12.

As we previously reported, 12 mg/day of astaxanthin supplementation for 12 weeks significantly shortened the reaction time of correct rejection (CR) in the 2-back working-memory task compared to the placebo, and significantly decreased POMS scores of the "Anger-Hostility (AH)", "Confusion-Bewilderment (CB)", "Fatigue-Inertia (FI)", and Total Mood Disturbance (TMD) versus pre-supplementation [1]. Although no significant differences were observed between the groups for the VAS, SPPB, 6MWT, and ISWT, when the subjects were stratified based on the median of changes in the ISWT walking distance of each group, the number of

correct reactions for unmemorized faces in the recognition memory task was increased by the astaxanthin supplementation [1].

We conducted correlation analysis between the cognitive function or mood conditions and the plasma astaxanthin concentration. The changes in the reaction time of CR in the 2-back working-memory task and in the number of correct reactions in the recognition memory task did not correlate with the plasma astaxanthin. Changes in the astaxanthin groups' POMS score for "FI" significantly correlated with plasma astaxanthin concentration. The correlation coefficient was -0.50 ($P = .03$). No correlations with plasma astaxanthin concentration were observed for "AH", "CB" or TMD. In the study on healthy adults supplemented with astaxanthin and sesamin (AS), it was shown that the changes in VAS score for fatigue significantly negatively correlated with the plasma astaxanthin, therefore the anti-fatigue effect of AS might be associated with the effect of astaxanthin [3]. Astaxanthin may contribute to the emotional health and mental stability in elderly people.

4. Conclusion

Twelve mg/day of astaxanthin supplementation for 12 weeks improved cognitive function in elderly people, especially the processing speed of short-term memory information. Astaxanthin supplementation also improved judgment for unmemorized faces in elderly people with greater adaptation in endurance. Negative mood scores in POMS were improved only in the astaxanthin group. Furthermore, the negative correlation between the changes in the score of "FI" and plasma astaxanthin concentration was observed. Astaxanthin may contribute to the cognitive ability and emotional health in elderly people.

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Synthesis of New Derivatives Similar to Carotenoids and Evaluation of Singlet Oxygen Quenching Ability

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Abstract: New carotenoid-like compounds in which the double bond of the polyene of astaxanthin was replaced by an aromatic ring or a heteroaromatic ring were synthesized, and their singlet oxygen quenching ability were evaluated. But none of the compounds exhibited singlet oxygen quenching activity. The reason for this is considered to be that the polyene became in a shorter state where π electron cannot be delocalized as astaxanthin judging from that the maximum absorption wavelength (λ_{max}) were shorter than 478nm of astaxanthin. So it is concluded that the π electron of aromatic ring cannot play the same role as that of the chain double bond of astaxanthin.

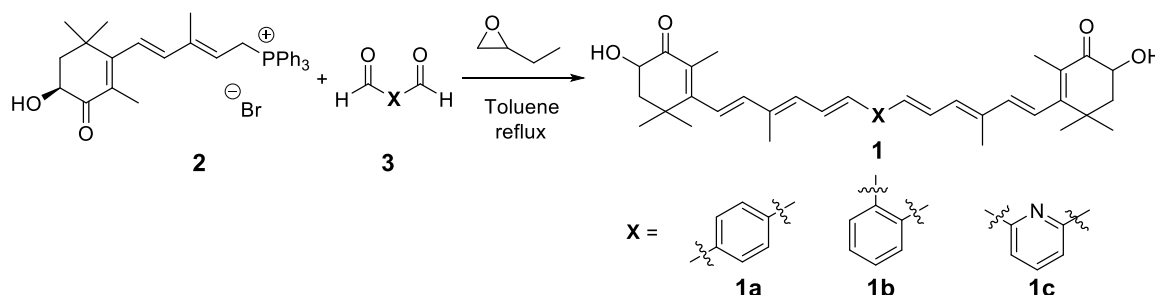


Fig.1 Synthesis scheme of new carotenoid derivatives

1. Introduction

Astaxanthin, one of carotenoids, is known to have extremely high antioxidant activity against active oxygen species, particularly singlet oxygen^[1], and is expected to be effective in treating various diseases caused by active oxygen species^[2]. Carotenoids have polyene structure in their molecule, which enable the presence of many geometric isomers. The presence of isomer bring about a big issue when seeking pharmaceuticalization of carotenoids. Therefore, for the purpose of reducing isomer we synthesized a compounds in which an aromatic ring was introduced in polyene structure, keeping the number of π

electrons of the polyene moiety. The aromatic ring fix the bonding position which reduced a production of geometric isomers. The physicochemical studies were carried out.

2. Material and methods

2.1 Synthesis of New Carotenoid-like Compounds

Ylide **2** was synthesized with reference to the method for the production of astaxanthin^[3], and wittig reaction was conducted with dialdehyde **3** in toluene, which has either an aromatic or heteroaromatic cycle. Although a cis form of **1** was formed during the reaction, the yield and purity were successfully improved by adding a catalytic amount of iodine in the residue, and by solidifying the trans form

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simultaneously with the isomerization of the *cis* form^[4].

2.2 Singlet oxygen quenching ability evaluation test

It is known that singlet oxygen was generated by irradiating visible light with methylene blue, which is a photosensitizer. Singlet oxygen reacts with methyl linoleate, one of an unsaturated fatty acid, to form an adduct having a conjugated diene^[5]. In this study, the quenching activity of the synthesized compounds were indirectly evaluated by utilizing the fact that the formation of the singlet oxygen adduct of methyl linoleate was suppressed by adding the singlet oxygen quenching agent. In this study, we tested whether the new compounds inhibit the formation of conjugated dienes in methyl linoleate compared with that of astaxanthin.

3. Results and Discussions

Compound	IC ₅₀ (μ M)
Astaxanthin	12.0
1a	none
1b	none
1c	none

Tabel1. Results of singlet oxygen quenching ability

Singlet oxygen quenching ability was evaluated. The IC₅₀ value of astaxanthin was 12 μ M, whereas none of the **1a~1c** exhibited singlet oxygen quenching activity. Generally, the longer the polyene is, the longer wavelength of λ_{max} becomes, but the **1a~1c** of the maximum absorption wavelength were 420nm, 348nm, and 434nm, respectively. The reason why the singlet oxygen scavenging activity was not observed is considered to be that the polyene became a short state and the localization of π electron was not similar to that of astaxanthin with λ_{max} of 478nm^[6]. So, it is believed that the π electron of aromatic ring cannot play the same role as that of the chain double bond of astaxanthin.

4. Conclusion

The new derivatives **1a~1c** in which an aromatic ring or a heteroaromatic ring was introduced into the central portion of polyene chain and the number of π electrons in the polyene portion was maintained as

that of astaxanthin, showed no singlet oxygen quenching activity. The reason for no efficacy is considered to be that the polyene became in a shorter state where π electron cannot be delocalized as astaxanthin even though the number of π electron is the same as astaxanthin. So it is concluded that the π electron of aromatic ring cannot play the same role as that of the chain double bond of astaxanthin. Time-resolved absorption spectroscopy is performed to determine whether these compounds receive energy from singlet oxygen and have an excited state.

