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CAROTENOIDS IN RED ALGAE*

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Key Word Index—Rhodophyceae; marine macroalgae; chlorophyll *a*; characteristic carotenoids; fucoxanthin; chemotaxonomy.

Abstract—The carotenoid composition of the following 8 species of red algae has been studied quantitatively and qualitatively: *Bangia fuscopurpurea, Nemalion helminthoides, Bonnemaisonia hamifera* (tetrasporophyte), *Gigartina stellata, Rhodymenia palmata, Ceramium rubrum, Polysiphonia brodiaei*, and *Polysiphonia urceolata*. Naturally occurring material of *G. stellata, R. palmata*, and *P. brodiaei* was investigated, while monoalgally cultured material was obtained from the remaining 5 species. α- and β-carotene, lutein, zeaxanthin, and small amounts of α- or β-cryptoxanthin were commonly present, but the two species *P. brodiaei* and *P. urceolata* were devoid of carotenoids containing α-ionone rings. Fucoxanthin was detected in naturally occurring material of *B. fuscopurpurea, N. helminthoides, C. rubrum*, and *P. brodiaei*, but could not be found in cultured material of the 3 first-mentioned species. The possible origin of the fucoxanthin is discussed as well as the biochemical and phylogenetic implications of the results obtained. As judged from the electronic spectrum of the total extracts, chlorophyll *d* was not present in any of the algae investigated. The total amounts of carotenoids and the ratio carotenoids:chlorophyll *a* were similar to those reported in previous work.

INTRODUCTION

The main carotenoids of the Rhodophyceae (*sensu* Christensen [1], but including *Cyanidium caldarium*) have repeatedly been identified as α-carotene, β-carotene, lutein, and zeaxanthin [2–21]. Nine further carotenoids seem to have a more casual occurrence: antheraxanthin (detected in *Acanthophora spicifera* and *Gracilaria lichenoides*) [16], aurochrom (probably) (*Gelidium corneum*) [7], auroxanthin (*G. corneum*) [7], α-cryptoxanthin (*Lenormandia prolifera*) [17], β-cryptoxanthin (*A. spicifera, Batrachospermum* sp., *G. lichenoides, Porphyridium aerugineum, P. cruentum*) [16,20], fucoxanthin (*Polysiphonia nigrescens*) [3], neoxanthin (with doubt) (*Antithamnion plumula, Nemalion helminthoides* (= *N. multifidum*) [22]) [11], taraxanthin (*Ceramium rubrum, Dilsea carnosa* (=*D. edulis*) [22], *G. corneum, Grateloupia filicina, G. proteus, Rhodymenia palmata, Schizymenia dubyi*) [2,4,7], and violaxanthin (*Halosaccion glandiforme*) [8]. Unlike the Cyanophyceae (blue-green algae) the Rhodophyceae have accordingly evolved the α-cyclase enzyme, whereas the glycosidic carotenoids commonly encountered within the Cyanophyceae [23] have still not been detected within the class. Epoxidic carotenoids have been hypothesized to be present in all oxygen-evolving organisms [24], and some are supposed to fulfil an important function in most algal classes by the participation in the light-induced reversible xanthophyll cycle [25]. The great majority of the Rhodophyceae, however, do not seem to contain detectable amounts of epoxidic carotenoids, as revealed by the numerous analyses performed by Strain [8,15]. The few exceptional species (see above) are all belonging to the subclass Florideophycidae. When the investigations were quantitative, both antheraxanthin [16] and taraxanthin [7] were among the main carotenoids.

The identification of rhodophycean carotenoids has until now mainly relied on chromatographic properties, cochromatography with authentic samples, and electronic spectra. Lutein from dried *Porphyra yezoensis* ("nori") has been characterized by IR [14], otherwise IR, MS, and PMR have not been applied for identification. The use of unsatisfactory identification methods has resulted in repeated misidentifications of the acetylenic xanthophylls of the Raphidophyceae (probably), Xanthophyceae, and Euglenophyceae with their non-acetylenic analogues (see [23]). A renewed investigation of the rhodophycean carotenoids using more modern methods, notably MS, therefore seemed justified.

