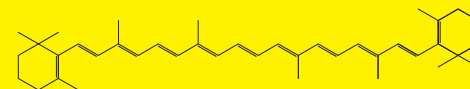


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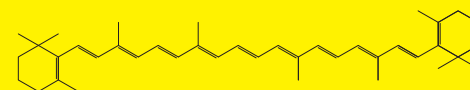
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Annual Meeting on*

Carotenoid Research

*Held in Tokyo, Japan
24-25 November 2023*



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CAROTENOID SCIENCE

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KIWASEI GAKUIN UNIVERSITY

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EDITOR

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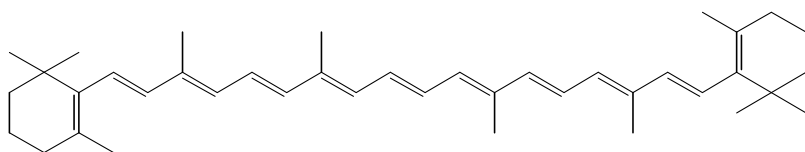
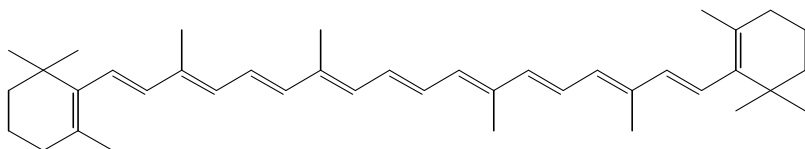
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Japan Carotenoid Society

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Japan Carotenoid Society

Preface for the 35th Annual Meeting

The 35th Annual Meeting of the Japanese Carotenoid Society took place at the Mejiro Campus of Japan Women's University in Tokyo on November 24 – 25, 2023 co-hosted with the 17th Annual Meeting of the Japanese Society for Astaxanthin. This year's scientific program was a testament to the thriving field of carotenoid research, featuring five captivating invited lectures by distinguished speakers: Dr. Yasuhiro Nishida (Fuji Chemical Industries), Prof. Tatsuya Sugawara (Kyoto University), Prof. Ritsuko Fujii (Osaka Metropolitan University), Dr. Ryuju Suzuki (National Institute of Technology, Sendai College), and Dr. Chisato Tode (Kobe pharmaceutical University). The event also featured 25 stimulating oral presentations, bringing together a total of 65 participants.

On November 26, we hosted the Annual Meeting of Young Carotenoid Scientists, providing attendees with invaluable insights. This post-meeting program featured two enlightening tutorial lectures by Dr. Ayako Osawa (Kanagawa Institute of Technology) and Dr. Masaki Honda (Meijo University). These lectures were moderated by Dr. Takashi Maoka (Research Institute for Production Development) and Prof. Kazutoshi Shindo (Japan Women's University), bringing together a total of 32 participants.

The presentations encompassed a diverse spectrum of research areas related to carotenoids and apocarotenoids, including physics, physical chemistry, synthetic and analytical chemistry, molecular biology, genetics, omics, pathway and metabolic engineering, as well as the nutritional and medical aspects of carotenoids. Collectively, these presentations vividly showcased the state of the art in carotenoid research, underscoring the remarkable growth and high standards upheld by Japanese carotenoid scientists.

I would like to extend my sincere gratitude to all the participants for their invaluable contributions, insightful comments, and for fostering a congenial atmosphere throughout the meeting. Additionally, I would like to express our deep appreciation to our generous sponsors:

- **Japan Business Association for Carotenoids (JBAC)**
- **San-Ei Gen F.F.I. Co., Ltd.**
- **Gulico Nutrition Co., Ltd.**
- **Fuji Chemical Industries Co., Ltd.**
- **Alnur Co., Ltd.**
- **Sun Blight Co., Ltd.**
- **Kemin Industries, Inc.**
- **Eneos Techno Materials Corporation**

Over the years, this meeting has played a pivotal role in promoting cross-disciplinary collaboration among Japanese carotenoid researchers from both academic and industrial backgrounds. Let us ensure that this tradition continues to thrive! During the COVID-19 pandemic, remote conference participation became commonplace. Therefore, we offered a hybrid format, combining in-person and Zoom participation, to accommodate the diverse needs of our members, just like last year.

We eagerly anticipate your participation in the 36th Annual Meeting of the Japanese Carotenoid Society, scheduled to be held at Hirosaki University on September 14 – 15, 2024.

Sincerely,

Kazutoshi Shindo

Professor, Japan Women's University

Meeting Chair of the 35th Annual Meeting of the Japanese Carotenoid Society

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The potential of marine carotenoids as functional food ingredients: Benefits in the prevention of skin aging and inflammation

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Abstract: As potentially renewable resources, marine organisms contain a variety of nutrients and bioactive substances that have beneficial effects on human health. Carotenoids found in marine organisms, such as astaxanthin, fucoxanthin and siphonaxanthin, have unique structures not found in land organisms. Our recent study indicated that dietary marine carotenoids can accumulate in the dermis and epidermis and appear to prevent intrinsic and extrinsic aging of the skin. Marine carotenoids are expected to have unique structure-dependent biological activities compared to terrestrial ones. Multivariate analysis of anti-inflammatory activity revealed that the C8-keto structure of carotenoids such as fucoxanthin and siphonaxanthin is important for their inhibitory effects. In addition, absorption of dietary carotenoids has also recently been shown to involve several transporters expressed in small intestinal epithelial cells. The recent findings open new avenues for future research on the utilization of marine carotenoids as functional food ingredients. Further investigation, especially clinical studies, are needed to validate the reported findings.

1. Introduction

Carotenoids are lipophilic pigments that are widely distributed in nature and have a yellow to red color. They are biosynthesized *de novo* by photosynthetic autotrophs. In general, animals cannot biosynthesize carotenoids, but they can partly convert the chemical structure of carotenoids, especially the functional groups, after oral intake. Therefore, a wide variety of carotenoids exist in nature and there is no doubt that they are consumed daily as general food ingredients. In particular, carotenoids such as β -carotene, lycopene and lutein, which are found in vegetables and fruits, have antioxidant and anti-inflammatory properties and are attracting attention for their potential health benefits against many diseases, including cardiovascular disease, age-related macular degeneration, and some types of cancer [1]. In addition, a wide variety of other carotenoids have unique biological activities depending on their characteristic structures. For highly utilization of

natural carotenoids in foodstuff, we have been studying their potential as functional food ingredients, paying particular attention to their unique structures. The purpose of this review is to discuss the intestinal absorption and functions of dietary marine carotenoids, focusing on the benefits in the prevention of skin aging and inflammation.

2. Unique structures of carotenoids in marine organisms as food ingredients

Marine organisms are particularly favored for their environmental sustainability as a potential renewable resource. They contain a variety of nutrients and bioactive substances that have beneficial effects on human health including carotenoids, dietary fibers, amino acids, essential fatty acids, vitamins, and minerals. Marine algal and animal carotenoids have particularly unique structures not found in terrestrial ones.

Various algal carotenoids, including astaxanthin, fucoxanthin and siphonaxanthin, have recently been candidates as nutraceuticals and pharmaceuticals

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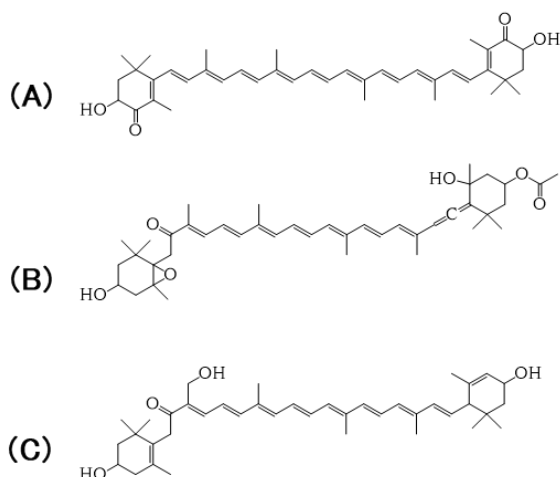


Fig. 1. Structures of astaxanthin (A), fucoxanthin (B) and siphonaxanthin (C).

(Fig.1). Astaxanthin is mainly found in seafood such as salmon, shrimp, crab and fish roe. The green microalgae *Hematococcus pluvialis* stores high concentrations of astaxanthin under several stress conditions and is a major source of astaxanthin as a food and cosmetic ingredient. It is well known to have higher antioxidant activity than that of α -tocopherol and other carotenoids including β -carotene and possesses many highly potent pharmacological effects, including anti-diabetic, anti-atherosclerotic and anti-inflammatory activities [2-5]. One of the major algal carotenoids is fucoxanthin, found in brown macroalgae, such as *Undaria pinnatifida* and *Laminaria japonica*. Its chemical structure consists of a specific allenic bond and a 5,6-monoepoxide. In recent years, many reports have been published on the beneficial effects of fucoxanthin on health, including its anticancer, antioxidant, and anti-obesity effects [6]. We previously found that fucoxanthin and its deacetylated product, fucoxanthinol, effectively suppress angiogenesis using *in vitro* and *ex vivo* assays [7,8]. In addition, siphonaxanthin is a specific keto-carotenoid of siphonaceous green algae, which is considered to help in absorbing green and blue-green light available in water. Siphonaxanthin possesses an additional hydroxyl group on the 19th carbon atom in its structure and is found in several green algae such as *Codium fragile* and *Caulerpa lentillifera* which are consumed as part of the Japanese diet. We have originally focused on the potential of siphonaxanthin as a bioactive compound and attempted to clarify its novel bioactivities [9].

Marine animals accumulate various carotenoids as dietary metabolites. Most of the carotenoids in bivalves involve alloxanthin, fucoxanthin, peridinin, and diatoxanthin, which are derived from microalgae as dietary sources, and their metabolites [10]. Halocynthiaxanthin was isolated from ascidian, *Halocynthia roretzi*, as a metabolite of fucoxanthin. Echinenone is a well-known major carotenoid in sea urchins.

3. Effect on the skin feature and function

The skin plays an important role in maintaining homeostasis of the mammals by insulating the body from the external environment. As the outermost and first layer, the skin can protect the body from environmental stimuli, prevents water loss, and regulates body temperature. The structure of the skin is composed of multiple cells, including keratinocytes, melanocytes, and Langerhans cells in the epidermis and fibroblasts in the dermis. Since skin is a tissue that changes significantly with age, it naturally contributes to the appearance of wrinkles, spots, and other signs of aging. Skin aging is classified into intrinsic aging and extrinsic aging (photoaging) [11].

Skin photoaging is the result of prolonged and repeated exposure to ultraviolet (UV) radiation and is clinically characterized by deep wrinkling, a dry, loose appearance, and seborrheic keratosis. The wavelength of UV radiation is 200-400 nm and can be further divided into three sections: UVA (320-400 nm), UVB (280-320 nm), and UVC (200-280 nm). UVA, which accounts for about 95% of terrestrial ultraviolet radiation from sunlight, reaches the dermis through the epidermis and damages collagen and elastin in the dermis. It has been reported that astaxanthin can prevent UVA-induced DNA damage in cultured human skin fibroblasts. To evaluate the effect of dietary astaxanthin derived from *H. pluvialis* on skin photoaging, we assessed various parameters in UVA-irradiated hairless mice [12]. As the results, we found that dietary astaxanthin can accumulate in the dermis and epidermis and appears to prevent the photoaging caused by UVA irradiation. Analysis of gene expression revealed that dietary astaxanthin exerted anti-photoaging effects, possibly by modulating natural moisturizing factor (NMF) synthesis and desquamation

in the epidermis, and by inhibiting the UVA-induced degradation of collagen fibers in the dermis. In subsequent studies, we have confirmed similar effects for fucoxanthin from microalgae and siphonaxanthin from green algae at lower doses (*submitted*). Our data indicate the potential application of dietary marine carotenoids as functional food ingredients for skin care against photoaging. There is strong interest in the relationship between the chemical structure of carotenoids involving the metabolites and effective dosage.

Intrinsic skin aging is a chronological decline in the texture and function influenced primarily by genetic factors and characterized by wrinkles, dryness, roughness, and loss of luster, along with dysfunctional changes in the skin barrier. In the epidermis, aging weakens the proliferative capacity of basal cells and thins the entire epidermis. In contrast, the stratum corneum thickens due to impaired desquamation process, resulting in delayed epidermal metabolism. In the dermis, the number and biosynthetic capacity of fibroblasts decreases with age, as does collagen content. Since the deterioration of skin function due to aging is physiologically inevitable, one important strategy may be to prevent aging and slow its progression through habits and diet in daily life. In this context, we evaluated the effects of dietary astaxanthin on intrinsic skin aging in aged mice. As a result, dietary supplementation of astaxanthin significantly alleviated skin viscoelasticity defects and reduced wrinkle formation caused by aging (*submitted*). Further analysis revealed that dietary astaxanthin can mediate genes related to skin cell proliferation and differentiation, degradation of extracellular matrix and dermal-epidermal junction proteins, synthesis of natural moisturizing factors, and maintenance of skin barrier function. Interestingly, dietary astaxanthin markedly attenuated the decrease in the expression level of collagen XVII A1 (COL17A1) in the aged skin of mice. COL17A1 plays a central role in the regulation of epidermal proliferation and is an excellent marker for epidermal stem cells, reflecting the potential and quality for self-renewal and skin homeostasis. Although effective dosage and the effects of other carotenoids need to be investigated, our study shows that dietary astaxanthin has protective effects on intrinsically aged skin.

4. Anti-inflammatory effects

Mast cells play a pivotal role in allergic reactions and inflammation by secreting biologically active substances such as histamine, eicosanoids, and cytokines. In the case of type I allergy, antigen-induced mast cell degranulation induced by antigen leads to local inflammation, therefore, natural compounds that can attenuate this degranulation are attracting attention. We previously found that astaxanthin and fucoxanthin, in addition to β -carotene and zeaxanthin, significantly inhibited antigen-induced mast cell degranulation due to suppressing the aggregation of Fc ϵ RI followed by intracellular signaling [13]. Furthermore, in a mouse model of dinitrofluorobenzene-induced contact hypersensitivity, ingestion of these carotenoids significantly suppressed ear swelling and significantly reduced TNF- α and histamine levels, suggesting that these carotenoids, including astaxanthin and fucoxanthin, exert their anti-inflammatory effects by suppressing mast cell degranulation *in vivo* [14].

In addition, chronic inflammation is one of the important pathophysiological factors in many diseases including atherosclerosis, diabetes and hypertension. Inflammation is generally characterized by the excess release of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6), as well as inflammatory mediators, including nitric oxide (NO) and prostaglandin E₂ (PGE₂) in immune cells, such as macrophages. There are many reports on the anti-inflammatory effect of astaxanthin, indicating a reduction of inflammatory cytokines and mediators [3-5]. Especially, astaxanthin decreases the expression of pro-inflammatory mediators such as PGE₂, TNF- α , and IL-1 via suppression of I κ B-dependent NF- κ B activation [15]. In an animal model, dietary astaxanthin significantly inhibited the occurrence of colonic mucosal ulcers, dysplastic crypts and colonic adenocarcinoma via modulation of inflammatory factors such as NF- κ B, TNF- α , IL-1 β , IL-6 and COX-2 [16]. In a clinical study, the possible immunostimulatory, anti-oxidative, and anti-inflammatory activity of dietary astaxanthin in healthy adult was found [17]. On the other hand, fucoxanthin also inhibited the inducible nitric oxide synthase (iNOS) and COX-2 protein expressions and reduced the levels of NO, PGE₂, TNF- α , IL-1 β , and IL-6 via suppression of NF- κ B activation and the phosphorylation of mitogen-activated protein kinases in

macrophages stimulated by LPS [18]. Alloxanthin and diatoxanthin isolated from *Halocynthia roretzi* significantly suppressed the expression of IL-1 β and IL-6 mRNA in cultured macrophages [19].

As mentioned above, the anti-inflammatory activity of carotenoids, especially against lipopolysaccharide-induced inflammatory responses, has been demonstrated in numerous studies. In addition to LPS, multiple inflammatory stimuli and their respective receptors are involved in the biological response to external attack. Therefore, we comprehensively investigated the anti-inflammatory effects of carotenoids with various chemical structures by using multiple inflammatory inducers (pathogen-associated molecular patterns, proinflammatory cytokines, degenerated proteins, and chemical irritants) and focusing on the signaling pathways involved in the immune response. We conducted a multivariate analysis to clarify the differences among eight carotenoids including astaxanthin, fucoxanthin and siphonaxanthin [20]. Our results indicated that C8-keto structures of carotenoids (siphonaxanthin and fucoxanthin) were might be crucial for their inhibitory effects on the activation of interferon regulatory factor.

5. Digestion, absorption and metabolism

To elucidate the potential of carotenoids as food functional components, it is generally important to understanding the mechanism of absorption and the bioactivity of metabolites. Passive diffusion has been thought to be important for carotenoid absorption, but a facilitative absorption mechanism mediated by the transporters such as scavenger receptor class B type 1 (SR-B1) and Niemann-Pick C1-like 1 (NPC1L1) has recently been demonstrated [21]. In the case of siphonaxanthin, NPC1L1 facilitates its transport across the intestinal epithelium, but SR-B1 does not [22]. In mice, three possible dehydrogenated metabolites of siphonaxanthin were detected after oral administration [23]. We have been studying intensively to elucidate the biological activity of siphonaxanthin metabolites and the enzymes involved in their metabolism. On the other hand, the involvement of transporters in the absorption of fucoxanthin is unknown, but it has been shown to be hydrolyzed to fucoxanthinol in the gastrointestinal tract and converted to amarouciaxanthin A in the body, which is stored mainly in adipose tissue [6,24,25].

Since the bioavailability of carotenoids from ingested foods is low, promoting their absorption is also expected to help carotenoids fulfill their biological functions. We found that fermented milk can enhance dietary carotenoids (β -carotene, lycopene and lutein) bioavailability in humans [26]. Exopolysaccharides (EPS) contained in fermented milk may enhance absorption of dietary carotenoids by simple diffusion mechanisms. It would be interesting if it could also be shown to enhance absorption of marine carotenoids such as fucoxanthin and siphonaxanthin.

6. Conclusions and future perspectives

Marine organisms, plants in particular, are the source of novel bioactive molecules for human health. Marine carotenoids have a unique structure compared to terrestrial ones and are expected to have biological activities. The recent findings open new avenues for future research on the advanced use of marine carotenoids as bioactive substances, but further investigation, especially clinical studies, are needed.

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My Chemical and Biochemical Studies of Natural Carotenoids Over 45 Years

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Abstract : Since 1978, I have dedicated myself to the chemical, biochemical, and analytical research of natural carotenoids. Over the years, I have developed advanced methods for the isolation and structural elucidation of these compounds, utilizing techniques such as HPLC, MS, and NMR analysis. My work has led to the identification of more than two hundred new carotenoids. Additionally, I have explored the functions of carotenoids in both animals and plants from chemical, biochemical, and ecological perspectives. In this review, I will present an overview of my carotenoid research conducted over the past 45 years.

Key Words: Natural carotenoids, History of my research, HPLC, NMR, MS, Chemical studies, Biochemical studies, Ecological studies

1. History of my carotenoid research

In 1978, I embarked on carotenoid research under the guidance of Professor Takao Matsuno in the Department of Natural Product Chemistry at Kyoto Pharmaceutical University. For my graduation research in 1979, I focused on the isolation and identification of β,γ -carotene from the red dragonfly, *Sympetrum frequens*. Using Al_2O_3 column chromatography, I successfully isolated approximately 0.5 mg of β,γ -carotene from 2,000 specimens of the red dragonfly.

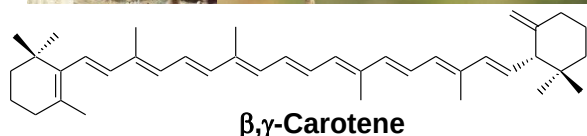


Fig. 1. Isolation of β,γ -carotene from the red dragonfly involved using Al_2O_3 column chromatography.

The identification of this carotenoid was confirmed by comparing the UV-VIS spectral data and TLC with semisynthetic β,γ -carotene, which was derived from β -carotene through chemical reactions (**Fig. 1**).

Later, this structure was confirmed by MS, CD, and ^1H -NMR spectral data. However, the origin of β,γ -carotene in the red dragonfly could not be explained by the metabolism of carotenoids in insects at that time. Subsequently, my quest to unravel the origin of β,γ -carotene began over 40 years ago [1, 2].

In my master's thesis in 1981, I focused on the structural determination of pectenol (7',8'-didehydro- β,β -carotene-3,4,3'-triol), which was isolated from marine shellfish. The structure of pectenol was elucidated through UV-VIS, IR, ^1H -NMR (80MHz), CD, EI-MS spectral data, and chemical reactions [3]. Pectenol marked the first carotenoid whose structure I determined (**Fig. 2**).

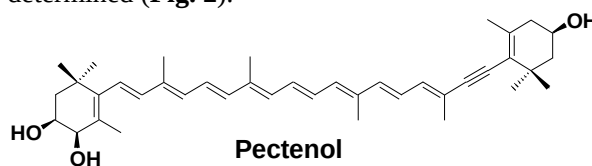


Fig. 2. Chemical structure of pectenol.

Afterward, I became a research associate at Kyoto Pharmaceutical University, where I was assigned to the Department of Natural Product Chemistry and continued my research on carotenoids. During this period, I developed separation methods for optical isomers of carotenoids using chiral phase HPLC (**Fig. 3**) [4], and successfully isolated several new stereoisomers of lutein, zeaxanthin, and others from marine animals [5].

In 1990, I earned my Ph.D. degree from Kyoto Pharmaceutical University with a thesis titled "Separation of Stereoisomers of Natural Carotenoids."

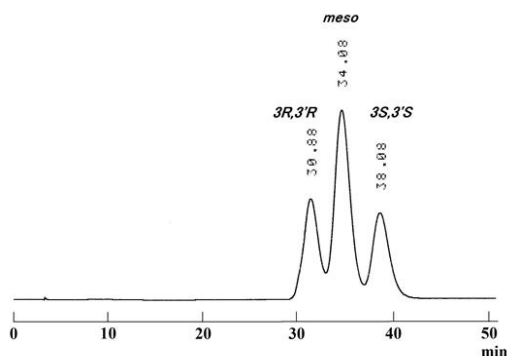


Fig. 3. The separation of three stereoisomers of astaxanthin was achieved using chiral phase HPLC.

I was instrumental in establishing the Japanese Society of Carotenoid Research. The inaugural symposium of the society took place in Kyoto in 1987. In May 1990, I participated in the 9th International Symposium on Carotenoids held at the Hieizan Hotel in Kyoto, organized by Prof. Matsuno and Prof. Tsukida. At this symposium, I contributed as an assistant and presented my research through poster sessions.