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Pharmacokinetics of Astaxanthin Derivative in Knee Osteoarthritis Models

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Abstract: We evaluated the pharmacokinetics of a new astaxanthin (AX) derivative FAX-051T, which was effective in the knee osteoarthritis (OA) model. When radiolabeled ^{14}C -FAX-051T was administered into the knee joint (intra-articularly: I.A.) of normal rats, it was considered that FAX-051T was transferred from the knee joint via the lymph to the blood as a transfer route. There were no significant differences in the pharmacokinetics of FAX-051T in the knees between normal rats and mono-iodoacetate (MIA) induced rats. No species differences were observed in the persistence of intra-knee concentrations after I.A. administration of FAX-051T in rats and rabbits, which were evaluated for efficacy. The toxic risks of systemic exposure were assessed when FAX-051T was administered I.A., because it was also transferred in the blood. As a result, the risk of toxicity from systemic exposure was considered to be extremely low, since the plasma concentrations after I.A. administration were lower than those in the toxicity studies.

1. Introduction

Recently, the potential therapeutic effects of AX on experimentally-induced OA in animals has been reported. AX itself, however, is not suitable for injection due to its poor water solubility. We newly synthesized an AX derivative FAX-051T with improved solubility in water and reported the pharmacological effects of I.A. injected FAX-051T on the experimentally-induced OA at The 33rd Annual Research Meeting of the Japanese Orthopedic Association (2018) [1]. Pharmacokinetics of carotenoids after I.A. administration are not well understood. In this study, radiolabeled ^{14}C -FAX-051T was administered I.A. to rats to evaluate the systemic distribution and the transfer route from the knee joint. FAX-051T was administered I.A. to rats and rabbits, which are animals for evaluation of drug efficacy, to evaluate the differences in pharmacokinetics between species and the presence or absence of disease states. In addition, the risk of toxicity was also assessed by comparing the plasma concentrations after I.A. administration with the plasma concentrations at the no-observed-adverse-effect level (NOAEL) in the

intravenous toxicity study.

2. Experimental

2.1 compound

FAX-051T and the radioactive label ^{14}C -FAX-051T are synthetically produced by Asta Pharmaceuticals Co., Ltd. and Sekisui Medical Co., Ltd, respectively.

2.2 Animal study

Under isoflurane inhalation anesthesia, ^{14}C -FAX-051T was administered I.A. to normal rats using syringes with injection needles, and the rats were euthanized by carbon dioxide inhalation at 0.25, 1, 24, 72, 168, and 336 hours after administration. Animal sections were prepared for quantitative whole-body autoradiography. Tissue radioactivity concentrations were measured and the distribution of radioactivity from the injection site to blood and tissues was assessed.

Rat MIA models were produced by administering I.A. 2 mg of MIA to rats. FAX-051T was administered I.A. at a dose of 0.768 mg/body to normal rats and rat MIA models. Blood was collected under inhaled isoflurane anesthesia at 0.25, 1, 24, 72, 168, and 336

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hours after administration. Blood was centrifuged to obtain plasma. The knee joint was collected.

Blood was collected at 0.25, 1, 24, 72, 168, and 336 hours after administration of 0.768 mg/kg of FAX-051T into the knees of normal rabbits. Blood was centrifuged to obtain plasma. After the blood collection, the rabbits were euthanized under thiamylal sodium anesthesia, and the muscle around the joint in the knee joint position was removed.

A bead-bombarded cell fragmentation device was used to fracture the collected knee joints, and phosphate buffered saline solution was added to prepare homogenate samples.

2.3 Quantitative whole-body autoradiography

After exposure of radioactivity in animal sections for a period of time, radioactivity images on imaging plates and photo-stimulated luminescence (PSL) were read using Bio-imaging Analyzer Systems to produce radioluminograms. Radioactivity in tissues was converted into luminescence intensity per unit area (PSL/mm), and tissue radioactivity concentrations were calculated based on the subtraction of background values corresponding to the evaluated area.

2.4 Measurement of FAX-015T concentrations in Knee Joint Cavity and Plasma

FAX-051T concentrations in knee joint cavity and plasma were measured by LC-MS/MS, with calibration curves ranging from 0.2 to 200 µg/g and from 1 to 1000 ng/mL, respectively.

3. Results and Discussions

After I.A. administration of ¹⁴C-FAX-051T to rats, the knee joint cavity radioactivity concentration persisted at high concentration for a long time, and disappeared with the half-life of about 45 hours. Radioactivity from the injection site was transferred to the blood via the lymph, but was less distributed to systemic tissues. Pharmacokinetics in normal rats and rat MIA models showed no significant differences in FAX-051T concentrations in knee joint cavity and persisted at high concentrations for a long time. Pharmacokinetics in rabbits showed that FAX-051T concentrations in knee joint cavity were sustained with the half-life of approximately 47 hours, which was similar to the half-life of approximately 44 to 61 hours

in rats. Plasma concentrations after I.A. administration of FAX-051T in rats and rabbits were lower than those at the NOAEL in the intravenous toxicity studies.

It was suggested that the FAX-051T migrated from the knee joint via the lymphatics to the blood. There were no significant differences in the pharmacokinetics of FAX-051T in knee joint cavity between normal rats and rat MIAs. In rats and rabbits, there were no species differences in the concentration persistence in the knee joint cavity, which is the site of drug effect. In addition, the risk of systemic toxicity was considered to be extremely low, since the plasma concentrations after I.A. administration were lower than those in the toxicity studies.

4. Conclusion

There were no significant differences in the pharmacokinetics between the presence and absence of disease states. In rats and rabbits, there were no species differences in the concentration persistence in the knee joint cavity.

The risk of systemic toxicity was considered to be extremely low, since the plasma concentrations after I.A. administration were lower than those in the toxicity studies.

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Investigation of anti-inflammatory mechanism of apo-fucoxanthinoids

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1. Introduction

Fucoxanthin is known to be metabolized to amarouciaxanthin A and fucoxanthinol in mice. In our recent study, paracentrone having allenic bond was detected in tissues of fucoxanthin-fed mice. This suggests that dietary fucoxanthin is metabolized to cleavage products apo-fucoxanthinoids after incorporation into the body. However, physiological function and mechanism of apo-fucoxanthinoids are unclear. In this study, as health beneficial function of apo-fucoxanthinoids, we investigated anti-inflammatory effects leading to the prevention against various chronic diseases.

2. Methods

Murine macrophage-like RAW264.7 cells were treated with apofucoxanthinoids and then induced inflammation by lipopolysaccharide (LPS). After LPS stimulation, total RNA was extracted from the cells, and mRNA expression levels of inflammatory factors were quantified by RT-qPCR method. Inflammatory cytokine IL-6 and nitric oxide levels in the culture media were analyzed by ELISA and Griess method, respectively. Anti-inflammatory signaling pathways were analyzed by Western blotting.

