Preface

The work presented in this thesis was carried out at the Department of Chemistry at the Norwegian University of Science and Technology (NTNU) during the period January 2000 to May 2004. The thesis is based on 14 papers, of which 11 were published or in press when the thesis was completed. The thesis represents an overview of these papers, and the three main chapters are organised according to the topics of the papers. The papers, containing a more thorough discussion of the results, as well as all the experimental details, are included at the end.

The first chapter gives a brief introduction to carotenoid chemistry.

The second chapter deals with the isolation and analysis of carotenoids from natural sources. Two phylogenically related extremophilic bacteria, and a marine yeast are treated here.

The third chapter describes the use of NMR spectroscopy for the characterisation and structure determination of a series of carotenoids and a new hopanoid isolated from oakmoss, and the use of CD spectroscopy for attempted determination of optical activity of chiral *Z*-isomers of *meso*-zeaxanthin.

The fourth chapter is a summary of the work on charge delocalised carotenoid cations. For the first time, the detailed structures and reactivity of charged carotenoids have been determined. These charged carotenoids are also the most delocalised polyene cations for which a complete structure has been determined. The results have been used for the determination of the width of a free soliton. The work on the carotenoid cations has been performed in collaboration with two graduate students. They have done much of the work on the reactions and characterisation of the products. Their contribution is reflected by the order of the authors on each paper.

I would like to thank my supervisor, Professor em. Dr. techn. Dr. h. c. Synnøve Liaaen Jensen, for sharing her knowledge and enthusiasm, and for guiding me through this work.

Professor Jostein Krane is thanked for help in acquiring the NMR spectra of the carotenoid cations, and for spending hours in the NMR-lab shimming the NMR instruments with these difficult samples. Associate Professor Dr. techn. Helge Kjøsen is thanked for help with mass spectrometry, and for the elegant solutions to all kinds of practical problems in the lab.

I would also like to thank the two students who graduated in the group; siv. ing. Liv Bruås, who did the initial work on the preparation and reactivity of carotenoid cations prepared with BF_3-etherates, and siv. ing. Geir Kildahl-Andersen, who joined the group at a later stage, and obtained a large amount of results, especially on the reactivity and structure of carotenoid cations (including NMR) prepared with Brønsted acids.

Siv. ing. Berit G. Petersen is thanked for her assistance in finding typos and improving the readability of this thesis.

The doctoral fellowship was given by the Department of Chemistry, NTNU. Hoffmann-La Roche, Basel, is thanked for additional economical support. Travel grants for participation in several international conferences, received from various sources, are gratefully acknowledged.

Cover photo from supplement to Adresseavisen 01.04.2004 and Aftenposten 02.04.2004

Contents

Abbreviations

COSY	Correlation Spectroscopy (NMR technique)
CD	Circular Dichroism
EPR	Electron Paramagnetic Resonance
GC	Gas Chromatograhy
HMBC	Heteronuclear Multiple Bond Correlation (NMR technique)
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence (NMR technique)
IUPAC	International Union of Pure and Applied Chemistry
MS	Mass Spectrometry
NIR	Near Infrared
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy (NMR technique)
ROESY	Rotating frame Overhauser Effect Spectroscopy (NMR technique)
R_T	Retention time
TIC	Total Ion Current
TLC	Thin Layer Chromatography
UV	Ultra Violet
VIS	Visible light

1 Introduction

In this brief introduction to the carotenoid field, a short definition of carotenoids, the numbering of the carotenoid carbon skeleton, and key references to carotenoid literature are given. It is intended mainly for organic non-carotenoid chemists.

Carotenoids are a class of tetraterpenes with a characteristic isoprenoid polyene chain connecting the two end groups which may be acyclic or cyclic. Carotenoids are synthesised *de novo* by all photosynthetic plants and bacteria, and by some heterotrophic bacteria, yeasts and fungi. Certain animals are able to modify the structure of the carotenoids in their diet.

To date, around 800 naturally occurring carotenoids have been encountered,[1] in addition to numerous carotenoids prepared synthetically. The isolation and analysis,[2] spectroscopy,[3] synthesis,[4] biosynthesis and metabolism,[5] and health aspects[6] of carotenoids have been treated in recent monographs.

Formally, all carotenoids are derivatives of ψ,ψ-carotene (lycopene, **1**), formed by hydrogenation, dehydrogenation, oxidation, cyclisation or cleavage of a carbon bond. Some compounds containing other than 40 carbon atoms are also classified as carotenoids, particularly some triterpenes and pentaterpenes containing the typical polyene system. A complete definition and nomenclature of the carotenoids was given by IUPAC.[7] Figure 1 shows the structures of the two basic carotenoids lycopene (**1**) and β,β-carotene (**2**), with the numbering of the carbon skeleton included. Also shown is the structure of 2*S*,2'*S*-bacterioruberin (**3**) as an example of a C_{50}-carotenoid.

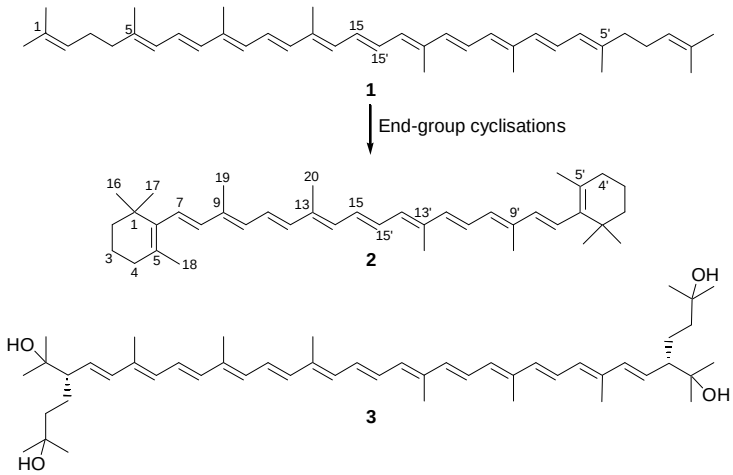


Figure 1. Structures of lycopene (**1**), β,β-carotene (**2**) and 2*S*,2'*S*-bacterioruberin (**3**) with numbering of the carbon skeleton indicated.

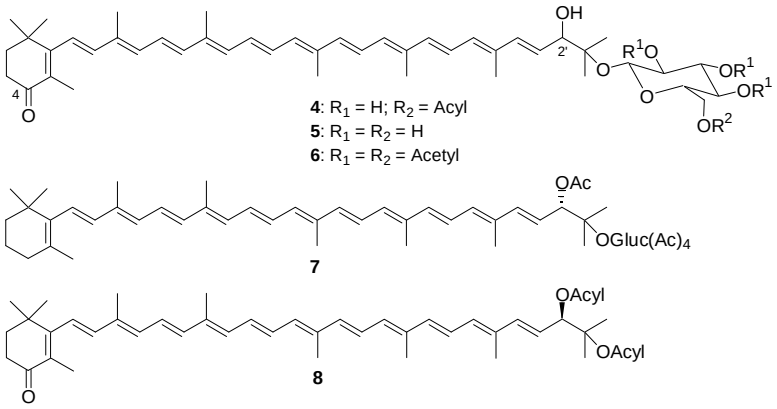
A semi-systematic nomenclature for carotenoids has been approved by IUPAC.[7] However, trivial names are still widely used in the carotenoid field, since the semi-systematic names often become long and complicated.

2 Isolation and Analysis of Carotenoids from Natural Sources

2.1 Carotenoids from *Salinibacter ruber*

The extremely halophilic eubacterium *Salinibacter ruber* was recently isolated from saltern crystalliser ponds in Southern Spain.[8,9] Saltern ponds are often coloured red due to the presence of the green microalgae *Dunaliella*, containing β,β-carotene (**2**), or halophilic *Archaea*, containing bacterioruberin (**3**). This novel bacterium was also coloured brightly red due to the presence of carotenoids. Professor A. Oren at The Hebrew University of Jerusalem provided lyophilised cells for the determination of the carotenoids of *S. ruber*.

After lysis of the cells and extraction by methanol/acetone, HPLC analysis of the crude extract revealed the presence of one major carotenoid (>96% of total). The carotenoid was isolated in a series of purification steps (column chromatography and preparative TLC), that were complicated by large amounts of lipids in the extract. The carotenoid was identified from the NMR and MS data as the carotenoid glucoside **4**, esterified in the C-6 position of the sugar moiety.

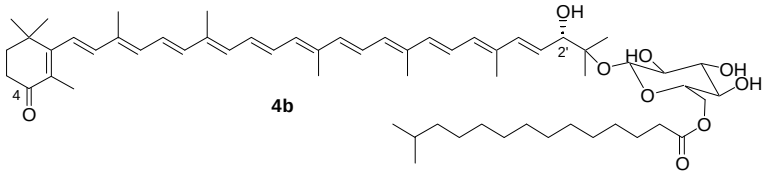


The structure was confirmed by NMR analysis of the free glucoside **5**, prepared by alkaline hydrolysis of **4**, and by MS of its tetraacetate **6**, formed by acetylation of **5**. VIS spectra of **4**, its $NaBH_4$ reduction product, and the product formed by allylic oxidation with *p*-chloranil were compatible with the dodecaenone chromophore, as shown in **4**.