Since 1993, I have been employed at the Research Institute for Production Development (**Fig. 4**). Established in 1947 as a general incorporated foundation in Shimogamo, Sakyo-ku, Kyoto, this institute has been my professional home.

I established the Laboratory of Food Function and Chemistry (**Fig. 5**) within this institute and introduced advanced analytical instruments such as a 500MHz NMR, LC/MS, UV-VIS, CD, IR, and preparative HPLC systems to support the Bio-oriented Technology Research Advancement Institution. At the institute, my research has encompassed both fundamental carotenoid science and the application of these findings to industry. I collaborate extensively with researchers from universities, national research centers, and industries. In 2017, I was appointed as the director of the institute.



Fig. 4. The Research Institute for Production Development (Seisan Kaihatsu Kenkyujo) is an organization established in 1947 as a general incorporated foundation located in Shimogamo, Sakyo-ku, Kyoto.

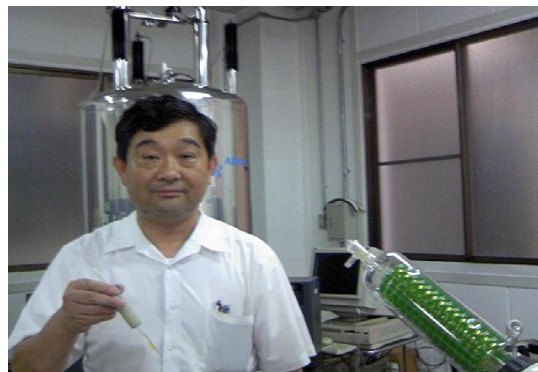


Fig. 5. My research laboratory located within the Research Institute for Production Development.

I have received awards from the Japanese Society for Astaxanthin in 2008 and the Japanese Society of Carotenoid Research in 2011. In 2017, I was honored as a fellow of the International Carotenoid Society. I have participated in the 15th (June 2008, Okinawa) and 19th (July 2023, Toyama) International Symposium on Carotenoids as an invited speaker and member of the local organizing committee.

In 2023, I was recognized as one of the top 2% most influential scientists in the analysis spanning from 1960 to 2022 by Stanford University [6].

2. Development of isolation and structural elucidation methods for carotenoid research

I have developed methods for the isolation and structural elucidation of natural carotenoids. Purification is a crucial step in the structural determination of carotenoids present in very small amounts. To achieve this, I integrated normal phase, reverse phase, and gel filtration HPLC techniques [7].

NMR spectroscopy is the primary tool for structural elucidation. However, in 1990, approximately 1 mg and 5 mg of sample were typically required for ^1H - and ^{13}C -NMR measurements, respectively. Therefore, I pioneered microscale NMR analysis for carotenoids, considering the limited amounts obtained from natural sources. Microscale analysis has been essential in advancing the study of natural carotenoids. For example, **Fig. 6** depicts the ^{13}C -NMR spectrum of a new peridinin derivative isolated from oysters, acquired using the Nano ProbeTM with overnight acquisition of a sample as small as 500 μg .

In 1995, Dr. Englert described experimental NMR techniques for the structural analysis of carotenoids and compiled 174 types of ^1H -NMR and 108 types of ^{13}C -NMR data on carotenoid end groups in Carotenoid Vol. 1B (1995). In 2016, I expanded upon Dr. Englert's work by introducing new NMR experimental techniques such as COSY,

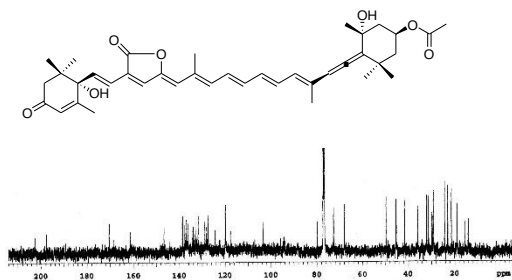


Fig. 6. ^{13}C -NMR analysis (in CDCl_3) of 500 μg of a new peridinin derivative, a carotenoid from oysters, was conducted using the NanoProbeTM.

NOESY, HSQC, and HMBC for carotenoid structural analysis. Additionally, I developed a method for determining the absolute configuration of hydroxyl groups in carotenoids using a modified Mosher method [7]. I also compiled data on 44 types of ^1H -NMR and 28 types of ^{13}C -NMR data for new carotenoid end groups [7].

In collaboration with the Japanese Society of Mass Spectrometry, I compiled electron ionization (EI), fast atom bombardment (FAB), electrospray ionization (ESI), and MS/MS spectra data for over 100 carotenoids, which were published in the society's database [8, 9].

3. Structure determination of carotenoids

Since the initial structural determination of the carotenoid pectenol in 1981, I have identified 167 different natural carotenoids from various sources [10, 11]. The 167th carotenoid I identified is roretziaxanthin, distinguished by its unique 2,6-cyclo- ψ -end group, isolated from the sea squirt *Halocynthia roretzi* [12].

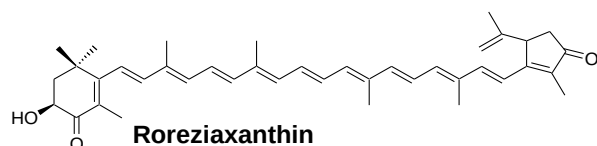


Fig. 7. Chemical structure of roretziaxanthin.

Additionally, I determined the structures of 22 types of reaction products of carotenoids with reactive oxygen species, as well as 13 types of carotenoids modified through pathway engineering [10, 11].

4. Animal carotenoids from food chain and metabolic studies

I have studied animal carotenoids from chemical, biochemical, and ecological perspectives [13, 14]. Generally, animals do not synthesize carotenoids *de novo*; instead, they acquire them directly from food or modify them through metabolic reactions. Carotenoids such as fucoxanthin and peridinin possess various functional groups such as allene bonds, epoxides, and

carbonyl groups. Consequently, several metabolites of these carotenoids are found in various marine animals, which I have identified [13, 14].

I conducted investigations on carotenoids in the sea angel *Clione limacine* from an ecological standpoint. *C. limacine*, a small floating sea slug inhabiting under drift-ice in the Sea of Okhotsk, is exposed to intense sunlight. Its transparent, gelatinous body exhibits bright orange-red gonads and viscera due to carotenoids like pectenolone. As a carnivore, *C. limacine* ingests diatoxanthin through its food chain and metabolically converts it into pectenolone, introducing a carbonyl group that changes the carotenoid's color from yellow to red. This modification enhances its antioxidative and photoprotective properties, leading to pectenolone accumulation in the gonads [15].

Additionally, I explored the biological function of the red carotenoid mytiloxanthin, a metabolite of fucoxanthin found in shellfish and tunicates. Through metabolic conversion from fucoxanthin, mytiloxanthin exhibits significantly increased antioxidative activities comparable to astaxanthin. This suggests that marine animals metabolize dietary carotenoids into more active antioxidative forms and accumulate them in their bodies and reproductive organs [16].

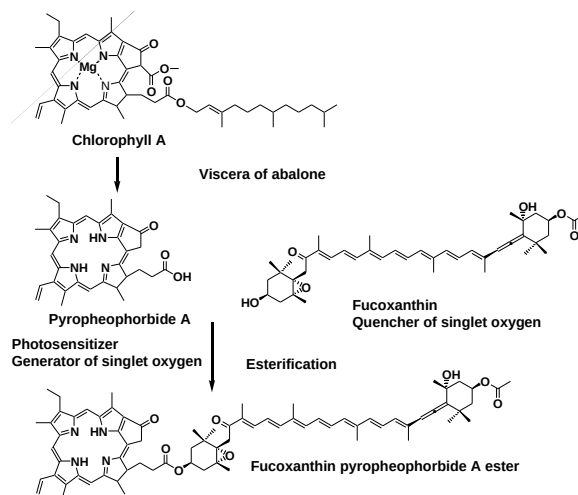


Fig. 8. Chemical structure of fucoxanthin pyropheophorbide A ester and its putative biosynthetic pathway.

During my studies on carotenoids in marine animals, I discovered several interesting carotenoid structures. One notable compound is a novel fucoxanthin pyropheophorbide A ester, which I isolated from the viscera of the abalone *Haliotis diversicolor aquatilis* (**Fig. 8**). The abalone primarily feeds on brown algae, where fucoxanthin is the major carotenoid. Pyropheophorbide A, a metabolite of chlorophyll *a* found in the abalone's viscera, acts as a singlet oxygen generator. Conversely, fucoxanthin is an excellent

quencher of singlet oxygen. It is intriguing that these compounds, which function as singlet oxygen generators and quenchers, are linked by ester bonds. As a result, fucoxanthin pyropheophorbide A ester exhibits weaker singlet oxygen generation compared to pyropheophorbide A alone [17].

The origin of β,γ -carotene in red dragonflies was uncovered in 2021 through collaboration with ecological, biochemical, and genetic research groups at Ishikawa Prefectural University. Our ecological observations revealed that red dragonflies accumulate β,γ -carotene from aphids, their primary diet. Generally, insects cannot synthesize carotenoids *de novo*. However, aphids can produce carotenoids with a γ -end group, such as β,γ -carotene and γ,γ -carotene, due to carotenoid synthesis genes horizontally transferred from fungi. Prof. Norihiko Misawa's group elucidated this carotenoid biosynthetic pathway in aphids using gene technology. They successfully produced carotenoids with the γ -end group in *Escherichia coli* by introducing carotenoid synthetic genes from aphids [2].

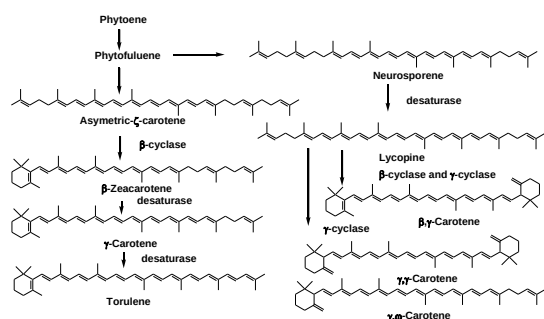


Fig. 9. The origin of β,γ -carotene in red dragonflies and the biosynthetic pathway of carotenoids in aphids

5. Plants carotenoids, structure and these functions

I also discovered several intriguing carotenoid structures in plants. *Pittosporum tobira* is a shrub-like evergreen tree that grows in the southern regions of Japan. In summer, its seeds are covered with a capsule and appear yellow. In autumn, the seeds are exposed to sunlight and change color from yellow to red. The major carotenoid in the yellow seeds is violaxanthin. In contrast, the primary carotenoid in the red seeds is a red seco-carotenoid named tobiraxanthin. During the autumn season, violaxanthin undergoes oxidative cleavage of its epoxide bonds, converting into tobiraxanthin. This bioconversion results in an eleven-

conjugated double bond system, which imparts the red color (**Fig. 10**). Tobiraxanthin exhibits a significantly stronger singlet oxygen quenching activity induced by sunlight compared to violaxanthin. Additionally, the red color of the seeds acts as an attractant for birds, facilitating seed dispersal (**Fig. 10**) [18].

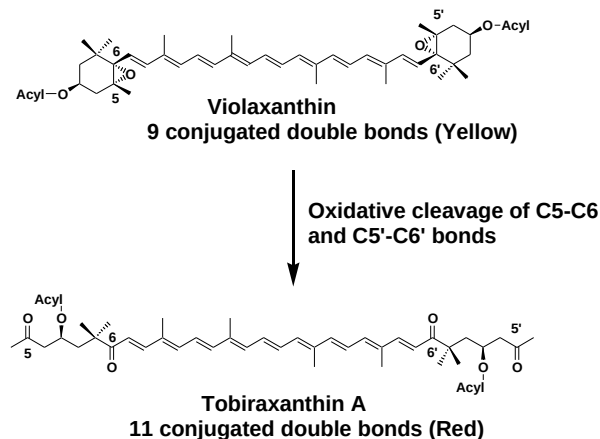


Fig. 10. The formation mechanism of tobiraxanthin A from violaxanthin in the seed of *Pittosporum tobira*.

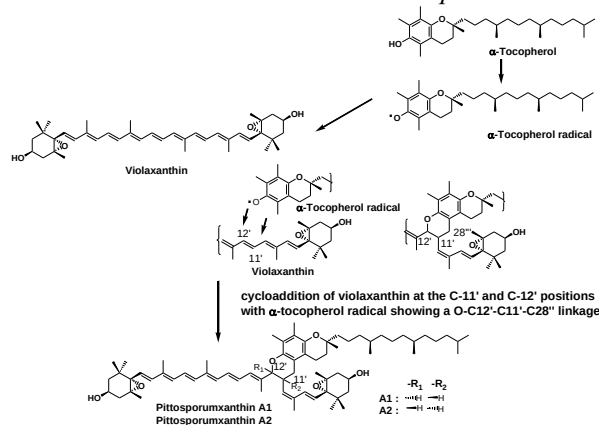


Fig. 11. The formation mechanism of pittosporumxanthins from violaxanthin in the seed of *Pittosporum tobira*.

I first isolated novel carotenoid and α -tocopherol complexes, named pittosporumxanthins, from the red-colored seeds of *Pittosporum tobira*. Pittosporumxanthin A1 and A2 are diastereomeric pairs formed by the cycloaddition of violaxanthin at the 11' and 12' positions with α -tocopherol. The α -tocopherol radical is generated from α -tocopherol by quenching or scavenging reactive oxygen species in the seed. This radical can become a pro-oxidant, causing oxidative damage. To mitigate this, carotenoids capture α -tocopherol radicals, forming adduct products (**Fig. 11**). This process suggests that carotenoids can quench phenoxy radicals by reacting with their polyene chains [19].

6. Chemical mechanisms of scavenging and quenching reactive oxygen species by carotenoids

I have researched the scavenging and quenching mechanisms of reactive oxygen species by carotenoids. Astaxanthin forms nitro-astaxanthins through reaction with peroxynitrite, effectively inhibiting the nitration of tyrosine in an in vitro model. These findings demonstrate that astaxanthin can capture peroxynitrite molecules and protect against the nitration of tyrosine (Fig. 12) [20].

Our research group has also discovered that astaxanthin protects against lipid peroxidation through the formation of apo-astaxanthins (Fig. 12) [21]. We investigated the reaction products of astaxanthin with hydroxyl radical, superoxide anion radical, and singlet oxygen using LC/MS and ESR spectrometry. The ESR study revealed that astaxanthin can quench not only singlet oxygen but also superoxide anion radical and hydroxyl radical. Meanwhile, the LC/MS study showed the formation of astaxanthin epoxides resulting from reactions with superoxide anion radicals and hydroxyl radicals. Additionally, astaxanthin endoperoxides were identified as reaction products with singlet oxygen (Fig. 13) [22].

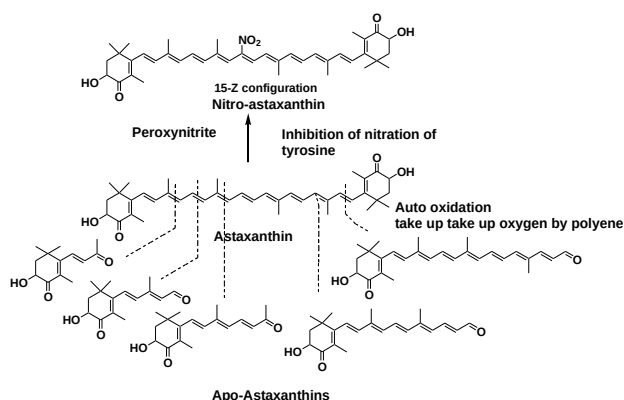


Fig. 12. Reaction products of astaxanthin with peroxynitrite and autooxidation products of astaxanthin.

These findings demonstrated that astaxanthin can neutralize reactive oxygen species through chemical reactions and exhibits antioxidative activity against these oxidative agents.

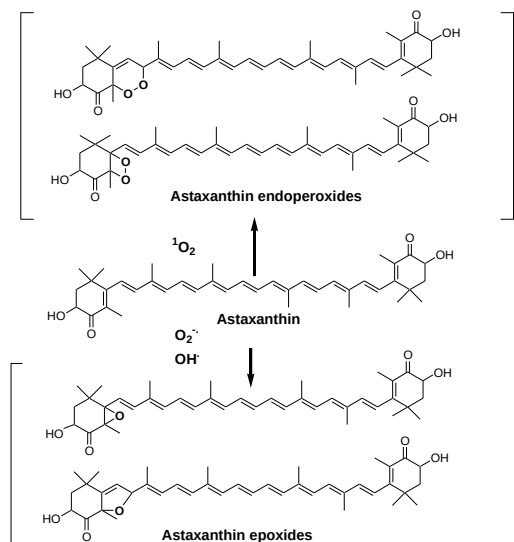


Fig. 13. Reaction product of astaxanthin with superoxide anion radical, hydroxyl radical, and singlet oxygen.

7. Conclusion

Over my 45-year research career, I have developed methods for the isolation, analysis, and structural elucidation of carotenoids. Continuous improvement in analytical techniques has enabled the elucidation of structures even for trace carotenoids in nature. Fascinating new structures of carotenoids continue to be discovered in nature, providing insights into their functions, metabolism, and roles in food chains.

I am committed to continuing my studies on carotenoids for as long as possible, and I am grateful to all my collaborators in this field of research.

This review is based on my presentation at the 19th International Symposium on Carotenoids in Toyama (2023).

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Potential applications of Z-isomer-rich carotenoids in the food, cosmetic, and feed industries

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Abstract: In nature, carotenoids occur predominantly in their all-*E*-configurations and they have very low bioavailability, whereas their *Z*-isomers exhibit greater bioavailability. Furthermore, recent studies have shown that carotenoid *Z*-isomers exhibit higher tissue accumulation efficiency and biological activities than the all-*E*-isomers. Therefore, the isomerization technology is gaining more and more attention in the food and cosmetic industries as a way to enhance the health benefits of carotenoids. Furthermore, the high absorption property of carotenoid *Z*-isomers was shown to be effective for the pigmentation of hen egg yolks and seafood body color. This suggests that carotenoid *Z*-isomers may also be useful in the feed industry. This review highlights the recent advancements in isomerization technology for carotenoids as well as future challenges for the practical application of materials rich in carotenoid *Z*-isomers in food, cosmetic, and feed industries.

1. Introduction

Carotenoids are naturally occurring lipid-soluble pigments responsible for imparting color to plants and animals, and their dietary intake is associated with a lower risk of various diseases, such as cancers, cardiovascular diseases [1, 2]. Thus, they have a wide range of applications in the food, cosmetic, and animal feed industries [3, 4]. As carotenoids contain multiple conjugated double bonds in the molecule, a large number of geometric (*E/Z*, *trans/cis*) isomers are theoretically possible (**Figure 1**). In general, (all-*E*)-carotenoids are the most predominant geometric isomer in nature, and they have high crystallinity and low solubility in various mediums, including edible oils and organic solvents, resulting in their low bioavailability and low processing efficiency [5, 6]. These unique properties of (all-*E*)-carotenoids limit their use in the food, cosmetic, and feed industries.

Technological developments for improving the bioavailability and processing efficiency of carotenoids utilizing the *Z*-isomerization have attracted a lot of

attention in recent years [5, 7]. For example, *in vivo* studies in rats and guinea pigs showed that the oral administration of a *Z*-isomer-rich astaxanthin diet

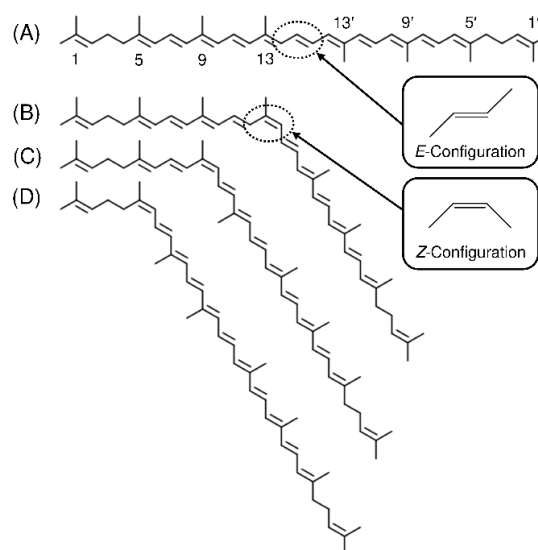


Figure 1. Chemical structures of lycopene isomers: (A) (all-*E*)-lycopene, (B) (13*Z*)-lycopene, (C) (9*Z*)-lycopene, and (D) (5*Z*)-lycopene.

resulted in a >25-fold increase in plasma astaxanthin concentration compared to an all-*E*-isomer-rich diet [8, 9]. *Z*-Isomerization of carotenoids induces a significant change in their physicochemical properties (e.g., solubility and crystallinity), leading to improved processing efficiency [5, 10]. For example, Gamlieli-Bonshtein et al. (2002) [11] reported that (9*Z*)- β -carotene in *Dunaliella* algae showed approximately 4 times higher extraction efficiency by SC-CO₂ than the all-*E*-isomer. For the practical use of *Z*-isomer-rich carotenoid materials, the development of efficient isomerization methods and an acute understanding of the changes in biological activity are required. This review highlights the recent advancements in carotenoid isomerization technology and in the development of new and useful application for *Z*-isomer-rich carotenoids. Additionally, research challenges for the practical application of carotenoid *Z*-isomers are discussed.

2. Methods for carotenoid isomerization

To date, thermal treatment, light irradiation, microwave irradiation, and catalytic treatment are well documented for *E/Z*-isomerization of carotenoids [5, 12, 13]. Since the isomerization efficiency of carotenoids varies widely among the methods, it is important to understand the characteristics of each isomerization method. Carotenoid isomerization is generally carried out in organic solvents or edible oils because the reaction is efficient in their dissolved states [5, 14]. For example, the thermal treatment of tomato powder at

140 °C for 5 min induced negligible *Z*-isomerization of lycopene (< 10% of total *Z*-isomer ratio), whereas the thermal treatment of (all-*E*)-lycopene solution dissolved in ethyl acetate at 140 °C for 30 s induced efficient *Z*-isomerization (> 50% of total *Z*-isomer ratio) [15, 16]. The heating isomerization method mentioned above is considered to be an inexpensive and efficient method with affinity to the food, cosmetic, and feed industries.

Light irradiation promotes the carotenoid isomerization, primarily from the *Z*-isomer to the all-*E*-isomers [17, 18]. Thus, light irradiation is not considered a suitable method to enhance the ratio of carotenoid *Z*-isomers.

Microwave irradiation also promotes *Z*-isomerization of carotenoids, but caution should be exercised because it also promotes rapid degradation [19, 20]. In addition, microwave processing has a low throughput and a high cost of equipment, which would make it difficult to use this method on an industrial scale; therefore, industrial application of this method may be difficult.