3. Results and Discussion

Apofucoxanthinoids down-regulated mRNA expression of IL-6, IL-1 β and inducible nitric oxide synthase (iNOS) in LPS-stimulated RAW264.7 cells. Both IL-6 and nitric oxide secretion levels also decreased by apofucoxanthinoids treatment. Under LPS stimulation, major inflammatory modulator NF- κ B p65 is known to translocate into nuclear and activate gene transcription of inflammatory factors such as interleukins. Apofucoxanthinoid markedly suppressed p65 nuclear accumulation. On the other hand, apofucoxanthinoids significantly increased anti-oxidative enzyme HO-1 and NQO-1 mRNA expression. It has been reported that transcription factor Nrf2 regulates these anti-oxidative enzyme expression (J. Biol. Chem. 1999, 274:26071). In Western blot analysis, apofucoxanthinoid dose-dependently increased Nrf2 nuclear accumulation. Further, co-treatment with apofucoxanthinoid and Nrf2 inhibitor N-acetyl cysteine (NAC) suppressed HO-1 mRNA induction through inhibition of Nrf2 nuclear accumulation. The suppressive effect of apofucoxanthinoid against LPS-induced inflammation was also cancelled under NAC treatment. These results suggest that Nrf2 play a critical role for anti-inflammatory action of apofucoxanthinoids.

Synthetic study of ζ -Carotene -Part 1-

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Abstract: A synthetic study of various geometrical isomers of $\square$ -carotene was performed. C20-aldehyde and C20-Wittig salt were prepared from commercially available geranyl acetone, and then these were treated with a base, and isomers of $\square$ -carotene were given.

1. Introduction

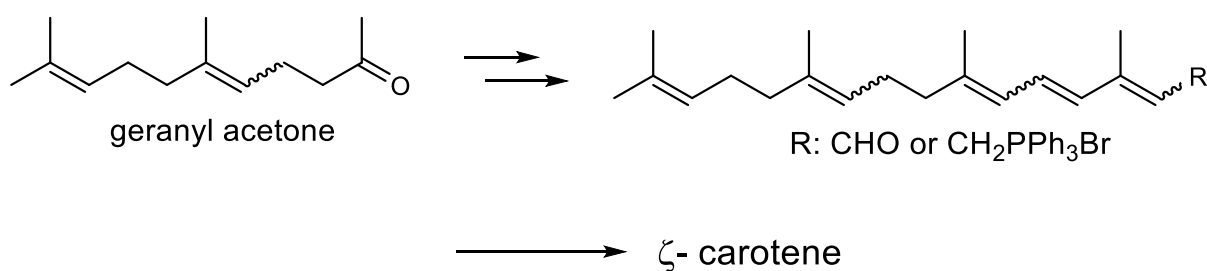
Carotenoids exist widely in nature and, about 750 kinds of carotenoids were reported till now. Carotenoid are de novo synthesized in plants and several bacteria. However, animals cannot synthesize carotenoids *in vivo*, and so those found in animals are either directly obtained from food or partly modified through metabolic reactions.

Phytoene is C40 carotenoid biosynthesized from isopentenyl pyrophosphoric acid (IPP) given by Mevalonate pathway or non-Mevalonate pathway. Lycopene and ζ -carotene are formed from phytoene by desaturation and are sometimes found mixture of geometrical

isomers in plants and bacteria. ζ -Carotene are key intermediate of this biosynthetic pathway. However, geometrical structure of ζ -carotene is not determined because of instability. In order to determine geometrical structure of ζ -carotene in plants and bacteria, several authentic geometrical isomers of ζ -carotene are needed. So, we tried to synthesize some geometrical isomers of ζ -carotene.

2. Experimentals

ζ -carotene has already a total synthesis method [1], but only all-trans type can be synthesized by this method. So, we aimed for total



synthesis by another method. In order to synthesize 15-*cis* form at first, C20 units were obtained using commercially available geranyl acetone as a starting material, and finally ζ-carotene was get by Wittig reaction.

3. Results and Discussion

Commercial geranyl acetone is a mixture of isomers at the 5-position, but the reaction proceeded as it was and the C20 units were obtained in a reasonable yield. Finally,

C20-aldehyde and C20-Wittig salt were reacted with NaOMe as a base to synthesize ζ-carotene. Since it was possible to infer from the NMR data there were several isomers in the obtained compound, we commissioned the collaborators to analyze it. As a result, it was unexpected that most were all-*trans* form. Currently, we are trying another synthesis method.

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Synthesis of (*R*)- and (*S*)- α -Carotenes and Their HPLC Separation

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1. Introduction

α -Carotene (**1**) is widely distributed carotenoid in nature. The absolute stereochemistry of natural α -carotene was determined to be (*R*) by comparison of specific optical rotation of synthetic (*S*)- α -carotene (**1b**) 50 years ago [1]. Recently, Takaichi et al. reported that unusual cyanobacteria *Acaryochloris* contains (*S*)- α -carotene having an opposite configuration at C6' [2]. Thus, in order to easily determine the absolute configuration of naturally occurring α -carotene, we synthesized both enantiomers of α -carotene and established their HPLC analysis method by using a column carrying a chiral stationary phase.

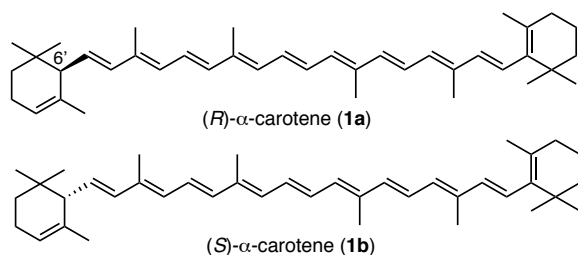


Figure 1. Structure of α -carotene enantiomers (**1a,b**).

2. Results

Separation of geometric isomeric mixture of α -carotene (**1**) by column chromatography was expected to be troublesome due to the lack of polar groups. Thus, we planned stereoselective syntheses of **1a,b** by Wittig reactions of C₁₀-phosphonium salt **2** with (all-*E*)-C₃₀-apocarotenals **3a,b** (Figure 2).

The apocarotenals **3a,b** were prepared starting from (*R*)- and (*S*)-C₁₅-alcohols **4a,b**, respectively, which were obtained by direct separation of racemic alcohol **4** by use of preparative HPLC column carrying a chiral stationary phase. The optically active alcohols **4a,b** were converted into the aldehydes **5a,b**, which were transformed into apocarotenals **3a,b** via twice Emmons-Horner reactions by use of phosphonates **6**

and **7**, respectively. Wittig reactions of aldehydes **3a,b** with phosphonium salt **2** proceeded stereoselectively to afford (all-*E*)- α -carotenes (**1a,b**) in good yields, respectively. CD spectra of synthetic **1a,b** were in good accordance with those reported [2].

Finally, a HPLC analytical method for **1a,b** was successfully established using a chiral column (CHIRALPAK IB; Daicel) as shown in Figure 3.

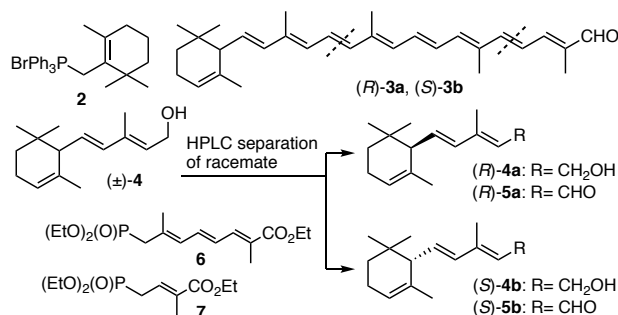


Figure 2. Synthesis of α -carotene enantiomers (**1a,b**).

