0014-4754/94/070684-03$1.50 + 0.20 © Birkhäuser Verlag Basel, 1994

Okadaxanthin, a novel C_{50}-carotenoid from a bacterium, *Pseudomonas* sp. KK10206C associated with marine sponge, *Halichondria okadai*

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Received 30 November 1993; accepted 6 January 1994

Abstract. To study the origins of biologically active substances in marine sponges, a carotenoid produced by a marine bacterium, *Pseudomonas* sp. strain number KK10206C, which was associated with a marine sponge, *Halichondra okadai*, was investigated. A visible absorption spectrum-guided isolation procedure led to the isolation of a novel C_{50}-carotenoid, okadaxanthin. Its structure, 2,2′-bis(4-hydroxy-2-methyl-2-butenyl)-ε,ε-carotene, was elucidated mainly by spectroscopic methods. Okadaxanthin turned out to be a potent singlet oxygen quencher, approximately 10 times as strong as α-tocopherol.
Key words. Carotenoid; marine sponge; *Halichondria okadai*; sponge associated bacteria; *Pseudomonas* sp.; okadaxanthin; singlet oxygen quencher.

In the course of our search for biologically active substances from marine organisms, carotenoids have been noted as quenchers and/or scavengers of active oxygen species[1]. For example, astaxanthin, a typical marine carotenoid, shows quenching activity on singlet oxygen that is approximately 500 to 1000 times as strong as that of α-tocopherol, a well-known quencher in mammals[1]. Thus, carotenoids have potential practical uses in the pharmaceutical and food industries. A number of interesting carotenoids such as renieratene, isorenieratene, renierapururine and other aromatic carotenoids have been isolated from marine sponges[2,3]. Their origins remain unknown, especially whether sponges can modify dietary carotenoids into suitable structures through enzymatic bioconversion or whether the precursors and/or carotenoids themselves are biosynthesized by some symbiotic or co-existing microorganisms before being transferred to the host sponge and stored. In the present study, we intended to isolate a sponge-associated bacterium which showed the characteristic yellow color of carotenoids and to elucidate the structure of the compounds, to get a clearer idea of the transfer of substances between a host sponge and its associated bacteria.

Materials and methods

Host sponge. A marine sponge, *Halichondria okadai*, which is commonly found on the southern part of the Japanese coast, was collected from the Numazu area of Suruga Bay, Shizuoka Prefecture in 1990, and used as the host organism.

Isolation and identification of bacteria. The fresh sponge (5 g) was homogenized with 10 ml of sterilized seawater and a 10-fold dilution series prepared. Each dilution sample was seeded onto agar medium in a mixture of 750 ml seawater (pH 7.7), 250 ml distilled water, 5.0 g Bacto-pepton (Difco), 1.0 g Bacto-yeast extract (Difco), 15.0 g agar (Nacalai tesque) and 0.01 g $FeSO_4$. After incubation at 20 °C for 4 days, all colored colonies of whatever pigmentation were chosen for isolation. Isolated bacteria were cultured in the medium described above without agar and with supplement of glucose (2 g) at 20 °C for 4 days. Among the isolates, a bacterium tentatively identified as strain number KK10206C was found to produce an interesting yellowish pigment. The strain was identified according to Bergey's Manual of Systematic Bacteriology[4].

Isolation of carotenoid. One liter of the 4-day-incubated culture broth of the bacterium KK10206C was centrifuged at 8000 rpm for 15 min to give a pellet, which was extracted with acetone. The extract was concentrated under reduced pressure and separated into two layers by addition of ethyl acetate and distilled water. The pigment was recovered in the ethyl acetate layer, which was then washed with distilled water several times, dried over anhydrous sodium sulfate and concentrated in vacuo to an oily drop. Visible absorption spectrum-guided isolation of the concentrate by silica gel column chromatography (Silica gel 60, 230-400 mesh, Merck), eluted with a mixture of 20% of acetone in hexane, resulted in the isolation of yellowish oily pigment. Further purification by HPLC on silica gel (Cosmosil 5SL, Nacalai tesque) using the same solvent described above, led to the isolation of 3 mg of the pigment as a yellowish oil.

Structural elucidation of the pigment. The structure of the pigment was elucidated mainly by spectroscopic analysis as follows: the visible absorption spectrum (VIS) in hexane was recorded on a Shimadzu UV-2100S recording spectrophotometer. The circular dichroism (CD) spectrum was recorded on a JASCO J-600 spec-

tropolarimeter using a mixture of diethylether, iso-pentane and ethanol in the ratio 5:5:2 (EPA) as a solvent. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum, ^{1}H-^{1}H shift correlation (COSY) spectrum and correlation spectrum for nuclear Overhauser effect (NOESY) were recorded on a Varian Unity 500 Spectrometer using $CDCl_3$ as a solvent. Electron impact mass spectrometry (EIMS) was measured by a JEOL JMS-SX102 mass spectrometer.

Results and discussion

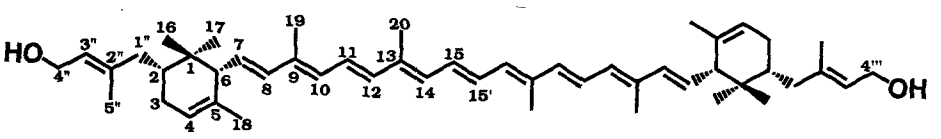
Identification of the bacterium. The colony of the bacterium with tentative strain number KK10206C was circular and raised. The strain was an aerobic Gram negative straight-rod (1.7 × 0.4 μm), and motile by a polar flagellum. Catalase test was positive, whereas O/F, oxidase, glucose and geletin tests were negative. The strain required seawater salts for growth, and GC content was 67.5–69.5 mol%. From these observations and the description of the genus *Pseudomonas* in Bergey's Manual of Systematic Bacteriology[4], this strain was identified as *Pseudomonas* sp.