To eliminate carotenoid contributions from biological contaminants (notably representatives of the Chromophyta) analyses of naturally occurring red algal material should whenever possible be controlled by analyses of cultured material also. This is specially important in relation to the possible presence of fucoxanthin within the Rhodophyceae. This biosystematically important caro-

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Table 1. Chlorophyll *a* and carotenoids of the Rhodophyceae

Algae Subclass Order species	analysed amounts of algae (g dry-weight)	Pigments (μg/g dry-wt) chlorophyll *a* ($E^{1\%}_{1cm}$ = 890[26])	α-carotene ($E^{1\%}_{1cm}$ = 2720[27])	β-carotene ($E^{1\%}_{1cm}$ = 2592[28])	α-cryptoxanthin ($E^{1\%}_{1cm}$ = 2640[29])	β-cryptoxanthin ($E^{1\%}_{1cm}$ = 2386[30])	lutein ($E^{1\%}_{1cm}$ = 2550[27])	zeaxanthin ($E^{1\%}_{1cm}$ = 2350[31])	carotenoids (total amount)**(mg/g)	Carotenoids: Chlorophyll *a*
Bangiophycidae										
Bangiales										
Bangia fuscopurpurea	23·0	4·5		170			780	250	2·70	0·60
Florideophycidae										
Nemaliales										
Nemalion helminthoides	18·5	7·9	10	110			410		1·97	0·25
Bonnemaisonia hamifera[c]	1·43	3·1	40	20			170	+	0·71	0·23
Gigartinales										
Gigartina stellata	61·0	0·13		1·4			8·3	1·4	0·07	0·54
Rhodymeniales										
Rhodymenia palmata	20·0	3·2	80	30			240		0·80	0·25
Ceramiales										
Ceramium rubrum	24·5	0·90	14	22	+		37	2	0·23	0·25
Polysiphonia brodiaei	44·0	0·60		71		12		87	0·28	0·47
Polysiphonia urceolata[c]	1·21	4·2		30		+		160	1·22	0·29

* Naturally occurring material was used except where shown by [c] = cultured material: for the latter the dry-weight determined on pigment-extracted residue. ** Calculated from the total extract ($E^{1\%}_{1cm}$ = 2020 at 470–475 nm). + Trace amount.

tenoid has earlier been isolated from naturally occurring red algae [3,57,58].

RESULTS

The chlorophyll and carotenoid contents of the 8 red algae analyzed are compiled in Table 1. The calculations of the amounts of chlorophyll *a* were based purely on the electronic spectra of the total extracts while the carotenoid content was calculated on the basis of the spectra of the separated pigments. The total amount of carotenoids, however, used to calculate the carotenoid:chlorophyll *a* ratio, was based on the spectrum of the total extract. If not otherwise stated, the identity of the carotenoids was based on co-chromatography both on paper and TLC, electronic spectra, and MS. The carotenoids of the cultured material of *B. fuscopurpurea*, *N. helminthoides*, and *C. rubrum* were identified by co-chromatography and electronic spectra only. The purpose of these analyses was to ascertain that the carotenoids isolated from naturally occurring material were synthesized by the red algae themselves and not by epipytes such as brown algae and diatoms. These results have therefore not been included in Table 1. For reasons given in the discussion, the fucoxanthin isolated from naturally occurring *B. fuscopurpurea* (3% of the total carotenoid content), *N. helminthoides* (43%), *C. rubrum* (18%), and *P. brodiaei* (3%) has been omitted from the table.