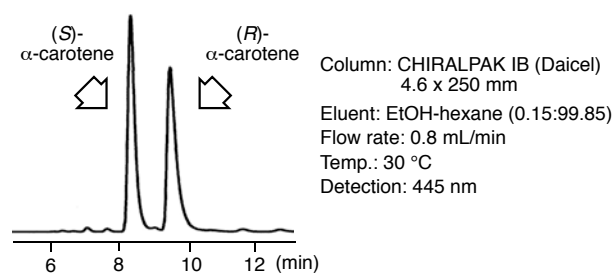


Figure 3. HPLC elution profile of α -carotene enantiomers (**1a,b**).

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Ultrafast Excitation Energy Transfer Dynamics of Carotenoids bound to Photosynthetic Reaction Centers from Green Sulfur Bacteria *C. tepidum*

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Carotenoids are photosynthetic pigments used for harvesting or dissipation of light energy in pigment-protein complexes in all the photosynthetic organisms. There is an enormous range of carotenoid species; most of them are well characterized chemically and spectroscopically by photochemical property and photosynthetic function [1]. Some of carotenoid are glycosylated at one or two edges of a polyene backbone mostly with glucoside or its fatty acid ester. However, carotenoid glycosides have remained virtually unknown about their function, especially effect of the glycosylation on photosynthesis. OH- γ -carotene glucoside ester and OH-chlorobactene glucoside ester are carotenoid glycosides bound specifically to the photosynthetic reaction center complex of the green sulfur bacterium *Chlorobaculum tepidum* [2]. Other carotenoids of *C. tepidum* are not glycosylated and not bound to the RC complex, and thus the glycosylation of OH- γ -carotene and OH-chlorobactene would be required specifically for some RC functions. In our previous work, we constructed a *C. tepidum* mutant strain, $\Delta cruC$, in which the *cruC* gene encoding glycosyl transferase of carotenoid [3] is insertionally inactivated. Photosynthetic function of the glycosylation was explored by fluorescence lifetime analysis in the $\Delta cruC$ mutant. When the most upper antenna system, chlorosome, in the *C. tepidum* cell was excited dominantly, the mutational effect was observed on fluorescence decays only at longer wavelength regions attributable to energy transfers to bacteriochlorophylls *a* in the reaction center complex. The glycosylated carotenoids would have a crucial role for effective quench of excess excitation energy in the *C. tepidum* reaction center complex.

In this study, we performed femtosecond pump-probe spectroscopic measurements on the isolated RCs from *C. tepidum* wild type and $\Delta cruC$ mutant to clarify roles of carotenoid glucoside in photosynthetic excitation energy transfer taking place on fs~ps timescale.

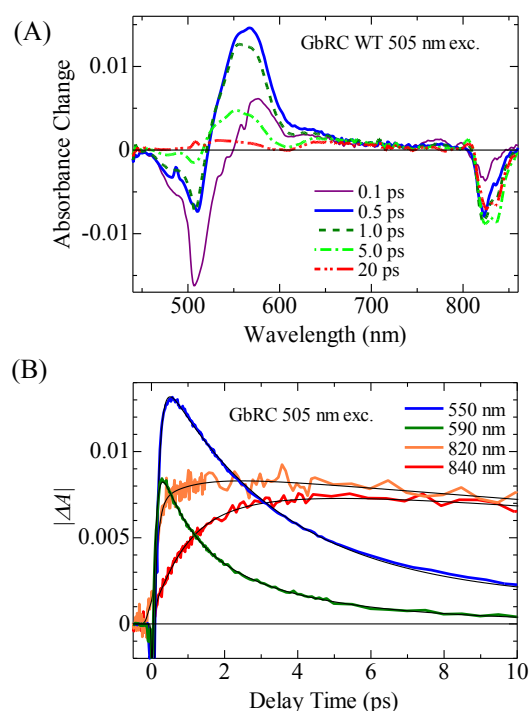


Figure: (A) Photo-induced absorption spectra of the isolated RC form *C. tepidum* wild type upon excitation of carotenoids at 505 nm. (B) Kinetic traces of the transient signals probed at different wavelength.

Figure (A) shows the photo-induced absorption spectra of the isolated RC from *C. tepidum* wild type upon excitation of carotenoids at 505 nm. Negative absorbance changes are due to the depletion of the ground state of carotenoids (450~520 nm) and bacteriochlorophylls (800~850 nm). A pronounced positive signal around 550 nm has been assigned to the transient absorption from lowest-lying singlet excited S_1 state to a higher-lying S_n state [4]. Figure (B) shows the kinetic traces of the S_1 - S_n transient absorption of carotenoids and the bleaching signals of bacteriochlorophylls. The bleaching signal at 820 nm rises with a time scale of 100 fs, while the rise of the bleaching at 840 nm agrees well with the decay of the S_1 transient absorption, suggesting the excitation energy transfer from carotenoids S_1 to bacteriochlorophylls. The results imply the efficient energy transfer from carotenoids to bacteriochlorophylls even in RC. Photosynthetic functions of the glycosylation will be discussed by comparison of the energy transfer dynamics between wild type and $\Delta cruC$ mutant strain.

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Control of an Intramolecular Charge Transfer Character of Artificial Short-Polyene Carotenoid

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In the primary photosynthetic processes, carotenoids bound to photosynthetic antenna pigment-protein complexes absorb sunlight in the blue-green region of the spectrum and the energy is rapidly and efficiently transferred to nearby (bacterio)chlorophylls [1, 2]. The transferred energy is eventually converted to electrochemical energy in reaction centers. In addition, carotenoids protect biological system against photo-destruction caused by excess amount of light exposure [2, 3]. The excited states of carotenoids are traditionally described by a three level electronic system (S_0 , S_1 , and S_2) [4]. The optically-allowed S_2 ($1^1B_u^+$) state of carotenoids is generated by a one-photon transition from the S_0 ground state ($1^1A_g^-$), and rapidly decays to the lower-lying dark S_1 ($2^1A_g^-$) state within 200 fs depending on the length of conjugation. In contrast, nonradiative relaxation from S_1 to S_0 is comparatively slow, occurring on the scale of ps~ns [1]. The excitation energy transfer from the carotenoid S_1 state to (bacterio)chlorophyll in a photosynthetic pigment-protein complex is often inefficient depending on how the optically forbidden S_1 state is involved in the excitation energy-transfer process [1, 2]. However, carotenoids containing a carbonyl group along their conjugated polyene backbone, such as fucoxanthin and peridinin, generate an excited state in which intramolecular charge transfer (ICT) occurs just below S_1 in polar environments, leading to the generation of a strongly coupled S_1 /ICT state [5]. The S_0 - S_1 /ICT transition dipole moment of carbonyl carotenoids is increased due to this charge transfer character, and efficient energy transfer from carbonyl carotenoids to chlorophyll via the S_1 /ICT state is realized in light-harvesting photosynthetic antennae.