Catalytic isomerization of carotenoids is traditionally performed and is known to be an efficient method. However, most previous studies have used toxic heavy metal catalysts, such as copper chloride and titanium tetrachloride [21, 22]. These catalysts are generally not approved for use in food manufacturing processes. In recent years, our group has found that certain food ingredients, such as onions, garlic, and broccoli, have *Z*-isomerization-promoting effects, and the causative agents were identified as isothiocyanates,

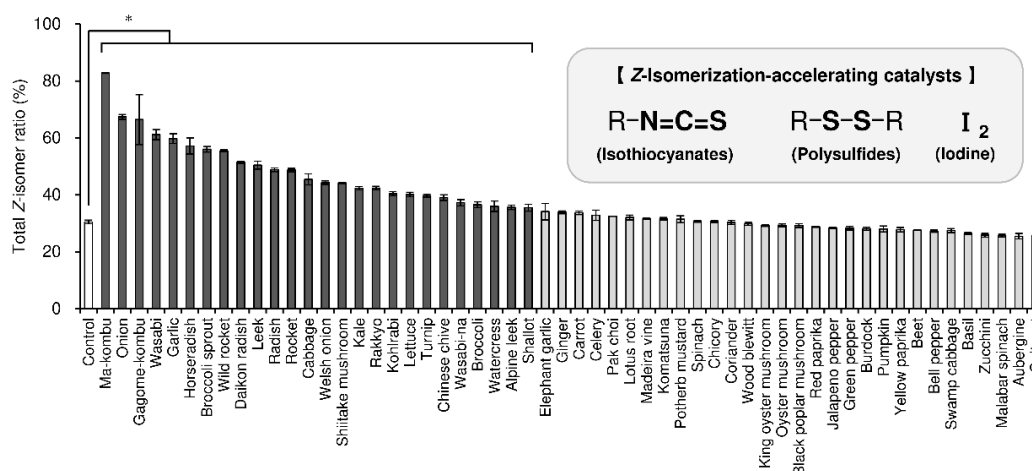


Figure 2. Effect of food ingredients on the total *Z*-isomer ratio of lycopene in tomato puree after heating and chemical structures of plant-derived *Z*-isomerization-accelerating catalysts for lycopene isomerization. Asterisk (*) indicates a statistically significant difference from the control group at $p < 0.05$ [23].

polysulfides, and iodine (**Figure 2**) [23, 24]. Furthermore, based on these findings, we have developed a new heterogeneous catalyst, i.e., isothiocyanate-functionalized silica [25]. This heterogeneous catalyst promoted *Z*-isomerization of (all-*E*)-carotenoids efficiently, e.g., when lycopene and astaxanthin solutions were incubated at 50 °C with this catalyst, their total *Z*-isomer ratios increased by approximately 80 and 50 %, respectively. These findings could be used to efficiently isomerize carotenoids in a manner suitable for the food, cosmetic, and feed industries.

3. Health benefits of carotenoid *Z*-isomers

Many clinical trials have demonstrated that improving carotenoid levels in the body can reduce the risk of various diseases [26, 27]. Therefore, the most anticipated health benefit of (*Z*)-carotenoid consumption is the increase in carotenoid concentrations in the body due to their increased bioavailability. The higher bioavailability of (*Z*)-carotenoids compared to the naturally predominant (all-*E*)-carotenoids has been demonstrated not only in *in*

vitro and *in vivo* studies but also in clinical trials [28–30]. For example, Cooperstone et al. (2015) [30] investigated the effects of consuming red tomato juice (total *Z*-isomer ratio of lycopene = 10%) and tangerine tomato juice (total *Z*-isomer ratio of lycopene = 94%) on plasma lycopene concentrations in humans, and found that lycopene derived from tangerine tomato juice had approximately 8.5-fold higher bioavailability than that derived from red tomato juice. Furthermore, *in vivo* studies have shown that (*Z*)-carotenoids have been shown to be more readily accumulated in the body than (all-*E*)-carotenoids [8, 29]. For example, oral administration of a *Z*-isomer-rich lycopene diet (total *Z*-isomer ratio of lycopene = 95.1%) to rats resulted in a >5-fold increase in lycopene concentrations in specific tissues (e.g., liver, kidney, adrenal, and prostate) compared to levels obtained after administration of an all-*E*-isomer-rich lycopene diet (total *Z*-isomer ratio of lycopene = 2.3%) (**Figure 3**) [8]. Several recent studies suggest that (*Z*)-carotenoids are superior not only in terms of bioavailability but also in terms of several biological activities [5, 7]. Specifically, (*Z*)-carotenoids have been reported to have greater antioxidant (e.g., DPPH radical and hydroxyl radical scavenging

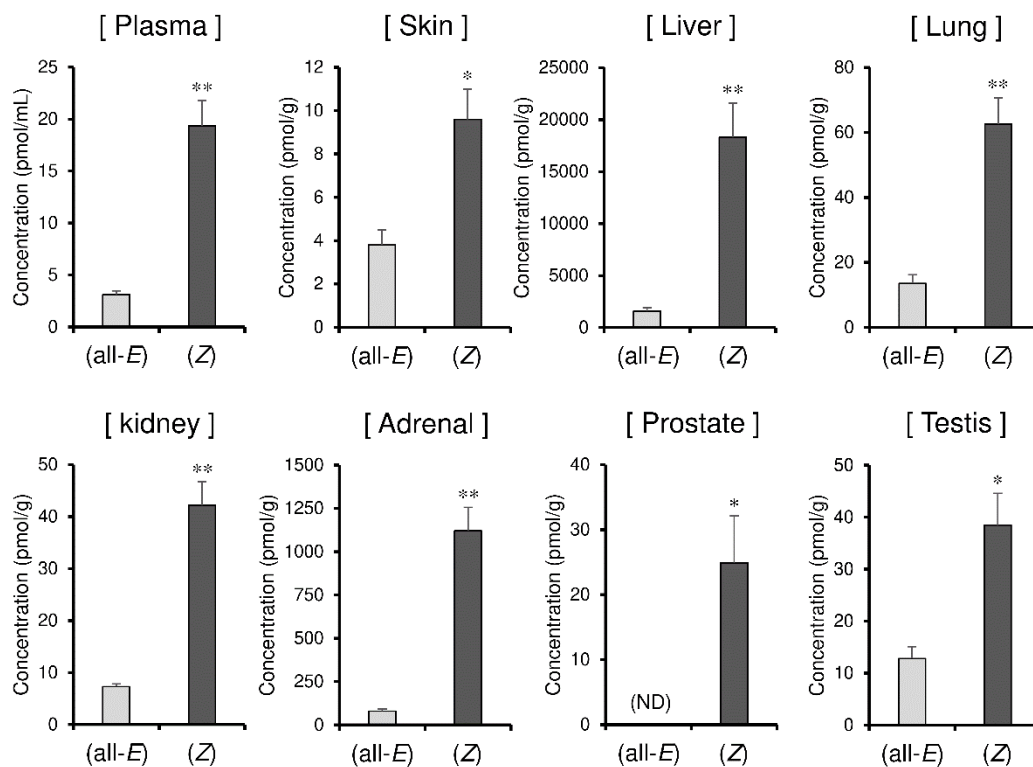


Figure 3. Lycopene concentrations in plasma and tissues/organs after 2 weeks of oral administration of an all-*E*-isomer-rich lycopene diet (total *Z*-isomer ratio = 2.3%) and a *Z*-isomer-rich lycopene diet (total *Z*-isomer ratio = 95.1%) to rats. Asterisk (*) indicates a statistically significant difference at * $p < 0.05$ or ** $p < 0.01$ [29].

activities), anti-inflammatory, skin-quality improving (e.g., anti-elastase, hyaluronic acid production-promoting, and anti-melanin formation activities), and anticancer activities than their all-*E*-isomers [31–35]. (*Z*)-carotenoids are also superior to their all-*E*-isomers in physical UV-light-shielding ability. However, as most of these reports are *in vitro* studies, further evaluation in *in vivo* and human studies is needed. It should be noted that *Z*-isomerization of carotenoids may have negative effects in some cases. For example, in the case of β -carotene, the *Z*-isomers might have lower bioavailability and provitamin A activity than the all-*E*-isomer [36, 37].

4. Potential application field of carotenoid *Z*-isomers

As mentioned above, carotenoid *Z*-isomers have greater bioavailability and potentially higher biological activities than their all-*E*-isomers. Thus, the application of (*Z*)-carotenoids in health foods and dietary supplements may provide greater health benefits than conventional (all-*E*)-carotenoid-rich ones. Furthermore, recent evidence has shown that the *Z*-isomers exhibit higher UV-light-shielding ability and skin-related biological activities, such as anti-elastase and anti-melanin formation activities, than the all-*E*-isomers [32, 33]. Thus, *Z*-isomer-rich carotenoids have the potential for application in sunscreen and skin care cosmetics.

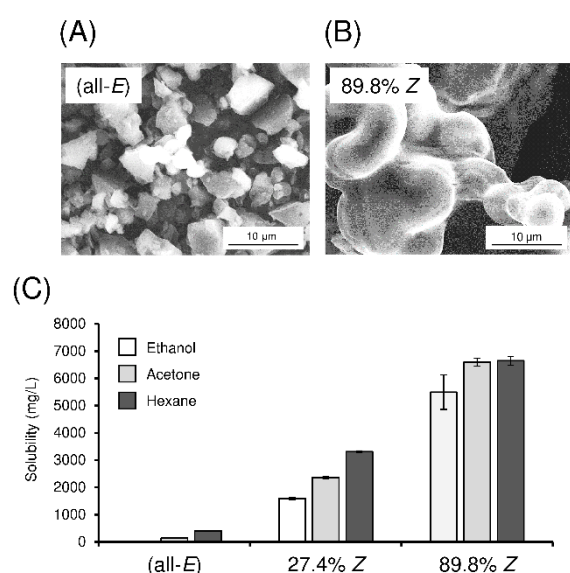


Figure 4. SEM images of (A) (all-*E*)- β -carotene and (B) *Z*-isomer-rich β -carotene, and (C) solubility of (all-*E*)- β -carotene and *Z*-isomer-rich β -carotene in different organic solvents [10].

The isomerization process may increase the production cost of carotenoid materials. However, this problem may be solved by utilizing the changes in physical properties associated with the *Z*-isomerization of carotenoids [5, 12]. In general, (all-*E*)-carotenoids, the predominant geometric isomer in nature, have high crystallinity and low solubility in various media, resulting in low processing efficiency, e.g., extraction, micronization, and emulsification. The *Z*-isomerization of carotenoids decreases their crystallinity and increases their solubility [10, 38] (**Figure 4**). It has been shown that these changes in the physical properties of carotenoids can be utilized to improve the efficiency of carotenoid processing [5, 12]. For example, we reported that when lycopene was extracted from tomato powder with ethanol, lycopene recovery was approximately 12 times higher with the *Z*-isomerization pre-treatment than without the pre-treatment [39]. This isomerization pre-treatment has also been reported to improve the efficiency of micronization, emulsification, and water-soluble powder formulation [40–42]. Since carotenoid materials used in food and cosmetic industries generally undergo the processing steps of extraction, emulsification, and sometimes undergo micronization and water-soluble powder formulation steps, improving the efficiency of these steps through *Z*-isomerization pre-treatment may counteract the increased production costs associated with the isomerization process.

In the carotenoid market, it is estimated that the feed segment accounted for the largest share of the market in 2021 [43]. Carotenoids, especially astaxanthin, are used as colorants for the exoskeleton of crustaceans, the flesh and skin of fish, and hen egg yolk. In recent years, several studies have demonstrated that astaxanthin *Z*-isomers are more effective for the pigmentation of rainbow trout (*Oncorhynchus mykiss*) and Pacific white shrimp (*Litopenaeus vannamei*) bodies and egg yolks of laying hens than the all-*E*-isomer (**Figure 5**) [44–46]. Furthermore, for two-spotted cricket (*Gryllus bimaculatus*), which is an edible insect, feeding a diet rich in astaxanthin *Z*-isomer resulted in a higher astaxanthin concentration in the body compared to feeding a diet rich in the all-*E*-isomer (**Figure 5**) [47]. This higher pigmentation efficiency of carotenoid *Z*-isomers would be attributed to their high bioavailability. These results indicate that *Z*-isomer-rich carotenoid materials are useful in the feed industry as an efficient body and egg yolk color improver.

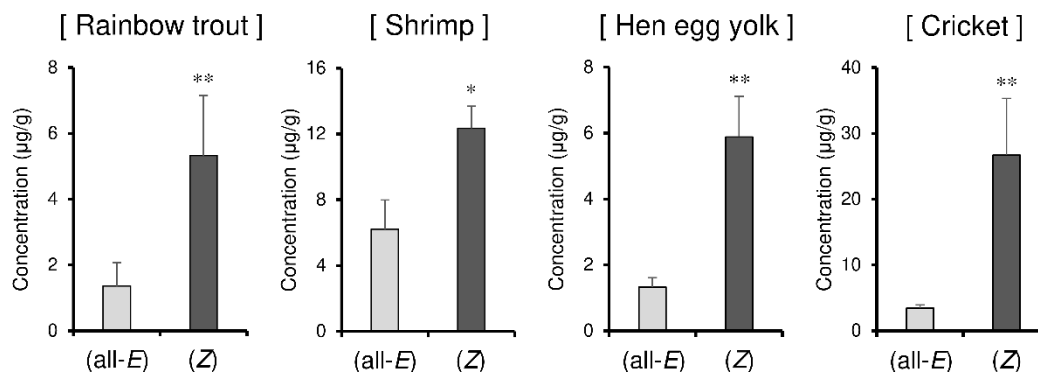


Figure 5. Astaxanthin concentrations in rainbow trout, Pacific white shrimp, hen egg yolk, and two-spotted cricket after feeding an all-*E*-isomer-rich astaxanthin diet and a *Z*-isomer-rich astaxanthin diet. Asterisk (*) indicates a statistically significant difference at $*p < 0.05$ or $**p < 0.01$ [44–47].

5. Future challenges for practical application of materials rich in carotenoid *Z*-isomers

The following challenges (1–4) need to be addressed to make *Z*-isomer-rich carotenoid material practical.

5.1. Development of efficient and environmentally friendly *Z*-isomerization methods:

Thermal treatment, light irradiation, microwave irradiation, and catalytic treatment are well documented for the *Z*-isomerization of carotenoids, and among these isomerization methods, thermal treatment is considered the most suitable method in terms of efficiency and industrial affinity [5, 12, 13]. To realize the practical application of thermal isomerization of carotenoids, technological developments like isomerization with less toxic media (e.g., ethanol, supercritical CO₂, and vegetable oils) and continuous isomerization are required. It is also important to develop processes and conditions that minimize the degradation of carotenoids during thermal isomerization.

5.2. Improvement of storage stability:

Carotenoid *Z*-isomers are less stable than the naturally predominant all-*E*-isomers, i.e., prolonged storage at room temperature or higher will result in all-*E*-isomerization of the *Z*-isomers [48, 49]. Thus, improving their stability is an important aspect to overcome for the practical use of carotenoid *Z*-isomers. Recent studies have reported that the *Z*-structures of carotenoids are stabilized by their formulation with ester-based nonionic emulsifiers,

poly(vinylpyrrolidone), and 2-hydroxypropyl- β -cyclodextrin [42, 50, 51]. Therefore, it may be possible to produce stable *Z*-isomer-rich carotenoid materials by utilizing formulation technology.

5.3. Ensuring safety:

Carotenoid *Z*-isomers are widely distributed in foods and are considered safe based on long dietary experience. However, the safety of ingesting high concentrations of the *Z*-isomer has not been adequately studied and should be investigated as necessary. Several safety evaluation studies (e.g., the bacterial reverse mutation test, oral toxicity test, and histopathological examination) on the *Z*-isomers of lycopene and β -carotene have confirmed that they are not toxic [52, 53].

5.4. Creating more value:

In recent years, several studies have demonstrated the usefulness of carotenoid *Z*-isomers. However, there is still limited information on their biological activities and the mechanisms of their beneficial effects are also largely unknown. In addition, many biological activity evaluations have been performed in *in vitro* studies. Therefore, extensive animal and human studies should be conducted in the future to further improve the value of carotenoid *Z*-isomers.

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Quantitative Analysis of Supplement Components Using Quantitative NMR

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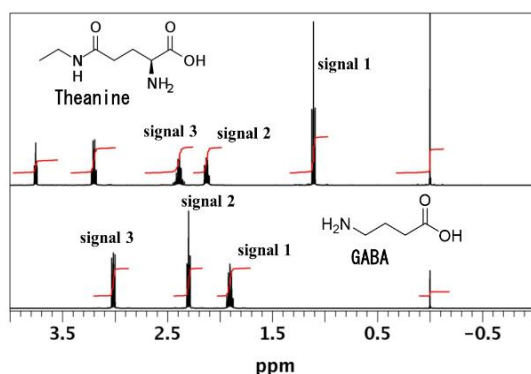
Abstract: Most quantitative methods rely on the absolute calibration curve method using HPLC or LC-MS. However, it is often difficult to prepare standard products for trace amounts of useful components in natural products, making accurate quantification challenging. In recent years, quantitative methods using ^1H quantitative Nuclear Magnetic Resonance (qNMR) have gained attention in addressing this issue. This method allows for accurate quantification of various measurement targets by utilizing certified standard products with precise purity values in the internal standard method. We employed this method to quantify amino acids and vitamins, the active ingredients in supplements.

1. Introduction

Approximately 80% of the Japanese population includes health supplements in their daily routines to support well-being. Unlike pharmaceuticals, these products are not subject to strict legal oversight, leaving quality and content regulation to manufacturers and sellers. Traditional quantitative methods, such as HPLC and MS, require the preparation of standard substances and the laborious creation of calibration curves. In contrast, qNMR compares integral values of calibrated reference substances with analyzed samples, eliminating the need for extensive calibration efforts. This study utilizes qNMR to quantitatively analyze components in various health supplements. Additionally, we explore which compounds should be measured under specific conditions.

2. Methods and Results

2.1. Quantification of Theanine and GABA [1]



Scheme 1. ^1H NMR spectra of commercially available theanine and GABA

To initiate the study, we quantified the amino acids theanine and GABA, which are known for their sleep-improving effects and are believed to undergo minimal structural changes during measurement. We crushed one tablet of commercially available theanine from

three different products (samples A to C) and two GABA products (samples D and E) in a mortar. Subsequently, we accurately measured 10 mg of the resulting powder using an ultra-microbalance and dissolved it in 1 mL of commercially available quantitative NMR DSS- d_6 in D_2O (500 mg/L) standard solution. This solution served as the measurement sample, and we prepared three samples for each product. The measurements were conducted using a JEOL-ECZ400, following the 17th revision of the Japanese Pharmacopoeia. The parameters included a pulse angle of 90° , a pulse delay (d1) of 60 s or more, ^{13}C decoupling, an accumulation of eight or more scans, and measurement in the non-spin mode.

Initially, three samples each of sample A to E were measured under the conditions of a d1 of 60 s and an accumulation of 32 scans (resulting in a measurement time of approximately 40 minutes). Quantitative values were calculated by manually drawing integral curves for signals 1 to 3 in each sample using JEOL DELTA software. The quantities obtained consistently fell below the labeled amounts for all products (see Table 1). Subsequently, extending the d1 to 180 s resulted in nearly all products showing a content level equal to or higher than the labeled amount (see Table 2). Typically, it is considered sufficient for d1 to be around 7 times the T1 relaxation time of each hydrogen. The condition of d1=60 s, which is more than seven times the T1 of theanine and GABA, seemed sufficiently long for quantification. However, it turned out that the length of d1 was insufficient for accurate quantification.

2.2. Quantification of Vitamin C (Ascorbic Acid)

To further investigate which compounds require attention when setting the d1 based on the results of 2.1, we conducted a survey of the content of vitamin C-containing dietary supplements, including six commercially available products (samples F to K). Sample preparation was conducted as described in 2.1, and measurements were carried out with d1 set at four

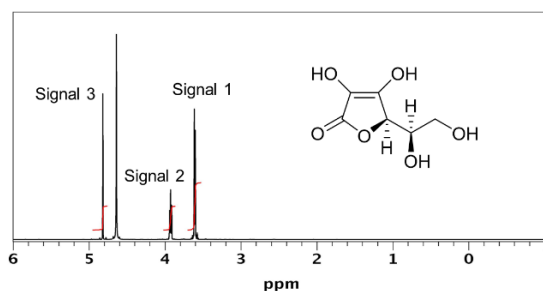
Sample A	Content rate calculated from each signal area (%)		Average content (%)
A-1	Signal 1	43.3	42.8
	Signal 2	43.2	
	Signal 3	42.9	
A-2	Signal 1	44.8	42.7
	Signal 2	43.9	
	Signal 3	45.6	
A-3	Signal 1	43.5	45.2
	Signal 2	45.8	
	Signal 3	45.5	

Table 1 : Results of measuring the content rate of theanine in sample A at d1=60s

	Average content (%) d1=60s	Displayed ratio (%)	Average content (%) d1=180s
Sample A (theanine)	43.6	47.6	48.7
Sample B (theanine)	7.7	10.0	9.5
Sample C (theanine)	15.8	17.9	18.9
Sample D (GABA)	56.4	62.5	64.6
Sample E (GABA)	12.9	13.9	15.5

Table 2 : Results of measuring the content rate of sample A-E at d1=180s (n=3)

different levels, ranging from 60 s to 150 s. As a result, it was found that, for vitamin C, a d1 of 60 s was sufficient for accurate measurements (see **Table 3**). This information is valuable for understanding the specific considerations needed when setting d1 for the quantification of various compounds.



Scheme 2. ¹H NMR spectrum of commercially available vitamin C

Sample F-1	60s	90s	120s	150s	Displayed ratio (%)
Signal 1	86.2	84.5	86.3	85.6	84.0
Signal 2	85.4	85.9	85.0	84.7	
Signal 3	82.7	82.6	82.9	82.8	
Av	84.8	84.3	84.7	84.4	

Table 3 : Results of measuring the content rate of vitamin C in sample F at d1=60-150s

2.3. Quantification of Vitamin B Complex (B₁, B₃, B₆)

We conducted a survey of the content of vitamin B complex (B₁, B₃, B₆) in three commercially available vitamin dietary supplements (samples L to N). Sample preparation was carried out as described in 2.1 and 2.2, and measurements were performed with d1 set at three levels: 60, 120, and 180 s (see **Table 4**). The results revealed that a d1 of 60 s was insufficient, and the use of 120 s produced better outcomes for quantifying the

vitamin B complex (see **Table 5**). This finding highlights the importance of selecting an appropriate d1 value for accurate measurements, particularly when dealing with different compounds.

