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Animal Carotenoids

3.* The Carotenoids of *Actinia equina* — Structure Determination of Actinioerythrin and Violerythrin

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Dedicated to Professor dr.techn. *Nils Andreas Sørensen* on his recent 60th birthday.

Actinioerythrin, the main carotenoid of the red variant of the sea anemone *Actinia equina* L., is shown to possess structure *1* (2,2′-bisnorastaxanthin diester) on the basis of chemical and spectroscopic data obtained for some thirty derivatives. This compound which contains two cyclopentenolone rings is the first example of a nor-carotenoid with ring contraction and constitutes a mixture of several closely related esters, of which the alcohol moiety, actinioerythrol, is in common.

The derivative violerythrin (*20*) with cyclopentenedione rings, the first blue carotenoid, shows exceptional behaviour on alkali treatment, which has been studied in some detail. The spectroscopic properties of carotenoids with substituted cyclopentene end-groups and the stereochemistry of violerythrol (*14*) are discussed. The utility of mass spectra of through-conjugated carotenoids like bisquinoxaline derivatives of α,β-diketones for exact location of in-chain methyl groups is mentioned.

The presence of two further carotenoids in *A. equina* is demonstrated. One of these is shown to be an astaxanthin diester (*24*).

The presence of carotenoid pigments in the sea anemone *Actinia equina* L. was assumed by Abelooz-Parize [1] more than forty years ago.

From the red variant Lederer and Fabre [2,3] isolated a new crystalline carotenoid designated actinioerythrin and considered it to be an ester of a

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coloured acid.[2–4] The green variant contained a more yellow, unnamed carotenoid ester, present as a protein complex and responsible for the green colour, whereas a brown-green variant contained a mixture of these two carotenoids. Small amounts of α- and β-carotene were present in all colour variants.

Independently, Heilbron, Jackson and Jones [5] isolated crystalline actinioerythrin from the red variant, and under carefully controlled conditions of saponification obtained from it a crystalline, blue derivative (m.p. 191—192°C, abs. max. 540, 572, and 625 nm in carbon disulphide) designated violerythrin. Violerythrin was thought to be the stable keto-form of an initially formed enol. Karrer and Jucker [6] have questioned whether the blue violerythrin belongs to the carotenoid series.

More recently de Nicola and Goodwin [7] reported a quantitative analysis of the carotenoid composition of *A. equina*, red variant (see Table 1). Beside actinioerythrin small amounts of hydroxy-actinioerythrin, β-carotene, and two minor carotenoids A_2 and A_3 were encountered. Actinioerythrin was stated to be present as a protein complex. A summary of the earlier reports is given in Table 1.

Table 1. Summary of earlier reports on the carotenoid composition of *A. equina*.

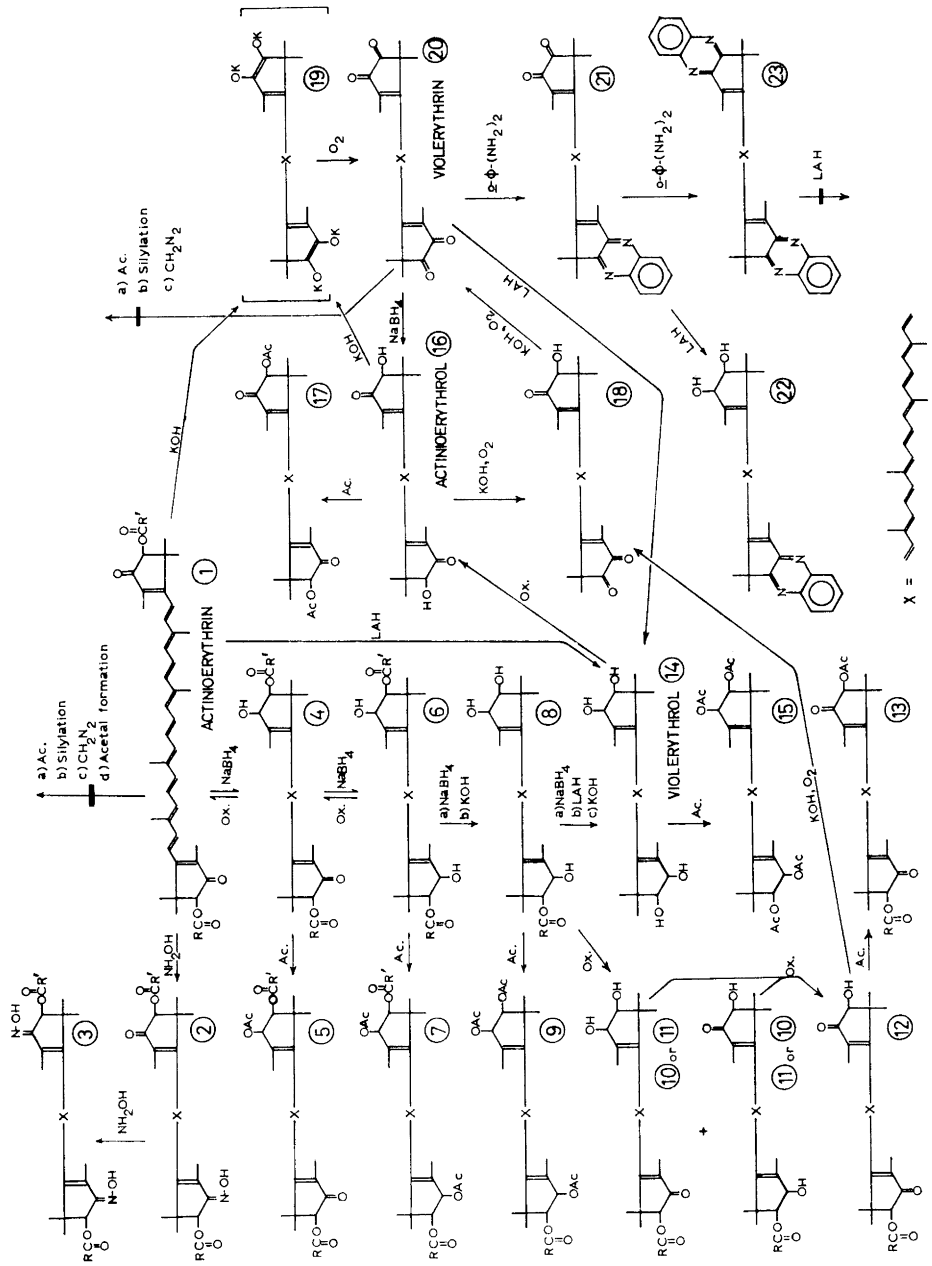
Red variety	Green variety
Actinioerythrin [2–4,7] (*a*) [7] 70—80 % [7]	Carotenoid abs. max. 472, 504 nm
m.p. 75°C,[2] 85°C,[3] 83°C [4]	CS_2 [2,3] (*a*) [2,3]
abs. max. 470, 497, 534 nm petroleum ether [3]	α-Carotene [2,3]
495, 533, 574 nm CS_2 [3]	β-Carotene [2,3]
497, 538, 574 nm CS_2 [4]	
Hydroxy-actinioerythrin; 5 % [7]	
α-Carotene [2,3]	
β-Carotene [2,3,7]; trace [7]	
A_2; 6 %, abs.max. 472 nm petroleum ether [7]	
A_3; 9 % abs.max. 488 (520) nm petroleum ether [7]	

(*a*) Present as protein complex.