α-carotene. The electronic spectrum of non-crystalline α-carotene from *B. hamifera* had λ_{max} (petrol.) at 421, 444, and 473 nm and a III/II-value [32] (in %) of 67. This is consistent with a chromophore system of 9 in-chain double bonds in conjugation with 1 cyclohexenyl double bond [33,34]. Informative MS peaks of the same sample (compare [35]) were observed at *m/e* 536 (100% of the base peak) (M, the molecular ion), 480 (1%) (M-56, retro-Diels–Alder cleavage characteristic for carotenoids with α-rings[36]), 444 (4%) (M-92, in-chain elimination of toluene [37]), and 430 (1%) (M-106, in-chain elimination of xylene [37]).

β-carotene. Non-crystalline β-carotene from *B. hamifera* had λ_{max} (petrol.) at (428), 449, and 476 nm and a III/II-value of 28. This is characteristic of a chromophore system of 9 in-chain and 2 cyclohexenyl conjugated double bonds [33,34]. The IR of a crystalline sample from *P. brodiaei* possessed the expected absorption bands [28]. The same sample gave prominent MS peaks (compare [35]) at *m/e* 536 (100%) (M), 444 (10%) (M-92), and 430 (0·6%) (M-106). β-carotene from *B. hamifera* was not characterized by MS.

α-cryptoxanthin. Non-crystalline α-cryptoxanthin from cultured *C. rubrum* had λ_{max} (petrol.) at (421), 441, and 469 nm and a III/II-value of 48. Both the positions of the maxima and the fine structure were somewhat lower than expected for an α-carotene-like chromophore system, which is probably explained by the small amounts available of this carotenoid and the lack of a satisfactory method for the separation of its stereo isomers. The MS (compare [38]) of a non-crystalline sample from naturally occurring *C. rubrum* possessed peaks at *m/e* 552 (19%) (M), 534 (9%) (M-18, loss of water [36]), 460 (2%) (M-92), and 446 (1%) (M-106). The MS contained peaks from contaminating colourless lipids. α-cryptoxanthin from both biological samples gave a monoacetate by acetylation. In the co-chromatographic tests of α-cryptoxanthin and its acetate an isolate from *Capsicum annuum flavum* was used as an authentic sample. This carotenoid was originally believed to be β,ε-caroten-3′-ol [29], but was later revised to β,ε-caroten-3-ol [38,39].

β-cryptoxanthin. Non-crystalline β-cryptoxanthin from *P. brodiaei* had λ_{max} (petrol.) at (426), 447, and 472 nm and a III/II-value of 12. As was the case with the α-analogue, *all-trans*-β-cryptoxanthin did not separate completely from its *cis*-isomers by paper chromatography. This may explain the somewhat low absorption maxima and the reduced fine structure observed. The MS (compare [38]) of the same carotenoid sample showed informative peaks at *m/e* 552 (100%) (M), 534 (1%) (M-18), and 460 (12%) (M-92). The intensity of the 534-peak was considerably lower than reported in [38] (13%), but was consistent with the value obtained by Leuenberger (*fide* [40]) (0·6%). The identity of the oxygen function as a hydroxyl group was confirmed by a positive acetylation test. The β-cryptoxanthin from *P. urceolata* was identified by co-chromatography only.

Lutein. Crystalline lutein from *R. palmata* had λ_{max} (diethylether) at 422, 443, and 472 nm and a III/II-value of 67. The MS (compare [41]) of this crystalline sample showed the simple elimination reactions already treated for the preceding carotenoids and also combinations of these. Of special diagnostic value were the peaks at *m/e* 568 (100%) (M), 550 (25%) (M-18), 532 (0·4%) (M-18-18), 512 (0·2%) (M-56), 489 (1·2%) (M-79, probably in-chain elimination of a methyl cyclopentadienyl radical from the polyene chain [36]), 476 (11%) (M-92), 462 (8%) (M-106), 458 (1·8%) (M-18-92), 444 (2·3%) (M-18-106), and 410 (1·4%) (M-158, in-chain elimination of dimethylcyclodecapentaene [37]). The allylic position of one of the hydroxyl groups was confirmed by the formation of a monomethylether by treatment with hydrochloric methanol [42]. The MS of the reaction product (compare [36]) possessed the expected peaks at *m/e* 582 (100%) (M) and 550 (21%) (M-32, methoxyl elimination with hydrogen transfer [36]). *Cis*-lutein always accompanied the *all-trans* isomer on paper chromatograms and served as an additional identification tool during the co-chromatographic tests of the lutein.