	60s	120s	180s	Displayed ratio (%)
Sample L	9.5	9.9	10.0	9.3
Sample M	6.6	7.3	7.3	8.0
Sample N	8.3	9.0	8.9	10.0

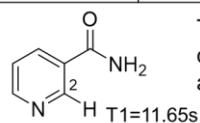


Table 4 : Results of measuring the content rate of vitamin B₃ in sample L-N at d1=60-180s (n=3)

		B ₁	B ₃	B ₆
Sample L	qNMR (%)	7.0	10.3	7.5
	Displayed ratio (%)	6.9	9.3	6.9
Sample M	qNMR (%)	7.4	8.5	5.1
	Displayed ratio (%)	8.0	8.0	6.0
Sample N	qNMR (%)	10.4	10.6	7.9
	Displayed ratio (%)	10.0	10.0	7.7

Table 5 : Results of measuring the content rate of vitamin B complex in sample L-N at d1=120s (n=3)

2.4. Quantification of Carotenoid (Lutein) [2]

We conducted a survey of the lutein content in two commercially available health supplements (sample O: tablet, sample P: soft capsule) containing this carotenoid. As lutein is lipophilic, CDCl₃ was used as the measuring solvent for quantitative NMR, and 1,4-BTMSB (1,4-Bis(trimethylsilyl)benzene) was employed as the internal standard substance, commercially available as a certified reference material. Sample O (tablet) was crushed, and approximately 60 mg was dissolved in 1 mL of commercially available

CDCl_3 for qNMR to prepare the measurement sample. Measurements were conducted at two levels: 60 s and 120 s (see **Table 6**). The results indicated that measurements were adequately possible with a d1 of 60 s. However, due to the inherently low lutein content in the sample, the signal-to-noise ratio was exceptionally poor, and the measured values deviated significantly from the labeled amount. Therefore, adjustments to the sample preparation method need to be devised to address these challenges. Finally, sample P (soft capsule) was dissolved in CHCl_3 , washed with brine, and the solvent was distilled off to prepare the measurement sample. As shown in **Table 6**, it was found that if the content ratio was around 5%, it could be quantified using the extraction method.

	60s	120s	Displayed ratio (%)
Sample O	0.2	0.2	1.6
Sample P	5.7		5.0

Table 6 : Results of measuring the content rate of lutein in sample O, P at d1=60s or 120s (n=1)

3. Discussion

Based on the results of the above experiments, it is suggested that for compounds containing nitrogen atoms in their molecules, it is advisable to set a relaxation delay (d1) longer than seven times the T1 relaxation time. Moving forward, in the measurement of various health supplements, we intend to continue investigating and optimizing the setting of appropriate relaxation delays to ensure accurate and reliable results.

4. References

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Analysis of Geometrical Isomers of Astaxanthin using Ion Mobility MS

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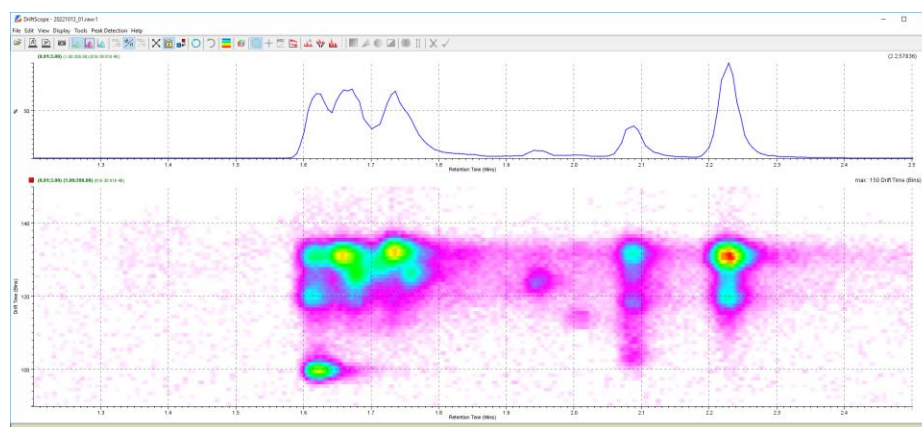
Several geometrical isomers of carotenoids are presented in nature. Analysis of geometrical isomers of carotenoids has been used by normal and reversed phase HPLC methods. Ion mobility spectrometry is the gas-phase separation of ions in an electric field on the basis of size and shape. When coupled with mass spectrometry, a dimension of high-speed separation can be added to mass spectrometric analysis. Therefore, ion mobility MS has been applied to separation of several geometrical isomers of fatty acid. However, there are a few reports for analysis of geometrical isomers of carotenoids, except for application of carotenes¹. In the present study, we applied ion mobility MS for analysis of geometrical isomers of astaxanthin.

Experiment

Geometrical isomers of astaxanthin was prepared by thermal isomerization (80°C, 5 hrs. in CH₂Cl₂ solution). Ion mobility MS was measured by SELECT SEREIS Cyclic IMS with following conditions: MS mode, ESI positive ion mode; capillary voltage, 3.5 kV; cone voltage, 40V; desolvation gas, 1,000 L/hr (500°C); cone gas, 50L/hr; ion source heater, 120 °C. HPLC was performed with ACQUITY UPLC CORTECS C18 as a stationary phase and H₂O:CH₃CN (5:95) as a mobile phase, flow rate of 0.4 mL/min. In ESI MS, sodium adduct ion (M+Na⁺) is observed predominantly in astaxanthin. Therefore, 10 μM NaOH solution was added (50 μL/min) by post column, in order to form sodium adduct ion.

Results and Discussion

Three peaks were observed in ODS HPLC of geometrical isomers of astaxanthin. The first peak contained all-*trans* and several *cis* isomers. The second peak contained 9-*cis* astaxanthin and third peak contained 13-*cis* astaxanthin. 2D ion mobility MS chromatogram of M+Na⁺ of geometrical isomers of astaxanthin was showed below. Some geometrical isomers contained in peak 1 were separated by ion mobility MS chromatogram. Furthermore, 5 times of pass in ion mobility cell, these geometrical isomers were clearly separated as shown here.



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Astaxanthin production by *Haematococcus* algae cultivation using LED

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Astaxanthin is a type of carotenoid compound and is a red pigment contained in various organisms, mainly seafood such as salmon and shrimp. It exhibits extremely strong singlet oxygen scavenging and lipid peroxidation inhibiting effects, and has been reported to have a wide variety of physiological activities, including improving eye strain, beautifying the skin, and preventing arteriosclerosis. As a result, its use in supplements, cosmetics, and food products is expanding, and global demand is expected to further expand in the future.

Currently, *Haematococcus* algae, a type of unicellular green algae, is widely used for the commercial production of natural astaxanthin for humans. It has the property of forming cysts under stress environments such as strong light, high salt concentration, and nutrient starvation, and accumulating astaxanthin within the cells to protect itself. *Haematococcus* algae can accumulate astaxanthin in high concentrations (about 3-5%).

Large-scale cultivation methods for *Haematococcus* algae include outdoor open ponds and outdoor glass tube systems, but our company uses an indoor photobioreactor system. By constantly exposing cells to strong light in a reactor with controlled indoor conditions, it is possible to stably produce high-quality astaxanthin with few impurities.

From the perspective of energy conservation, there is a trend worldwide to stop using fluorescent lights and switch to LEDs. Compared to fluorescent lights, LEDs are more energy efficient and have a longer service life. It is thought that it is possible to reduce the environmental burden by using LEDs in the cultivation of *Haematococcus* algae.

In this presentation, we will introduce astaxanthin production by *Haematococcus* algae and *Haematococcus* algae cultivation using LED.

Application of natural astaxanthin derived from *Haematococcus* algae to functional foods

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Astaxanthin is an orange to red carotenoid pigment that is found in foods that have been eaten extensively, such as the muscles of salmon and trout, fish and crustaceans with red skin, and birds. Industrially, it is produced from a highly safe green algae, called *Haematococcus* algae, and in recent years astaxanthin has been widely used as a supplement and functional food. We offer a variety of dosage forms, including oil extracted from *Haematococcus* algae, as well as astaxanthin powder and water-soluble bulk using our unique emulsification technology to meet others' needs to use in tablets, drinks, and gummies.

In addition, many functional claims have been approved for supplements and functional foods that use our astaxanthin raw material. Typical functional claims are in the eye care and beauty fields, but in recent years, it has also been confirmed to reduce irritation and maintain mental alertness and concentration (i.e. improving rate of calculation errors due to temporary mental fatigue) as an effect on mental fatigue.[1] Based on this effect, a functional claim notification was accepted for the first time for a function that alleviates temporary stress (irritability). In the future, astaxanthin is expected to be effective not only in the eyes, skin, and stress relief, but also in many other areas.

In this forum, we will introduce the characteristics and manufacturing methods of each of these dosage forms of astaxanthin bulk, as well as various functional claims using these bulks.

Reference

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Industrial production of carotenoids using *Paracoccus carotinifaciens* and carotenoid applications for animal feed

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1. Industrial production of carotenoids using *Paracoccus carotinifaciens*

Paracoccus carotinifaciens (Fig.1) is an aerobic Gram-negative bacterium that was isolated from soil early in the 1990s[1]. It mainly produces (3S,3S')-astaxanthin, which is in free form without esterification. To achieve the commercial production of astaxanthin, we conducted mutation breeding and optimized media components, culture conditions, and manufacturing processes. Selective breeding has been performed using classical mutation methods using chemical treatment and UV irradiation. Genetic engineering technology hasn't been used because consumers have preferred non-GMO products. As a result, its productivity has increased over 500 times more than that in original conditions. The astaxanthin production medium consists of carbon and nitrogen sources, inorganic salts, vitamins, and amino acids. The optimal concentration of each ingredient has been determined by bacterial growth and astaxanthin productivity. and we successfully started the industrial production of astaxanthin in 2000s.[2]

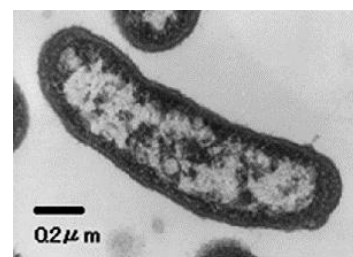


Fig.1 *Paracoccus carotinifaciens*

The carotenoid biosynthetic pathway of *P. carotinifaciens* is thought to be the same with the *Paracoccus* sp. N81106, elucidated by Misawa et al.[3]. *P. carotinifaciens* is cultured under optimized conditions and internally accumulates more than 10 kinds of carotenoids, such as adonirubin, canthaxanthin, and adonixanthin. The carotenoid composition can be controlled by the concentration of dissolved oxygen (DO) in the culture. Lower DO increases canthaxanthin and adonirubin accumulations, whereas higher DO leads to adonixanthin productivity (Fig. 2). These phenomena are brought by the activities of 2 conversion enzymes, CrtW and CrtZ. In addition, through the mutation breeding process, we could find some strains which has no or less functional CrtW or CrtZ and can produce only specific carotenoid, such as zeaxanthin or canthaxanthin. Hence, *P. carotinifaciens* are suitable for producing desired several kinds of carotenoid.

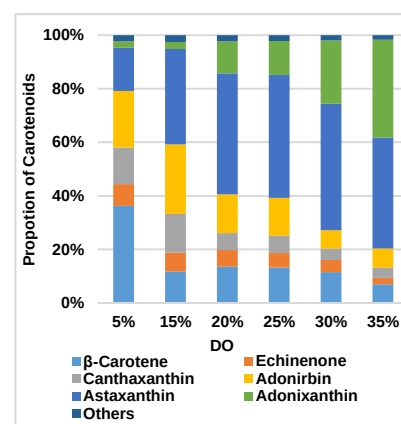


Fig.2 Carotenoid composition on each DO conc.

2. Carotenoid applications for animal feed

Dehydrated *P. carotinifaciens* is sold under the name Panaferd® as a pigment for fish, crustaceans and egg yolk. Since almost all carotenoids included in Panaferd® are transferred to animals, Panaferd® imparts natural pigmentation, which is favored by consumers. We also explored that Panaferd® has additional effect on improving productivity, releasing stress, and increasing immunity of animals. Panaferd® is not only an source for pigmentation, but a multi-functional feed additive.

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Temporal changes of blood carotenoids in whale shark (*Rhincodon typus*)

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The whale shark (*Rhincodon typus*) is the largest extant fish belonging to the monotypic family Rhincodontidae within the Order Orectolobiformes. Whale sharks appear to be widely distributed in tropical and warm temperate seas in the Atlantic, Pacific, and Indian Oceans. It is believed to have a wide-ranging migratory pattern, from coastal to open ocean regions. The whale shark is a filter-feeder that appears to feed on a wide variety of planktonic and nektonic prey as well as crustaceans, fish eggs, surface-dwelling shoaling fish, and relatively large fish. The whale shark is listed by the IUCN as vulnerable, much of its biology and habits remained uncertain, including its method of reproduction.

Research on carotenoids in fish has predominantly focused on Osteichthyes, with numerous reports on new carotenoids and metabolic pathways. However, in cartilaginous fish, including the whale shark, there are only reports on carotenoid of the Spiny Dogfish (*Squalus acanthias*), which is commercially harvested, and little information regarding carotenoid content is available. Therefore, this study investigated the carotenoid content in the blood plasma of five whale sharks kept at the Osaka Aquarium Kaiyukan and the Osaka Aquarium Biological Research Institute Iburi Center (Iburi Center), along with its temporal variations.

One female (No.1) and four males (No.2 to No.5) of whale sharks were kept after being accidentally bycaught in gillnets off the coast of Kochi Prefecture and transported to the Iburi Center for maintenance. No.1 and No.2 were transferred to the Kaiyukan after being kept at the Iburi Center for one month. They were primarily fed with Antarctic krill, North Pacific krill, and *Neomysis* sp., and occasionally provided with whitebait, Sakura shrimp and artificial feed (Shark and Ray Gel Diet, Mazuri) for cartilaginous fish. No.1 and No.2 had their blood samples collected one month after being transported to the Iburi Center, while No.3 to No.5 had their blood samples collected regularly from the time of arrival at Iburi Center. The collected blood was frozen at -30°C after plasma separation and stored for carotenoid analysis. Carotenoid analysis was performed using LC/MS. Similarly, carotenoid analysis was also conducted for the feed materials being used.

From the blood plasma of all samples, astaxanthin, canthaxanthin, β -carotene, and lutein (including zeaxanthin) were isolated, with astaxanthin comprising the majority of these carotenoids. In the plasma of No.3-5 at the time of arrival, high levels of astaxanthin and canthaxanthin were detected, and a significant increase in astaxanthin content was observed in all individuals between 3 and 6 months after arrival. High concentrations of astaxanthin were detected in feed materials other than whitebait, suggesting that the astaxanthin in whale shark plasma is likely derived from the diet. Furthermore, since astaxanthin and lutein (zeaxanthin) exhibited similar dynamics, it was presumed that lutein is a metabolite of astaxanthin that has undergone reduction in the whale shark's body. Canthaxanthin was found in *Neomysis* sp., Sakura shrimp, and artificial feed, but since not all individuals were fed these materials throughout the entire study period, it was considered both a potential metabolite of astaxanthin and possibly derived from the diet. This suggests that whale sharks, like other fish, have the ability to metabolize carotenoids.

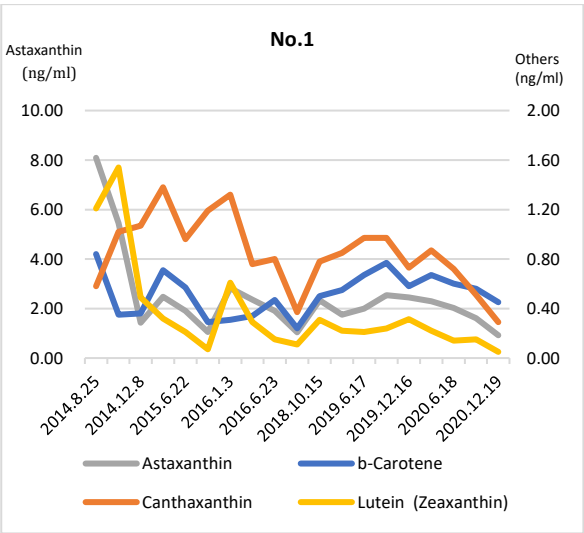


Fig.1 Changes in blood plasma carotenoid levels of No.1.

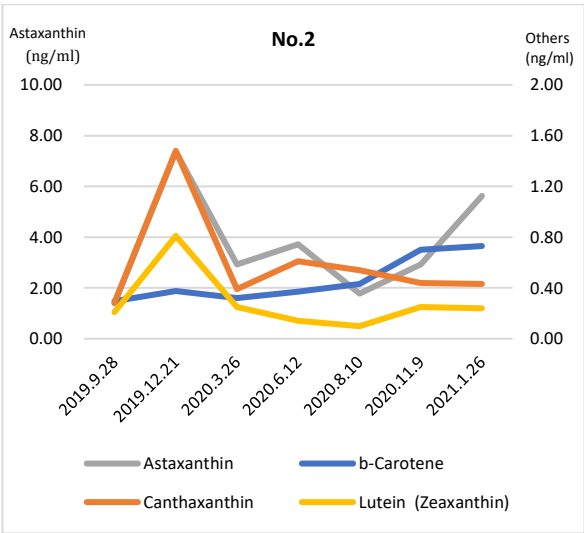


Fig.2 Changes in blood plasma carotenoid levels of No.2.

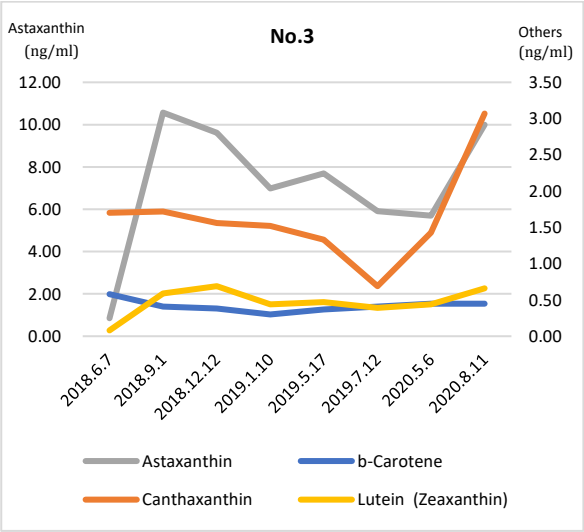


Fig.3 Changes in blood plasma carotenoid levels of No.3.

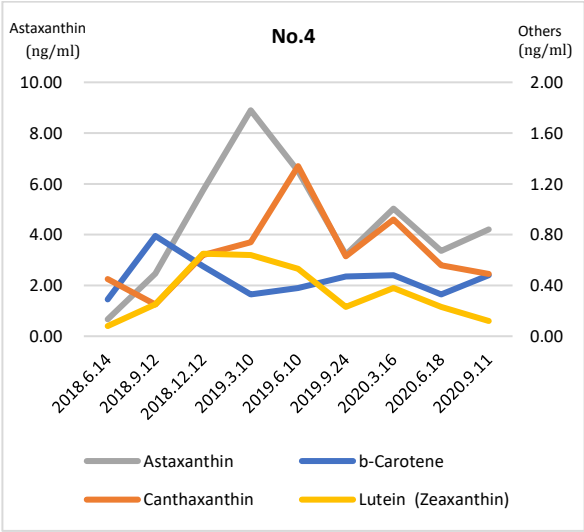


Fig.4 Changes in blood plasma carotenoid levels of No.4.

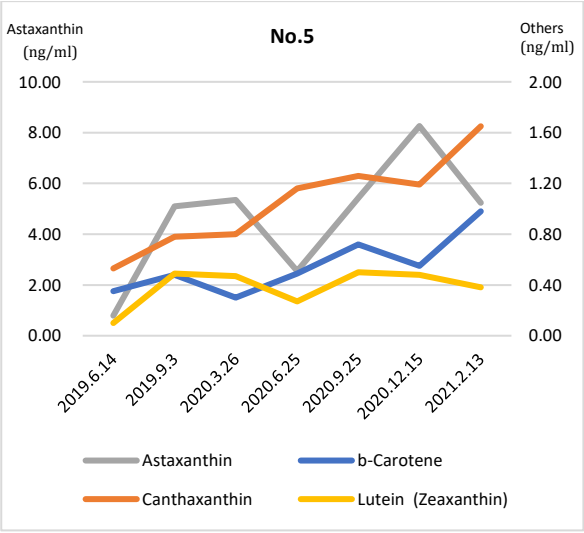


Fig.5 Changes in blood plasma carotenoid levels of No.5.

Table1. Carotenoid contents in each feed

	Whitebait	Antarctic krill	North Pacific krill	Neomysis sp.	Sakura shrimp
β-Carotene	0	0	0	0	0
Tunaxanthin	0.02618	0	0	0	0
Lutein	0.05457	0	0	0	0
Epilutein	0.04301	0	0	0	0
Zeaxanthin	0.04233	0	0	0	0
Astaxanthin diester	0	1.34067	11.8437	3.2292	8.0441
Astaxanthin monoester	0	2.83475	14.5209	10.1088	12.1561
Astaxanthin	0	1.97432	1.8333	1.7004	4.6003
Canthaxanthin	0	0	0	0.1872	0.4626
Others	0.00391	0.52026	0.9021	0.3744	0.4369
Total Carotenoid (μg/g)	0.17	6.67	29.1	15.6	25.7

Absorption and metabolism of fucoxanthin from diatoms in rats

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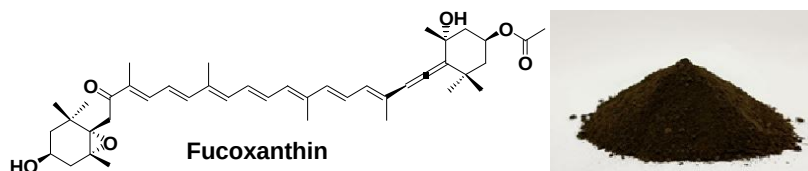
Fucoxanthin is a marine carotenoid with an allenic bond, an epoxide group, a conjugated carbonyl group and an acetylenic group in the molecule. Brown algae and some microalgae, such as diatoms, produce fucoxanthin. Fucoxanthin is known for its various health benefits such as anti-diabetic, anti-inflammatory, anti-obesity, and anti-non-alcoholic steatohepatitis. Therefore, the demand for fucoxanthin reagents has increased. However, there are high costs to prepare fucoxanthin from natural sources. In the presentation, we report fucoxanthin production by cultured microalgae and the absorption and metabolism of fucoxanthin produced by microalgae in rats.

Production of fucoxanthin by microalgae

Pure fucoxanthin reagent is difficult to procure. Until now, pure fucoxanthin reagents have rarely been available on the market due to their high scarcity value, as they are only contained in trace amounts in brown algae, but we have succeeded in mass culturing microalgae that contain fucoxanthin in percentage order.