The esterifying fatty acid was isolated after hydrolysis of **4** and methylated by treatment with BF_3-dee/methanol. Analysis by GC-MS revealed a saturated C_{15} fatty acid, which was shown by co-chromatography with authentic samples to be the branched 13-methyltetradecanoic acid.

The chirality at C-2' was determined by CD spectroscopy. Whereas a free hydroxyl in the 2' position of β,ψ-carotenes only give a very weak CD signal, the 2' acetates are known to exhibit a strong Cotton effect.[10,11] The CD spectrum of peracetylated **4** (**6**) was thus compared to that of pheixanthophyll pentaacetate (**7**) and plectaniaxanthin diester (**8**), revealing 2'*S* configuration for **4**.

The structure of the carotenoid isolated from *S. ruber* was thus determined as (all-*E*, 2'*S*)-2'-hydroxy-1'-[6-*O*-(13-metyltetradecanoyl)-β-D-glucopyranosyloxy]-3',4'-didehydro-1',2'-dihydro-β,ψ-caroten-4-one (**4b**). The carotenoid was given the trivial name salinixanthin (**4b**).



The isolation and structure determination of the carotenoid in *Salinibacter ruber* is presented in Paper I.

2.2 Carotenoids from *Rhodothermus marinus*

The moderately halophilic thermophilic eubacterium *Rhodothermus marinus* was first isolated from a submarine hot spring in Isafjarðardjúp in NW Iceland.[12] It has later been found in effluents of geothermal powerplants in the Blue Lagoon (Iceland), and in hot-springs in the Azores (Portugal) and Naples (Italy).[13-15] Freeze-dried bacterial cells were provided by Dr. S.K. Pétursdóttir at Prokaría Ltd, Reykjavík. The isolation and structure determination of the carotenoids of *R. marinus* was started by Dr. Å. Strand at Sør-Trøndelag University College, but the project was delayed due to problems with the large amounts of lipids in the extract. After the completion of the salinixantin (**4b**) structure, it was of interest to resume the project because of the similar chromatographic (TLC, HPLC) and spectroscopic (VIS) properties of the carotenoids of the two bacteria, and because of the recent finding that *S. ruber* is the closest known relative of *R. marinus*, based on the bacterial 16S rRNA genes.[9,16]

The carotenoids were extracted with methanol/acetone from the freeze-dried bacteria, which had been ground in a mortar. Reversed phase HPLC of the extract showed two peaks with polarity as expected for carotenoid glycosides and a series of peaks with polarity compatible with carotenoid glycoside esters, see Figure 2.

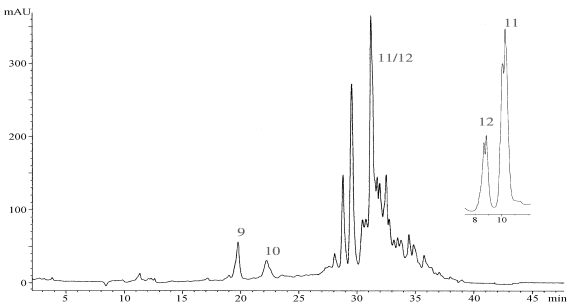
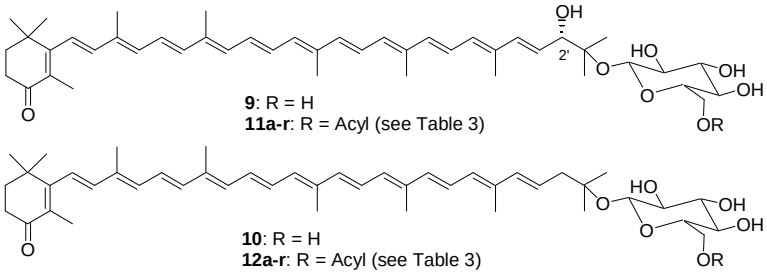


Figure 2. HPLC chromatogram of the raw extract from *R. marinus*. Inserted is shown the normal-phase separation of the carotenoid glucosides esters **11** from its hydroxy substituted analogue **12**.

After hydrolysis, the carotenoids eluted in two peaks with the same retention time as the two peaks of the non-hydrolysed sample, which were suspected to be carotenoid glycosides. The two hydrolysed carotenoids were separated by preparative HPLC to give the carotenoid glucosides **9** and **10**, as judged by ^{1}H NMR and VIS data, as well as ^{1}H NMR, MS, VIS and CD data for the peracetylated analogues.



By normal-phase preparative HPLC, the non-hydrolysed carotenoid glucoside esters **11** and **12** could be separated into two fractions, see insert in Figure 2. After re-chromatography in the same HPLC system, the carotenoid fatty esters were re-esterified by methoxide in methanol. The fatty acid methyl esters thus formed were analysed by GC-MS to provide the composition of the esterifying fatty acids, Table 1.

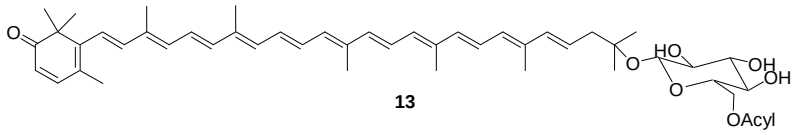
Table 1. Composition of fatty acid methyl esters derived from the carotenoid acyl glucosides **11** and **12** by GC-MS based on the intensity of the McLaffery ion at *m/z* 74 and of the TIC.

				11		**12**	
	R_T (min)	M		TIC (%)	*m/z* 74 (%)	TIC (%)	*m/z* 74 (%)
a	5.06	200	*iso*-C11:0		traces		
b	5.13	200	*anteiso*-C11:0		traces		
c	6.00	214	*iso*-C12:0	9	10	9	10
d	6.32	214	*n*-C12:0	1	1	1	1
e	6.89	228	*iso*-C13:0	13	14	16	17
f	6.95	228	*anteiso*-C13:0	16	14	18	15
g	7.18	228	*n*-C13:0		traces		
h	7.70	242	*iso*-C14:0	8	9	6	8
i	7.98	242	*n*-C14:0	4	5	4	5
j	8.48	256	*iso*-C15:0	10	10	10	11
k	8.55	256	*anteiso*-C15:0	19	15	19	15
l	8.74	256	*n*-C15:0	1	1	1	1
m	9.21	270	*iso*-C16:0	7	8	5	6
n	9.47	270	*n*-C16:0	6	7	6	7
o	9.91	284	*iso*-C17:0	1	2	1	2
p	9.96	284	*anteiso*-C17:0	3	3	3	3
q	10.64	298	*iso*-C18:0		traces		
r	10.83	298	*n*-C18:0	0	1	1	1

The analysis of the carotenoids in *Rhodothermus marinus* is presented in Paper II.

2.3 Structure and function of carotenoids in extremophilic bacteria

In the two bacteria studied here, *S. ruber* and *R. marinus*, closely related carotenoids were found, which is in agreement with their close phylogenic relationship. When compared to the carotenoids of other bacteria living under extreme conditions (high or low temperature, high salinity, high or low pH and strong light) the high occurrence of carotenoid glycosides and carotenoid glycoside esters is striking. The carotenoids found in these organisms include 1'-glucopyranosyloxy-3,4,3',4'-tetradehydro-1',2'-dihydro-β,ψ-caroten-2-one acylated in the 6''-position (**13**), zeaxanthin mono- and diglucoside esters (**14**, thermozeaxanthins), and the C_{30}-carotenoid acyl glucoside **15**, from *Meiothermus ruber*,[17] *Thermus thermophilus*,[18] and *Heliorestis spp*.,[19] respectively.



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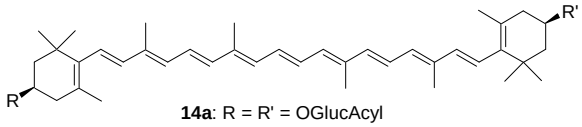
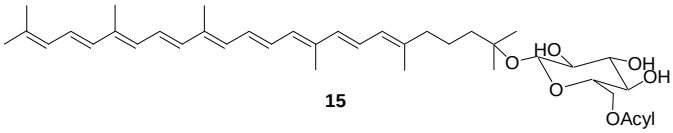


14a: R = R' = OGlucAcyl
14b: R =OGlucAcyl, R' = OH
14c: R =OGlucAcyl, R' = H
14d: R = R' = OGluc
14e: R = R' = ORham



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In addition to the general function of these carotenoids to protect against the strong oxidising environments under which these bacteria are living, it has been suggested that they play a special role as membrane stabilisers.[18,20,21] C_{50}-carotenoids, such as bacterioruberin (**3**),[20,21] have a suitable length to fit into the lipid membranes. Also for the thermozeaxanthins (**14**),[18] a model has been developed for the incorporation of these carotenoids into the membrane, see Figure 3.