We previously synthesized an unique compound 2-(all-*trans*-retinylidene)-indan-1,3-dione with five conjugated double bonds n , denoted as C20Ind and clarified its photophysical properties by femtosecond pump-probe spectroscopy [6]. Although C20Ind has two carbonyl groups within the indan group as shown in Fig. 1(A), its excited state signatures did not exhibit a pronounced ICT character. In the present study, we synthesized a short polyene compound C15Ind with three conjugated double bonds ($n=3$) as shown in Figure (A). It has

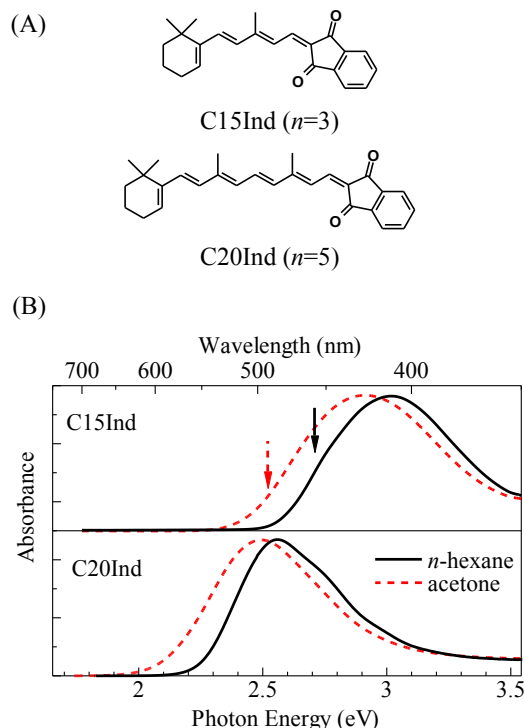


Figure: (A) The chemical structures of C15Ind and C20Ind. (B) Steady-state absorption spectra of C15Ind and C20Ind in *n*-hexane and acetone.

been reported that carbonyl carotenoids with a short polyene has a strong ICT character. Thus, characteristic spectral signatures are expected such as stimulated emission and transient absorption due to the ICT character of C15Ind. Figure (B) shows the steady-state absorption spectra of C15Ind in *n*-hexane and acetone together with those of C20Ind. Previous study has proposed that the asymmetric broadening of carbonyl carotenoids in polar solvents can be attributed to both inhomogeneous band broadening and variation of the ground and/or excited state conformations (inhomogeneous distribution model). Thus, the spectra imply an ICT character of C15Ind. Fs pump-probe spectroscopic analyses with 100 fs time-resolution were performed on C15Ind to elucidate ultrafast dynamics of an excited state having an ICT character.

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Construction of the pathways toward non-natural retinoids

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Beta-carotene oxygenases (BCOs) cleaves 15-15' bonds of the beta-carotene to produce two molecules of retinals. Retinal is the direct precursor of retinol (vitamin A) and retinoic acids, which play a variety of biological roles in higher animals. Also, it is the core component of rhodopsins/ bacteriorhodopsins that work as light-sensors, light-driven proton/ ion transporters. Nature harbors >900 natural carotenoids, and synthetic biologists have created numbers of non-natural carotenoids. By testing BCO for possible capacity to act on these carotenoids, we should be able to access a variety of novel apocarotenoids with new biological activity and/or photochemical properties.

For years, we have created various artificial pathways toward non-natural carotenoids with novel structures. We have systematically assembled all the carotenoid biosynthetic enzymes and their mutants to a single, pACYC184-basee plasmid. By tweaking the relative expression level mainly by tuning translation efficiency of each individual genes, we established the pathways for various natural/non-natural carotenoids with decent yield and selectivity. In this talk, we report our current efforts to test mouse beta-carotene monooxygenase and the bacterial beta-carotene dioxygenase derived from non-culturable marine bacteria 6603 for their possible activity toward various carotenoids.

Yellow xanthophylls in shrimp belonging to *Penaeus*

Structures and Metabolism

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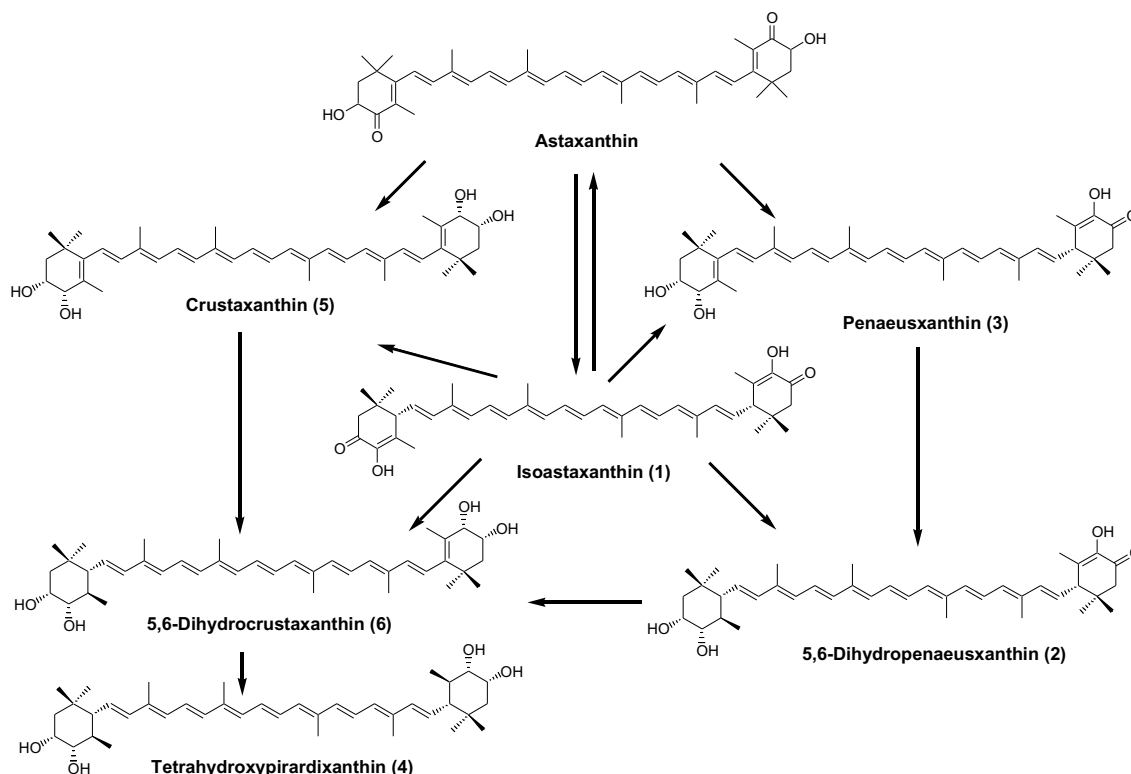
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In the previous symposium, we reported the presence of yellow xanthophylls, isoastaxanthin (1), 5,6-dihydropenaeusxanthin (2), penaeusxanthin (3), tetrahydroxypirardixanthin (4), crustaxanthin (5) as a metabolites astaxanthin in *Penaeus japonicus*. In the present symposium, we present the isolation and structural elucidation of a new carotenoid, 5,6-dihydrocrustaxanthin (6), from *Penaeus vannamei*.

Furthermore, distribution and metabolism of these yellow xanthophylls (1-6) in *Penaeus* are presented.