Since then, it has become possible to supply fucoxanthin pure reagent in grams stably (Alnur Co., Ltd. Sales price 1,000mg bottle of fucoxanthin pure reagent 200,000 yen). At present, Alnur Co., Ltd. always has about 100g of pure fucoxanthin reagent with almost 100% purity in stock. Stable mass production is essential for the fucoxanthin market. Currently, in collaboration with Alnur Co., Ltd., which has culture equipment technology, and TOPPAN Inc., which is planning to operate its own mass production facility, we are working to improve the functionality and mechanism of action by supplying pure fucoxanthin reagents.

Elucidation of the health benefits mechanism and cost reduction through stable mass production for actual commercialization are two pillars of market expansion.



Absorption and metabolism of fucoxanthin produced by microalgae in rats

The experimental diet contained 0.3% fucoxanthin dissolved with MCT oil in CE-2 food (CLEA Japan, Inc.). SD rats (8-week-old males) were fed this experimental diet ad libitum for seven days. After seven days of feeding, the carotenoid content and composition in plasma, liver, small intestine, large intestine, kidney, lung, heart, and pancreas of the rats were analyzed by VIS absorption and LC/PDA/MS methods. The carotenoid content and composition in plasma and organs of rats are shown in **Table 1**. Metabolites of fucoxanthin were detected in plasma, liver, small intestine, large intestine, kidney, lung, heart and pancreas of rats.

Fucoxanthinol was presented as a component (58~93%), and amarouciaxanthin A as a minor component (2~4%) in plasma and organs. These fatty acid esters were also presented as minor components. ¹H-NMR analysis revealed that the hydroxy group at 3' in fucoxanthinol was esterified with fatty acids. ESI MS showed that the fatty acids esterified with fucoxanthinol were palmitic, oleic, linoleic, and linoleic acids. Amarouciaxanthin A was also esterified with fatty acids. The composition of amarouciaxanthin A was tiny (less than 1% except for the liver). The metabolic pathway of fucoxanthin in rodents is shown in **Fig. 1**

The carotenoid compositions in plasma, small intestine, large intestine, kidney, lung, heart and pancreas were almost similar. These results suggested that intake fucoxanthin was converted to fucoxanthinol by hydrolysis in the

digestive tract and part of it was metabolized to amarouciaxanthin A in the small intestine of rats. These carotenoids were also esterified with fatty acids in the small intestine. These carotenoids were transferred by plasma and accumulated in the kidney, lung, heart and pancreas. About 40% of carotenoids in the liver were presented as fatty acid esterified form. It was assumed that rats accumulated polar xanthophyll, fucoxanthinol as fatty acids esterified form to reduce polarity.

In the case of mice, much of fucoxanthinol was converted to amarouciaxanthin A and accumulated in these organs¹⁾. Compared with mice, the metabolic capacity of fucoxanthin to amarouciaxanthin A in rats was tiny.

In the present investigation, we demonstrated that fucoxanthin prepared from microalgae accumulated in rats and is well used for animal experiments.

Table 1. Accumulation of fucoxanthin derivatives in rats.

		Composition		%	
	Carotenoid	Free type		Esterified type	
	content	Fucoxanthinol	Amarouciaxanthin A	Fucoxanthinol	Amarouciaxanthin A
Plasm	1.87µg/mL	87.2	3.1	8.9	0.8
Liver	64.9 µg/g	58.6	3.1	30.1	8.2
Small intestine	87.7 µg/g	>90	~4.5	~5	~0.5
Large intestine	8.46 µg/g	90.2	2.1	6.8	0.9
Kidney	13.81 µg/g	90.8	2.1	6.8	0.3
Lung	14.69 µg/g	88.2	3.2	8.2	0.4
Hart	18.65 µg/g	89.2	2.5	7.9	0.4
Pancreas	23.67 µg/g	93.1	2.9	3.5	0.5

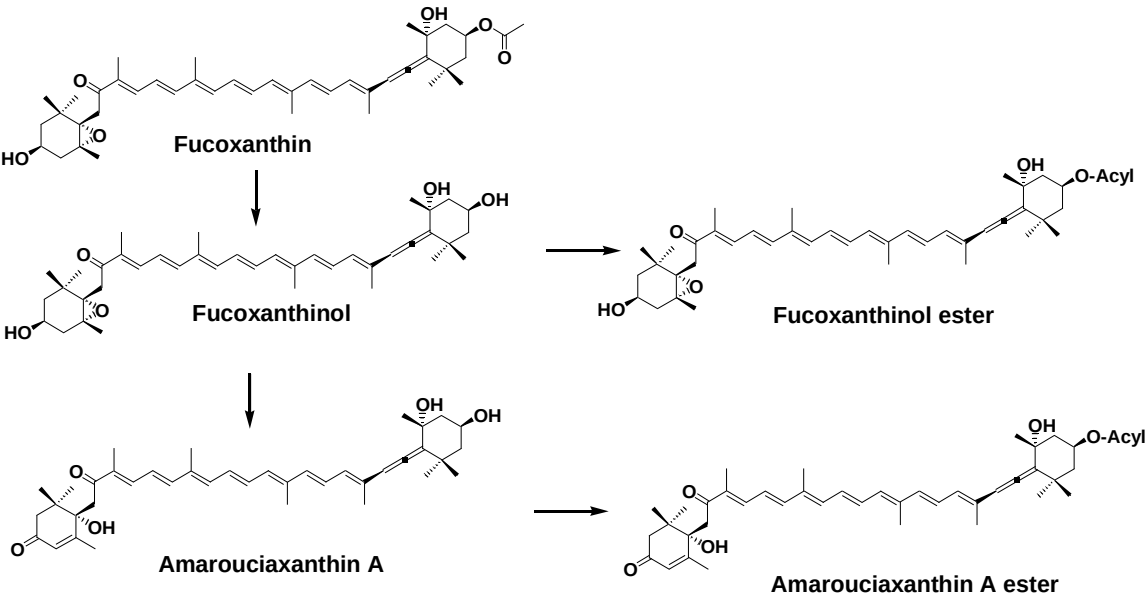


Fig. 1 Metabolism of fucoxanthin in rodents.

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Photoprotective function of reaction centers from the purple photosynthetic bacterium *Blastochloris viridis*

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[Introduction] Photosynthetic bacteria have been actively employed as research subjects to elucidate the primary processes of photosynthesis due to their relatively simple photosystems and the ease of preparing biochemical samples. While there are numerous studies on light-harvesting systems containing bacteriochlorophyll (Bchl) *a*, there is a limited body of research on light-harvesting pigment-protein complexes containing Bchl *b*¹⁾.

The photosystem of the purple photosynthetic bacterium *Blastochloris (Blc.) viridis* is exclusively composed of the LH1-RC complex, where the light-harvesting pigment-protein complex of LH1 envelops the reaction center (RC). It contains two carotenoid pigments (1,2-dihydroneurosporene and 1,2-dihydrolycopene) and a Bchl *b* dimer 1,2). Notably, it exhibits a Q_y absorption band in the near-infrared region (at 1010 nm), which is in a longer wavelength region compared to the Bchl *a*-type LH1 complex (see Fig. 1).

Purple photosynthetic bacteria can produce reactive oxygen species that are capable of damaging cells when exposed to excess light energy. However, the LH1-RC complex effectively prevents cell damage through photoprotection. This process involves deactivating excess energy and dissipating it as heat into the surroundings. In a prior study, concerns were raised about whether the two carotenoid pigments in LH1 serve a photoprotective function³⁾. In this study, we conducted an investigation into the spectroscopic properties and energy transfer pathways of carotenoids in RCs isolated from *Blc. viridis* and assessed their photoprotective function.

[Experimental] Cells of the *Blc. viridis* DSM 133 strain were cultivated photosynthetically in C-succinate media under anaerobic conditions at approximately 27°C for a duration of 5 days to 1 week. Subsequently, the cells were harvested through centrifugation, and the cell membranes were disrupted using a French Press to isolate the photosynthetic membranes. These photosynthetic membranes were then solubilized with lauryldimethylamine N-oxide (LDAO) to ultimately obtain Reaction Centers (RCs) from *Blc. viridis*.

Subnanosecond time-resolved absorption spectroscopic measurements of the obtained RCs were conducted, with excitations at the Bchl *b* Q_y bands using accessory Bchl *b* (at 832 nm) and the special pair Bchl *b* (at 970 nm) to investigate the process of excitation energy transfer from Bchl *b* to carotenoids.

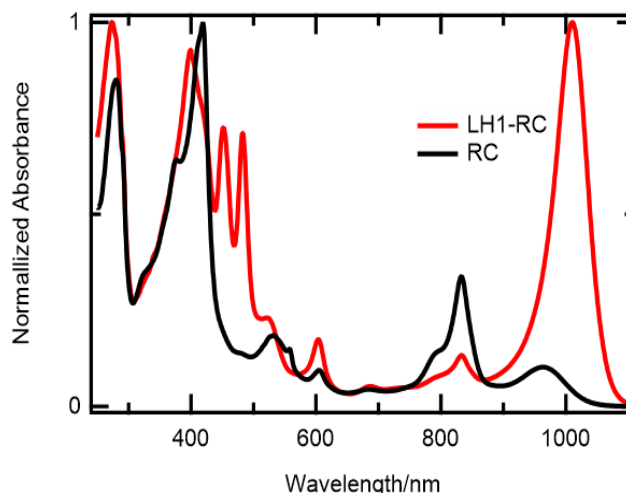


Fig 1. Absorption spectra of the LH1-RC and RC complexes isolated from *Blc. viridis*.

[Results] A global analysis of the acquired transient absorption (TA) data was conducted to generate Evolution Associated Difference Spectra (EADS). This analysis helped elucidate the spectroscopic properties and energy transfer pathways of carotenoids in the Reaction Centers (RCs) purified from *Ble. viridis*.

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Light-harvesting and photoprotection process by the dark-side carotenoid in the LH1 complex from purple photosynthetic bacteria, *Rhodospirillum rubrum*

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In the primary process of purple photosynthetic bacteria, solar energy that is captured by the light-harvesting (LH) complex is transferred to the reaction center (RC), where charge separation takes place [1]. Both LH complex and RC have carotenoids that play important roles as the light harvesters (to boost the light-harvesting capacity) and the photoprotectors (to dissipate the excess energy delivered to bacteriochlorophyll *a* (Bchl *a*)) [1]. One carotenoid (Car) molecule and 2/3 Bchl *a* molecules consist a pair of heterodimer. The arrangement of heterodimers is different for the species of photosynthetic organisms or the type of LH complex. The most typical arrangement is ring-like structure (**Fig. 1**). For example, 16 heterodimers line up in the circle in the LH1 complex from *Rhodospirillum* (*Rsp.*) *rubrum*. Besides, there are some exceptions like open-ring-like structure with 17 heterodimers in the LH1 complex from *Blastochloris viridis*, or the s-shape structure with the dimer of the 14 heterodimers in the LH1 complex from *Rhodobacter* (*Rba.*) *sphaeroides*.

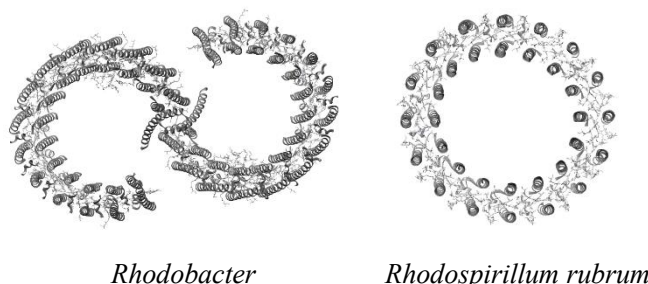


Fig.1. Schematic illustration of LH1 complex

Photosynthetic organisms synthesize different carotenoids for each species in order to realize the optimized light-harvesting and photoprotection process by carotenoids.

In this study, we have extracted and purified spheroidene from *Rba. sphaeroides* strain 2.4.1 and reconstituted it to the carotenoidless LH1 complex from *Rsp. rubrum* strain G9+. We have investigated the excited energy transfer (EET) efficiency from Car to Bchl *a* in the reconstituted LH1 and found that it was relatively low compared to the native LH2 complex from *Rba. sphaeroides* 2.4.1, but was still high (77%) [2]. Also, from femto second time resolved absorption spectroscopy, we have found that spheroidene conducts light-harvesting in the reconstituted LH1 complex as well as in the native LH2 complex. However, from sub-nano second time resolved absorption spectroscopy (pumped at 880 nm), free long-lived Bchl *a* cation which shorten the lifetime of the Car triplet was observed. Through these results, we concluded that spheroidene in the closed ring LH1 complex performs well as a light-harvester, but does not perform well as a photoprotector because it cannot fully dissipate the extra energy on the Bchl *a* and cause unstability of the complexes' conformation. That is why carotenoids of spheroidene chain are not synthesized in *Rsp. rubrum*.

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Isolation and purification of lutein isomers and their femtosecond time-resolved spectroscopic measurements

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【Introduction】

Xanthophylls are carotenoids that play an important role in photosynthesis. They are light-harvesting pigments that strongly absorb solar energy in the 450-550 nm wavelength range. Light in this wavelength range is not efficiently absorbed by chlorophyll, another photosynthetic pigment, so the presence of carotenoids enables the use of light in a wide wavelength range in photosynthesis. The characteristic absorption of xanthophylls in the visible light range is due to the transition from the ground state, S_0 , to the second excited state, S_2 , suggesting the existence of an additional excited singlet state, called S_1 or S^* , which is an optically forbidden singlet excited state in the relaxation process¹⁾. In this study, spectroscopic measurements of lutein isomers produced from high-purity lutein were performed to investigate the spectral features derived from the structural differences between the *trans* and *cis* forms.

【Experiments and Results】

A pure lutein microcrystalline sample was obtained by purifying marigold oil, which contains a mixture of several carotenoids. This sample was dissolved in benzene, iodine was added as a catalyst, and photoisomerization was performed by irradiation with halogen lamp light for 50 minutes to produce various *cis* isomers. From the solution containing a mixture of these isomers, each isomer was isolated and purified using HPLC with a column of OSAKA SODA SILICA, SG120 5 μm (10 mm i.d. $\times$ 250 mm) with benzene : ethyl acetate = 8 : 2 as the expansion solvent. Femtosecond time-resolved spectroscopic measurements were then performed on the five isomers obtained. Analysis of the results yielded spectral variations and different excited-state lifetimes for the different isomers. In this talk, we will report the results for the isomers in which particularly striking features were observed.

【Acknowledgments】

We thank Koyo Shokai Co., Ltd. for providing us with marigold oil.

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Real-time spectroscopy on fucoxanthin using sub-10 fs laser pulses

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Carbonyl carotenoids are common in marine algae and they participate in photosynthetic light-harvesting processes [1]. Characterization of the photophysical properties of carbonyl carotenoids is necessary to understand the highly efficient nature of energy transduction in the light-harvesting systems of marine organisms. The S_0 ground state of all-*trans*-carotenoids is denoted as $1^1A_g^-$, assuming C_{2h} symmetry of its polyene backbone. Strong absorption band in the visible region is attributed to the one-photon transition from S_0 to the S_2 ($1^1B_u^+$) state. A one-photon transition from S_0 to the lowest-lying excited singlet state, S_1 ($2^1A_g^-$), is optically forbidden because S_0 and S_1 have the same A_g^- symmetry. Ultrafast spectroscopic studies on carbonyl carotenoids have revealed several unique features that distinguish them from carotenoids without carbonyl groups. These include the dependence of the S_1 lifetime on solvent polarity which has been accounted for an intramolecular charge transfer (ICT) state located near S_1 [2]. However, it has not yet been clarified whether S_1 and the ICT state are strongly coupled or distinct electronic states.

When relaxation events following electronic excitation are considered, electron-molecular vibration interaction is a key issue. It is now possible to observe real-time oscillations of molecular vibrations of carotenoids under impulsive excitation using sub-20 fs laser pulses [3, 4]. Since the dephasing of coherent oscillations can reflect the interaction between the surrounding environment and electronic excitations, this dephasing provides important information when discussing the relaxation processes. In addition, time-resolved spectroscopy using sub-20 fs laser pulse can reveal the extreme ultrafast S_2 - S_1 /ICT relaxation reported to be shorter than 50 fs in fucoxanthin [5, 6]. In the present work, we performed 10 fs pump-probe measurement on fucoxanthin in solvents, since the dynamics of the electronic excitations as well as the coherent oscillations can be monitored simultaneously.

Home-built noncollinear optical parametric amplifier (NOPA) whose pulse width is about 8 fs was utilized as pump/probe pulses. **Fig. 1** shows the steady-state absorption spectrum of fucoxanthin in methanol and cyclohexane together with the output spectrum of NOPA. The red-tail of absorption band of fucoxanthin was excited by the blue-tail of NOPA spectrum. The pump-probe spectroscopic measurements using sub-10 fs pulses were performed based on a Michelson-type interferometer. The output pulses from NOPA were divided into pump and probe pulses. The pump pulse was modulated at 500 Hz by an optical chopper. The probe pulse was detected by a CMOS linear image sensor through a spectrometer [7, 8]. The instrumental response function and the precise zero-time delay were determined by the auto-correlation between pump and probe pulses using a 25 μm -thick type-I BBO crystal.

Fig. 2(A) and **(B)** respectively present the wavelength-time 2D counter plot and the photo-induced absorption spectra of fucoxanthin in methanol. In the spectra, the negative bleaching signal was observed due to the depletion of the ground state below 520 nm at earlier delay (<1 ps). Then, the broad positive change

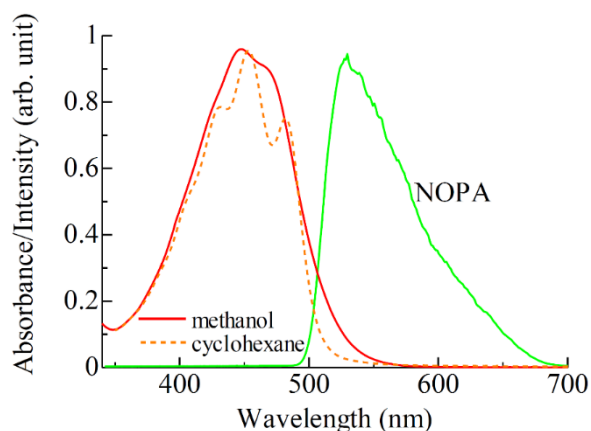


Fig.1: Steady-state absorption of fucoxanthin in methanol and cyclohexane and NOPA spectra.

appeared in the spectral range from 520 to 680 nm. This signal has been assigned to the transient absorption from the S_1 /ICT state to a higher lying excited state [9]. The S_1 /ICT transient absorption of fucoxanthin in methanol is composed of the S_1 -like band around 540 nm and ICT-like band around 630 nm [10]. Furthermore, short period oscillatory behavior was obviously observed in the all spectral range. Spectral interferometric patterns were observed in the photo-induced absorption spectra at the delay times of 0.0 and 0.05 ps as shown in **Fig. 2(B)** and a strong spike appeared around time zero in the kinetic trace shown in **Fig. 2(C)**. This signal has been assigned to the coherent signals including two-photon absorption and dynamical Stark effect without a generation of excited state population [11, 12]. The thin black line denotes the best fit curves using exponential decay/rise components and instantaneous response, convoluted with the instrument response function assuming Gaussian. The instantaneous response around time zero due to coherent signals agrees well with the instrument response function (12.3 fs) determined by the auto-correlation as shown in **Fig. 2(C)**. Four exponential components of 43 fs, 216 fs, 779 fs, and 18 ps were required to fit fully. Since the decay of the S_1 /ICT transient absorption is much longer than the delay range in this study (2 ps), the longest time constant of 18 ps is referred to the previously reported S_1 /ICT lifetime [5, 13]. The shortest time-constant of 43 fs is assignable to the S_2 lifetime. The S_1 /ICT transient absorption band shifted to shorter wavelength in the delay time ranging from 0.1 ps to 1.0 ps. Therefore, it is likely that the time-constants of 216 fs and 779 fs originate from vibrational relaxation in the S_1 /ICT.

The coherent oscillation with a period of about 20–30 fs was superimposed on a slowly varying exponential components (black line). The clear decay profile of the coherent oscillation was observed after the subtraction of the slowly varying exponential component (black line) from the raw kinetic trace, as shown in the inset in **Fig. 2(C)**. **Fig. 2(D)** exhibits the fast Fourier transform (FFT) intensity spectrum of the coherent oscillation shown in the inset in **Fig. 2(C)**. In the FFT intensity spectrum of fucoxanthin in methanol detected at 600 nm, three peaks were observed at 1033, 1150, 1533 cm^{-1} . Based on the steady-state Raman spectrum (not shown), these observed vibrational modes are assigned to CH_3 rocking, C-C stretching, and C=C stretching mode of fucoxanthin. As shown in **Figs. 2(A)** and (B), the S_1 and ICT-like transient absorption band coincidentally appeared following the excitation. Therefore, it can be concluded that the coupled S_1 /ICT state of fucoxanthin having two minima of potential energy surface of S_1 and ICT states are coincidentally populated from S_2 with the time-constant of 43 fs.

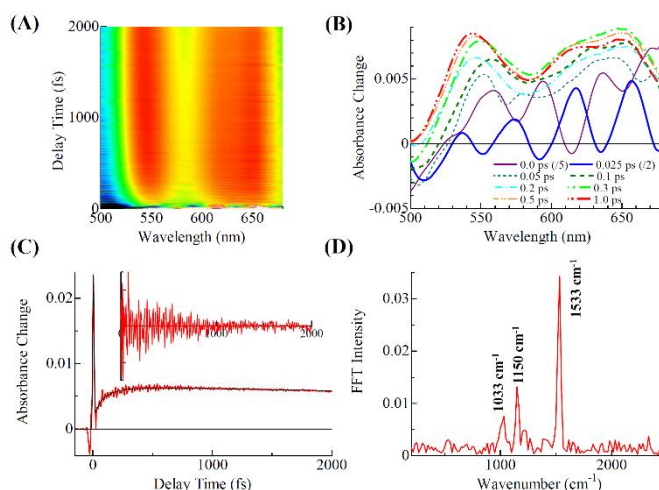


Fig.2: (A) Wavelength-time 2D counter plot and (B) photo-induced absorption spectra of fucoxanthin in methanol obtained by 10 fs pump-probe measurement. (C) Kinetic trace of the photo-induced absorption detected at 600 nm. Thin black line is the best fit curve. The inset shows the oscillatory component that is obtained by subtracting the slowly varying exponential components (black line) from the kinetic trace. (D) Fast Fourier transform (FFT) power spectrum of the coherent oscillation shown in the inset in Fig. 2(C).

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Investigation of ICT State of Carotenoid in Light-Harvesting Function by Femtosecond Absorption and Emission Spectroscopy

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We have interests on the light-harvesting function of carotenoids (Car) in the photosynthesis. The visible light is absorbed by the S_2 state of Car and the energy is transferred to bacteriochlorophyll (BChl) with high efficiency. However, the S_2 state relaxes quickly to the optically forbidden S_1 state in Car. Fucoxanthin (Fx) is characterized by its intra-molecular charge transfer (ICT) state in polar solvents. The excitation energy transfer (EET) from ICT to BChl is considered to be more efficient than that from S_1 , because ICT is the optically allowed state [1].