The present paper deals with the structure determination of actinioerythrin and related compounds obtained from the mauve-coloured *A. equina* from Norwegian waters. A preliminary report has already been published.[8]

RESULTS AND DISCUSSION

The mauve-coloured variant of *A. equina* common in Norwegian waters appears to be richer in total carotenoid than those previously examined;[2–5] average carotenoid content 0.12 % of the dry weight. Actinioerythrin (*1*) was the dominant carotenoid (*ca.* 90 % of total). A minor ester X (*ca.* 5 % of total), tentatively identical with A_3 of de Nicola and Goodwin,[7] and a still less abundant ester Y (*24*, *ca.* 2 % of total), presumably identical with A_2



Scheme 1. Chemical reactions of actinioerythrin (*1*).

of the same authors,[7] were also present. No carotenes or other carotenoids were detected. Their hydroxy-actinioerythrin may be an artefact produced by hydrolysis on the alumina column.

Actinioerythrin (*1*)

Actinioerythrin, m.p. 91°C, total yield *ca.* 40 mg, was isolated after extraction with acetone and repeated chromatography of a petroleum ether extract on calcium carbonate columns. Evidence for structure *1* for actinioerythrin will be presented, see Scheme 1.

No free hydroxyl groups were present in *1* as judged by its polarity and the failure of acetylation and silylation reactions. Two carbonyl functions were indicated by the formation of a monoxime (*2*) and a dioxime (*3*). The latter (*3*) provided a monoacetate and a diacetate on acetylation. The absorption spectra in visible light of *1*, *2*, and *3* were different (Table 3). Attempted acetal formation failed.

Careful reduction of actinioerythrin (*1*) with sodium borohydride gave a mono-ol (*4*), which gave a monoacetate (*5*) on acetylation, and reverted to actinioerythrin (*1*) on oxidation. Allylic oxidation was here and in subsequent experiments effected with oxygen in the presence of iodine.[9] Actinioerythrin (*1*) and the mono-ol (*4*) on further reduction provided a diol (*6*), which gave a monoacetate and a diacetate (*7*) on acetylation. On oxidation the diol (*6*) gave the mono-ol (*4*) and actinioerythrin (*1*). The absorption spectra of actinioerythrin (*1*), the mono-ol (*4*), and the diol (*6*), given in Fig. 1, confirmed the presence of two conjugated keto groups in *1* and one in *4*.

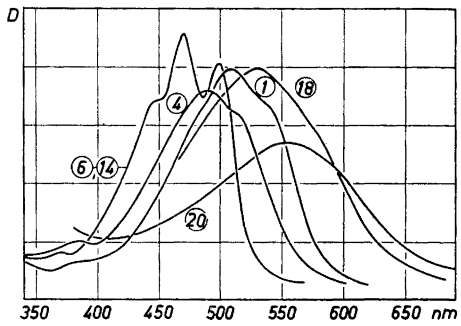


Fig. 1. Absorption spectra in visible light recorded in acetone solution of carotenoids with dicyclopentene, cyclopentenone-cyclopentene, dicyclo-pentenone, cyclopentenedione-cyclopentenone and dicyclopentenedione end-groups: violerythrol (*14*) and 4,4′-tetrahydro-actinioerythrin (*6*), 4′-dihydro-actinioerythrin (*4*), actinioerythrin (*1*), 3′-dihydro-violerythrin (*18*) and violerythrin (*20*).

On further treatment with sodium borohydride or alkali the diol (*6*) gave a triol (*8*). The presence of three hydroxyl groups follows from the chromatographic behaviour and the formation of three intermediary acetates in the acetylation of *8* to its peracetate *9*. Oxidation of the triol (*8*) gave three oxidation products, considered to be the monoketones *10* and *11*, and the diketone *12*. The monoketones (*10* and *11*) were spectroscopically similar to *4* and the diketone (*12*) to actinioerythrin (*1*), Table 3. However, the keto-products (*10*, *11* and *12*) were more polar than *4* and *1*. The diketone (*12*) gave a monoacetate (*13*).

Compound	Required eluent from		Developer on	R_F-value										
				Kieselguhr paper [a]						Aluminium oxide paper [b]				
	Cellulose column	Alumina column [c]	Calcium carbonate column	5 % AP	7 % AP	10 % AP	20 % AP	25 % AP	30 % AP	7 % AP	10 % AP	20 % AP	30 % AP	50 % AB
1	5–10 % AP	60–70 % EP	5–10 % EP	0.11		0.52					0.62	0.90		
2			2–3 % AP			0.43								
3			2–3 % AP			0.24	0.83							
3-monoacetate						0.51								
3-diacetate						0.70								
4						0.51					0.41	0.82		
5						0.58								
6	7–10 % AP					0.33	0.92					0.62		
6-monoacetate						0.62								
7						0.81								
8	10–20 % AP						0.46	0.55				0.08		0.19
9						0.80								
10											0.80			0.19
11														0.41
12	7 % AP													0.54
13						0.29						0.68		
14a	25 % AP							0.31	0.60					
b								0.48	0.70					
c								0.65	0.80					
14-acetonide *a*						0.21								
b						0.44								
15	20 % AP	60 % EP		0.31	0.52	0.67				0.27				
16	15–20 % AP							0.64	0.78					
16-monoacetate							0.60							
17						0.25	0.75							
18							0.52							
18-monoacetate							0.69							
20	20 % AP						0.57						(0.91)	
Product A	0 % AP					0.70								
B	5 % AP													
21						0.28						0.63		
22								0.42						
23		5–7 % EP				0.34						0.72		
24	5 % AP		0 % EP		0.65									
25						0.53								
26						0.41						0.65		
27									*ca.*0.72					
28				0.49							0.49			
29	5 % AP			0.58	0.73									

[a] ... [b] ... [c] ... No. 288. [d] Activity grade 3.

Table 3. Absorption maxima in visible light in three solvents of the *trans* isomer of the carotenoids studied.

Compound	Abs.max. in nm in								
	Petroleum ether			Acetone			Chloroform		
1	(470)	496	529	(480)	508	(538)		518	(550)
2	(468)	493	(523)		498	(530)			
3	(470)	489	518	(470)	495	(527)			
4					490	(525)			
5					490	(525)			
6				(444)	470	499			
8				(444)	470	499			
10					*ca.* 486				
11					489				
12					504				
14a	(445)	468	495	(446)	470	500	(455)	478	506
b	(445)	468	495	(446)	470	500			
c	(445)	468	495	(446)	470	500			
14-acetonide *a*				(445)	468	498			
b				(445)	468	496			
15	(440)	467	494	(444)	470	499	(450)	477	508
16							(490)	518	(550)
17					503	(535)		518	(550)
18					533				
20					556			580	
Product A	(358)	375	398						
B	(405)	428	453						
21					536				
22				(470)	503	(540)	(515)	545	(580)
23				(510)	530	(565)			
24					480				
25					482				
26					482				
28				(427)	453	480			
29					486				

Treatment with sodium borohydride or alkali of the diol (*6*) and the triol (*8*) gave a tetraol (*14*), designated violerythrol. The tetraol formulation was confirmed by mass spectrometry, see below. Acetylation of *14* gave a peracetate (*15*).