Structural elucidation of the pigment. The spectral data of the pigment were as follows: VIS (hexane) λ_{max} 414, 437, 467 nm; EIMS m/z 704 (M^+), 564 (M^+-140), 368; ^{1}H-NMR ($CDCl_3$, 500 MHz, ppm) δ 0.74 (6H, s; 16, 16″-CH_3), 0.93 (6H, s; 17, 17′-CH_3), 1.26 (2H, t; OH) 1.30–1.40 (2H, m; 2, 2′-CH), 1.52 (6H, s; 18, 18′-CH_3), 1.60–1.72 (2H, m; 3, 3′-CH), 1.67 (6H, s; 5″, 5‴-CH_3), 1.77–1.85 (2H, m; 1″, 1‴-CH), 1.93 (3H, s; 19, 19′-CH_3), 1.97 (3H, s; 20, 20′-CH_3), 2.01–2.10 (2H, m; 3, 3′-CH), 2.22–2.30 (2H, m; 1″, 1‴-CH), 2.44 (2H, d, J = 10.5; 6, 6′-CH), 4.01 (4H, d, J = 6; 4″, 4‴-CH_2), 5.41 (2H, t, J = 6; 3″, 3‴-CH), 5.45 (2H, brs; 4, 4′-CH), 5.53 (2H, dd, J = 10.5, 15; 7, 7′-CH), 6.13 (2H, d, J = 12; 10, 10′-CH), 6.16 (2H, d, J = 15; 8, 8′-CH), 6.25 (2H, d, J = 9; 14, 14′-CH), 6.36 (2H, d, J = 15; 12, 12′-CH), 6.63 (2H, dd, J = 12, 15; 11, 11′-CH), 6.60–6.68 (2H, m; 15, 15′-CH). All spectral details described above closely resemble those of decaprenoxanthin[5,6] [2,2′-bis(4-hydroxy-3-methyl-2-butenyl)-ε,ε-carotene], although a peak at δ 4.01 of the pigment was split into doublet. Moreover, the coupling constants of the peaks δ 4.01 and δ 5.41 were both 6 Hz, and this value is too large for long range coupling. These differences led to the structural elucidation of the carotenoid as 2,2′-bis(4-hydroxy-2-methyl-2)-butenyl-ε,ε-carotene, an isomer of decaprenoxanthin for the positions of two methyl groups. This elucidation was also supported by the proton-proton correlation between δ 4.01 and δ 5.41 clearly observed by COSY. This carotenoid is a novel compound, and was named okadaxanthin from the taxonomic name of the host sponge.

Absolute configuration of okadaxanthin. The stereochemistry of okadaxanthin at the C-2, C-2′, C-6 and C-6′ positions was deduced by NOESY and CD spectra, NOESY cross-peaks were observed between H_3 (16-C, δ 0.74) and H (6-C, δ 2.44), H_3 (17-C, δ 0.93) and H (2-C, δ 1.35), H_3 (17-C, δ 0.93) and H (1″-C, δ 1.82), H (1″-C, δ 1.82) and H (3″-C, δ 5.41), and H_2 (4″-C, δ 4.01) and H_3 (5″-C, δ 1.65). This observation suggested that the linkage of C-5 side chains at C-2 and C-2′ positions, and polyene chains at C-6 and C-6′ positions, to ε-ionon rings should both be *cis*-configuration. The CD data of okadaxanthin is shown below; CD [EPA, nm ($\Delta\varepsilon$)] 391 (−0.02), 358 (+1.20), 326 (+3.45), 314 (+2.30), 299 (+0.50), 266 (−19.52), 228 (+1.86). Finally, the absolute configuration of okadaxanthin was elucidated to be *2R, 6R, 2′R, 6′R* by comparison with that of decaprenoxanthin which was deduced from CD data[6], in particular the negative maxima of $\Delta\varepsilon$ of both carotenoids at around 263 to 266 nm, and positive maxima at around 326 nm. The structure of okadaxanthin with absolute configuration is shown in the figure.

Quenching effect against singlet oxygen. Quenching activity of okadaxanthin was examined according to Miki's method[1]. The ED_{50} of the carotenoid was approx. 120 nM, which was about 8 times higher than that of α-tocopherol.

Presumption of the biosynthetic pathway of okadaxanthin. Julia[7] suggested, based on a biomimetic synthesis of C_{50} carotenoid, that decaprenoxanthin might be biosynthesized by head to tail coupling of C_{40} carotenoid with a C_5 part of geranyl geraniol. Although the speculation is reasonable for the position of two methyl groups in this carotenoid, it is unlikely that okadaxanthin is formed this way. In the case of okadaxanthin biosynthesis, it is probable that the coupling of C_{40} carotenoid with a C_5 part of geranyl geraniol is not a head to tail but a head to head bioformation. Details of the biosynthesis will be clarified by cultivation of the bacterium using C_{40} carotenoid and geranyl geraniol in the medium. Okadaxanthin could not be detected in the host sponge. However, reductive deacylation of the carotenoid is considered to produce an aromatic carotenoid which is commonly found in sponges. Thus, C_{50}-carotenoids in sponge-associated bacteria may be the origin of aromatic carotenoids in host sponges through transference and bioconversion.



Acknowledgments. We are indebted to Drs K. Adachi and M. Nishijima, in our laboratories, for their help in the structural analyses and bacterial identification, respectively. We also wish to thank Prof. W. Fenical, Scripps Institution of Oceanography, and Dr. M. Endo, Director of our laboratories, for their valuable discussions.

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