The dynamics of EET in LH1 reconstituted with Fx have been investigated by the femtosecond absorption spectroscopy using tunable pump pulses [2]. The dynamics have two time constants. The faster process is assigned to the EET from the S_2 state. The slower process might be the EET from ICT, but the time constant is much shorter than the lifetime of ICT.

We have measured the dynamics of Fx in methanol by multi-pump transient absorption spectroscopy [3]. The initially generated S_2 state relaxes to the S_1 /ICT equilibrium state with the time constants of sub-picosecond and 5 ps. The lifetime of the equilibrium state is 20.3 ps. The transient dynamics of the equilibrium state might be important in the EET, but it is difficult to distinguish between S_1 and ICT by the absorption measurement.

In this study, the dynamics of ICT have been investigated by femtosecond emission spectroscopy and analyzed in detail together with the results of the femtosecond absorption spectroscopy. The 4 transient components with time constants of 0.11 ps, 0.52 ps, 8.3 ps, and 20.3 ps are found in the absorbance changes. However, the 8.3 ps component is not observed in the emission. The 0.11 ps, 0.52 ps, and 20.3 ps components are assigned to S_2 , ICT* (unrelaxed ICT state in the relaxation to the equilibrium state), and ICT of the equilibrium state, respectively, as shown in **Fig.1**. The 8.3 ps component is assigned to the relaxation in the electronic ground state. The efficient sub- picosecond energy transfer from Fx to BChl is assigned to occur from the transient state before the S_1 /ICT equilibrium state.

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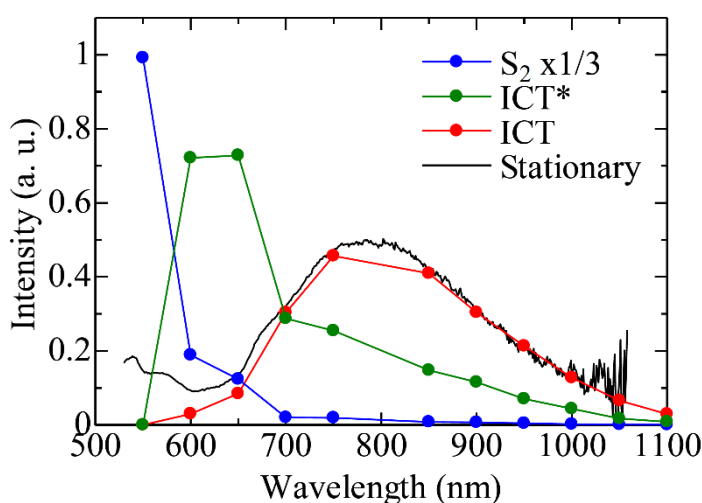


Fig. 1. Species associated emission spectra of fucoxanthin in methanol.

Solvent dependence on the emissive state of a carbonyl carotenoid, siphonaxanthin

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1. Introduction

The singlet energies of carotenoids are usually expressed as the S_0 ($1A_g^-$), S_1 ($2A_g^-$), and S_2 ($1B_u^+$) states based on the C_{2h} symmetry of polyene backbone. In this representation, "+" and "-" denote ionic and covalent states, respectively. The ionic state is sensitive to the external perturbations, whereas the covalent state is not. The fluorescence from the S_1 state is not observed because of symmetrically forbidden transfer. A carbonyl carotenoid, which has a carbonyl group asymmetrically attached to the conjugated backbone such as siphonaxanthin (Sx), has the intramolecular charge transfer (ICT) state (Figure 1). It is strongly associated to the shortening of the S_1 lifetime and observed only in polar solvent [1]. The phenomenon has been explained by a double minimum potential model of the S_1 state: Upon excitation to the S_2 state, the S_1 state is populated via internal conversion, and equilibrated to the ICT state. In polar solvent the ICT state is stabilized (low-energy shift) and more populated, but in nonpolar solvent, it is not [2]. According to this model, carbonyl carotenoids emit fluorescence from the S_1 state in nonpolar solvent but from both the S_1 and ICT states in polar solvent. However, the emission from the ICT state is expected to extend above 800 nm, which cannot be measured with common fluorometers, and this has not yet been observed. In this work, we used a home-made setup to measure the fluorescence spectra of Sx dissolved in a series of nonpolar and polar solvents at room and liquid nitrogen temperatures.

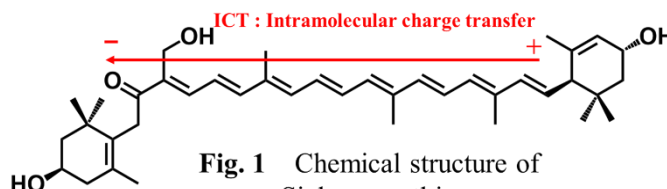


Fig. 1 Chemical structure of Siphonaxanthin

2. Experimental

Synthesized Sx [3] was purified using HPLC. Purified Sx was dissolved in a series of solvents shown in Table 1. The solution was poured into an NMR tube (4.24 mm i.d.), which was then submerged in liquid-nitrogen in a custom-made Dewar. The sample was excited with 475 nm light from an LED light source (SPECTRA X Light Engine, Lumencor). A photonic multichannel analyzer (PMA-12, C10027-02, Hamamatsu) was used to measure the emission up to 1,100 nm in L-format with a long-pass filter (Hoya) blocking the excitation light.

Table 1. Solvents and these dielectric constants.

	Solvents	Dielectric constant
Protonic polar solvents	Methanol	33
	1-Decanol	7.6
Nonprotic polar solvents	Acetonitrile	37.5
	Tetrahydrofran	7.4
Nonpolar solvents	<i>n</i> -Hexane	2.0
	Toluene	2.3

3. Result

Fluorescence spectra of Sx at room temperature represent clear difference between polar and nonpolar solvents as shown in Figure 2 (top). On the other hand, fluorescence spectra at 77 K are almost identical among the solvents measured and show clear vibrational structures (Figure 2 bottom). The peak maximum can be assignable to the $S_1 (v = 0) \rightarrow S_0 (v = 2)$ transition according to the literature [4], and the 0-0 transition energy of the S_1 state is determined to be $15,900 \text{ cm}^{-1}$. These results suggest that the fluorescence occurs from the S_1 state at 77 K, but from both the S_1 and ICT states at room temperature. In other words, the excitation is equilibrated between the S_1 and ICT states beyond the potential barrier at room temperature, whereas at 77 K this barrier cannot be overcome, and the excitation is localized in the S_1 state. This work is a first direct observation to support the double-minimum model of the S_1 /ICT state and provide a novel insight into the behavior of the enigmatic ICT state.

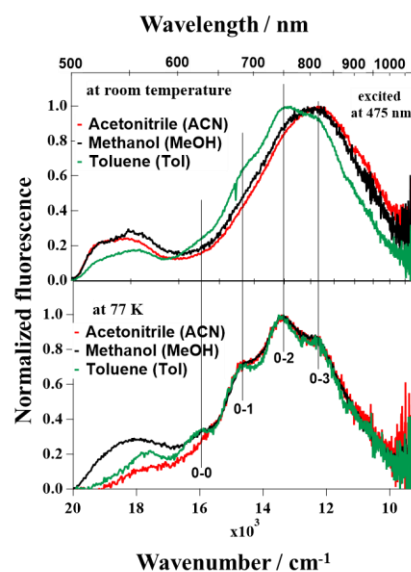


Fig. 2 Fluorescence spectra of Sx at room temperature (top) and at 77 K (bottom)

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The siphonaxanthin structure in photosynthetic antenna SCP from marine macroalga *Codium fragile*

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Light-harvesting complex II (LHCII) absorbs sunlight and transfers its excitation energy to the photosystems. Marine macroalga, *Codium fragile*, has unique LHCII, SCP (Siphonaxanthin-chlorophyll a/b binding protein), that can absorb blue-green light to adapt a weak blue-green light environment on the sea bottom¹. The green absorption of SCP is come from carbonyl conjugated carotenoid, Siphonaxanthin (Sx) and its fatty acid ester (Sn). However, the 3D-structure of SCP has not determined yet, the determination how such carotenoid have green absorption by binding to SCP complex.

Recently, we determined 3D-structure of SCP at 2.78 Å resolution by Cryo-EM². The structure of SCP was a trimer which is common in plants and green algae (**Fig. 1**). Compared to LHCII from higher plant, the binding pigment was partially replaced, and the two lutein-binding sites were replaced by Sx and Sn. The Sx-binding site was more polar than Sn, and the conjugation system was locally twisted (**Fig. 2**). In addition, two hydrogen bonds were present between the 8C=O of Sx and the cysteine residue, and between the 19OH group of Sx and the carbonyl of the main chain. Therefore, the Sx, but not Sn, is responsible for the green absorption in SCP. How such green absorption happens in pigment-protein complexes will be discussed.

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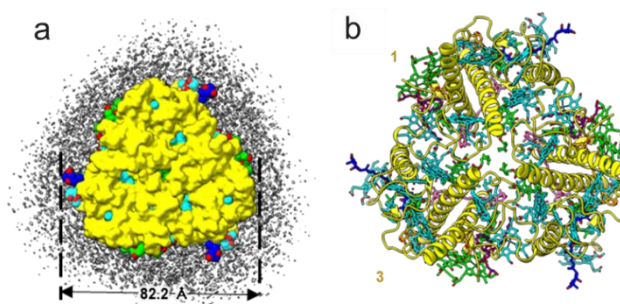


Fig. 1 The whole structure with maps (a) and only model (b)

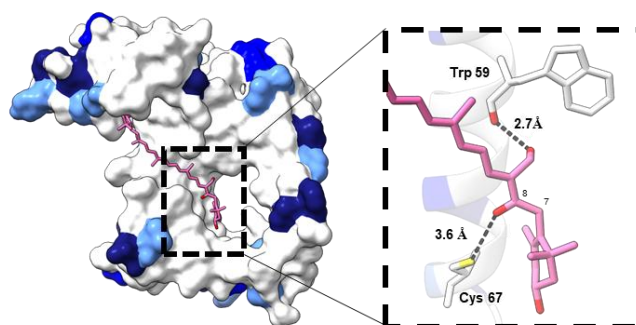


Fig. 2 The siphonaxanthin structure in SCP complex. The two hydrogen bonds have been formed in its binding site.

Synthesis and Biological Evaluation of 9-*cis*-Retinoic Acid Analogs Having a 2*H*-Chromene Ring

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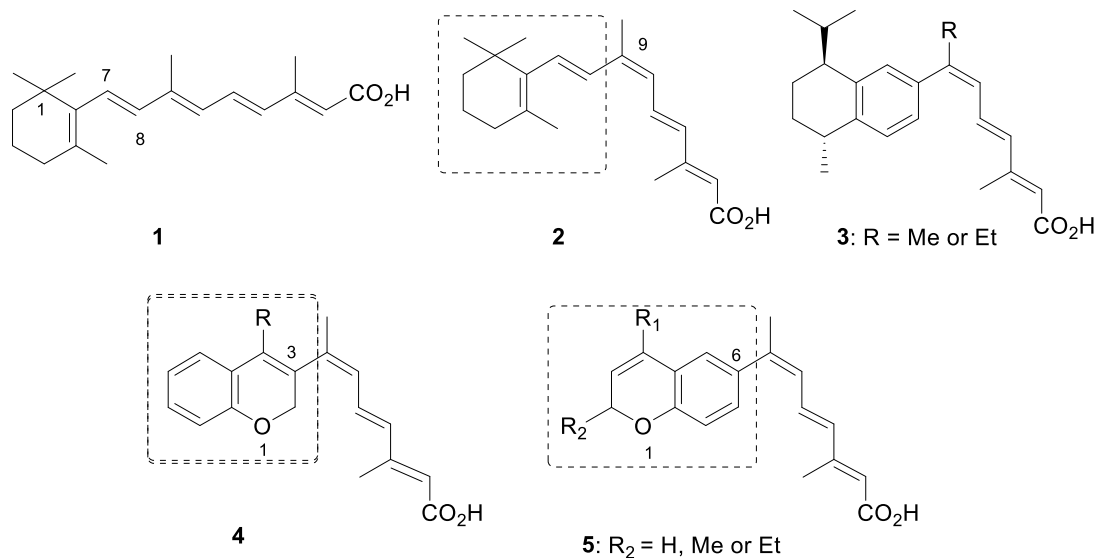
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All-*E*-Retinoic acid (ATRA, **1**) and 9*Z*-retinoic acid (9CRA, **2**) are metabolites of vitamin A and these compounds are the natural ligands of the retinoic acid receptors (RAR) and retinoid X receptors (RXR), which are members of nuclear receptor superfamily and exhibit the significant biological functions.¹⁾ Much effort has been directed toward for the preparation of receptor-selective retinoids in order not only to define the functions of each receptor but also to develop therapeutic agents. We have also studied the stereoselective synthesis and structure–activity relationship of retinoid analogs. Recently, we prepared 9*Z*-retinoic acid analogs, in which the cyclohexene ring and adjacent double bond in retinoic acid (**1**) were replaced with cyclic terpenoid moieties.²⁾ The analogs **3**, substituted by the benzene fused menthane skeleton, exhibited the higher activity than 9CRA.

It is well known that retinoic acid analog having a high lipophilic character causes undesirable side effects for clinical applications. So, in order to diminish the lipophilicity of analogs **3**, we prepared two kinds of analogs contain 2*H*-chromene skeleton **4**, **5** and evaluated their biological activity.



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Carotenoids in the LH1 complex of red photosynthetic bacteria and Interactions with the Surrounding Environment: Reconstructions and Spectroscopic Investigations

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In this study, we created an artificial LH1 complex artificially incorporating a specific carotenoid by using a "reconstitution method" on the light-harvesting pigment protein LH1 complex from red photosynthetic bacteria. Furthermore, we compared this artificial LH1 complex with carotenoids in organic solvents and investigated the interaction between the carotenoids and their surrounding environment using spectroscopic techniques. In the photosynthetic membrane of red photosynthetic bacteria, bacteriochlorophyll (Bchl) and carotenoids interact with membrane proteins (LH1- α and LH1- β polypeptide) to form the LH1 complex. Carotenoids play an important role in stabilizing the structure of the LH1 complex, absorbing light at wavelengths that Bchl *a* cannot absorb, and transmitting it to Bchl *a* [1].

The experimental procedure is as follows. The linear carotenoid neurosporene was reconstituted in the LH1 complex prepared from *Rhodospirillum rubrum* G9+ strain, a red photosynthetic bacterium lacking carotenoids. The steady-state spectrum of the reconstituted LH1 complex was measured and compared with the data of neurosporene dissolved in organic solvents. This comparison suggested that the steady-state absorption spectra of carotenoids in the LH1 complex and in benzene were similar (see **Fig. 1**). In addition, we attempted to gain detailed insight into the interaction between carotenoids and their surrounding environment by comparing the spectral shapes and lifetimes of the excited-state components obtained by time-resolved absorption spectroscopy measurements and global analysis.

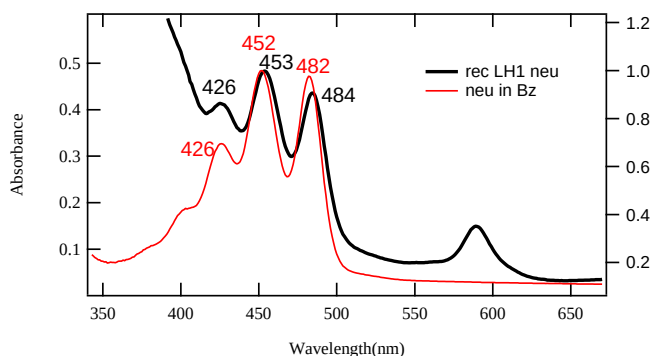


Fig. 1. Comparison of steady-state absorption spectra of neurosporene reconstituted in benzene solution (red) and in the LH1 complex.

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Reconstitution of carotenoids of the spirilloxanthin series in the LH2 complex from the purple photosynthetic bacterium *Rhodobacter sphaeroides* strain R26.1

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In the initial process of photosynthesis in purple photosynthetic bacteria, carotenoids absorb solar energy. The energy is converted to chemical energy and transferred to bacteriochlorophyll *a*, which moves to the reaction center, where the photosynthetic light reaction proceeds. In this process, carotenoids play a role in light-harvesting and photoprotection. The excitation energy transfer (EET) efficiency from carotenoids to bacteriochlorophyll varies from 28~92% depending on the type of carotenoid [1]. The carotenoids of the spirilloxanthin series, anhydrorhodovibrin and spirilloxanthin, used for reconstitution have longer conjugated double bonds (12 and 13, respectively) than other purple photosynthetic bacteria and absorb visible light in the long-wavelength region. The detailed study of EET is meaningful for the creation of artificial photosynthetic antennae and will contribute to the realization of artificial photosynthesis.

In this study, carotenoids of the spirilloxanthin series were isolated from the purple photosynthetic bacterium *Rhodospirillum rubrum* strain S1, which lacks the LH2 complex in its light-harvesting system, and reconstituted in the LH2 complex of the carotenoidless strain *Rhodobacter sphaeroides* R26.1 [2]. We confirmed that the carotenoids were reconstituted with absorption (Fig. 1) and fluorescence excitation spectra, and time-resolved absorption measurements were used to investigate the excitation energy transfer process between the carotenoids and bacteriochlorophyll.

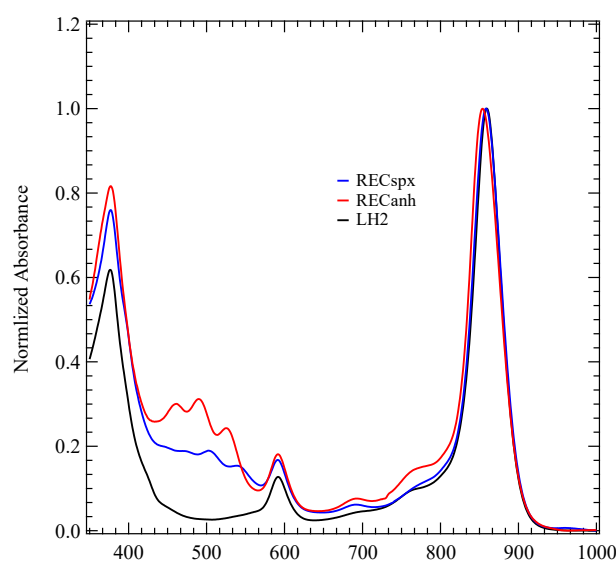


Fig 1. Absorption spectra of LH2 complex and spirilloxanthin reconstituted LH2 (RECsp) and anhydrorhodovibrin reconstituted LH2 (RECanh)

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A water-soluble blue carotenoprotein decorates marine blue sponge

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Marine animals display diverse vibrant colors, but the mechanisms underlying their specific coloration remain to be clarified. Blue coloration is known to be achieved through a bathochromic shift of the orange carotenoid astaxanthin (AXT) by the crustacean protein crustacyanin, but other examples have not yet been well investigated. Here, we identified a novel water-soluble blue carotenoprotein responsible for the coloration of the *Haliclona* sp. marine blue sponge.

A blue protein was purified as a secreted glycoprotein of a heterodimer of 40 kDa and a sponge blue body was found to be derived from this protein. We show that it binds orange AXT and mytiloxanthin (MXT) and exhibits a bathochromic shift. However, using reconstitution assays, we found that incomplete bathochromic shifts occurred when the protein bound to only AXT or MXT. Amino acid sequencing showed that the protein was a member of EPD superfamily of glycoproteins that are distinct from crustacyanin. EPDs were originally identified as piscine brain glycoproteins for memory consolidation and neuronal regeneration. EPD-related proteins of relatively unknown functions are widely distributed in eukaryotes. We identified a blue protein as the first carotenoprotein among EPDs, and named EBD-BCP1. X-ray crystallography of EPD-BCP1 revealed that two carotenoids are specifically bound to the heterodimer interface. However, carotenoids were aligned in parallel to each other like crustacyanin.

Taken together, we identified an EPD in a basal metazoan as the second blue protein that decorates the sponge body which are distinct from crustacyanin. We plan to study the possibility that EPDs constitute the body color of various organisms.

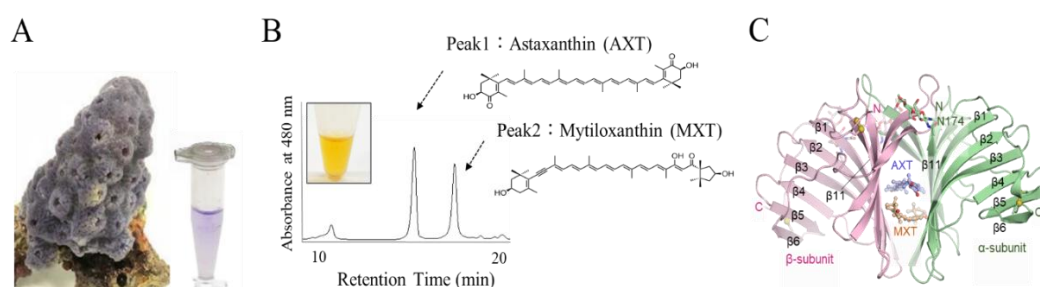


Fig. 1. Purified blue protein EPD-BCP1 from blue sponge and its binding carotenoids.

A, a blue marine sponge *Haliclona* sp. (left) and a purified blue protein EPD-BCP1 (right). B, binding carotenoids on EPD-BCP1 detected with a photodiode array detector and two binding carotenoids, identified as peak1: astaxanthin and peak2: mytiloxanthin. The extraction of carotenoids dissolved in acetone in the inset. C, X-ray crystal structure of EPD-BCP1.

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This is an abstract of the 35th Annual Meeting on Carotenoid Research, on 31 October 2023 at Japan Women's University, Bunkyo-ku, Tokyo, Japan.

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Promiscuous carotenoid pathways for colorimetric screening of intracellular IPP/DMAPP ratio

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Introduction

Isoprenoids, including carotenoids and terpenoids, are synthesized by the stepwise addition of IPPs (elongation units) to DMAPP (starter unit). The number of IPP and DMAPP consumed is unique for each of the isoprenoids, and it is therefore necessary to tailored the intercellular IPP/DMAPP ratio for the efficient production of each isoprenoid. In this report we report the construction of a system for visualizing intracellular IPP/DMAPP ratio.