This model can easily be adopted to fit also the carotenoids of the salinixanthin (**4b**) type found in *S. ruber* and *R. marinus*. A strengthening of the lipid bilayer by incorporation of natural thermozeaxanthins (**14**)[22] or by incorporation of a synthetically pure *iso*-C15:0 acylated thermozeaxanthin[23] has been reported. The high degree of *iso* and *anteiso* fatty acids in the esterifying fatty acids of the carotenoids from *R. marinus* is in agreement with the composition of fatty acids in these bacteria as determined previously.[15,24,25] The occurrence and function of such fatty acids in bacteria have been reviewed.[26]

The composition and function of carotenoids in extremophiles is discussed in Paper II.

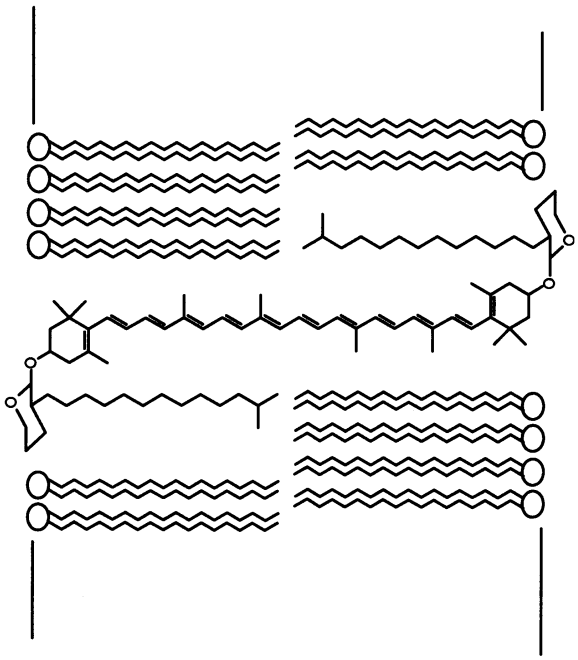
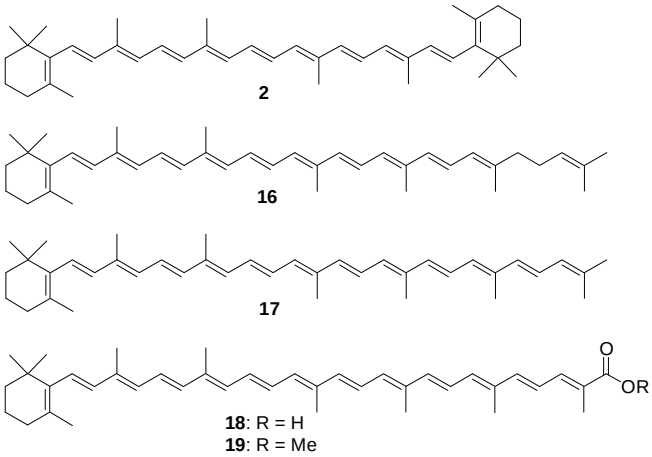


Figure 3. Model for the incorporation of carotenoid glucoside esters of the thermozeaxanthin (**14**) type in a lipid membrane. Figure from Yokoyama *et al*.[18]

2.4 Carotenoids from *Rhodotorula sp. nov*.

Dr. B. Landfall at the University of Tromsø recently isolated a new species of psycrophilic marine yeast of the genus *Rhodotorula*. Frozen yeast cells, whose cell walls had been disrupted using a French-press, were supplied for examination of the red carotenoid pigments.

The carotenoids were extracted with acetone, and distributed between hexane and alkaline, aqueous methanol. The non-polar carotenoids were separated and isolated by preparative TLC to yield three carotenoids, which were identified as β,β-carotene (**2**), β,ψ-carotene (γ-carotene, **16**) and 3',4'-didehydro-β,ψ-carotene (torulene, **17**) based on VIS and MS data, and co-chromatography with authentic samples by HPLC. The isolation of the polar, acidic carotenoid was hampered by large amounts of acidic, colourless contaminants, still the R_T (HPLC) and VIS data indicated that the carotenoid was 3',4'-didehydro-β,ψ-caroten-16'-oic acid (torularhodin, **18**). The acid was methylated with diazomethane and the resulting methyl ester isolated by preparative TLC. Subsequently, MS, VIS and co-chromatography with an authentic sample proved this compound to be methyl 3',4'-didehydro-β,ψ-caroten-16'-oic acid (torularhodin methyl ester, **19**).



This carotenoid complement is characteristic of *Rhodotorula spp*.[1,27-31] The effect on the carotenoid production and composition by variation of the growing conditions for *Rhodotorula spp* have been extensively studied, including aeration,[29] sugar supplementation,[28] illumination,[30,32-34] agro-industrial waste as substrate,[35] temperature[34,36] and carbon/nitrogen ratio of the medium.[37]

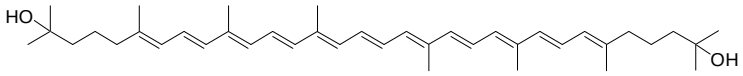
As the only known sources of torularhodin (**18**), the possibility of using *Rhodotorula spp*. for future isolation of the gene transferring torulene (**17**) to torularhodin (**18**) is pointed out. So far, no plausible intermediates (carotenol or carotenal) for this transformation have been unequivocally identified from natural sources.[1] The role of torularhodin as a scavenger against peroxyl radicals[38] and as a singlet oxygen quencher[32] have been investigated.

The study on the carotenoid composition of this new *Rhodotorula* species is presented in Paper III.

3 Spectroscopic Studies of Carotenoids and Related Compounds

3.1 Assignments of NMR chemical shifts for selected carotenoids

The new Carotenoids Handbook[1] was recently published, giving a selective review of the carotenoid literature. Included are references to NMR data for the naturally occurring carotenoids where complete or partial NMR data are available. Some of the carotenoids for which no NMR data are provided in the Carotenoids Handbook were available in synthetic form. This was the case for the bacterial carotenoids 1,2,1',2'-tetrahydro-ψ,ψ-carotene-1,1'-diol (OH-rhodopin, **20**), 1'-methoxy-1,2,1',2'-tetrahydro-ψ,ψ-caroten-1-ol (rhodovibrin, **21**), 1-methoxy-3,4-didehydro-1,2-didehydro-ψ,ψ-carotene (anhydrorhodovibrin, **22**), ϕ,ψ-carotene (chlorobactene, **23**) and for ϕ,ϕ-carotene (isorenieratene, **24**), which had been prepared by total synthesis (cf. Paper X). Complete ^{1}H and ^{13}C NMR data for these carotenoids (only ^{1}H for **25**) and for the synthetic carotenoids 1'-hydroxy-1-methoxy-3,4-didehydro-1,2,1',2'-tetrahydro-ψ,ψ-caroten-2-one (2-ketorhodovibrin, **25**) and 4',5'-didehydro-4,5'-*retro*-β,β-carotene (isocarotene, **26**) was obtained from ^{1}H, ^{1}H-^{1}H COSY, 1D and 2D ROESY, ^{13}C, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC NMR spectra recorded in $CDCl_3$ at 400, 500 or 600 MHz.



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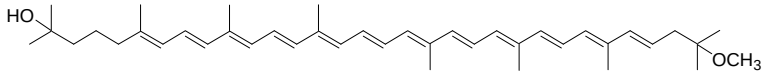

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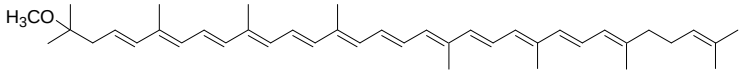

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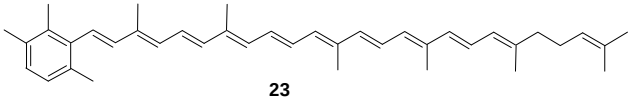

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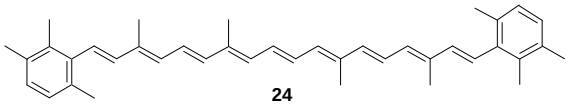

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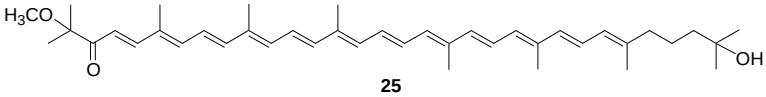

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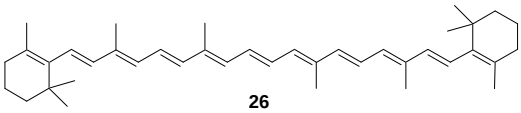

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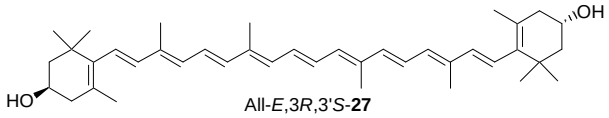
NMR data for these carotenoids are published in Paper IV (**20**-**23** and **25**) and in Paper X (**24** and **26**).