The above results supported the presence of two conjugated keto groups and two esterified hydroxyl groups in actinioerythrin. Ester groups are generally considered to be non-reactice on borohydride treatment.[10,11] However, exceptions are known,[10,12] and in the case of *6* and *8* hydrolysis with methanolic potassium hydroxide gave the same products. As expected, treatment of actinioerythrin (*1*) with lithium aluminium hydride gave the tetraol (*14*) in a fast reaction. The stereochemistry of violerythrol (*14*) will be discussed below.

The tetraol (*14*) decomposed in the presence of dicyano-dibenzoquinone,[13] and could like carotenoids with 3,4-dihydroxy-β-rings not be oxidized with *p*-chloranil.[14] Careful allylic oxidation with air in the presence of iodine [9] gave

the diketone *16*, designated actinioerythrol, which provided a monoacetate and a diacetate (*17*) on acetylation. Actinioerythrol (*16*) was spectroscopically (visible light) identical with actinioerythrin (*1*), hence favouring a chromophore comprising an ω,ω'- rather than an α,β-diketone structure.

The effect of alkali on actinioerythrin (*1*) shall now be considered. Careful alkaline hydrolysis in a two-phase system according to Heilbron *et al.*[5] resulted in the isolation of violerythrin (*20*), m.p. 236°C. The course of this transformation will be returned to later. Violerythrin (*20*) reacted very smoothly with *o*-phenylene diamine, *cf.* Refs. 15, 16, to give a mono- (*21*) and bisquinoxaline derivative (*23*). The monoquinoxaline derivative (*21*) gave a more polar product (*22*) on reduction with lithium aluminium hydride, whereas the bisquinoxaline derivative (*23*) could not be reduced on similar treatment. These data strongly suggested that violerythrin (*20*) possessed two α,β-diketone groupings, and it could thus be inferred that actinioerythrin (*1*) was the diester of a bis-α-ketol with the conjugated keto groups in ω,ω'-position to the polyene chain.

This hypothesis was confirmed by several transformations inter-relating the alkaline hydrolysis product violerythrin (*20*) and various derivatives made subsequent to borohydride reduction of actinioerythrin (*1*). Thus lithium aluminium hydride reduction of the tetraketone violerythrin (*20*) provided the tetraol violerythrol (*14*) described above, whereas brief treatment of *20* with borohydride permitted the quantitative transformation to the bis-α-ketol actinioerythrol (*16*), presumably as an inseparable mixture of epimers, and eventually furnished the tetraol (*14*). Analogies may be found in the reported selective reduction of 2-methyl-1,3,4-cyclopentanetrione to the 4-hydroxy compound,[17] and of 9-methyl- $\Delta^{5(10)}$-octalene-1,6-dione to the 1-hydroxy derivative.[18] Alkali treatment of the bis-α-ketol actinioerythrol (*16*) gave the intermediary triketone (*18*) and ultimately the tetraketone violerythrin (*20*), see Fig. 1. The triketone (*18*) gave a monoacetate on acetylation. Likewise the monoester of the bis-α-ketol (12) on alkali treatment provided the intermediary triketone (*18*) and violerythrin (*20*).

Turning now to a consideration of the chromophore of violerythrol (*14*) (Fig. 1, Table 3), this corresponded to slightly less than eleven spectroscopically efficient carbon-carbon double bonds in an aliphatic polyene chain, but could not be identified with any known chromophores. Nor had any of the other derivatives prepared spectra in visible light corresponding to those of known carotenoids, see Fig. 1, Table 3.

The conjugated carbonyl groups of actinioerythrin (*1*) exhibited infrared absorption at 1695 cm^{-1}, while carbonyl absorption of violerythrin (*20*) occurred at 1750 and 1675 cm^{-1} (see Fig. 2), and hence suggested five-ring ketones. The carbonyl stretching vibration of cyclopent-2,3-en-1-ones is reported near 1700 cm^{-1},[19,20] whereas non-enolic cyclopent-3,4-ene-1,2-diones exhibit carbonyl absorption for unconjugated carbonyl at higher frequency and for conjugated carbonyl near the one observed for the corresponding cyclopentenone.[16,21]

Since its mass spectrum (see below) revealed that violerythrin (*20*) was a C_{38}-compound and the NMR-spectrum of actinioerythrin (*1*) clearly demonstrated the presence of a total of ten methyl groups (four in-chain at τ 7.98,

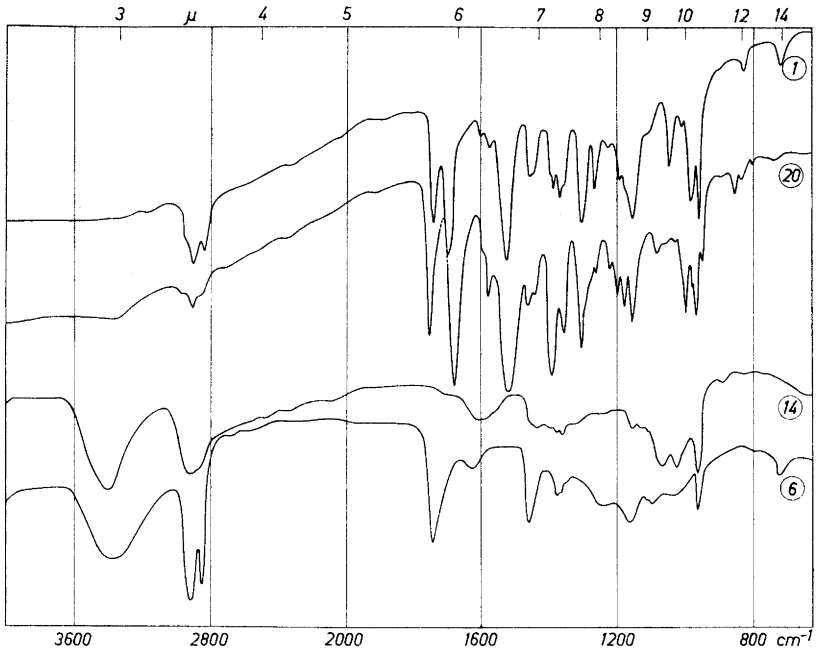


Fig. 2. Infrared spectra (KBr) of actinioerythrin (*1*), 4,4′-tetrahydro-actinioerythrin (*6*), violerythrol (*14*) and violerythrin (*20*).