Carotenoids of Insects belong to Hemiptera, Perspective of their origin, metabolism, chemical ecology

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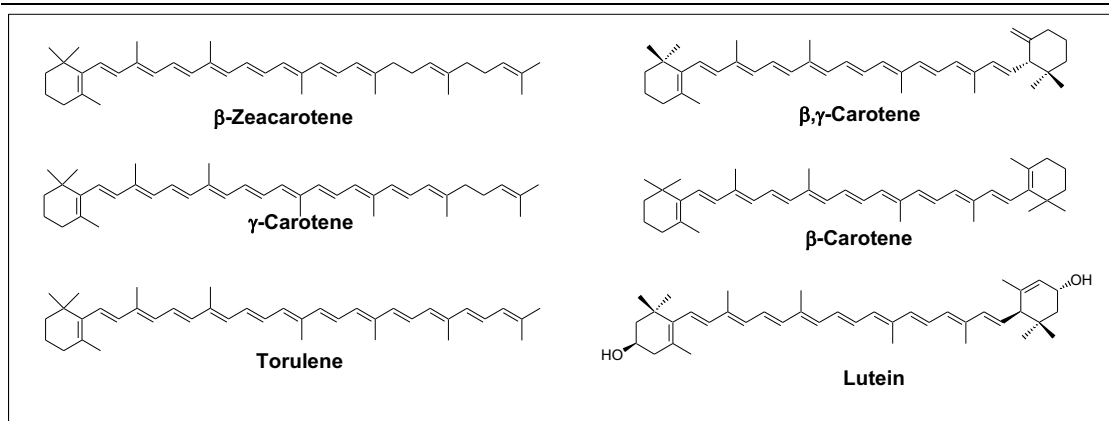
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Carotenoids of insects belonging to Hemiptera are investigated from the perspective of their origin, metabolism, and chemical ecology. These results are compiled in Table 1. This symposium, we present these results.

Table 1. Carotenoids of insects belonging to Hemiptera (Family of Aphidoidea, Heteroptera, Homoptera, and Cicadoidea).

Carotenoids	Aphidoidea	Heteroptera	Homoptera	Cicadoidea
β,β -carotene	++	++	++	—
β,γ -carotene	++	—	—	—
β -zeacarotene	+	—	—	—
β,ψ -carotene	+	—	—	—
γ,γ -carotene	+	—	—	—
γ,ω -carotene	++	—	—	—
β -cryptoxanthin	—	—	++	—
zeaxanthin	—	+	—	—
lutein	—	++	++	—



Carotenoid Fatty Acid Esters from Prokaryote:

Chloracidobacterium thermophilum

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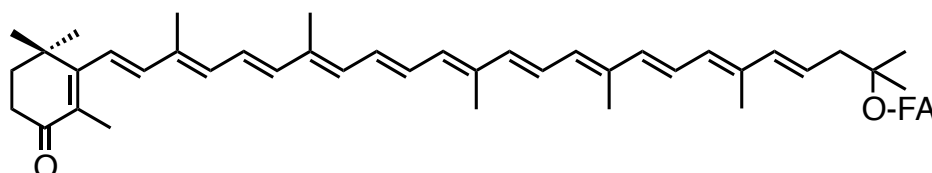
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Abstract:

Some carotenogenesis organisms can produce carotenoid derivatives. Carotenoid fatty acid esters have not been reported in Prokaryote. Recently, we found carotenoid fatty acid esters from *Chloracidobacterium thermophilum*, which is an aerobic phototroph and the seventh phylum of phototrophs.

It has been isolated from Yellow Stone National Park, USA. The major carotenoid was identified to be deoxyflexixanth ester and 1'-hydroxytorulene ester based on the retention time on HPLC, absorption spectrum in the HPLC eluent, LC-MS analysis, and saponification etc. Canthaxanthin and echinenone were also found as minor carotenoids. BChl *a* and BChl *c* were also found. Since the genome DNA sequences has been determined, we will functionally identify major carotenogenesis genes.

New species of *Chloracidobacterium validum*, which was isolated from a hot spring in Bulgaria (now preparing description paper), has almost the same carotenoids.



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This is an abstract of the 33th Annual Meeting on Carotenoid Research, on September 15-16, 2019 at Chiba University, Japan.

This will be published as a research article.

Dynamics of ICT State in Fucoxanthin Investigated by Multi-Pump Femtosecond Spectroscopy

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Abstract: We have applied multi-pump femtosecond spectroscopy to ultrafast dynamics in carotenoids. Fucoxanthin has intramolecular charge transfer (ICT) state additional to the S_2 and S_1 excited states. Initial dynamics of the S_1 /ICT equilibrium state have been investigated.

Carotenoids play important roles in photosynthesis. In the antenna complexes, they act as light-harvesting and photo-protecting pigments. Fucoxanthin has a dipole-allowed intra-molecular charge transfer (ICT) state in polar solvents additional to the S_2 and S_1 excited states. The ICT state makes an equilibrium state with the S_1 state (S_1 /ICT). Energy transfer from fucoxanthin to bacteriochlorophyll has been already reported in LH1 complex reconstituted with fucoxanthin, LH1(Fx) [1] and efficient energy transfer occurs from S_1 /ICT [2]. The initial dynamics of S_1 /ICT have two time constants, 350 fs and 3.8 ps [3]. However, the detailed dynamics of the equilibrium state has not been well-understood.

Figure 1 (a) shows absorbance change of fucoxanthin induced by 400 nm pump. A peak at 540 nm is assigned to S_1 and signal at longer than 600 nm is assigned to ICT. The spectra at 0.4 ps and 5.0 ps have similar structure, because fucoxanthin is in the S_1 /ICT equilibrium state. The lower figures show absorbance changes induced by 960 nm dump pulse which decreases population of ICT selectively. The dump at 0.4 ps (b) decreases both S_1 and ICT quickly. On the other hand, the dump at 5.0 ps (c) decreases S_1 a little later than ICT. It means that S_1 /ICT is in different condition at 0.4 ps and 5.0 ps after the pump.

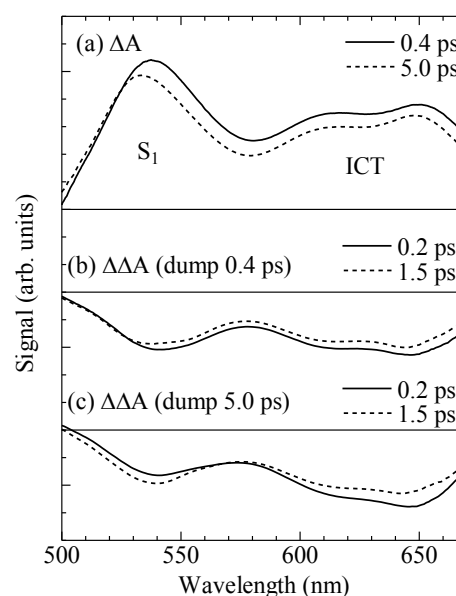


Fig. 1. Absorbance change of fucoxanthin in methanol (a) induced by pump, (b) induced by dump at 0.4 ps after pump, and (c) induced by dump at 5.0 ps.

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Carotenoid biosynthetic pathway of the filamentous anoxygenic phototrophic bacterium *Chloroflexus aurantiacus*

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Abstract:

Many carotenoid species are found among photosynthetic bacteria, and have functional roles of light-harvesting for photosynthesis and photo-protection against oxidative stress. Three groups of photosynthetic bacteria, filamentous anoxygenic phototrophic bacteria (FAPs), green sulfur bacteria, and chloracidobacteria, possess a large antenna system called chlorosome, which shows most efficient light-energy harvesting ability among phototrophic organisms. Although under aerobic condition, such an antenna also occurs oxidative stress, green sulfur bacteria and chloracidobacteria have a quenching system of the light-energy absorbed by chlorosome, for protection. While, FAPs does not possess this quenching system, and are considered that their carotenoids are important role to prevent the cells from photo-oxidative damage. However, the carotenoid biosynthesis of FAPs have not been studied in detail. Here, we focused on the functions of carotenoids in FAPs, and observed about the carotenoid biosynthesis of the FAP *Chloroflexus (Cfx.) aurantiacus*.