Zeaxanthin. Crystalline zeaxanthin from *P. brodiaei* had λ_{max} (petrol.) at (428), 449, and 476 nm and a III/II-value of 38. The high III/II-value is characteristic for crystalline β-carotenoids, and comparable values were obtained for crystalline samples of authentic β-carotene (41), β-cryptoxanthin (44), and zeaxanthin (40). The MS (compare [36]) of crystalline zeaxanthin from naturally occurring *B. fuscopurpurea* possessed diagnostically important peaks at *m/e* 568 (100%) (M), 550 (84%) (M-18), 532 (5%) (M-18-18), 489 (1%) (M-79), 476 (13%) (M-92), 462 (1%) (M-106), 458 (11%) (M-18-92), 444 (1%) (M-18-106), and 410 (5%) (M-158). Zeaxanthin from *B. hamifera* was identified by co-chromatography only.

Fucoxanthin. Non-crystalline fucoxanthin from naturally occurring *C. rubrum* had λ_{max} (petrol.) at (427), 447, and 473 nm and a III/II-value of 33. This is somewhat lower than may be calculated from the literature (40) [26] and obtained for the crystalline authentic sample (λ_{max} in petrol.: 426, 449, and 477 nm, III/II-value: 43), but is explained by the presence of a small amount of the *cis*-isomer (*cis*-peak at 330 nm). A complete loss of fine structure (λ_{max}: 458 nm) in $CHCl_3$ was observed and is characteristic of carotenoids with a carbonyl function in conjugation with the polyene chain [43]. The MS (compare [41]) at 250° of crystalline fucoxanthin from naturally occurring *N. helminthoides* possessed informative peaks at *m/e* 658 (2·5%) (M), 640 (5·8%) (M-18), 622 (3·4%) (M-18-18), 580 (1·7%) (M-18-60, elimination of water and acetic acid [41]), and 562 (1·5%) (M-18-18-60). The spectrum revealed the expected absence of the prominent peaks at *m/e* M-92 and M-106 so commonly encountered in the MS of other carotenoids. The positive epoxide test [44] and the lability towards alkali [26] were further supports for the correct identification of the fucoxanthin. $E^{1\%}_{1\,cm} = 1650$ [26] was used for the quantitative calculations.

Chlorophyll. The electronic spectrum of the total extract always possessed a symmetric peak at 659–662 nm (diethylether) or 661–666 nm (acetone). This peak is ascribed to chlorophyll *a* [45]. The presence of chlorophyll *d* in red algae has been disputed [15,46]. This chlorophyll absorbs at 688 and 692 nm in diethylether and acetone, respectively [47]. In the present investigation no peak at these wave lengths could be observed. Chlorophyll *d* is therefore absent (or present in trace amounts only, compare [15]) in the red algae analyzed.

Both the chlorophyll *a* content and the carotenoid: chlorophyll *a* ratio are in good agreement with the values that may be calculated from the literature [20,48].

DISCUSSION

The two-step TLC method applied (see Experimental) resulted in complete separation of the 8 following carotenoids: α-carotene, β-carotene, α-cryptoxanthin, β-cryptoxanthin, lutein, zeaxanthin, fucoxanthin, and neoxanthin, all of which have been reported in red algae. TLC-system I also differentiated between acetylenic (diatoxanthin and diadinoxanthin) and non-acetylenic (lutein, zeaxanthin, and antheraxanthin) carotenoids as the former possessed considerably lower R_f-values than the latter. Additional support for the identity of the isolated carotenoids was obtained by their visible spectra, IR, and MS, and by simple chemical methods. MS was the most valuable tool. Knowledge of the molecular weights distinguished non-acetylenic carotenoids conclusively from their acetylenic analogues. Fragment ions characteristic of unsubstituted aliphatic end groups [36], primary in-chain hydroxyl groups [49,50,51], epoxidic groups [52] or furanoids [52] were not observed in any of the spectra. Carotenoids with hydroxyl groups in the 2- or 2′-positions may be spectroscopically discerned by PMR only, but possess higher R_f-values than both the 3-OH and the 4-OH analogues in the paper-chromatographic systems applied [53].