3.2 Generation of optical activity by *E*/*Z* isomerisation of *meso*-zeaxanthin

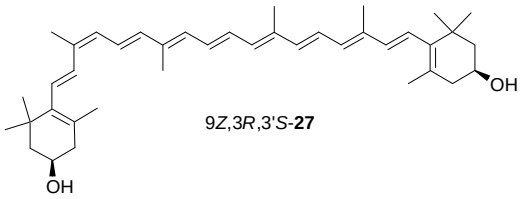
Due to a two-fold alternating axis of symmetry, the all-*E* isomer of the 3*R*,3'*S* diastereomer of β,β-carotene-3,3'-diol (zeaxanthin, **27**) is achiral, and thus optically inactive. This *meso* form is naturally occuring, together with the enantiomeric pair 3*R*,3'*R*-**27** and 3*S*,3'*S*-**27** in many animals, but not in plants.[1]



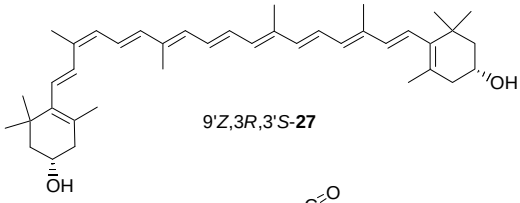
All-*E*,3*R*,3'*S*-**27**

The symmetry is lost upon the *E*/*Z* isomerisation of a double bond, which results in the formation of a chiral compound. In principle, this *Z*-isomer should be optically active. Since the origin of chirality in nature is still unknown, this would be an interesting case where optical activity is achieved by *E*/*Z* isomerisation of an achiral compound.

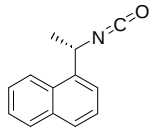
Catalytic stereoisomerisation of carotenoids using iodine as catalyst is known to give an equilibrium of *E*/*Z* isomers, in which the 9*Z* and and 13*Z* isomers are the most abundant due to the low steric interactions, and the stabilising effect of the methyl groups in these positions. For *meso*-zeaxanthin (3*R*,3'*S*-**27**), the isomerisation at C-9/9' results in the formation of a racemate of the two enantiomers 9*Z*,3*R*,3'*S*-**27** and 9'*Z*,3*R*,3'*S*-**27**.



9*Z*,3*R*,3'*S*-**27**



9'*Z*,3*R*,3'*S*-**27**



S-**28**

The racemic 9*Z* and 9'*Z* enantiomers were isolated from the other isomers in the equilibrium mixture by preparative HPLC on a C_{30} column. As expected, the racemate showed no optical activity, as determined by CD spectroscopy. Separation of the two enantiomers by chiral HPLC was unsuccessful. However, after derivatisation with the

chiral icocyanate, *S*-(+)-1-(1-naphtyl)ethylisocyanate (*S*-**28**), to two diastereomeric dicarbamates, separation and isolation of the two 9*Z*/9'*Z* diastereomers was possible by chiral HPLC.

Attempted cleavage of the carbamate moiety to regain the free diols was unsuccessful, and the CD spectra therefore had to be obtained from the 9*Z*/9'*Z* isomers in the derivatised state. These spectra were correlated with spectra recorded of all-*E*-*meso*-zeaxanthin (3*R*,3'*S*-**27**) compared to the dicarbamates of 3*R*,3'*S*-**27** formed by reaction with the enantiomeric isocyanates *R*-**28** and *S*-**28**, and with the carbamate formed by reaction of methanol with *S*-**28**.

The study of the optical activity of *Z*-isomers of *meso*-zeaxanthin (3*R*,3'*S*-**27**) was publised in Paper V. It was concluded that no optical activity was induced despite the formation of a chiral compound.

3.3 A novel hopanoid from oakmoss (*Evernia prunastri*)

Evernia prunastri is a lichen growing primarily on oak trees. It is widespread in Southern Europe and Western Mediterranean Africa. The great interest in the chemical composition of this lichen is due mainly to its widespread use in perfume industry and to its use in the European standard test for diagnosing perfume allergy.[39,40] The chemical composition of *E. prunastri* has been extensively studied, and five triterpenes have been identified based on tandem MS studies.[41]

In the 1950's, the late Dr. T. Bruun at our Department studied the composition of terpenes in the residue of oakmoss extracts, which was provided by N.V. Chemische Fabriek Naarden (now Quest International), Naarden, The Netherlands. From a non-polar extract, a compound was isolated, whose structure could not be determined by the spectroscopic techniques available at the time.

MS of this compound showed a fragmentation pattern typical of triterpenes, and by high-resolution electrospray MS, the molecular formula was determined to be $C_{32}H_{54}O_5$. The electron impact MS spectrum also showed a typical fragment ion for a dihydroxypropyl moiety (*m*/*z* 75).

The ^{1}H and ^{1}H-^{1}H COSY spectra were very crowded in the 1 – 2 ppm region. ^{1}H-^{13}C HMBC proved to be a powerful technique for the structure determination of the triterpene, since long-range correlations through $^2J_{H,C}$ and $^3J_{H,C}$ coupling constants from the methyl groups defined most of the carbon skeleton, as shown with bold bonds in Figure 4.

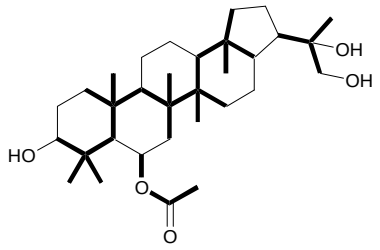
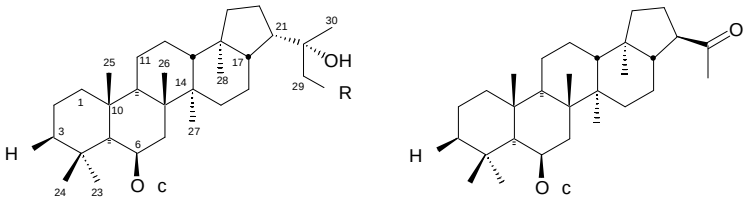


Figure 4. HMBC correlations (bold bonds) defining major part of the hopane skeleton.

From this and the other (^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY, NOESY, ^{1}H-^{13}C HSQC) NMR spectra obtained it was thus possible to assign all ^{1}H and ^{13}C chemical shifts of this novel hopanoid, 6-*O*-Acetyl-21*b*H-hopane-3*b*,6*b*,22,29-tetrol (**29**). The *S*-chirality at C-22 could be determined by correlation of the chemical shift of H-29a and H-29b of *O*-29-acetylated **29** (**30**) to the two C-22 epimers of 29-acetoxyhopan-22-ol.[42]



Attempted recrystallisation of the hopanoid **29** from CH_2Cl_2/MeOH resulted in the formation of a new hopanoid, formed by the formal loss of CH_4O. This hopanoid was identified as 30-nor-6-*O*-acetyl-3*b*,6*b*-dihydroxy-21*a*H-hopan-22-one (**31**), a compound which has previously been detected in an extract of *E. prunastri* in an MS/MS study.[41] A plausible mechanism for the loss of formaldehyde and H_2 through a 6-ring transition state is shown in Figure 5.

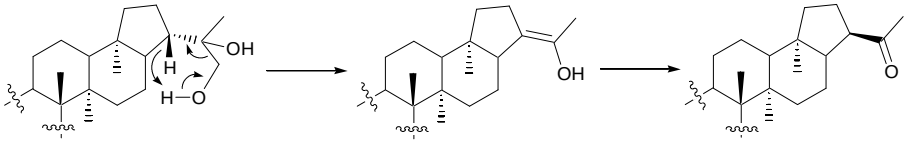


Figure 5. Plausible reaction mechanism for loss of CH_4O from hopane **29**.

No biological activity was found for the hopane **29** when tested against the human pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli*, or against the fish pathogenic bacteria *Vibrio anguillarum* and *Vibrio splendidus*.

The structure determination of the novel hopanoid from *E. prunastri* is presented in Paper VI.

4 Structure and Reactivity of Charged Carotenoids

The term charged carotenoids may refer to carotenoids containing charged functional groups[1] as well as to carotenoids with the charge on the carbon backbone. In this thesis, only carotenoids with a positive charge on the polyene backbone are considered. These carotenoid cations may be prepared either by oxidation (removal of π-electrons by chemical or electrochemical methods), by addition of a cation (usually proton), or by elimination of an allylic anionic moiety (usually hydroxide after protonation).

Carotenoid cation radicals have been detected in nature in the reaction centres of Photosystems I and II, which oxidises water to oxygen in photosynthesis. Charged polyene systems are also of interest for the understanding of electron transport in conducting organic polymers, and in the field of molecular design. This includes molecular wires, molecular switches, and push-pull polyenes with nonlinear optical properties. For further details on charged carotenoids, cf. the review article presented as Paper VII.