Firstly, the carotenoid compositions in this bacterium grown under phototrophic or aerobic conditions were compared. The phototrophic cells synthesized β -carotene, γ -carotene, and HO- γ -carotene glucoside as main pigments, while the aerobic cells changed them to keto-carotenoids, and possessed mainly canthaxanthin. Next, heterogenous expressions of carotenoid biosynthetic gene homologs found in the *Cfx. aurantiacus* genome, were performed in *E. coli*, indicating that the *crtE*, *crtB*, *crtI*, *crtY*, *cruF*, *cruC* and *cruD* were functional as well as carotenoid ketolase gene *crtO*. Semi-quantitative RT-PCR analysis of their genes transcripts with RNA isolated from *Cfx. aurantiacus* cells showed that the transcription of *crtO* especially was regulated and promoted under aerobic condition. We will discuss about carotenoids biosynthetic pathways of *Cfx. aurantiacus* and their alterations by growth condition.

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The construction of a strigolactone production system in cyanobacteria

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Abstract: Strigolactones (SLs), carotenoid-derived plant hormones, are known as germination stimulants of root parasitic weed *Striga* that is the most problematic on agricultural production in Africa and recognized to be highly effective in extermination *Striga* from field test. The amount of plant producing natural SLs is few and easy to inactivate, therefore there is an increasing demand to establish an efficient production system of SLs. SLs are synthesized from β -carotene by four enzymes: D27, CCD7, CCD8, and P450 homolog MAX1, which localize in mainly chloroplast in plant cells. Cyanobacteria, considering as an ancestor of chloroplast, contain high levels of β -carotene and is ideally suited to the function of chloroplast proteins. Here, we attempt to produce SLs using cyanobacterium *Synechococcus elongates* PCC 7942 (*S. 7942*). *D27*, *CCD7*, and *CCD8* genes were placed under the IPTG-dependent inducible promoter in the high copy plasmid and it was introduced into *S. 7942* cell. High levels production of carlactone (CL), an intermediate of SLs, was observed under the cultivation with IPTG. In addition, the levels of β -carotene in CL-producing strain was comparable to that in the wild type. Efficient CL and SL production using cyanobacteria will be discussed.

This is an abstract of the 33th Annual Meeting on Carotenoid Research, on September 15-16, 2019 at Chiba University, Japan. This will be published as a research article.

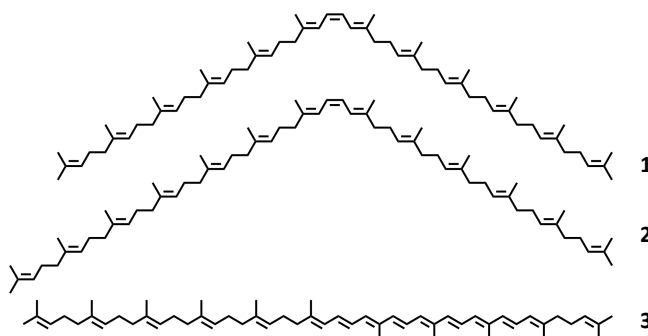
De novo construction of C60 carotenoid pathways

Takahiro Seki^{1*}, Takumi Ojima¹, Yusuke Otani¹, Takuya Hosoi¹, Shigeko Kawai-Noma¹, Li Ling¹, Takashi Maoka², Maiko Furubayashi³, Kyoichi Saito¹, and Daisuke Umeno¹

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Most of the natural carotenoids possess C₄₀ backbones, while some bacteria produce C₃₀ carotenoids. Several years ago, we have developed the artificial pathways for C₅₀ carotenoids via condensation of two molecules of C₂₅PP [1, 2]. Recently, we have additionally expressed C5-elongase to these pathways, resulting in the establishment of C₅₀ + C₅ + C₅ = C₆₀ carotenoids [3]. In this report, we present our latest efforts to construct another C60 carotenoids by using C₃₀PP or C₃₅PP as the building unites.

The size-shifted mutants of Staphylococcal carotenoid synthase CrtM were systematically mutated in their key residues consisting reaction pockets into Alanine. The resultant mutants were co-expressed with HexPS or HepPS, the C₃₀PP synthase and C₃₅PP Synthase in Escherichia coli, and the carotene fraction of each of the transformant cells was analyzed by HPLC-PDA-MS. Many of the CrtM variants exhibited detectable activity as C₃₀ + C₃₀ = C₆₀ synthase, and transformants accumulated C₆₀-phytoene (**1**), C₆₅-phytoene (**2**). *Pantoea* CrtI failed to desaturate them into pigments, but it could accept byproduct C₅₅-phytoene, resulting in the accumulation of novel carotenoids with 11 conjugated double bonds (**3**) [4].



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Transformation of *Freesia* × *hybrida* with astaxanthin biosynthesis genes and evaluation of generated transformants

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Abstract:

Freesia (*Freesia* × *hybrida*) is a member of the subfamily Ixioideae, the family Iridaceae, and is originated in the Cape Province in South Africa. It is a popular cut flower, because of flower forms, flower fragrance and wide petal color variations. In Ishikawa prefecture we have bred new varieties of freesia: 'Ishikawa fl ~ fl1' called 'Airy-Flora', which are distributed in market. In order to create novel flower color and other new characters, we tried to produce transgenic plants of 'Airy-Flora' by *Agrobacterium*-mediated transformation using astaxanthin biosynthesis genes.

In this study, we constructed a plasmid pUTR-crtZWI(pZH2B) containing a binary vector pZH2B (Kuroda *et al*, *Biosci Biotechnol Biochem* 74:2348-2351, 2010), in which three enzyme genes was inserted for β,β-carotenoid 3,3'-hydroxylase (CrtZ) and β,β-carotenoid 4,4'-ketolase (4,4'-oxygenase; CrtW) from a marine bacterium *Brevundimonas* sp. strain SD212, and isopentenyl diphosphate isomerase (type 2 Idi) from a marine bacterium *Paracoccus* sp. strain N81106, under the regulation of the CaMV 35S promoter and the transit peptide sequence of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) small subunit from pea. And the 5'-untranslated region (UTR) of the *Nicotiana tabacum* alcohol dehydrogenase gene (*NtADH*) was attached to the ATG of each gene, preceded with the transit peptide sequence.