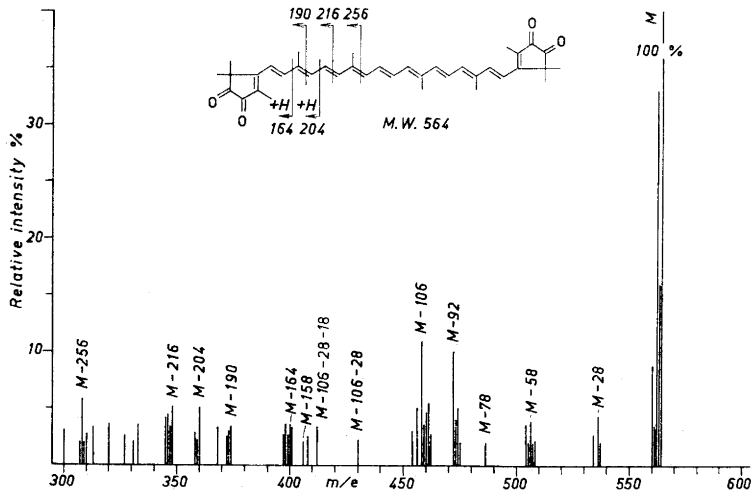


Fig. 3. Mass spectrum of violerythrin (*20*).

two end-of-chain at τ 8.08, and four *gem.* methyl groups at τ 8.58, 8.79), the formulation of actinioerythrin as a 2,2′-bisnorastaxanthin diester (*1*) with two five-rings was obvious.

The fragmentation patterns observed on electron impact of various derivatives of *1* substantiated this idea. The mass spectrum of violerythrin (*20*), Fig. 3, confirmed the molecular weight to be 564. The assignment of the molecular ion, here and in the compounds discussed below, was supported by the observation of M—92 and M—106 ions, which have been found to be typical of carotenoids.[22,23] The spectrum of violerythrin (*20*) showed several peaks corresponding to ions formed by cleavage of the in-chain bonds of the molecule. Hydrogen transfer is involved in most cases and the situation is that found in most carotenoids having cyclic end groups.[23] The presence of a prominent M—28 ion is consistent with the proposed α-diketone structure

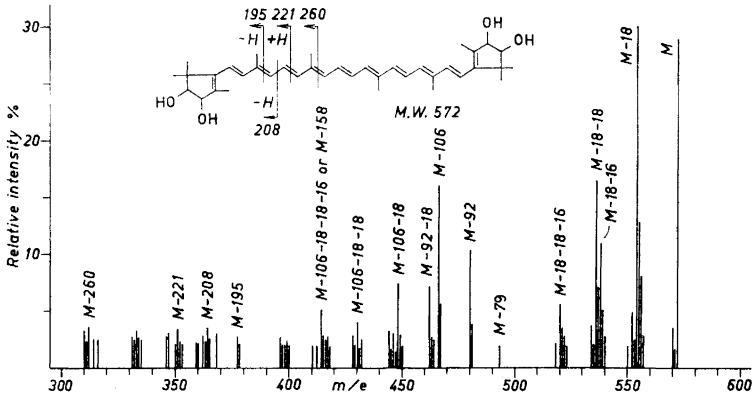


Fig. 4. Mass spectrum of violerythrol (*14*).

(*20*). The mass spectrum of violerythrol (*14*), Fig. 4, exhibits a prominent molecular peak and, in agreement with expectation, strong peaks due to the loss of water and oxygen from the molecular and the M—92 and M—106 ions. The loss of oxygen has previously been shown to occur in acyclic allylic carotenoid alcohols.[23]

The mass spectrum of violerythrin bisquinoxaline derivative, Fig. 5, is also consistent with the assigned structure (*23*). In addition to abundant molecular (*m/e* 708), M—92 and M—106 ions, peaks corresponding to the cleavage of all in-chain bonds are present. Similar cleavages were reported [23] for 3,4,3′,4′-tetradehydrolycopene which lacks the common more saturated carotenoid end-groups. The fragmentation of isorenieratene [24] provides an even better analogy.[25] The in-chain cleavages observed for the quinoxaline derivative (*23*) in fact confirm the so far assumed localization of the lateral methyl groups on the polyene chain. Losses of 15 mass units are not common in carotenoids,[22,23] but are observed for *23* as well as for the bisphenazine derivative (*29*) of astacene, Fig. 6, recorded for comparison.

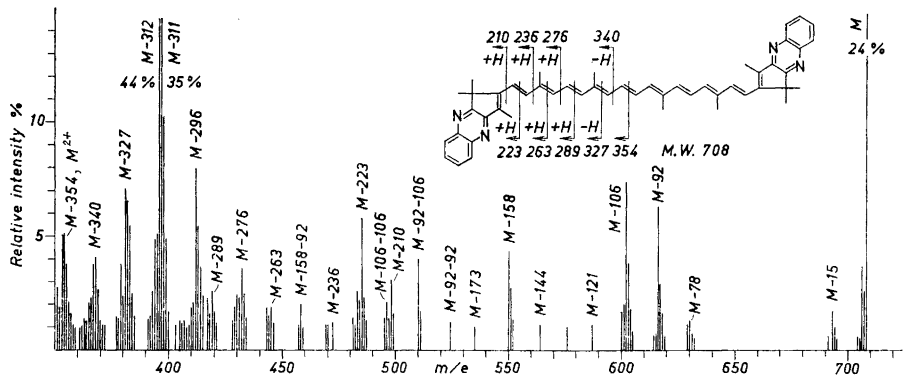


Fig. 5. Mass spectrum of violerythrin bisquinoxaline derivative (*23*).