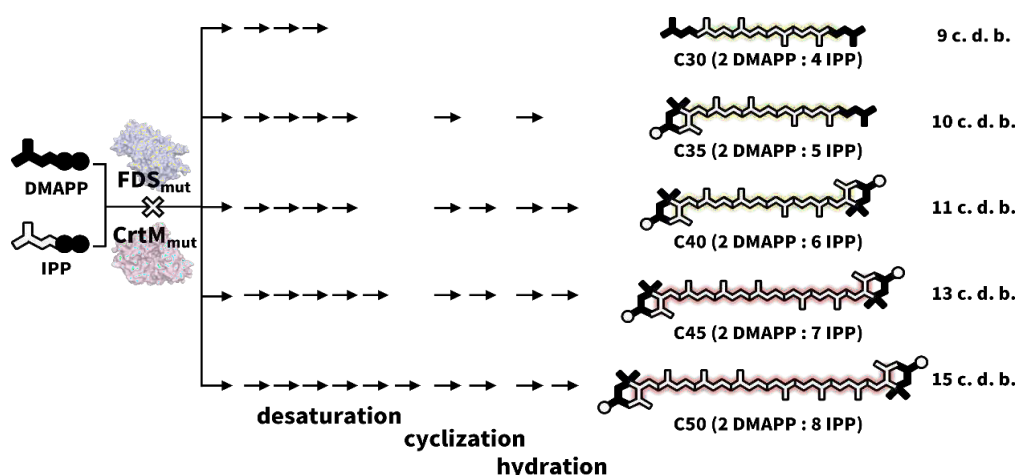


Fig. 1. Visualization of cellular IPP/DMAPP ratio through carotenoid pigment.

Results and Discussion

While natural carotenoid pathways are highly size-specific, artificial pathway for non-natural carotenoids are nonspecifically produces the mixture of carotenoids with different sizes in general. We co-expressed size mutants of farnesyl diphosphate synthase (FDS) and diapophytoene synthase (CrtM) in *Escherichia coli* and found that the pair of mutants FDS_{Y81A} and CrtM_{W38A} yielded mixture of phytoenes with C₃₀, C₃₅, C₄₀, and C₄₅ backbones. Additional expression of CrtN_{mut}, CrtIp, CrtY, and CrtZ resulted in the complex mixture of carotenoid pigments.

We attempted to forcefully alter the intercellular IPP/DMAPP ratio by additionally expressing isopentenyl monophosphate enzyme (IPK), which is known to produce DMAPP and IPP, by phosphorylating DMAOH and IOH, respectively, into the medium. We found that the color of colonies changed strikingly, depending on the ratio of DMAOH/IOH supplemented in the medium. This system was applied for high-throughput screening for the genetic factors that affect the intracellular IPP/DMAPP ratio.

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Laboratory Evolution of Mini-Carotenoid Biosynthetic Enzymes

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Research background

All natural carotenoids are synthesized either from C₃₀ and C₄₀ pathways. They have value as antioxidants, colorants, and nutraceuticals. But when it comes to their performance as specialty lubricants and SAF (Sustainable aviation Fuel) materials, smaller molecular weight (C₁₀-C₂₅ in size) is desirable. In this study, we attempted to establish a biosynthetic pathway for the "mini-carotenoids" by reconstructing the size specificity of the substrates and by the laboratory evolution of CrtM, a natural C₃₀ backbone synthase.

Results and Discussion

The smallest naturally existing carotenoids are biosynthesized from the C₃₀ skeletal phytoene (4,4'-diapophytoene) by bimolecular condensation of FPP (C₁₅PP) catalyzed by CrtM¹. In this work, several residues that constitute the bottom of the S2-site of *Staphylococcus aureus* CrtM was randomized to establish steric hindrance against binding of C₁₅PP and C₁₀PP only to the residues. Among the CrtM mutants thus created, we isolated a mutant that synthesized C₂₀-phytoene (a condensation product of C₁₅PP and C₅PP), albeit weakly. This mutant is the world's first carotenoid synthase that uses C₅PP as a substrate. In this presentation, we report our continuing effort to further evolve this mini-carotenoid biosynthetic enzyme using a novel selection system.

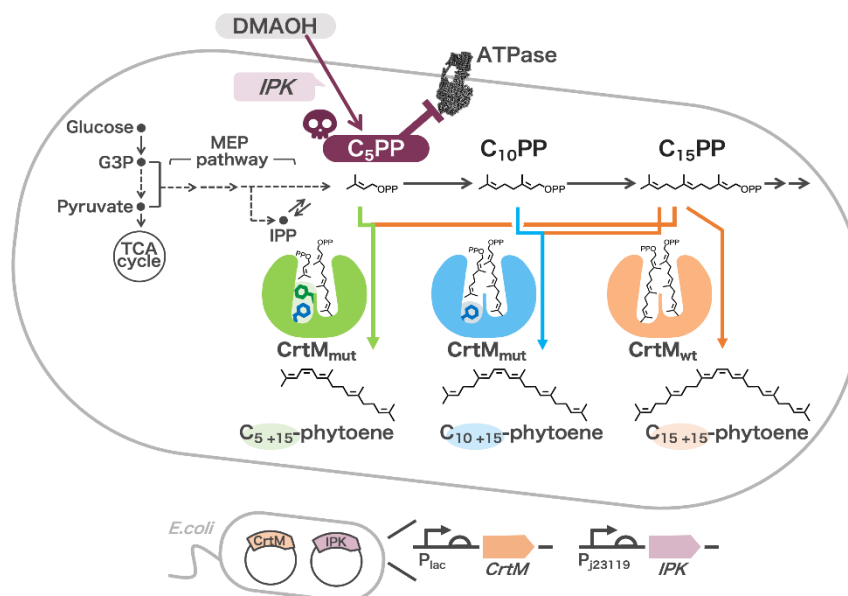


Fig1. Biosynthetic route to mini-carotenoids.

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Bioproduction of neoxanthin in *Escherichia coli*

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Violaxanthin, neoxanthin, and lutein are carotenoids (xanthophylls) biosynthesized specifically in land plants and part of algae. Nowadays, it is feasible to produce violaxanthin and lutein in *Escherichia coli* by pathway engineering, whereas there is no report to synthesize neoxanthin in *E. coli*. In this study, we tried to produce neoxanthin in the genetically engineered *E. coli* using several neoxanthin synthase genes. So far, several genes have been reported to code for neoxanthin synthases, e.g., NSY (NXS), ABA4 and VDL, which were assigned to catalyze a reaction for forming neoxanthin from violaxanthin. However, neither gene of these was common in plants or algae that biosynthesize neoxanthin, nor was confirmed by the *E. coli* complementation system. In this study, we found that the *E. coli* cells failed to generate neoxanthin, when the *NSY* or *ABA4* gene was introduced. On the other hand, the algal *VDL* gene (*PtVDL1*) was functional in recombinant *E. coli* cells accumulating violaxanthin to produce neoxanthin. Moreover, deepoxyneoxanthin was also produced in this *E. coli*. This result notes that *VDL* is one of veritable neoxanthin synthase genes.

(This work was supported by JSPS KAKENHI Grant Number 21K05773.)

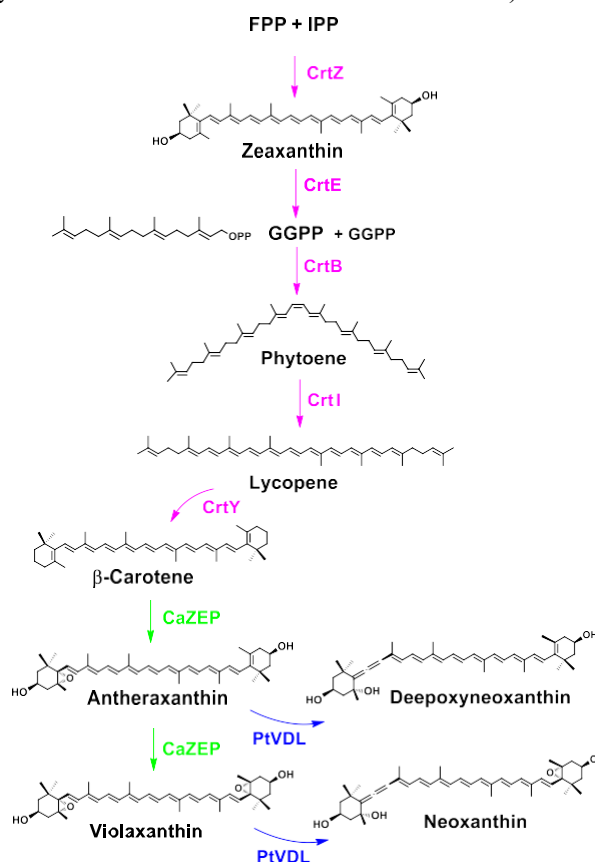


Fig. 1. Biosynthetic pathway of neoxanthin and deepoxyneoxanthin

Production of a rare carotenoid ‘4-ketolutein’ using new combination of known C40 carotenoid biosynthesis genes and investigation of its $^1\text{O}_2$ quenching activity

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【Purpose】

We are currently conducting research to produce rare and antioxidant carotenoids by recombinant *E. coli* transformed with known carotenoid biosynthesis genes in a new pattern. In this study, we aimed to produce the rare carotenoid ‘4-ketolutein’ which was expected to possess potent antioxidant activity due to its chemical structure.

We report about 1) preparation of recombinant *E. coli*, 2) culture of recombinant *E. coli*, 3) isolation of the produced carotenoids, 4) structural determination of the isolated carotenoids, and 5) evaluation of antioxidant ($^1\text{O}_2$ quenching) activities of the produced carotenoids including 4-ketolutein.

【Method】

1) Preparation of recombinant *E. coli*

We cultured recombinant *E. coli* carrying the biosynthesis gene of α -carotene (*HpIDI*, *crtE*, *crtB*, *crtI*, *MpLCYb*, *MpLCYe*), a ketolase gene which oxidize C-4 in β -ionone ring (*crtW*), and hydroxylase genes which introduce OH at C-4 in β -ring (*crtZ*) and C-3 in ϵ -ring (*MpCYP97C*) as shown in **Fig. 1**.

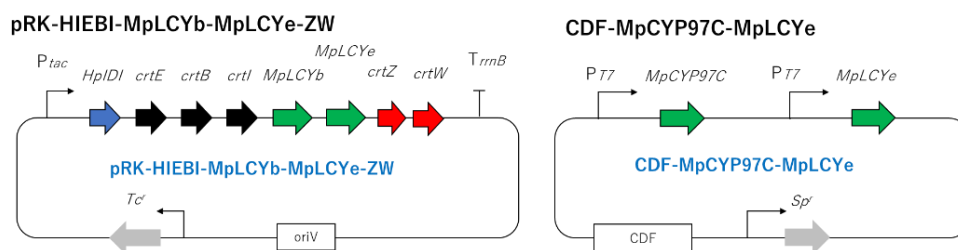


Fig. 1. Plasmid composition

2) Culture of recombinant *E. coli*

The recombinant *E. coli* was cultured in sakaguchi flasks using 2YT + fructose medium containing tetracycline 10 mg/L, spectinomycin 100 mg/L and chloramphenicol 30 mg/L mM at 27°C for 48-72 hours.

3) Isolation of the produced carotenoids

The cells from 2 L culture were collected by centrifugation and the produced carotenoids were extracted with CH_2Cl_2 -MeOH (1:1). Preliminary DAD HPLC analysis of the extract proved the production of four carotenoids (1-4). The extract was concentrated to small volume and partitioned with *n*-hexane/90% MeOH, and 90% MeOH layer containing the produced carotenoids were concentrated to dryness to afford orange syrup (1.1 g). The orange syrup was chromatographed by preparative ODS HPLC (column : Develosil C30-UG 2.0 x 25 cm, solvent : MeOH:THF (9:1), flow : 8.0 ml/min, detection : 200~700nm(DAD)), and the peaks of t_R 15 min, t_R 17min and t_R 19 min were collected. In this chromatography, pure 1 (2.0 mg) and 4 (1.1 mg) were obtained from the peaks of t_R 15 min and t_R 19 min, respectively. Since the peak of t_R 17 min was still mixture of 2 compounds, this peak was further purified

by preparative silica HPLC(column : nacalai tesque cosmosil 5SL-II, solvent: hexane:acetone (4:1), flow: 3.0 ml/min, detection : 200~700nm(DAD)), and the peaks of t_R 16 min, t_R 19min were collected to afford pure **2** (0.6 mg) and **3** (0.6 mg).

4) Structural determination

The structures of isolated **1–4** were analyzed by HRESI-MS (+) and 1D (^1H and ^{13}C) and 2D (^1H - ^1H DQF COSY, HSQC, HMBC, and NOESY) NMR spectra.

5) The $^1\text{O}_2$ quenching activities of **1–4**

When a test tube containing methylene blue and linoleic acid solution was irradiated with light, singlet oxygen is generated by methylene blue and oxidation of linoleic acid progress to afford conjugated linoleic acid which possess an UV absorption maximum at 235 nm. When carotenoids which possess singlet oxygen scavenging properties exist in the tube, the production of conjugated linoleic acid decrease. Thus, we can evaluate the singlet oxygen scavenging activity of carotenoids by the decrease rate of OD_{235} after light irradiation.

【Results and Conclusions】

Compounds **1–4** were identified as 4-ketolutein (**1**), 3,3'- β , β -caroten-4-one (**2**), lutein (**3**), and zeaxanthin (**4**), respectively by the detailed analyses of MS and NMR spectra. The structures of **1–4** were shown in Figure 2.

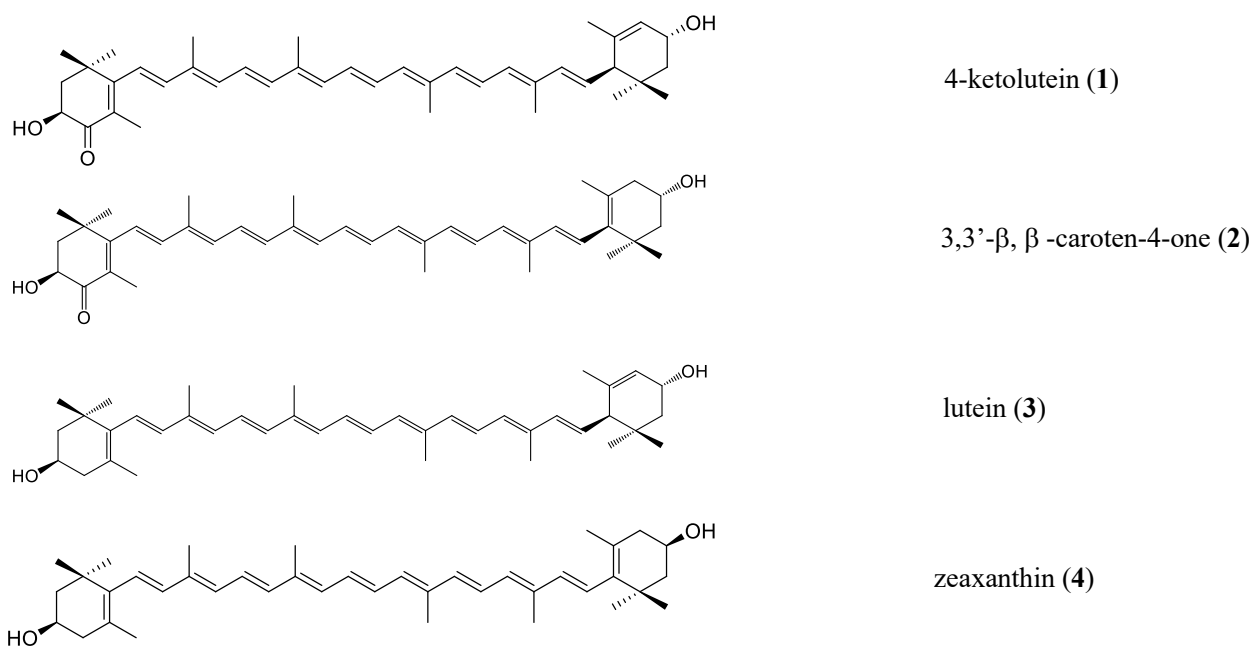


Fig. 2. Chemical structures of compound **1–4**

All compounds possessed the structures that could be produced using the biosynthetic genes used in this study. This is the first study reporting the production of these rare carotenoids in recombinant *E. coli*. The singlet oxygen scavenging activity of **1–4** were evaluated. The IC_{50} of compounds **1–4** were 4.6 μM (**1**), 4.7 μM (**2**), 9.2 μM (**3**), and 12.2 μM (**4**) respectively. (Astaxanthin IC_{50} 4.3 μM)

Myxol production in *E. coli* using gene clusters from *Nonlabens spongiae*

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Myxol is a monocyclic carotenoid with β -end group and is one of the rare carotenoids isolated from several microorganisms such as marine flavobacteria and cyanobacteria. Myxol has been reported to possess stronger antioxidative activity than β -carotene and zeaxanthin with dicyclic structure, but due to their rarity, little studies on the physiological functions and biological activities. In addition, the biosynthetic pathway is not well understood [1,2,3]. We have previously shown that marine flavobacteria *Nonlabens spongiae* JCM 13191 produced myxol as major carotenoids and isolated a carotenoid biosynthesis gene cluster for myxol production from this flavobacteria [4]. In this study, we performed functional analysis of the candidate genes encoding carotene hydroxylase (CrtZ), carotenoid 3,4-desaturase (CrtD), carotenoid 1,2-hydorataase (CruF), lycopene cyclase (CrtYm), and carotenoid 2'-hydroxylase (CrtA-OH), which are responsible for biosynthesis from lycopene to myxol, and investigated the production of myxol in *E. coli* using the reconstructed carotenoid biosynthesis gene cluster of *N. spongiae*.

To test the catalytic activity, the carotenoid biosynthesis genes of *N. spongiae* were introduced into lycopene-accumulating *E. coli* strains, which contained the plasmid pACCRT-EIB with *crtE*, *crtB*, and *crtI* from *Pantoea ananatis* [5]. When *cruF* or *crtYm* of *N. spongiae* was introduced into the lycopene-accumulating *E. coli* strain, dihydroxylycopene or β -carotene was mainly produced, respectively, and when both *cruF* and *crtYm* were introduced, 1'-hydroxy- γ -carotene is mainly produced. In addition to *cruF* and *crtYm*, when *crtD* or both *crtD* and *crtA-OH* of *N. spongiae* were introduced into the lycopene-accumulating *E. coli* strain, 1'-hydroxytorulene or deoxymyxol was mainly produced, respectively. Furthermore, when *cruF*, *crtYm*, *crtD*, *crtA-OH*, and *crtZ* of *N. spongiae* were introduced into the lycopene-accumulating *E. coli* strain, myxol was produced. Based on these results, we propose a biosynthetic pathway for the myxol in *N. spongiae* (Fig.1)

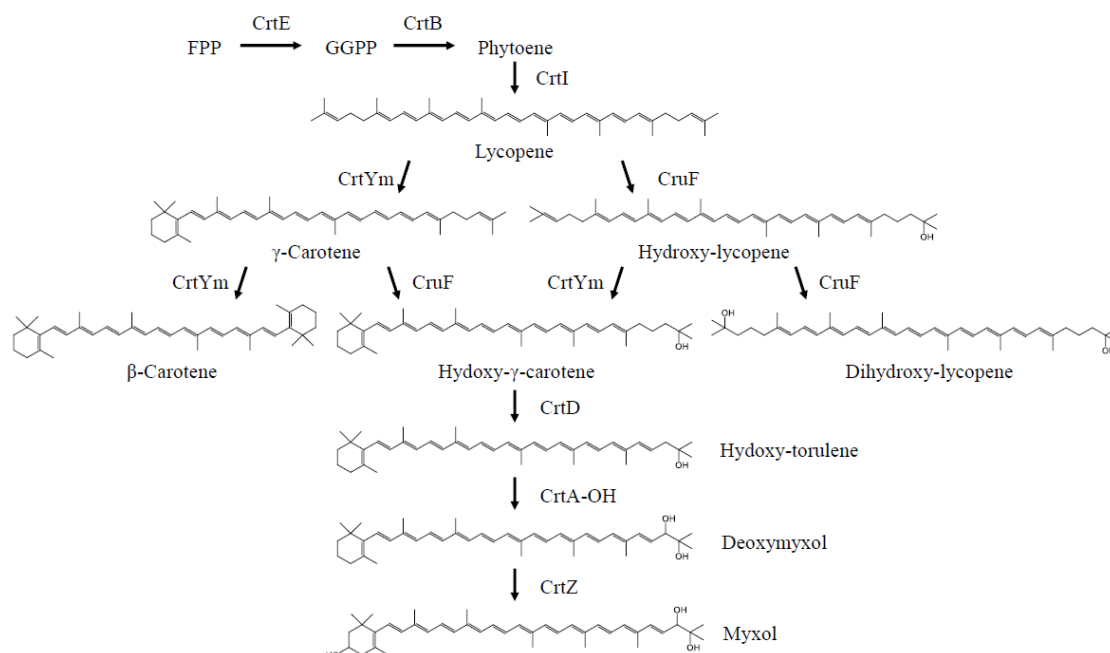


Fig.1 Proposed biosynthetic pathway of the myxol in marine flavobacteria *Nonlabens spongiae* JCM 13191

Next, the carotenoid biosynthesis gene cluster from *N. spongiae* was reconstructed by reversing the orientation of *crtA-OH* so that all genes in the cluster had the same transcription direction and adding *crtE* encoded geranylgeranyl diphosphate synthase, which was isolated at a different locus from the carotenoid biosynthesis gene

cluster. When the reconstructed gene cluster was introduced into *E. coli*, myxol was accumulated predominantly, indicating that this gene cluster could be used for myxol production in *E. coli*.

This is an abstract of the 35th Annual Meeting on Carotenoid Research, on 24-26 November 2023 at Japan Women's University.

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Preparation of astaxanthin-derived apocarotenoids and evaluation of their biological activities

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1. Introduction

Carotenoid cleavage products, i.e., apocarotenoids, are formed through enzymatic or nonenzymatic reactions. In recent years, various apocarotenoids derived from carotenes and xanthophylls have been found in human and rodent tissues. Furthermore, it has been reported that some carotenoids in foods are converted to apocarotenoids during manufacturing and storage. However, information regarding the biological activities of apocarotenoids is limited. In this study, we prepared some apocarotenoids derived from astaxanthin (Ax) and evaluated their effects in suppressing inflammation and adipocyte differentiation, which are involved in the development of various chronic inflammatory diseases.

2. Materials and Methods

Structural analysis of apocarotenoids produced by oxidation reaction of Ax with potassium permanganate (KMnO₄) was performed using LC-MS and NMR. To evaluate the anti-inflammatory effects of the apocarotenoids, inflammation was induced by LPS stimulation of mouse macrophage-like RAW264.7 cells or co-culture of mouse adipocytes 3T3-L1 and RAW264.7 cells, and then mRNA expression in the cells and the amounts of secreted proteins in the culture media were determined by quantitative PCR (qPCR) and ELISA, respectively. Furthermore, to evaluate the suppressive effect on adipocyte differentiation, 3T3-L1 preadipocytes were differentiated using a standard protocol, and then the accumulation of lipid droplets and mRNA expression of differentiation marker genes in the adipocytes were measured using oil red O staining and qPCR, respectively.