The present investigation has supported the earlier view on rhodophycean carotenoids. The class is able to synthesize bicyclic carotenoids belonging to both the α- and the β-series, and the xanthophylls are simple non-acetylenic mono- and di-hydroxy derivatives of α- and β-carotene. Only exceptionally (see the introductory part) have more highly oxygenated xanthophylls been reported. The rhodophycean carotenoids thereby bear greatest resemblance to the carotenoids of the Chlorophyceae (for the following discussion compare [23]) but are more primitive in the sense that they are generally not so highly oxidized. They deviate, however, from the acetylenic xanthophylls found in most classes of the Chromophyta and in the Euglenophyceae. It is also noteworthy that each of the 3 phycobilin-containing classes (Cyanophyceae, Rhodophyceae, and Cryptophyceae) possess their own characteristic carotenoid composition. A few representatives of the Cyanophyceae (see [54]), however, seem to lack glycosidic carotenoids and thereby

attain a carotenoid composition more similar with that of *P. brodiaei* and *P. urceolata* (present investigation).

A key substance in the chemotaxonomy of algae based on carotenoids is fucoxanthin. This carotenoid has been reported both in classes with acetylenic (Dinophyceae (exceptionally) (see [55]), Chrysophyceae, Haptophyceae (not always [56]), and Bacillariophyceae) and non-acetylenic (Rhodophyceae (exceptionally [3]), and Phaeophyceae) main xanthophylls. Within the Rhodophyceae fucoxanthin has been detected in *Callithamnion pikeanum* [57], *Ceramium rubrum* [58], and *Polysiphonia nigrescens* [3]. Diatoms were considered as a possible source of the fucoxanthin in the two first-mentioned algae while the possibility of biological contaminants was not discussed for *P. nigrescens*. In the present investigation the amounts of fucoxanthin found in naturally occurring *B. fuscopurpurea*, *C. rubrum*, and *P. brodiaei* were comparable with the amounts expected to be contained in the epiphytic diatom flora (species of *Cocconeis* and *Licmophora*) present in the material. The conclusion that the fucoxanthin was not synthesized by the 3 red algae themselves was supported by the absence of fucoxanthin in the cultured material of the two first-mentioned species. *N. helminthoides*, however, contained almost 400 times more fucoxanthin than could be accounted for by the diatoms present. Endophytic diatoms, which have sometimes been reported as abundant both in the Rhodophyceae [59] and the Phaeophyceae [60] could not be detected by microscopic examination of numerous squash-preparates. Analysis of the cultured material was negative for fucoxanthin as well as for the polar carotenoid tentatively identified as neoxanthin in a previous work [11]. *N. helminthoides* accordingly requires a reinvestigation, but according to the results obtained for the cultured sample fucoxanthin has been excluded from Table 1.

The α-carotene/β-carotene and the lutein/zeaxanthin ratios have been claimed [8] to express the main differences in the carotenoid composition within the Rhodophyceae. Similar results were obtained for the carotenes by Larsen and Haug [6]. In the present investigation *P. brodiaei* and *P. urceolata* were outstanding as they contained neither α-carotene nor lutein. These two algae may therefore be unable to perform the α-cyclizing step in the carotenoid biosynthesis.

The biosynthetic intermediates α-cryptoxanthin or β-cryptoxanthin were detected in 3 species (Table 1) in so small amounts that they may be easily overlooked. α-cryptoxanthin has been supposed [17] to be restricted to a few species within the Rhodomelaceae or even to the Amansia group. The isolation of this carotenoid from *C. rubrum* (family: Ceramiaceae) disproved this suggestion, and also represents the first isolation of this carotenoid from a monoalgally cultured red alga.