The transformation method was a modification of that described (Uwagaki *et al*, *Plant Biotechnol* 32:165-168, 2015). The cormlets of freesia cultivar 'Ishikawa f6' were cut aseptically into ca. 5-mm cubes. The cubes were infected to *Agrobacterium tumefaciens* EHA101 harboring pUTR-crtZWI(pZH2B), and co-cultured in the dark at 23°C. After three days of co-cultivation, they were transferred onto MS basal medium supplemented with 5,6-dichloro-1H-indole-3-acetic acid, thidiazuron (TDZ), hygromycin, meropenem, 3% sucrose, and gellan gum (selection medium). The cultures were carried out at 22°C in the dark. After around 90 days of cultivation, generated hygromycin-resistant calli were transferred onto regeneration medium, which was selection medium without 5,6-dichloro-1H-indole-3-acetic acid. The cultures were carried out at 22°C under a 16-h light/8-h dark photoperiod. After around 30 days of cultivation, these were subsequently cultured on PGR-free MS medium containing hygromycin. Consequently, seven hygromycin-resistant plantlets were regenerated from the totals of 985 cormlets. Two types of plantlets were observed, *i.e.*, green lines (4 plantlets) and orange lines (3 plantlets). HPLC-PDA and HRMS analysis was used to determine carotenoid profiles of the wild-type and transgenic freesia leaves (one orange plantlet line). As a result, astaxanthin was detected in high ratio in addition to minor carotenoids such as 4-ketoantheraxanthin and fritschiellaxanthin in the transgenic orange-line leaf. We are awaiting their flowering.

Phytochemical genomics: from Arabidopsis to medicinal plants

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Plants produce a number of useful chemicals ranging from medicine, food additives, and industrially valuable chemicals. To efficiently and freely produce these materials, it is important to understand how this huge chemical diversity is accessed in limited evolutionary timescale.

To achieve this, it is important to establish novel platform, where functional genomics is tightly coupled with metabolomics. Years ago, our group has developed, for the first time, of such, and applied to the identification of important genes, metabolites, and metabolic networks of model organism *Arabidopsis thaliana*, then of various medicinal plants. In this lecture, I explain how this new field, so called phytochemical genomics, has been established, and how it is successfully applied to understand the nature of plant secondary metabolism, as well as to utilize the knowledges thus obtained. (Summarized by Japanese Society of Carotenoids)

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Necessity is the mother of extension? Polymeric adsorbents created using graft polymerization

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Ever since 1984, when I first met radiation-induced graft polymerization, I have been developing functional materials with this method. Based on the wide applicability and on the strong support/expectation from industrial sectors, this technology has been successfully applied to various purpose in variety of the field. In my >35 years of career as a specialist of graft polymerization, I have witnessed at least three major leaps of this technology. All of these leaps came out from our struggle to solve the tough and urgent real-world problems brought by companies. In this presentation, I would like to introduce the stories behind the development of the three following materials/systems.

1. The ultra-high-throughput, high-capacity antibody purification systems, Qyu-SPEED™ (with Asahi-Kasei Medicas)
2. Electrodialysis membrane tailored for salt-production (with AGC and Salt Industry Center of Japan)
3. High-capacity Cesium absorbent, GAGA, developed for decontamination of Fukushima Daiichi Nuclear Power Station (with Kankyo Joka Kenkyusho)

(Summarized by Japanese Society of Carotenoids)

Reference: K. Saito, K. Fujiwara, T. Sugo, Innovative Polymeric Adsorbents, Springer Singapore (2018).

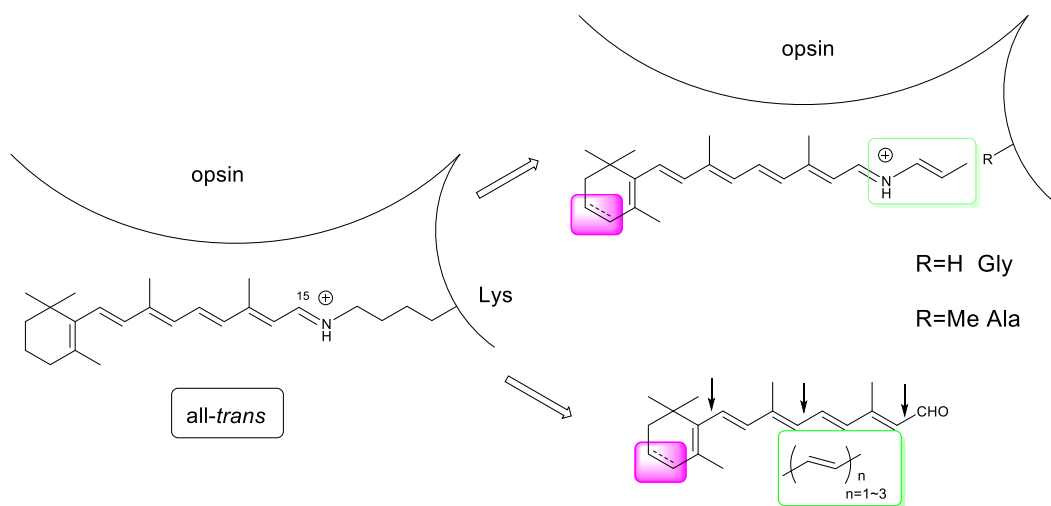
Development of Red-Shifted Channelrhodopsin Using Long Conjugated Retinal Analog as Chromophore

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Abstract

Optogenetics is a new technology to control neural activity by light using channelrhodopsins (ChRs), and has become a powerful tool to clarify the neural circuits and the mechanism of various neural diseases.¹ ChRs now used in optogenetics are mostly sensitive to blue-green light (430-550 nm) and have several limitations, therefore, the development of new red-shifted ChRs, which are sensitive to the long-wavelength light, are eagerly sought.² We prepared new chromophores having long conjugated polyene system than retinal (A1) and 3,4-didehydroretinal (A2) by two methods. One method was construction of conjugated double bond in the enamine side of the Schiff base part, another method was an elongation of conjugation with one double bond inserted into the polyene side chain of A1 and A2. In the binding assay of newly produced chromophores, all analogs bound with opsins (mutated-C1C2, ReaChR and ChrimsonR) to afford the new red-shifted ChRs.



¹ a) Deisseroth, K., Feng, G., Majewska A.K., Miesenböck G., Ting A., Schnitzer M.J., *J. Neurosci.*, **2006**, 26,10380. b) Yizhar, O., Fenno, L. E., Davidson, T. J., Mogri, M., Deisseroth, K. *Neuron*, **2011**, 71, 9.

² Lin J. Y., Knutsen P. M., Muller A., Kleinfeld D., Tsien R. Y., *Nat. Neurosci.*, **16**, 1499-1508 (2013).



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CAROTENOID SCIENCE

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Types of contributions

Original research papers, letters to the editor, and review papers are welcomed. All of them should contain an Abstract (of up to 200 words) and a Conclusions section, which, particularly in the case of theoretical papers, translates the results into terms readily accessible to most readers. All the submitted manuscripts are subjected to critical reading by the reviewers who are authorized in the relevant field of the work. It is to be noted that the authors(s) cannot insist the priority of the work published in Carotenoid Science.

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2. Manuscripts should be typed on A4 size (21.0 cm × 29.7 cm) papers.
3. Times or Times New Roman font should be used for the text of the manuscripts.
4. In principle, there is no limitation for the length of manuscripts. However, it is recommended to obey the following page limit: up to 10 pages for the research papers, up to 5 pages for the letters to the editor, and up to 20 pages for the review papers.
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All mathematical symbols representing physical quantities should be typed in italic.

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- (iv) One line space should separate the end of a previous section or subsection with the next subsection or section's title.
- (v) Sections and subsections should not normally begin on a new page.
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- (vii) References in the text should be cited with square brackets ([1], [2]). Two references in one bracket should be cited with a comma in between ([1, 2]), and more than two references should be cited with a hyphen in between ([1–3]).
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