By including the charged carotenoids, the region of light absorption of carotenoids ranges from *ca*. 230 – 1200 nm, see Figure 6.

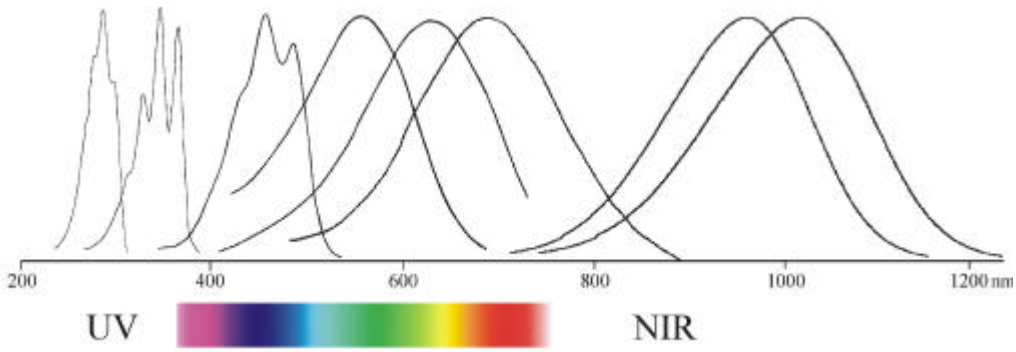


Figure 6. UV/VIS/NIR absorption spectra of selected carotenoids, from left phytoene (conjugated triene), phytofluene (conjugated pentaene), β,β-carotene (undecaene, **2**), violerythrin (undecaenetetrone), crustacyanin (Chapter 4.3), fucoxanthin oxonium ion,[43] β,β-carotene dication (Chapter 4.1.1), and β,β-carotene-iodine solvent complex (Chapter 4.6.2).

4.1 Carotenoid cations prepared with BF_3-etherates

4.1.1 The b,b-carotene dication

Treatment of a concentrated solution of β,β-carotene (**2**) in chloroform or dichloromethane with BF_3-etherate immediately results in a colour change from orange to black. In a diluted solution of **2**, the colour of the solution disappears. In the VIS/NIR spectrum this colour change is observed as a bathochromic shift from 450 nm in the VIS region to around 1000 nm in the NIR region.

The reaction of β,β-carotene (**2**) with BF_3-etherate was studied by in the 1950's by Zechmeister.[44,45] Based on the products formed upon addition of water to the reaction mixture, it was postulated that the reaction proceeded *via* BF_3-substituted resonance stabilised cationic species.

In the present work, NMR spectroscopy of the reaction mixture at –25 °C showed one symmetrical carotenoid with large downfield shifts for the polyene protons. All ^{1}H and ^{13}C NMR chemical shifts were assigned from the ^{1}H, ^{1}H-^{1}H COSY, 1D and 2D ROESY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC spectra recorded, see Figure 7. The total ^{1}H and ^{13}C downfield shifts, $\Sigma\Delta\delta_H$ and $\Sigma\Delta\delta_C$ of 35.82 and 504 ppm were, when compared to downfield shifts for other cations,[46,47] compatible with the formation of a carotenoid dication.

The coupling constants between the protons in the polyene chain were determined from the resolution enhanced ^{1}H NMR spectrum, combined with spectrum simulation in the WinDaisy software, see Figure 7. At first, the coupling constants in the central AA'BB' type four-spin system were interpreted as if the central bond had adopted a *Z*-configuration. This was invalidated, however, by the ROESY spectra, which showed that the central bond was still in the *E*-configuration. The ROESY spectra also showed that the C-6,7 double bond had adopted the s-*trans* configuration, which is not present in neutral carotenoids. A better overlap of the π-orbitals, causing a somewhat higher degree of charge delocalisation, may explain this unusual feature.

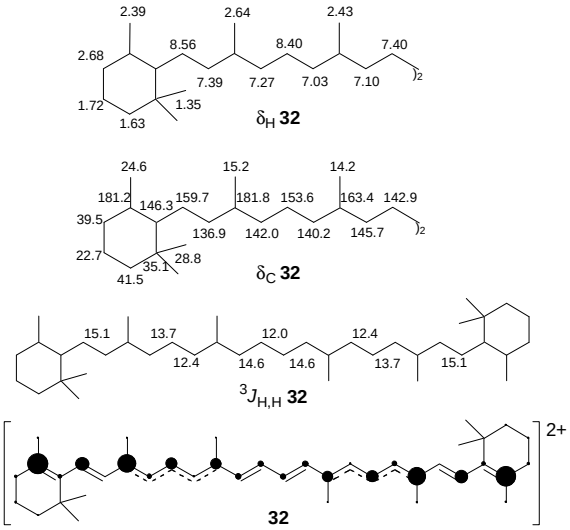


Figure 7. ^{1}H and ^{13}C NMR chemical shifts, coupling constants, and the charge distribution illustrated by the diameter of the filled circles for the β,β-carotene dication (**32**). Dotted bonds indicate regions of bond inversion.

The charge distribution of the β,β-carotene dication (**32**) was determined from the change in chemical shifts at each carbon atom, as discussed in Chapter 4.4. The charge distribution is illustrated by the diameter of the filled circles in Figure 7.

The formation of the dication **32** was monitored by EPR spectroscopy at –25 °C, showing that free radicals were involved. The EPR signal showed a linewidth of 15-

16 G, which is in agreement with previous results for the β,β-carotene cation radical.[48] The presence of cation radicals in the reaction mixture indicates that the reaction proceeds in a one-electron process, where one electron is abstracted from β,β-carotene (**2**) at a time.

The structure of the β,β-carotene dication (**32**) with the charges separated by the coulombic charge repulsion, and with two areas of double bond inversion, is in agreement with the soliton theory,[49-51] see also Chapter 4.4. The structure for **32** is also compatible with previous AM1 calculations, although the rotation of the end groups around the C-6,7 single bond was not treated in these calculations.[52-55]

The preparation and structure elucidation of the β,β-carotene dication (**32**) is treated in Papers VIII and IX.

4.1.2 Cations from other carotenoids

In addition to the β,β-carotene dication (**32**), it was desirable to prepare carotenoid dications of other carotenoids for comparison. Experiments were performed with lycopene (**1**), β,β-carotene-4,4'-dione (canthaxanthin, **33**), 3,3'-dihydroxy-β,β-carotene-4,4'-dione (astaxanthin, **34**) and ϕ,ϕ-carotene (isorenieratene, **24**). In order to avoid the strong decoupling pulses in the NMR experiments, which were necessary when using BF_3-dimethyl etherate, this reagent was replaced with BF_3-THF-d_8 etherate. This reagent was also easier and less expensive to prepare than deuterated BF_3-dimethyl etherate.

Despite the immediate formation of cationic carotenoids as judged by the rapid colour change and VIS/NIR data, the NMR spectra of all the carotenoid cations investigated, except the cation from **24**, were not interpretable. For astaxanthin (**34**), also the solubility was a problem, since large amounts of black precipitates were formed.

The isorenieratene (**24**) reaction mixture, however, provided a set of sharp signals in the NMR experiments. Analysis of the obtained spectra revealed that this was not the desired dication, but rather a monocation formed by the formal addition of a proton to isorenieratene (**24**). The structure of this monocation, 7-hydro-ϕ,ϕ-carotenyl monocation (**35**), with the charge distribution indicated, is shown in Figure 8

Figure 8. Structure and charge distribution of the 7-hydro-ϕ,ϕ-carotenyl monocation (**35**).

The unsuccessful oxidation to dications of carotenoids other than β,β-carotene (**2**) might be due to the fact that β,β-carotene (**2**), as the only one of these carotenoids (data not available for isorenieratene (**24**)), has a lower oxidation potential for the second than for the first oxidation step.[56,57]

The attempted formation of dications from lycopene (**1**), canthaxanthin (**33**), astaxanthin (**34**) and isorenieratene (**24**) with BF_3-etherates is treated in Paper X.

4.2 Carotenoid cations from allylic carotenols

4.2.1 Monocations from isocryptoxanthin

As an alternative method for preparation of carotenoid cations, the treatment of allylic carotenols with strong Brønsted acids was attempted. The reaction of β,β-caroten-4-ol (isocryptoxanthin, **36**) with HCl, providing 4',5'-didehydro-4,5'-*retro*-β,β-carotene (isocarotene, **26**), has been used as a diagnostic reaction for allylic carotenols.[58,59]

The reaction of isocryptoxanthin (**36**) with HCl in $CHCl_3$ was monitored by VIS/NIR spectroscopy, but proceeded too quickly to the product **26** for the intermediate cation to be studied by NMR. By changing the acid to CF_3COOH, the reaction stopped after the elimination of water. The VIS/NIR spectrum revealed a complete conversion to a cation with λ_{max} 1028 nm at –20 °C, which showed considerable stability. This monocation was studied by NMR (^{1}H, ^{1}H-^{1}H COSY, 2D ROESY, ^{1}H-^{13}C HSQC and HMBC) at –10 °C. The NMR data showed that the monocation from isocryptoxanthin (**36**), formally the 4-dehydro-β,β-carotenyl monocation **37**, was present as the two diastereomers **37a** and **37b**, which differs in the configuration of the C-6,7 double bond. Also present were the two minor conformers **37c** and **37d**, with the opposite conformation of the C-6',7' single bond.