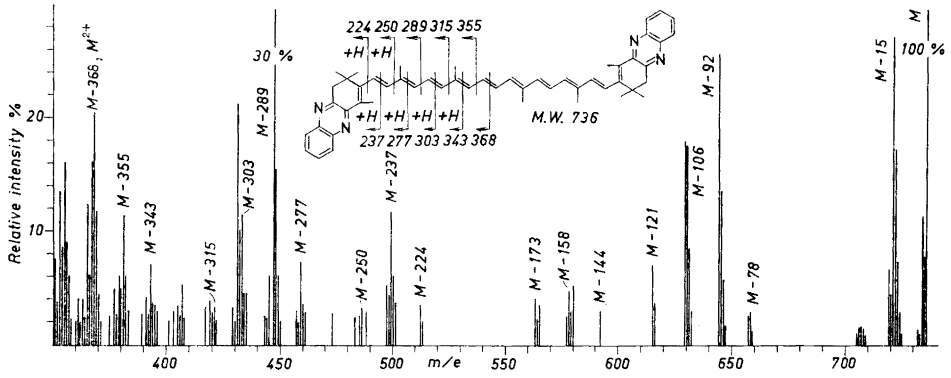
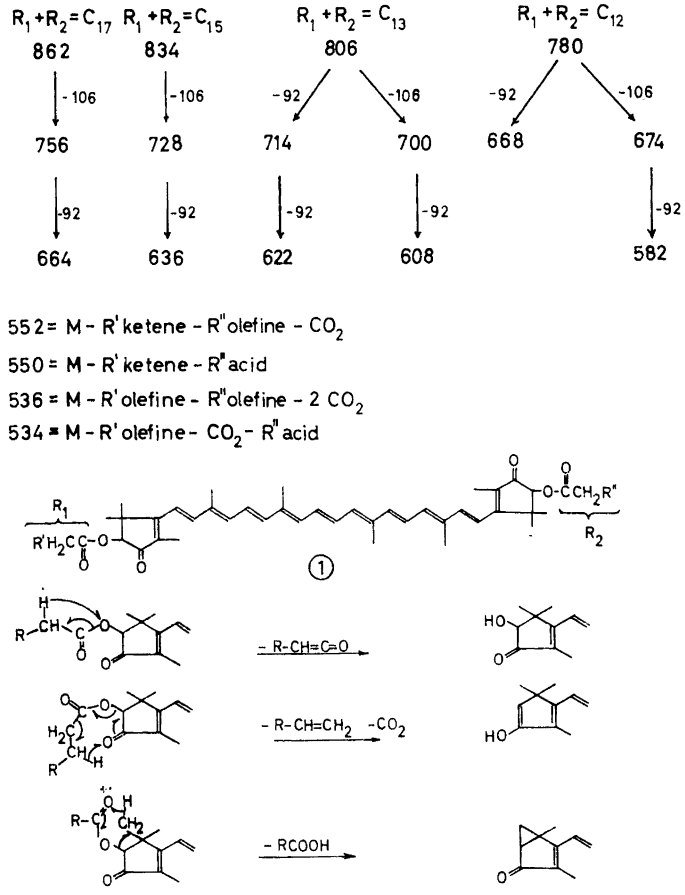


Fig. 6. Mass spectrum of astacene bisphenazine derivative (*37*).

The mass spectrum of actinioerythrin (*1*) suggests that it is largely a mixture of four esters. This is supported by the chromatographic separation of crystalline *1* into six ester components on paraffin-impregnated paper. Molecular weights of 862, 834, 806, and 780 are ascribed to the four main esters. In each case a loss from the molecular ion of 106 mass units and a subsequent loss of 92 mass units are observed. The more intense molecular ions at *m/e* 806 and 780 show also initial losses of 92 mass units. The situation is diagrammatically summarized in Scheme 2. At considerably lower mass numbers four very intense peaks were observed at *m/e* 552, 550, 536, and 534, and these are envisaged as being formed by combination of losses of RHC=C=O, RCOOH, and $RCH=CH_2$ plus CO_2, see Scheme 2.

Actinioerythrol diacetate (*17*) may be considered a synthetic analogue of actinioerythrin (*1*). The mass spectrum of *17* confirms the molecular weight (M=652, M—92, M—106) and the presence of acetyl groups (M—58, M—60).



Scheme 2. Peaks observed in the upper mass region of the mass spectrum of actinioerythrin (*1*), and their presumed origin.

The occurrence of peaks at *m/e* 536 (M−58−58) and 534 (M−60−58) which are also present in the spectrum of actinioerythrin (*1*) is in accordance with the structural similarity of the two compounds, and similar mechanisms might be expected to operate in each case since only cleavage of different ester functions is involved.

Turning now to the esterified acids of *1*, the low melting point of *1* and the R_F-values of actinioerythrol diacetate (*17*) and actinioerythrin (*1*) were suggestive of fatty acids of higher molecular weight than acetic acid. The molecular weights proposed for the four main ester components of *1* from mass-spectrometric evidence (Scheme 2), indicate that the R_1 and R_2 groups together contain 17, 15, 13, and 12 carbon atoms, in the last case a double bond would be needed in one of these groups to explain the molecular weight. The NMR-

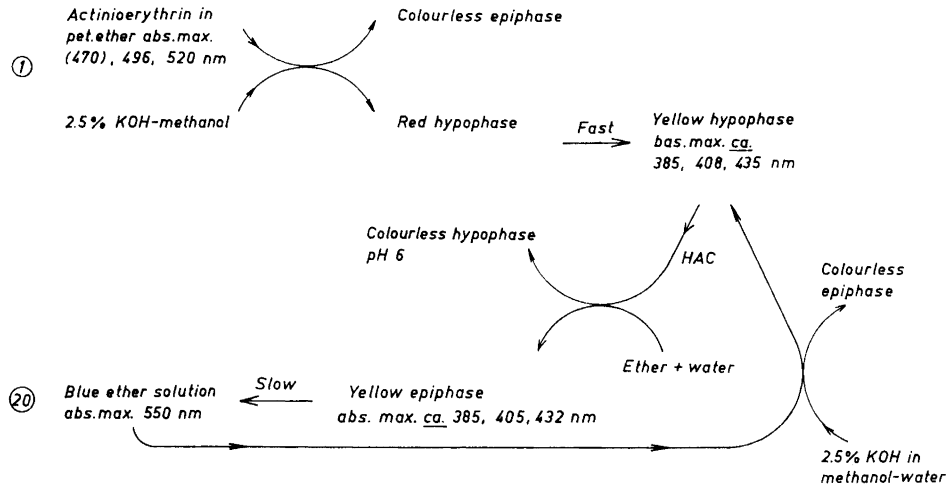
spectrum of *1* also provides some evidence for the latter requirement (signal at τ 4.65). The NMR-spectrum further supports the presence of methylene groups adjacent to the carboxyl function (multiplet at τ 7.6, *ca.* 4 H), methylene in saturated environment (τ 8.72, *ca.* 28 H) and terminal methyl (τ 9.10, *ca.* 6 H) attached to methylene, thus indicating that R_1 and R_2 together in average contained *ca.* 17 carbon atoms, based on saturated fatty acids alone. Four or more adjacent methylene groups in one or more of the esterifying acids were indicated by IR-absorption of *1* at 710 cm^{-1}. For isolation of the fatty acids saponification of the diol (*6*) rather than of actinioerythrin (*1*) was preferred, because of the complicated phase behaviour of the carotenoid products in the latter case, *cf.* Scheme 5. The fatty acids were methylated with diazomethane and the methyl esters examined by i) gas chromatography and ii) combined gas chromatography-mass spectrometry. Only methyl esters of capric (C_{10}), undecanoic (C_{11}) and lauric (C_{12}) acid were identified unambiguously and methyl caproate (C_6) tentatively by gas chromatography alone. Since the pH by transfer of the acids to ether after saponification of *6* was too high (pH *ca.* 5), some short-chain and unsaturated acids with lower K_a-values[26] may have escaped detection. Natural xanthophylls esterified by a variety of fatty acids have been reported: acetic,[27,28] caprylic,[29] capric,[29–33] lauric,[31–37] myristic,[30–34,37] palmitic,[29–31,34–40] stearic,[34,36,37,39] palmitoleic,[39] oleic,[39] and linoleic acid;[29,41] lauric and myristic acid being among the more common ones.

The stereochemistry of natural actinioerythrol (*16*=unesterified actinioerythrin) poses a single problem of configurational assignment of the esterified hydroxy groups. Whether these are α or β (according to steroid nomenclature) is not known.