3. Results and Discussion

Six Ax-derived apocarotenoids were identified in the Ax oxidation products generated by KMnO₄. These apoastaxanthinals suppressed the increase in mRNA expression of inflammatory cytokines *Il6* and *Il1b* induced by LPS stimulation in RAW264.7 cells. Especially, apo-12'-astaxanthinal and apo-14'-astaxanthinal, which are cleaved near the central site of the polyene chain, exhibited strong anti-inflammatory activity [1]. Furthermore, the apoastaxanthinals suppressed adipocyte differentiation. Among them, apo-15'-astaxanthinal, which was cleaved at the central site of the polyene chain, showed the most potent activity. These activities of the apoastaxanthinals against macrophages and adipocytes were higher than those of the parent compound Ax, suggesting that cleavage of Ax may enhance its anti-inflammatory activity and ability to suppress adipocyte differentiation.

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Exploration of aerial microalgae have high carotenoid production

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Carotenoids, a group of compounds with a 40-carbon basic skeleton, are accumulated in almost all organisms that exist in aerobic environments exposed to sunlight. Aerial microalgae live on land, forming green or red microbial mats on walls, trees, and rock surfaces, and grow under severe environmental stresses such as intense light, drought, temperature changes, and nutrient source depletion. All carotenoid-producing strains currently in practical use are microalgae found in the hydrosphere. Although algal strains have been found on microbial mats formed on walls, screening under various combinations of culture conditions is expected to reveal promising strains with high carotenoid production from the surface of terrestrial substrates. In this presentation, we isolated land-dwelling aerial microalgae and searched for carotenoid-rich algal strains.

Cell suspensions were obtained by sampling with cotton swabs wetted with sterile water from rock surfaces near stairs surrounded by trees on the University campus and from concrete surfaces under the shade of buildings. After washing with sterile water, the cell suspension was dropped near the center of the filter paper, stacked on 10 layers of filter paper, placed on a non-fat cotton pad containing Bold's Basal (BB) medium, and incubated for 14 or 28 days at a light intensity of $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a temperature of 25°C . Using a sterile toothpick, the colonies generated on filter paper were transferred to 96-well microplates and incubated for 28 days, and absorbance (turbidity) at 750 nm was measured with a plate reader. 12-well microplates were used to check the growth status, and algal strains that were successfully monocultured and proliferated were obtained. The algal strains were pre-cultured for 21 days, then dispensed into 96-well microplates and cultured for 7 days under standard conditions (light intensity of $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, pH 8.0, temperature 25°C , NaCl concentration 0.04 M , turbidity 0.5) (conditions considered: with or without nitrogen source, light intensity 0 or $170 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, NaCl concentration 0.15 , 0.5 or 1.5 M). After this incubation, absorbance from carotenoids and chlorophyll were measured and analyzed for carotenoid content by the WCA method [1]. A total of 25 strains of algae, 15 from rock surfaces and 10 from concrete surfaces, were successfully monocultured. These algal strains were cultured under various conditions. **Fig. 1** shows some of the results of the evaluation of the carotenoid accumulation of the isolated algal strains, and **Fig. 2** shows cell photographs of the selected strains. Nine isolates showed high carotenoid accumulation under the culture condition of 0.5 M NaCl with nitrogen source, and 16 isolates showed high carotenoid accumulation under the condition of high light with nitrogen source. We will analyze the 18S rRNA gene sequence of these algal strains to identify the species. We will also examine the carotenoid composition by HPLC analysis and explore the characteristics of the algal strains that showed high carotenoid accumulation.

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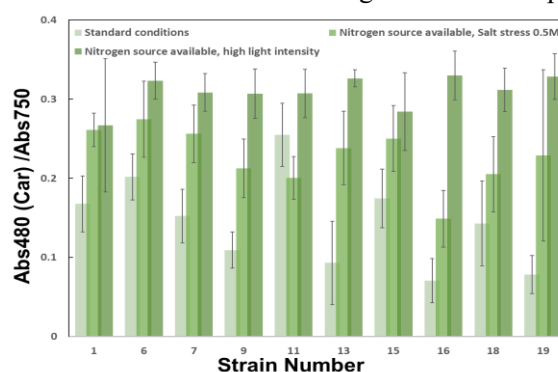


Fig 1. Evaluation of carotenoid accumulation of isolated algal strains.

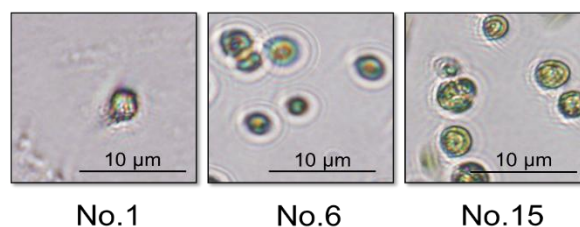


Fig 2. Photographs of cells

Eco-physiological significance of carotenoids for aerobic anoxygenic phototrophic bacteria in the ocean

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The photosynthetic apparatus of aerobic anoxygenic phototrophic bacteria (AAnPB) was originally adapted to photosynthetic reactions in a reducing anaerobic environment, and under oxidative aerobic conditions, it faces a higher risk of photo-oxidative stress due to the generation of reactive oxygen species through photosynthetic reactions. As a defense mechanism, attention has been given to the antioxidant effects of carotenoid pigments.

While the difference in chlorophyll pigments among photosynthetic organisms is relatively simple, carotenoid pigments exhibit high diversity. AAnPB and AnAnPB are characterized by possessing straight-chain carotenoids. The diversity of non-photosynthetic carotenoids, which do not directly participate in photosynthesis, is high. When considering both photosynthetic and non-photosynthetic carotenoids, AAnPB carotenoids can be classified into five groups (Takahashi, 2006; Takaichi, 2009). Focusing only on major photosynthetic carotenoids, they can be categorized into three types: spirilloxanthin type, spheroidenone type, and *Erythrobacter* type. Spirilloxanthin and spheroidenone function as light-harvesting carotenoids that transfer energy to the reaction center (RC), which is bacteriochlorophyll, BChl *a*. Additionally, excessive excitation of BChl *a*, caused by factors such as intense light, can lead to the formation of reactive oxygen species (ROS), specifically singlet oxygen. Normally, carotenoids take up this excited state, releasing the excess energy as heat to dissipate the excited state. Both spirilloxanthin and spheroidenone also function as photoprotective carotenoids by dissipating the excited state of BChl *a*. The difference lies in that spirilloxanthin prevents the generation of ROS by dissolving the triplet state of BChl *a* (by becoming triplet itself through energy transfer), whereas spheroidenone not only dissipates the triplet state of BChl *a* but also has a removal effect on ROS, making it more effective as an antioxidant. Taxonomically, spheroidenone type is limited to the Alpha-proteobacteria class within the Alpha-3 subclass. The previously mentioned AnAnPB, the purple bacteria, also belongs to the Alpha-proteobacteria class within the Alpha-3 subclass and shares the spheroidenone type limitation. All others are spirilloxanthin types, but within the Beta-proteobacteria class, there are genera like *Roseateles*, *Limnohabitans*, and *Rubrivivax* that produce both spirilloxanthin and spheroidenone.

The *Roseobacter* clade, one of the representative strains of AAnPB in marine isolation cultures, possesses spheroidenone. Additionally, the *Erythrobacter* clade also has a fascinating abundance of multifunctional carotenoids. The complex and diverse carotenoid pigments, in addition to their role in light harvesting during photosynthetic reactions, are believed to contribute to defense functions against high photo-oxidative stress in the distribution of AAnPB in the oceans. The multifunctionality and high efficiency of carotenoid production by AAnPB suggest that, despite the cost of production, photosynthesis complemented by carotenoids is a valuable and essential reaction for sustaining life and acquiring niches.

Comparative analysis of antioxidant activity between 4-ketomyxol and myxol

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We are currently analyzing pigments produced by bacteria isolated and cultured from marine and other unique aquatic environments to search for new and rare carotenoids. In this study, we confirmed production of a rare carotenoid ‘myxol (**Fig. 1a**)’ from *Flavobacteriaceae* and *Actinobacteria* species. We also found that strain *Hymenobacter setariae* (NBRC110362) produces a rare carotenoid ‘4-ketomyxol (**Fig. 1b**)’ in an independent study. 4-ketomyxol is produced by many species of the genus *Hymenobacter*, and its chemical structure contains a keto group in the β -ionone ring structure of myxol. Some antioxidant properties were reported for myxol in the previous studies, but no antioxidant activity was reported for 4-ketomyxol to date. Since 4-ketomyxol is expected to have better $^1\text{O}_2$ quenching activity than myxol due to the difference in chemical structure, we compared the some antioxidant activities of myxol and 4-ketomyxol in this study.

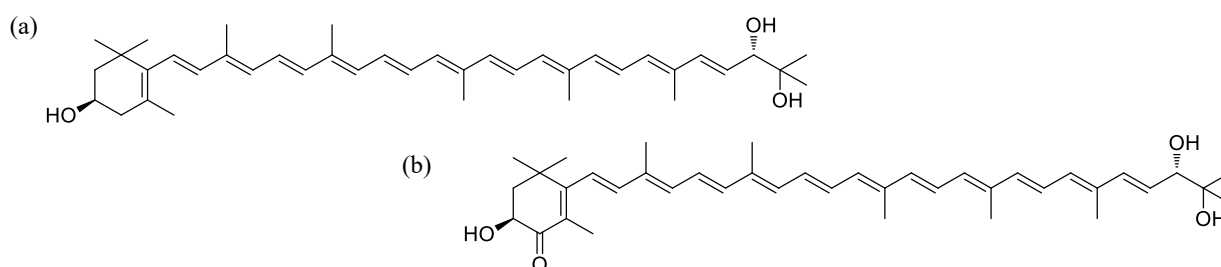


Fig. 1. Chemical structure of 4-ketomyxol and myxol

This study investigates and compares the antioxidant activities of two carotenoids, 4-ketomyxol and myxol, which are prominent in various natural sources. Both compounds are structurally related and belong to the xanthophyll class of carotenoids known for their potential health benefits. The evaluation of their antioxidant properties is crucial for understanding their potential contributions to human health and their applications in various industries.

Carotenoids are well-known for their antioxidant properties, playing a vital role in protecting cells from oxidative stress. Among these, 4-ketomyxol and myxol are two structurally related carotenoids found in diverse natural sources, including marine organisms, algae, and certain bacteria. While myxol has been extensively studied for its antioxidant potential, limited information is available regarding 4-ketomyxol. This study aims to compare the antioxidant activities of 4-ketomyxol and myxol, shedding light on their potential applications in health and industry.

Materials and Methods

Myxol was isolated from *Nonlabens ulvanivorans* NR17, and 4-ketomyxol was isolated from *Hymenobacter setariae* (NBRC110362). Extraction from cells with acetone-MeOH (7:2) followed by CH_2Cl_2 -MeOH (1:1) and two-phase partition with ethyl acetate/water was performed. Purification was achieved through preparative ODS HPLC.

Culturing Conditions: *Nonlabens ulvanivorans* NR17 was cultured in MB (Marine Broth 2216E) medium. *Hymenobacter setariae* (NBRC110362) was cultured in R2A + trehalose medium. Antioxidant Activity Tests: Singlet oxygen scavenging activities (IC_{50}) were determined for both myxol and 4-ketomyxol. Radical quenching activities were assessed.

Results: Since

Nonlabens ulvanivorans NR17 produced myxol with high titer (2.1 mg/L), we isolated myxol from this strain using chromatographic method. We also isolated 4-ketomyxol from *Hymenobacter setariae* (NBRC110362) using chromatographic methods.

Nonlabens ulvanivorans NR17 was cultured in MB medium and *Hymenobacter setariae* (NBRC110362) was cultured in R2A + trehalose medium. Each carotenoid was purified using following scheme 'extraction from cells with acetone-MeOH (7:2) followed by CH_2Cl_2 -MeOH (1:1) $\rightarrow$ two phase partition with ethyl acetate/water $\rightarrow$ preparative ODS HPLC'. From 30 L culture, pure myxol (24.8 mg) and 4-ketomyxol (15.8 mg) were obtained. Antioxidant activity tests (singlet oxygen quenching activity and radical quenching activity) were performed on each carotenoid.

The singlet oxygen scavenging activities (IC_{50}) of myxol and 4-ketomyxol were proved to be 50 μ M and 10 μ M, respectively. 4-ketomyxol was proved to possess more potent singlet oxygen quenching activity than myxol because 4-ketomyxol possess more conjugated double bond number than myxol.

Conclusion:

This study provides valuable insights into the antioxidant activities of 4-ketomyxol and myxol. Further investigations, including in vivo studies and exploration of potential synergistic effects with other antioxidants, are warranted to fully comprehend their impact on human health and their applications in various industries. This research contributes to the understanding of the antioxidant potential of these carotenoids and their possible applications in various fields.

Evaluation of the potential use of Z-isomer-rich astaxanthin in poultry, aquaculture, and insect feeds

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Abstract: Effects of feeding astaxanthin with different *E/Z*-isomer ratios on astaxanthin accumulation in egg yolk of hens (*Gallus gallus domesticus*), flesh of rainbow trout (*Oncorhynchus mykiss*), and whole bodies of Pacific white shrimp (*Litopenaeus vannamei*), migratory locust (*Locusta migratoria*), and two-spotted cricket (*Gryllus bimaculatus*) were investigated. Except for migratory locust, diets rich in astaxanthin *Z*-isomers showed greater accumulation of astaxanthin in egg yolk, flesh, and whole body than those rich in the all-*E*-isomer. These results may contribute to the practical use of *Z*-isomer-rich astaxanthin in the feed industry.

1. Introduction

Astaxanthin (C₄₀H₅₂O₄; 3,3'-dihydroxy- β,β -carotene-4,4'-dione; **Fig. 1**) is a functional carotenoid widely used in salmonid and crustacean aquaculture to provide the pink-red color characteristic of those species as well as the pigmentation of egg yolk of hens [1]. However, the low pigmentation efficiency is often an important issue in industry, which may be due to the low bioavailability of astaxanthin for living animals [2]. In recent years, the *Z*-isomerization has attracted attention for improving the bioavailability of carotenoids, including astaxanthin (**Fig. 1**) [3,4]. For example, an oral administration trial in rats showed that a *Z*-isomer-rich astaxanthin diet resulted in 36.6-fold and >10-fold increases in plasma concentrations and specific tissue concentrations (e.g., adrenal, lung, and prostate), respectively, when compared to an all-*E*-isomer-rich astaxanthin diet [4]. However, data on the absorption (pigmentation) advantage of astaxanthin *Z*-isomers in other animals is limited. Therefore, the aim of this study was to investigate the effect of astaxanthin with different *Z*-isomer ratios on the bioavailability and

pigmentation efficiency of egg yolk or body in several animals, i.e., hens (*G. gallus domesticus*), rainbow trout (*O. mykiss*), Pacific white shrimp (*L. vannamei*), migratory locust (*L. migratoria*), and two-spotted cricket (*G. bimaculatus*). This data should be useful for efficient pigmentation of animal bodies and egg yolks.

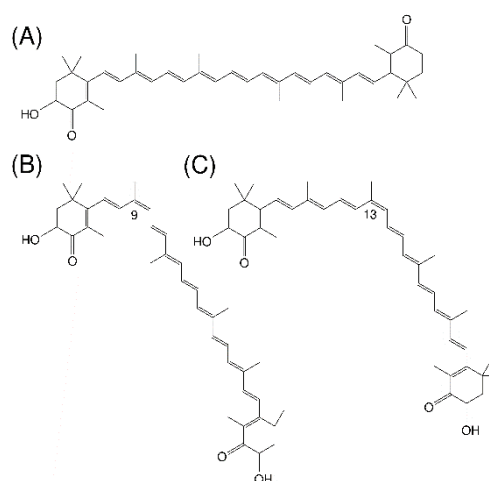


Fig. 1. Chemical structures of astaxanthin isomers: (A) (all-*E*)-astaxanthin, (B) (9*Z*)-astaxanthin, and (C) (13*Z*)-astaxanthin.

2. Methods

Astaxanthin rich in the *Z*-isomers was prepared from the all-*E*-isomer derived from synthetic or an aerobic Gram-negative microorganism, *Paracoccus carotinifaciens*, by thermal treatment as described previously [5,6]. The intact all-*E*-isomer-rich and prepared *Z*-isomer-rich astaxanthin were added to the basal diets to reach defined astaxanthin concentrations, i.e., 8 ppm for laying hens, 50 ppm for rainbow trout and Pacific white shrimp, and 100 ppm for migratory locust and two-spotted cricket. After feeding diets rich in (all-*E*)-astaxanthin (E-AST-D) and (*Z*)-astaxanthin (Z-AST-D) for a certain period of time (2 or 8 weeks), the concentration and *E/Z*-isomer ratio of astaxanthin in the egg yolk, flesh, and whole body were determined by normal-phase HPLC analysis [6]. In addition, the egg yolk color of laying hens and the flesh color of rainbow trout were evaluated using the DSM Yolk Color Fan and the Roche Salmo Fan, respectively.

3. Results and Discussion

When Z-AST-D was fed to laying hens, rainbow trout, Pacific white shrimp, and two-spotted cricket, the astaxanthin concentrations in the egg yolk, flesh, and whole body were significantly increased compared to those fed E-AST-D (Fig. 2). In addition, egg yolk color of laying hens and flesh color of rainbow trout were significantly raised. These results suggest that astaxanthin *Z*-isomers have higher bioavailability and/or body accumulation than (all-*E*)-astaxanthin in these species. On the other hand, in migratory locust, the feeding of astaxanthin isomers did not significantly affect the whole body astaxanthin concentration (Fig. 2), suggesting that its bioavailability is low in this species. Incidentally, regardless of the dietary astaxanthin composition, the total and individual isomer ratios of astaxanthin in these species remained almost constant. This trend has also been observed in some mammals [4,7], suggesting that animals have a mechanism to maintain a constant carotenoid isomer ratio. These results may contribute to the practical use of *Z*-isomer-rich astaxanthin in the feed industry.

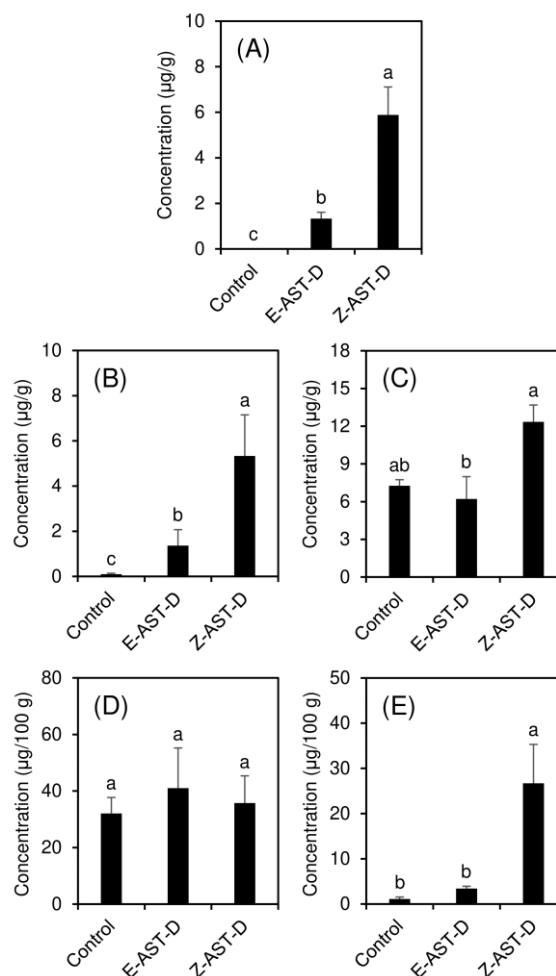


Fig. 2. Effects of feeding diets rich in (all-*E*)-astaxanthin (E-AST-D) and (*Z*)-astaxanthin (Z-AST-D) on astaxanthin concentration in (A) egg yolk of laying hens, (B) flesh of rainbow trout, and whole bodies of (C) Pacific white shrimp, (D) migratory locust, and (E) two-spotted cricket. The different letters on graph bars indicate a significant difference in each test ($p < 0.05$).

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Evaluation of Anti-Inflammatory Effect of Siphonaxanthin by Using Lipidomic Analysis

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Inflammatory responses are physiological responses to the entry of pathogenic microorganisms into the body, tissue damages, and exposure to irritants such as chemicals and ultraviolet light. Although they are essential for the maintenance of homeostasis in individuals, excessive or prolonged inflammatory responses can also damage one's own tissues, leading to the development of sepsis, atherosclerosis, and other diseases. We have been screening carotenoids for their anti-inflammatory effects and found that siphonaxanthin (Spx, **Fig. 1**) inhibits lipopolysaccharide (LPS)-induced inflammatory responses [1, 2]. However, molecular mechanisms underlying the anti-inflammatory effect of siphonaxanthin remained unknown. In this study, we evaluated effects of Spx on inflammatory responses including proinflammatory gene expression and lipid metabolism in LPS-exposed macrophages to gain insight into the mechanism of the anti-inflammatory effect.

According to our real time RT-PCR analysis, Spx treatment suppressed LPS-stimulated mRNA expression of various proinflammatory genes in mouse macrophage-like cell line RAW264. In fact, Spx suppressed almost all analyzed proinflammatory gene expression. The analysis of phospholipid and sphingolipid levels in RAW264 cells under the same experimental conditions showed that LPS-induced ceramide accumulation was ameliorated by Spx treatment. Whereas some changes induced by LPS were not affected by Spx, indicated that not all inflammatory responses were ameliorated by Spx treatment. Multivariate analysis of the data from the lipidomic analysis suggested that the increase in phosphatidylcholine (PC) with no or only one double bond in the molecule and the decrease in PC with two or more double bonds were characteristic changes induced by Spx. In support of these results, Spx significantly decreased mRNA expression of some fatty acid desaturases. In addition, Spx suppressed mRNA expression of genes related with fatty acid transport and cholesterol synthesis. Previous reports suggest that some of these changes could be responsible for the anti-inflammatory effects. Of course, further experiments are required, but the lipidomic analysis could provide clues to the mechanism underlying the anti-inflammatory effect of Spx.

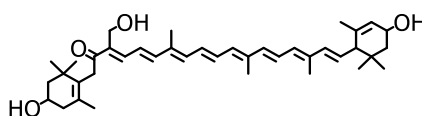


Fig. 1. Chemical structure of siphonaxanthin.

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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]).
- (viii) Figures and Tables should be numbered and placed in the text with captions, typed underneath, not at the end of the manuscript.
- (ix) References should be listed at the end of the text with their serial numbers in square brackets as cited within the text. Please refer to the recent issue of Carotenoid Science as an example.

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