The charge distribution, calculated as described in Chapter 4.4, of the monocations **37a** and **37b** is illustrated by the diameter of the filled circles in Figure 9.

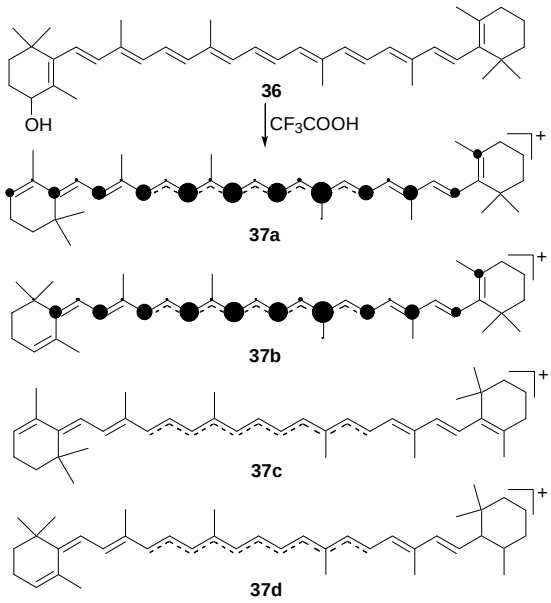


Figure 9. Formation of 4-dehydro-β,β-carotenyl monocations **37a-d** by treatment of isocryptoxanthin (**36**) with CF_3COOH.

4.2.2 Dications from isozeaxanthin

Using the same approach as for the preparation of the monocation from isocryptoxanthin (**36**), the formation of a dication from β,β-carotene-4,4'-diol (isozeaxanthin, **38**) was attempted. NMR studies of the cation formed showed that CF_3COOH was not a sufficiently strong acid to bring about the elimination of both hydroxy groups from **38**. One of the hydroxy groups was eliminated, while the other was still bound to the carotenoid, although in the protonated state. Figure 10 shows the structure of dication **39**, formed by treatment with CF_3COOH, with the charge distribution indicated.

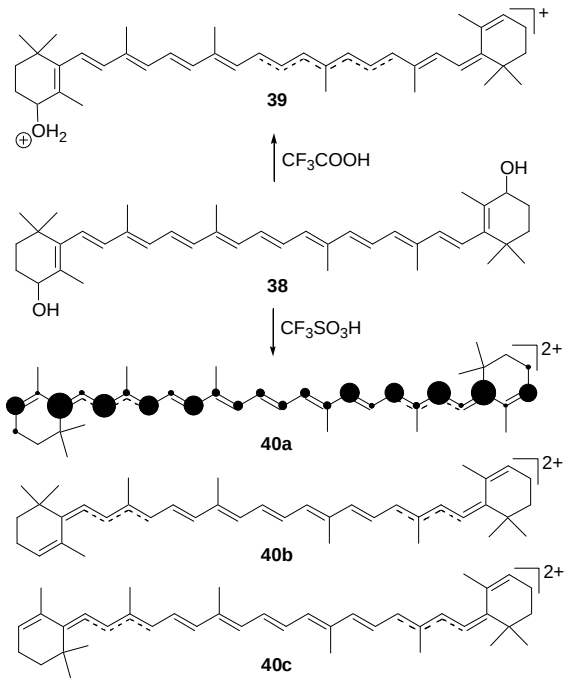


Figure 10. Formation of dications **39** and **40** from isozeaxanthin (**38**) by treatment with CF_3COOH or CF_3SO_3H.

By using the stronger acid CF_3SO_3H, the desired elimination of both allylic hydroxyl groups from isozeaxanthin (**38**) was accomplished. The resulting dication, formally the dication of isocarotene (**26**), was assigned the structure **40**, based on the NMR data obtained. The structures of the three diastereomeric dications **40a-c**, with the charge distribution indicated, are shown in Figure 10. The dication was present as the three C-6 diastereomers **40a**, **40b** and **40c**, in a 44:45:11 ratio.

The formation of carotenoid mono- and dications by elimination of allylic hydroxy groups using Brønsted acids is described in Papers X and XI.

4.3 Protonated canthaxanthins as models for astaxanthin in crustacyanin

A true carotenoprotein is a stoichiometric complex between a carotenoid (usually astaxanthin (**34**)) and a protein. α-Crustacyanin is a carotenoprotein which is found in the carapace of *Crustaceaea*. Crustaceans are covered by hard shells, and the best-known crustaceans include the lobsters, crabs, shrimp, and crayfish. α-Crustacyanin is responsible for the colour of the lobster shell, which turns red when the lobster is boiled because the protein denaturates, thus giving the colour of the free carotenoid, astaxanthin (**34**).

Astaxanthin (**34**) is not covalently bound in α-crustacyanin, and the X-ray structure of the β-crustacyanin subunit, which was recently published,[60] showed that the keto-groups of astaxanthin (**34**) in β-crustacyanin is located in molecular surroundings allowing hydrogen bonding to surrounding amino acids and water molecules, see Figure 11. NMR data for some of the carbon atoms of astaxanthin (**34**) in α-crustacyanin was also available from previous solid-state NMR experiments[61] with ^{13}C labelled astaxanthin (**34**) reconstituted in crustacyanin, showing downfield ^{13}C chemical shifts, compatible with positive charge on the polyene chain.

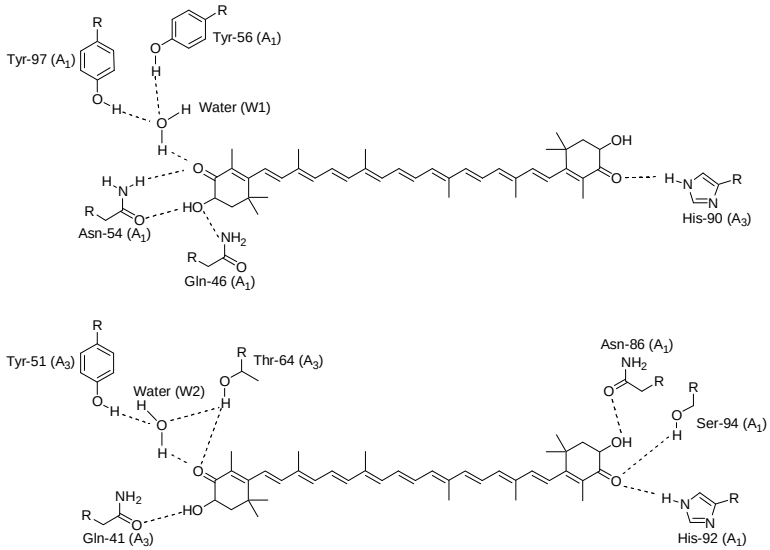


Figure 11. Molecular environment of astaxanthin (**34**) in the carotenoprotein β-crustacyanin. Modified from the X-ray structure.[60]

Protonation of astaxanthin (**34**) with CF_3COOH in $CHCl_3$ was attempted as a model for the protonation of the keto-groups, but failed due to low solubility of astaxanthin at low temperature and formation of black precipitates in the NMR tube. Canthaxanthin (**33**) with the same chromophore as astaxanthin (**34**), however, provided a mixture of monoprotonated cations upon treatment with CF_3COOH. The structures of the cations **41**-**43**, prepared from **33**, is shown in Figure 12.

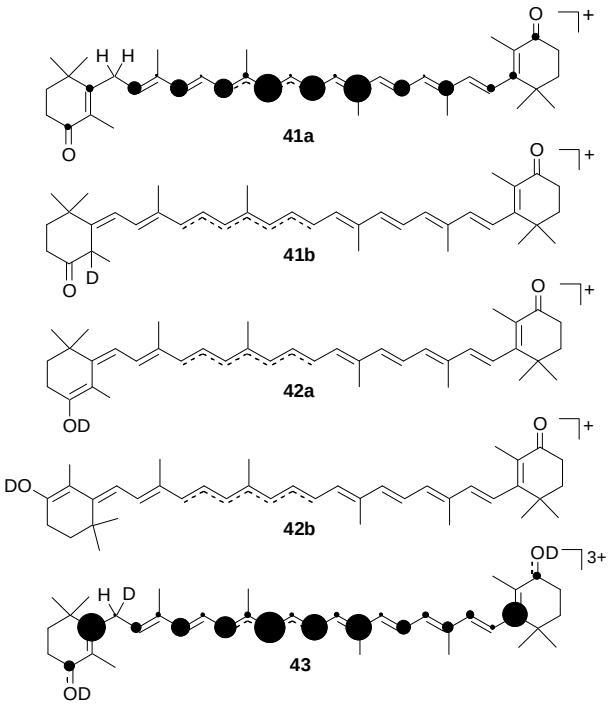


Figure 12. Mono- and triprotonated canthaxanthins. The charge distribution is indicated by the diameter of the filled circles when suitable neutral models for the charge determination was available.