Taraxanthin has been reported in both *R. palmata* [2] and *C. rubrum* [4] but could not be detected in any of these two algae during the present investigation. The taraxanthin reported was probably *cis*-lutein which is inevitably formed during usual chromatographic manipulations (compare [3]). However, this assumption cannot explain the large amounts of taraxanthin isolated by de Nicola and Furnari [7] from several red algae (70% of the total carotenoids in *Grateloupia filicina*).

EXPERIMENTAL

Biological material. The red algae were collected at the following localities: naturally occurring material: *Bangia fuscopurpurea* (Dillw.) Lyngb (Hvaler, Østfold; April 22nd 1971), *Nemalion helminthoides* (Vell. in With Batt.) (Eftang, Vestfold; August 7th 1971), *Gigartina stellata* (Stackh.) Batt. (Frøya, Sør-Trøndelag; June 23rd 1971), *Rhodymenia palmata* (L). Grev. (Trondheim, Sør-Trøndelag; May 9th–11th 1971), *Ceramium rubrum* (Huds.) C.Ag., (Sotra, Hordaland; July 17th 1971), and *Polysiphonia brodiaei* (Dillw.) Spreng. (Sotra, Hordaland; July 17th 1971). Monoalgally cultured material: *B. fuscopurpurea* (Rissa, Sør-Trøndelag; May 22nd 1971; yield of monoalgally cultured material after 236 days at 1200 lx: 1·12 g dry-weight). *N. helminthoides* (isolated from the Swedish west coast by L. Fries; provided amount: 0·17 g), *B. hamifera* Hariot; (Rissa, Sør-Trøndelag; January 29th 1972; yield after 300 days at 800 lx: 1·43 g), *C. rubrum* (Trondheim, Sør-Trøndelag; March 16th 1972; yield after 64 days at 1800 lx: 0·63 g), *P. urceolata* (Lightf. ex. Dillw.) Grev. (Sund, Hordaland; July 20th 1971; yield after 158 days at 800 lx: 1·12 g). The algae were extracted fresh or stored at −20° until use. The algae were cultured [61] at 12° in a 14 hr/10 hr light/dark regime. The light source was equal numbers of Phillips TL/33 and TL/55 fluorescent tubes. Petri-dishes of glass with diameter 90 mm were used as culture vessels. About 40 ml of culture medium were supplied to each Petri-dish, and the medium was renewed weekly.

The culture medium was an enriched sea water medium. The sea water was pumped from 30 m depth at the Biological Station, Trondheim, filtered twice through glass fibre paper (Whatman GF/C), and autoclaved at 120° for 20 min. The following additives were used (mg per liter of autoclaved sea water): Macro elements: $NaNO_3$ (75), $NaH_2PO_4.H_2O$ (5), $NaHCO_3$ (16·8); trace elements: [Na_2EDTA (4·36)], $FeCl_3.6H_2O$ (3·15), H_3BO_3 (0·246), $MnCl_2.4H_2O$ (0·18), $AlK(SO_4)_2.12H_2O$ (0·039), $ZnSO_4.7H_2O$ (0·022), $Cd(NO_3)_2.2H_2O$ (0·011), $NiSO_4.7H_2O$ (0·011), $CoCl_2.6H_2O$ (0·01), $CuSO_4.5H_2O$, (0·01), KJ (0·0067), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0·0055), $Cr(NO_3)_3.9H_2O$ (0·0032), $Na_2WO_4.2H_2O$ (0·0029), $VOSO_4.5H_2O$ (0·0019); vitamins: thiamin.HCl (0·2), Ca-D-pantothenic acid (0·1), nicotinic acid amide (0·1), pyridoxine.HCl (0·04), pyridoxamine.2HCl (0·02), *p*-amino-benzoic acid (0·01), riboflavin (0·005), folic acid (0·0025), vitamin B_{12} (0·001), biotin (0·0005). The composition of the medium was based on the media successfully applied in earlier works [62–64]. Silicate was omitted as an additive to give less favourable conditions for the growth of diatoms [65] during the isolation period. Other deviations from the media used in the references cited above were due to the immediate non-availability of the appropriate reagent.