Violerythrol (*14*) obtained from *1* by borohydride reduction was found to consist of three all-*trans* (polyene chain) stereoisomers *14a,b,c*, chromatographically distinguishable on kieselguhr paper. The same three isomers were obtained by lithium aluminium hydride reduction of *1*. However, in the former case *b* and *c* and in the latter case *a* and *b* were dominant. Since the stereochemistry of the 3,3′-hydroxy groups is given, these isomers are considered as *cis-cis*, *cis-trans*, and *trans-trans* di-α-glycols. Since *14a,b,c* gave an apparently chromatographically homogeneous tetraacetate, hydrogen bonding is considered the reason for the more facile chromatographic separation of *14a*, *b*, and *c*. The least polar one *14c* tentatively represents the *cis-cis*, *14b* the *cis-trans*, and the most strongly adsorbed *14a* the *trans-trans* di-α-glycol. This would imply that borohydride in this case should favour the formation of *cis* and aluminium hydride the *trans* α-glycol. When the carbonyl group is reduced prior to hydrogenolysis of the ester (shown to be the case with borohydride), *cis*-glycols are anticipated on the basis of favoured addition of hydrogen from the side of the carbonyl group opposite to the bulky ester group.[10] Our conclusion is in agreement with the result of Nicoara *et al.*[42] who claimed that reduction of natural astaxanthin diester (*cf. 24*) with sodium borohydride gave the *cis* di-α-glycol. Attempted acetonide formation of the tetraols (*14a,b,c*) gave low pigment recovery and was incomplete. Two presumed acetonides were detected chromatographically, but it was not possible to decide from which of the isomers *14a*, *b* or *c* the acetonides originated and

thereby to confirm which of these were *cis* α-glycols. Reports on successful separation of epimeric carotenols are few.[42,44]

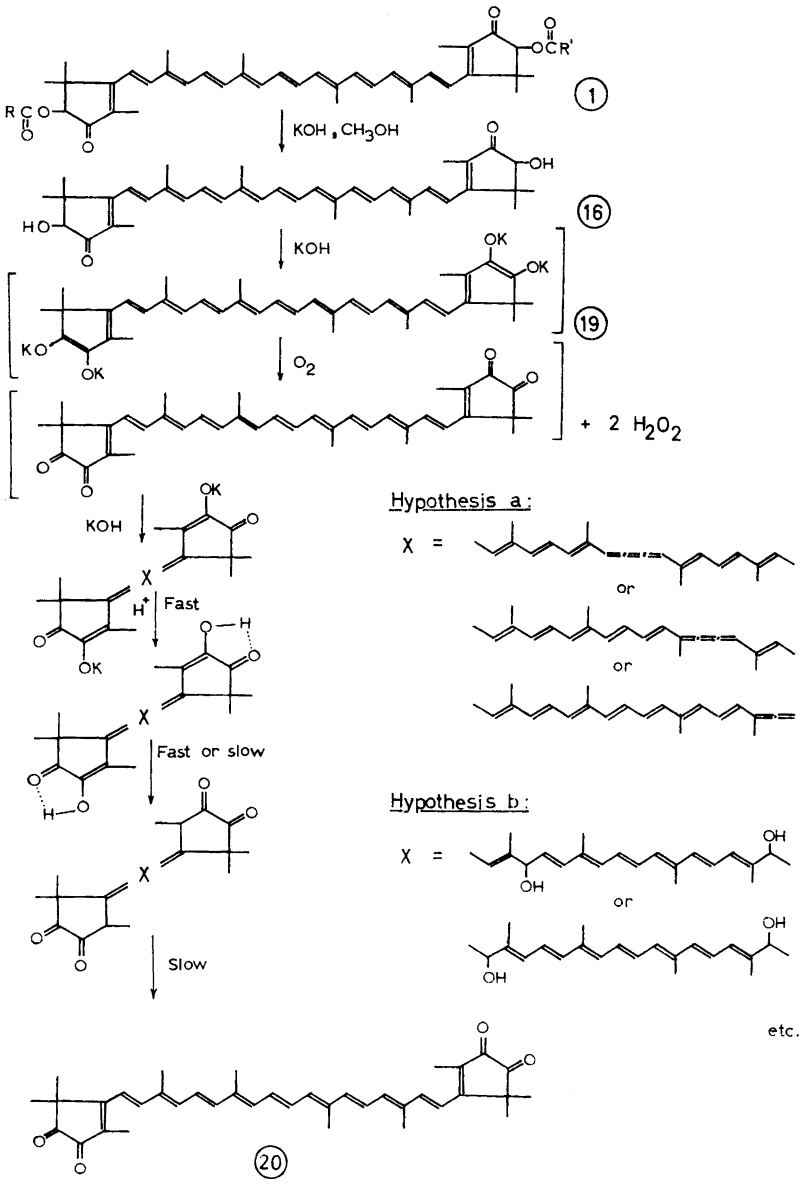
Models revealed that in violerythrol (*14*) there is no steric conflict between the 18-methyl group and the hydrogen atom at C_8. This explains the larger spectral contribution of the 5,6-double bond of the trimethylcyclopentene ring than of the related 1,1,5-trimethyl-cyclohexylidene ring, *cf.* visible light absorption spectra of β-carotene [45] and *14*. Models further indicate that the cyclopentenone and cyclopentenedione rings present in the compounds studied are planar, and thus explain the large spectral contribution of the ring carbonyl groups of, *e.g.*, *1* and *20*. It should also be remembered that simple cyclopentenediones are yellow compounds.[15,16]



Scheme 3. Colour changes and phase behaviour on alkali treatment of actinioerythrin (*1*) and violerythrin (*20*) in a two-phase system.

The exceptional colour changes on alkali treatment of actinioerythrin (*1*) and violerythrin (*20*) will now be considered. The colour changes and phase behaviour observed on alkali treatment of *1* and *20* and subsequent acidification are depicted in Scheme 3. Careful alkali treatment of violerythrin (*20*) results in yellow hypophasic products, which after neutralization are immediately transformed to yellow epiphasic products which slowly revert to violerythrin (*20*). The drastic colour changes from the blue violerythrin to the light yellow hypophasic products with visible light absorption corresponding to only eight or less conjugated double bonds cannot be visualized without assumig broken conjugation in the polyene chain. At least two theories, a) and b), can be entertained; see Scheme 4.

a) The observed colour change is ascribed to enolization of the 4,4′-keto groups resulting in products with cumulated double bonds, acting as insulators of the π-electron resonance system. On acidification the enolate turns epiphasic, now either a strongly bound diosphenol or an unconjugated bis-α-diketone



Scheme 4. Structural interpretation of the effect of alkali on actinioerythrin (*1*) and violerythrin (*20*).

which slowly isomerizes to the non-allenic, conjugated tetraketone (*20*). Resonance interaction is known to be prevented by an allenic grouping due to perpendicular double bonds. According to Celmer and Solomons [46] resonance interaction should still be possible in compounds containing an odd number of cumulated double bonds. The absorption region and shape of the electronic spectra of carotenoidal diphenyl polyenes synthesized by Karrer's school [47] confirm that a system of three cumulated double bonds does not break the resonance interaction in a sufficient manner to explain the spectra of the products obtained on alkali treatment of *1* or *20* by the allenic structures indicated in Scheme 3. Moreover, attempts to confirm such allenic structures by IR-spectroscopy failed. Consequently this hypothesis is abandoned.