Depending on the manufacture of the acid, the ratio of in-chain protonation (**41a**, **b**) to carbonyl protonation (**42a**, **b**) varied, but a selective protonation of the carbonyl groups could not be achieved. This acid only provided monoprotonated cations, in accordance with experience gained on cations from the allylic carotenols in Chapter 4.2. The stronger acid CF_3SO_3H was therefore employed, providing the trication **43** as the only product with the desired protonated carbonyl groups.

Analogous to the deduction of the charge distribution of a dication from the difference in ^{13}C chemical shift between the dication and the neutral molecule, the effect from protonation of the ketones could be determinted from the difference in chemical shifts between the trication **43** and the monocation **41a**. The downfield ^{13}C chemical shifts caused by protonation of O-4 and O-4' in **43** were both ca. 30 ppm, indicating that the partial charge delocalised from the carbonyl moiety is independent of the conjugation of the carbonyl.

By using the change in chemical shift of the carbons for C-1' to C-20', and correcting for the part of the charge located in the C-8 to C-15 region, a symmetrical model for the charge distribution in di-*O*-protonated astaxanthin (**34**) was devised, see Figure 13. It should be noted that the charge on the carbonyl carbons (C-4/4') can not be estimated from the change in chemical shift, because a rehybridisation of the C=O double bond occurs upon protonation.[62] The rehybridisation causes an upfield shift of ca. 260 ppm for the carbonyl oxygen upon protonation, since ^{17}O NMR chemical

shifts are much more sensitive to the hybridisation than to the charge on the oxygen atom.[62]

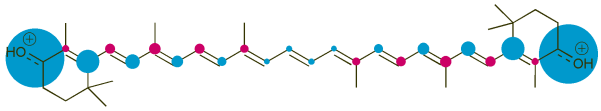


Figure 13. Model for *O*-4,4'-diprotonated astaxanthin (**34**) derived from mono- and triprotonated canthaxanthin (**41a** and **43**). The area of the filled circle indicates the distribution of the charge; 86% of the charge is located on the carbonyl moiety. For some carbons, a small negative charge was expected, indicated in red.

This model for protonated astaxanthin (**34**) may be seen as an extreme case, where both carbonyl groups are fully protonated. In the crustacyanins, a partial protonation is more likely, with the protons being partly associated with the astaxanthin (**34**) carbonyl and hydroxyl groups, and partly with the amino acids of the protein and adjacent water molecules. Also, the X-ray structure of β-crustacyanin shows that the carotenoid is unsymmetrically associated with the protein, thus making it likely that the two keto groups in the bound astaxanthin (**34**) is protonated to a different degree.

The studies of protonated canthaxanthin as a model for astaxanthin in crustacyanin is described in Paper XII.

4.4 Determination of charge distribution and the soliton width

The charge distribution of the cations described in the preceding Chapters 4.1-4.3 was calculated by correlating the total change in chemical shift, SDd_C, with the change in carbon chemical shift at each position, $Dd_{C,i}$

$$r_i = Dd_{C,i} / SDd_C * Q_{tot} \qquad (1)$$

where Q_{tot} is the total charge on the polyene.

The total ^{1}H and ^{13}C chemical shift changes of **37a** relative to an isocarotene (**26**)/β,β-carotene (**2**) neutral model were found to be 13.8 ppm and 254.2 ppm, respectively. Of the ^{13}C chemical shift change, 249.1 ppm, or 98%, was associated with the polyene chain.

Traditionally, the charge distribution of molecules with a charged π-electron system was calculated using the Spiesecke-Schneider relationship.[63] This was later extended to include sp^2 hybridised systems in general, but still restricted to planar, un-bridged and un-substituted hydrocarbons.[64] The correlation between ^{13}C chemical shift, d_C, and π-charge density, r, is given in Equation 2[65]

$$d_C = a\,r + d_0 \qquad (2)$$

where a is the total change in ^{13}C chemical shift and d_0 is the average ^{13}C chemical shift of all sp^2 carbons in the uncharged molecule.

The charge distributions calculated for monocation **37a** using Equation 1 and Equation 2 are shown in Figure 14 with black and red lines, respectively. From the soliton theory,[49-51] it may be deduced that the charge distribution should have the form of a wave function. It is seen from Figure 14 that using Equation 1 gives a charge distribution that is more symmetrically located, and has a more wave-like shape, compared to the charge distribution obtained from using Equation 2.

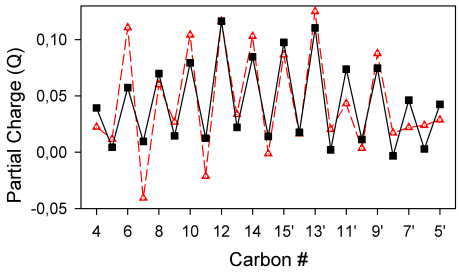


Figure 14. Charge distributions for monocation **37a**, determined using Equation 1 (black) and Equation 2 (red).

The accumulated charge, Q_i, on the polyene chain may be expressed by Equation 3.[66,67]

$$Q_i = Q_{tot}/2 * \tanh [(i-i_c)/l] \qquad (3)$$

Here, l is the soliton charge-wave half-width, i is the number of the carbon, and carbon number i_c gives the centre of the soliton charge-wave. In Figure 15 (black line) is plotted the accumulated charge, Q_i, for **37a** against the charge-carrying carbons. Curve fitting (Figure 15, red line) with Equation 3 then gives the half-width for the soliton l = 7.8, in good agreement with l = 7–9, found by Reimers *et al.*[66] using AM1 calculations. It should be noted, however, that an even longer polyene system is needed for determination of the width of a soliton that is entirely free of effects from the termini of the polyene chain.

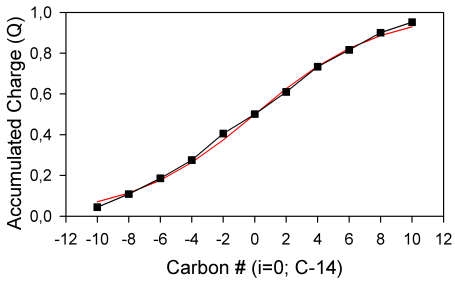


Figure 15. Accumulated charge (Q_i) for monocation **37a** *vs.* carbon number i, with middle of the soliton, i = 0, at C-14 (black), and regression curve for Q_i = 0,5 tanh(i/7.8), R^2 = 0.997 (red).

The determination of the charge distribution and of the soliton width is discussed in Paper X.

4.5 Nucleophilic reactions of carotenoid cations

The chemical reactions of the carotenoid dications described in the previous Chapters (4.1-4.3) were investigated using various O, N and S nucleophiles. For the carotenoid monocations, addition of the nucleophiles either resulted in the elimination of a proton or the addition of the nucleophile. For the carotenoid dications, also the combination of addition and elimination reactions was noted. When water was used as nucleophile, furanoxide products were formed from carotenoid dications by an addition-internal addition mechanism.

The addition of the nucleophile usually occurred at the end of the polyene chain, thus resulting in a formal substitution of the allylic hydroxy group. However, this substitution reaction proved to be of limited synthetic value, as the products were strongly isomerised, and the reaction usually resulted in a complex product mixture.

The reactions performed were not optimised for achieving the elimination products, but by quenching the cationic species by a non-nucleophilic base, good yields of the elimination products would be expected. The elimination reaction, however, is of limited interest, as it results in the loss of functionality from the parent carotenoids.

Major products formed upon treatment of β,β-carotene dication (**32**) with water as nucleophile is shown in Figure 16. Methanol as reagent furnished isocryptoxanthin (**36**) methyl ether as well as isocarotene (**26**).

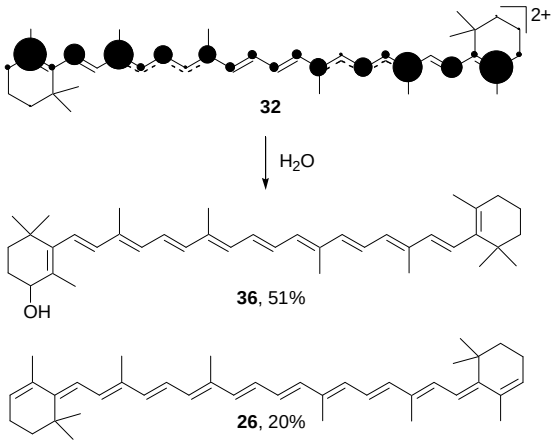


Figure 16. Major products formed upon treatment of β,β-carotene dication (**32**) with water as nucleophile. Only all-*E* isomers are shown.