Monoalgal cultures were achieved from monospores (*B. fuscopurpurea*), tetraspores (*C. rubrum*), and sprouting non-fertile shoot tips (2 mm long) (the remaining species). Larger biological contaminants (if any) were eliminated by repeated pushing of shoot tips through 1·75% solid agar [66]. Contaminating Cyanophyta were killed by treatment with antibiotics (Oxoid Multodiscs, code 30-2H and code 11-15F).

Physical and chemical methods. The commonly applied methods for isolation and identification of carotenoids have been used [40,43,67]. Electronic spectra (including the *cis*-peak region) were recorded in petrol. (exceptionally Et_2O) and $CHCl_3$. When the carotenoid amounts were less than 0·005 mg a Cary 17H Spectrophotometer was sometimes used. IR was recorded in KBr pellets. MS were obtained on an AEI MS 902 instrument by the application of the direct insertion technique. The ionization energy was 70 eV, and the temperature of the source block heaters 190° (215, 225, 230 or 250° for fucoxanthin).

The carotenoids of *C. rubrum* (naturally occurring material) and *P. brodiaei* were separated into carotenes, monohydroxy-xanthophylls, dihydroxy-xanthophylls, and fucoxanthin by column chromatography. The adsorbent was cellulose powder (S & S 123) with 20% (w/w) kiesel gel G nach Stahl, and the eluent was petrol. with increasing amounts of 5% (v/v) iso-propanol in acetone. The rough separation of the other extracts was performed on TLC-plates with kieselgel G or kieselgel G–$CaCO_3$ as 1:1 (w/w) as an adsorbent. The fractions

obtained were further purified by TLC. Plates with kieselgel G–$Ca(OH)_2$–MgO–$CaSO_4$, 10:4:3:1 (w/w), as an adsorbent and varying amounts of petrol., Me_2CO, and iso-propanol as eluents (TLC-system I) were most commonly applied. Plates with alumina G, alumina HF_{254}, or MgO–$CaSO_4$, 87:13 (w/w), were used exceptionally. One-dimensional circular paper chromatography [68] was used for co-chromatographic purposes [69] and to eliminate *cis*-isomers prior to electronic spectrophotometry. Alumina paper (S & S 288) was utilized to purify the carotenes [70] while a kieselguhr paper (S & S 287) was used for the xanthophylls [68]. The identity of all red algal carotenoids isolated was confirmed by co-chromatography both on TLC (TLC-system I) and on paper.

The authentic carotenoids were isolated from the following sources: α-carotene (*Daucus carota*), β-carotene (*D. carota*), α-cryptoxanthin (*Capsicum annuum flavum*), β-cryptoxanthin (*Physalis alkekengi*), lutein (*Medicago sativa*), zeaxanthin (*Arthrospira* sp.), and fucoxanthin (*Pelvetia canaliculata*). In addition, the chromatographic behaviour of the following algal carotenoids was ascertained: lutein epoxide (partial synthetic from lutein diacetate by epoxidation with *m*-Cl-perbenzoic acid [71–73]), antheraxanthin (*Lilium tigrinum*), violaxanthin, *all-trans* (*Viola tricolor*), violaxanthin, *cis* (isomerized violaxanthin, *all-trans*), diatoxanthin (*Amphidinium carteri*), diadinoxanthin (*Euglena gracilis*), and neoxanthin (*Hordeum vulgare*).

Iodine catalyzed isomerization [32], acetylation [32], selective methylation of allylic hydroxyl groups [42], and the epoxide test [44] were performed as recommended in the references cited. The lability of fucoxanthin towards alkaly was tested by treatment with equal amounts (v/v) of methanolic KOH (5% v/w) and diethylether for 12 hr.

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