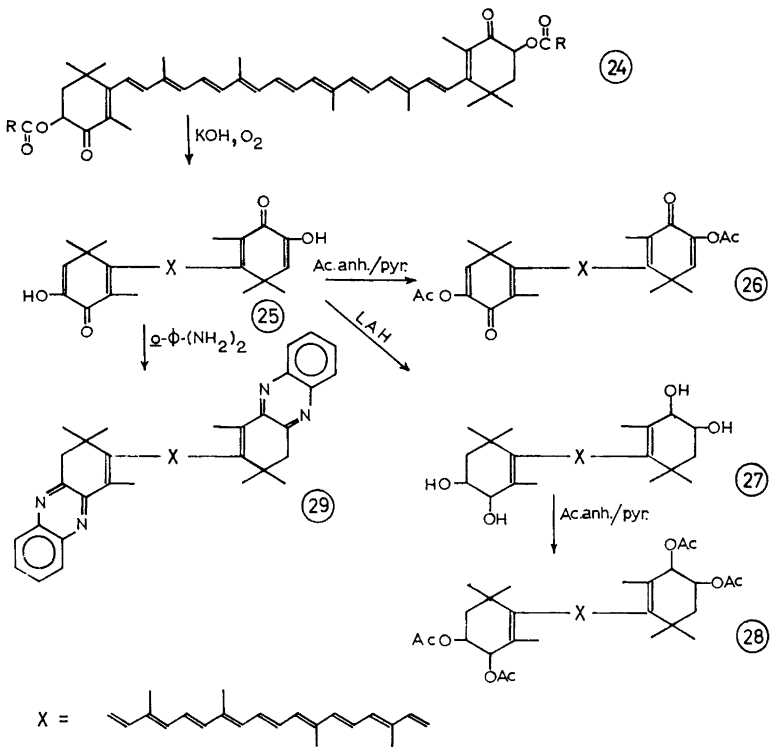
b) It is assumed [48] that the first step in the benzilic acid rearrangement of α,β-diketones involves addition of OH^- to one of the carbonyl groups, and it is conceivable that in the conjugated system present in *20* the addition could alternatively occur to alternating carbon atoms of the polyene chain leading by electron displacement to enolate ions at 4,4′-positions. According to this hypothesis the rate-determining step in the transformation of the yellow alkali-products to *20* after acidification should involve elimination of water. Experimental confirmation of such hydroxylated intermediates was not sought and will be further pursued.

Treatment of actinioerythrin (*1*) with weak alkali provides an unstable, red hypophasic compound, considered to be the α-ketol, actinioerythrol (*16*). In the presence of alkali *16* would give, by analogy with astaxanthin,[50] an unstable tetraenolate (*19*) which in the presence of traces of oxygen (the ordinary flushing with nitrogen does not provide sufficiently anaerobic conditions) would oxidize to the tetraketo astacene-analogue, violerythrin (*20*). However, as already discussed, *20* is not stable as such in the presence of alkali, and the further reactions leading to *20* would be those already discussed. Alkali treatment of *1* in the absence of oxygen was shown to give a blue-black product, considered to be the tetraenolate *19*, which turned brown-red on access to air, *cf.* Ref. 49a. Kuhn and Sørensen [49a] have demonstrated the oxygen uptake on alkali treatment of astaxanthin. It is also known that hydrogen peroxide is formed in that reaction.[49a] Evidence of oxygen uptake on alkali treatment of *1*, and lack of such on subsequent acidification, was obtained from manometric experiments.

Minor esters

The evidence obtained for ester X does not yet permit a structural assignment, and is not included.

Ester Y was isolated as such only in micro scale by paper chromatography, and proof of its structure rests on examination of the product obtained on alkaline hydrolysis, see Scheme 5. The hydrolysis product was identified as astacene (*25*) from its spectral properties (visible light, IR), acetylation to astacene diacetate (*26*), hydride reduction to 3,4,3′,4′-tetrahydroxy-β-carotene (*27*), acetylation of the latter to 3,4,3′,4′-tetraacetoxy-β-carotene (*28*) and transformation of *25* to its bisphenazine derivative (*29*). Each derivative was identified by means of chromatographic and spectral (visible light) comparison



Scheme 5. Chemical reactions of ester Y (*24*).

with authentic material. The mass spectrum of astacene bisphenazine derivative (*29*) derived from *A. equina* (Fig. 6, also indicating observed fragmentations) was identical with that of an authentic sample.

From these data it is inferred that ester Y is an astaxanthin diester (*24*).

A possible mechanism for the biosynthetic formation of the 2,2′-bisnorastaxanthin diester (*1*) by benzilic acid rearrangement of a hypothetic triketone derived from astaxanthin has already been considered.[8] Studies on the biosynthesis of actinioerythrin (*1*) are in progress.

After this paper was written, Holzel, Leftwick and Weedon[49b] have reported a chemical conversion of astacene (*25*) to violerythrin (*20*), guided by the structures given in our preliminary report.[8] The conversion was effected by manganeous dioxide oxidation, and they assumed a reaction sequence involving benzilic acid rearrangement analogous to the biosynthetic route suggested for actinioerythrin (*1*).[8]

EXPERIMENTAL

Materials and methods. When not stated to the contrary these were as summarized elsewhere.[14] IR-spectra were recorded on a Perkin Elmer Model 257 grating spectrometer and mass spectra on an LKB-9000 mass spectrometer. Gas chromatography was carried out on a Pye Argon Gas Chromatograph Model 1201 or on the LKB gas chromatograph fitted to the mass spectrometer. Adsorptive properties for the *trans* isomers are compiled in Table 2, and absorption maxima in visible light in Table 3. Further reference to these data is generally not given in the text.

Biological material. Mauve-coloured *Actinia equina* L. specimens of 3–5 cm diameter were collected on the beach near Vanvika, Trondheimsfjord, in June 1967 and at the Biological Station, Espegrend near Bergen in December 1967 and March 1968.

Isolation of the carotenoids. The pigments were extracted with acetone in a Waring blender and transferred to ether. The ether extract was taken to dryness and white contaminants partly removed by precipitation from a concentrated petroleum ether solution. Occasionally violet needles of actinioerythrin (*1*) crystallized at this stage. These were collected by extraction of the total precipitate with acetone.

From four batches in total 402 mg pigment were extracted, corresponding to 0.08–0.16 % carotenoid of the dry, extracted cell residue of some two-three hundred animals in total; Fabre and Lederer [3] extracted *ca.* 5 mg from 30 specimens.