Treatment of the isocarotene dication (**40**) with water or methanol provided a complex mixture of addition and elimination products. The dication **39**, with a protonated hydroxy group, however, provided mainly 4,4'-disubstituted β,β-carotenes (**44**), see Figure 17. Treatment with water gave a 66% recovery of isozeaxanthin (**38**).

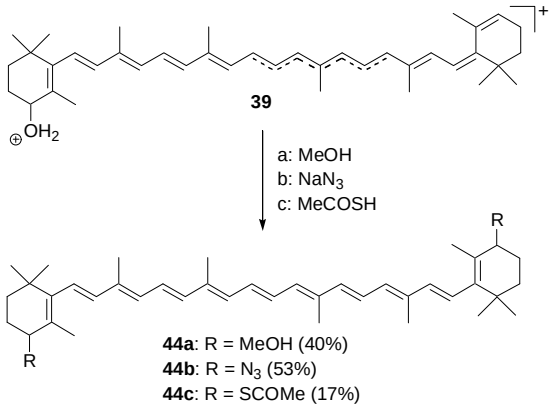


Figure 17. Preparation of 4,4'-dimethoxy-β,β-carotene (**44a**), 4,4'-diazido-β,β-carotene (**44b**) and 4,4'-diacetylthio-β,β-carotene (**44c**) from the dication **39**.

The monocation **37**, which was obtained from isocryptoxanthin (**36**) by treatment with CF_3COOH, provided isomerised isocryptoxanthin (**36**) upon quenching with water.

The studies on nucleophilic reactions of carotenoid cations are treated in Papers IX and XIII.

4.6 β,β-Carotene–iodine complexes

The reaction between β,β-carotene (**2**) and iodine is a classical reaction, first described by Arnaud in 1886,[68] who used the complex formed for the determination of the molecular formula of carotene extracted from carrots. However, despite an extensive literature on the subject, the structural knowledge about the complex is limited. In the present work, the preparation and structure of carotenoid–iodine complexes were reinvestigated using modern methods. Depending on the solvent used during the preparation, a solvent or a solid β,β-carotene (**2**)–iodine complex was formed, see Figure 18.

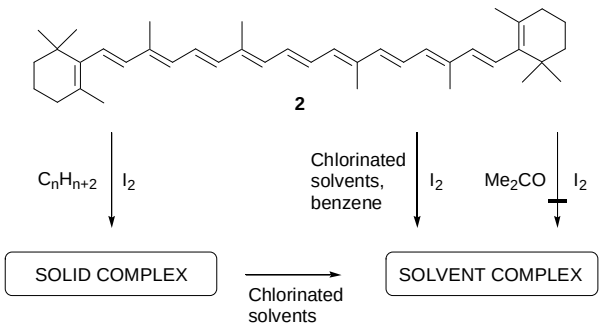


Figure 18. Solvents used for the formation of the β,β-carotene (**2**)–iodine complex.

4.6.1 The solid complex

According to elemental analysis, the composition of the solid complex was $C_{40}H_{56}I_4$. The solid complex showed three strong IR absorption bands in the 900–1500 cm^{-1} region. Three strong bands in this region have previously been observed for charged molecules, and have been ascribed to charged self-localised excitations.[69] Similar IR bands have been observed for β,β-carotene (**2**) doped with iodine in the solid state,[52,53,70] but contrary to the iodine doped β,β-carotene (**2**), the solid complex prepared here did not show any conductivity.

Mass spectroscopy data showed no covalently bound iodine in the solid complex. Solid-state ^{13}C NMR showed downfield shifts compatible with carotenoid monocations. The strong signal broadening in the spectrum and the short T_1 relaxation time observed for the solid complex indicated that there is a radical contribution in the complex.

4.6.2 The solvent complex

The IR spectrum of the solvent complex in chloroform was similar to that of the solid complex, with three strong absorption bands in the 900–1500 cm^{-1} region. The VIS/NIR spectrum showed an absorption at 1014 nm (in $CHCl_3$), similar to that of the carotenoid cations discussed in Chapters 4.1-4.3, and with absorptions also at 294 and 363 nm, compatible with triiodide (I_3^-). No NMR spectrum could be obtained of the solvent complex. The EPR spectrum had linewidth and g-factor compatible with previous calculations for the cation radical of β,β-carotene (**2**).[71] The amount of radicals in the β,β-carotene (**2**)–iodine solvent complex has previously been determined to be less than 2%.[72-74]

The major cleavage product from the solvent complex obtained by treatment with thiosulfate or acetone was isocarotene (**26**). Reaction of the complex with methanol gave isocarotene (**26**) and isocryptoxanthin (**36**) methyl ether.

In total, the acquired data for the β,β-carotene (**2**)–iodine complex indicates that:

- Iodine is not covalently bound.
- The β,β-carotene (**2**) molecule has a +1 charge.
- The counter ion is I_3^-.
- A minor part of the carotenoid is present in a radical state.
- The reaction products are compatible with a dication, cf. Chapter 4.5.

The combined data are thus compatible with iodine bound in a π-complex to β,β-carotene (**2**). In Figure 19, the complex is drawn as a tetrahapto (h_4) π complex with the radical cationic resonance structure indicated, but from the obtained data it cannot be decided whether iodine is associated in a monohapto, dihapto or tetrahapto complex. For the benzene–iodine complex, however, the monohapto complex has been found energetically favoured using computational methods.[75] This structure can also account for the structure of the solid complex, by assuming that the solid complex is a $(\text{carotenoid–iodine})^+$ – triiodide^- salt, which is insoluble in the apolar alkane solvents.

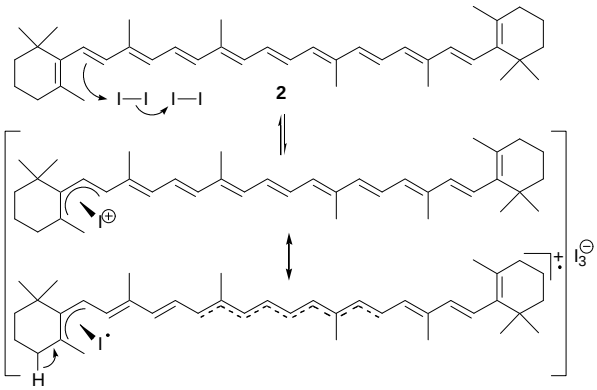


Figure 19. Formation of and suggested structure for the β,β-carotene (**2**)–iodine π complex, exemplified by a tetrahapto (h_4) π complex.

The structure elucidation of the carotenoid–iodine complex is treated in Paper XIV.

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Symposium presentations

1 lycopene
2 beta-carotene
3 bacterioruberin - Ikke gitt struktur
4 Carotenoid glucoside ester (opprinnelig 4)
5 Hydrolysert 4 (oppr 5)
6 acetylert 5
7 pheixanthophyll pentaacetate
8 plectaniaxanthin diester
9 Ikke-hydroxy carotenoid glucosid fra R. marinus (oppr 10)
10 2'-OH car. Glucosid fra R. marinus (oppr 11)
11 acylert 10
12 acylert 11
13 1'-glucopyranosyloxy-3,4,3',4'-tetradehydro-1',2'-dihydro-β,ψ-caroten-2-one acylated in the 6''-position
14 zeaxanthin mono- and diglucoside esters
15 C_{30}-carotenoid acyl glucoside
16 β,ψ-carotene
17 3',4'-didehydro-β,ψ-carotene
18 3',4'-didehydro-β,ψ-caroten-16'-oic acid
19 methyl 3',4'-didehydro-β,ψ-caroten-16'-oic acid
20 1,2,1',2'-tetrahydro-ψ,ψ-carotene-1,1'-diol
21 1'-methoxy-1,2,1',2'-tetrahydro-ψ,ψ-caroten-1-ol
22 1-methoxy-1,2-didehydro-ψ,ψ-carotene
23 chlorobactene
24 isorenieratene NYTT NR
25 1'-hydroxy-1-methoxy-3,4-didehydro-1,2,1',2'-tetrahydro-ψ,ψ-caroten-2-one
26 isocarotene NYTT NR
27 meso-zeaxanthin
28 1-(1-naphtyl)ethylisocyanate
29 (22*S*)-6-*O*-Acetyl-21βH-hopane-3β,6β,22,29-tetrol oppr. 23
30 *O*-29-acetylated 23
31 30-nor-6-O-acetyl-3β,6β-dihydroxy-21αH-hopan-22-one
32 beta-carotene dication
33 canthaxanthin
34 astaxanthin
35 7-hydro-ϕ,ϕ-carotenyl monocation
36 isocryptoxanthin
37 monocation fra isocryptoxanthin
38 isozeaxanthin

39 Protonert dication fra isozeaxanthin.
40 Isocarotene dication
41 In-chain monoprotonated canthaxanthin
42 carbonyl monoprotonated canthaxanthin
43 triprotonert canthaxanthin
44 4,4’-disubstituerte β,β-carotener.