Chromatographic purification was attempted on deactivated alumina, magnesium oxide, kieselgel G, and calcium carbonate columns. Adsorption on alumina caused the formation of products more strongly adsorbed than *1*. Some decomposition to yellow compounds was observed on magnesium oxide. Kieselgel G was effective in removing colourless oil from the extract prior to chromatography on calcium carbonate, although repeated chromatography (*ca.* eight times) was required to remove oily contaminants on the calcium carbonate columns generally used.

Actinioerythrin (*1*)

Crystallization. 1 comprised generally *ca.* 90 % of the total carotenoid. Violet needles with a metallic sheen crystallized from concentrated petroleum ether solutions, m.p. 90–91°C (*cf.* Table 1), total yield *ca.* 40 mg. Fabre and Lederer [3] obtained 1.5 mg from 30 specimens and Heilbron *et al.*[5] 30 mg from 500 specimens.

Solubility. 1 was readily soluble in petroleum ether, ether and carbon disulphide, less soluble in chloroform, acetone, and pyridine and rather insoluble in methanol.

Adsorptive properties, see Table 2. Separation of crystalline *1* (chromatographically homogeneous on ordinary kieselguhr paper) into six ester components was achieved by a modified method of Egger [50] on circular paraffin-impregnated paper developed with acetone:methanol:water (15:3:1), R_F-values 0.28, 0.33, 0.45, 0.53, 0.62 (major component), and 0.74. Separation on thin-layer plates was less satisfactory. Neither method was applicable on a preparative scale.

Visible light absorption spectra. Absorption data are given in Table 3 and Fig. 1, in petroleum ether $E(1\ \%,\ 1\ \text{cm}) = 1370$ (for $M = 860$, $\varepsilon = 118\ 000$) at 496 nm; Fabre and Lederer reported $\varkappa^{51} = ca.\ 66\ 000$.[3] For comparison canthaxanthin has $\varepsilon = 124\ 000$ [52] at λ_{max} in petroleum ether and β-apo-2′-carotenyl (C_{37}) acetate [53] abs. max. 495 and (520) nm and C_{30}-dialdehyde [52] (470), 493, and (533) nm in acetone.

Infrared spectrum. In KBr-disc abs. max. (Fig. 2) were located at 2920, 2850 (CH), 1738 (saturated ester), 1695 (α,β-unsaturated five-ring ketone); 1600, 1587, 1520 (conj. double bonds), 1430, 1420 (CH_2), 1392, 1385 (methyl, *gem.* dimethyl); 1300, 1265, 1215, (1195), (1187); 1155 (ester C–O); 1045 (ester); 990, 960 (*trans* disubst. double bonds); 815 (*trans* trisubst. double bonds) and 710 ($(CH_2)_4$) cm^{-1}.

For comparison astaxanthin diacetate exhibited carbonyl absorption for saturated ester at 1745 cm^{-1} and for α,β-unsaturated six-ring ketone at 1668 cm^{-1}.

Proton magnetic resonance spectrum. At 60 Mc/sec in $CDCl_3$ signals were located at τ 3–3.7 (multiplet, *ca.* 11 H, calc. 14 H, conjugated olefinic); 4.65 (triplet, $J = 7$ cps., *ca.* 3 H, olefinic, in esterifying acids?); 4.82 (singlet, *ca.* 2 H, calc. 2 H, O=C–CH–OOCR); 7.6 (multiplet, *ca.* 4 H, calc. 4 H, $-CH_2CO-$ in esterifying acid); 7.98 (*ca.* 11 H, calc. 12 H, in-chain methyl); 8.08 (*ca.* 5 H, calc. 6 H, end-of-chain methyl); 8.58 (*ca.* 6 H,

calc. 6 H) and 8.79 (*ca.* 6 H, calc. 6 H), *gem.* dimethyl; 8.72 (*ca.* 28 H, CH_2 in esterifying acids), 9.10 (6 H, multiplet, CH_3-CH_2 in esterifying acids).

Mass spectrum. In the upper mass region peaks were observed at *m/e* 862, 834, 806, 780, 756, 742, 728, 714, 700, 688, 686, 674, 672, 664, 650, 636, 622, 620, 618, 582, 568, 552, 550, 536, 534, 519, 460 (552−92), 458 (550−92), 446 (552−106), and 444 (550−106); base peak at *m/e* 28.

Partition ratio. In petroleum ether/95 % methanol *1* had partition ratio 97:3; Heilbron *et al.*[5] reported completely epiphasic partition in petroleum ether/90 % methanol.

Stereoisomerization. The iodine catalyzed stereomutation mixture consisted at equilibrium of *trans* (61 % of total, $R_F=0.29$), neo A (22 %, $R_F=0.33$, abs. max. (395), 493 nm) and neo B (17 %, $R_F=0.40$, abs. max. (395), 492 nm). R_F-values refer to kieselguhr paper (7 % acetone in petroleum ether) and abs. max. to acetone solutions.

Acetylation. 1 (7.56 mg) was submitted to acetylation conditions; pigment recovery was 90 %. The recovered pigment was inseparable from, and exhibited after column chromatography and crystallization the same IR-spectrum as unacetylated *1*.

Silylation. 1 (0.16 mg) was submitted to silylation conditions. The recovered pigment consisted exclusively of *1*.

Attempted methylation. 1 (0.34 mg) in ether was treated with diazomethane for one day at 5°C, pigment recovery was 91 % consisting of unreacted *1*.

Attempted acetal formation.[55] *1* (0.11 mg) in methanol (1 ml) was treated with conc. HCl (0.1 ml) for 30 min; pigment recovery was 38 % consisting of unreacted *1*.

Oxime formation.[55] *1* (3.70 mg) in dry pyridine (4 ml) was treated with hydroxylamine hydrochloride (100 mg) for 5 h at 40°C, then at room temperature overnight; pigment recovery was 95 %. The recovered pigment, separated on a calcium carbonate column, consisted of unreacted *1* (48 %), the monoxime (*2*, 40 %) and the dioxime (*3*, 12 %).

Actinioerythrin monoxime (*2*). *2* was converted to the dioxime (*3*) on further treatment with hydroxylamine and proved particularly unstable.

Actinioerythrin dioxime (*3*). Acetylation of *3* (0.1 mg) overnight gave a presumed diacetate (90 %) and a monoacetate (10 %), see Table 2.

Sodium borohydride reduction of 1. One of several experiments was followed by paper chromatography: *1* (7.2 mg) in benzene (3 ml) and ethanol (3 ml) was reacted with $NaBH_4$ (*ca.* 100 mg). After 10 min unreacted *1*, a mono-ol (*4*), a di-ol (*6*) and a tri-ol (*8*) were present in the reaction mixture. After 20 min *1* was completely converted to a mixture of the mono-ol (*4*, *ca.* 5 % of total), di-ol (*6*, *ca.* 70 %), tri-ol (*8*, *ca.* 20 %) and the tetra-ol (*14*, *ca.* 5 %). The reaction was interrupted by transfer to ether; pigment recovery was 70 %.

4′-Dihydro-actinioerythrin (*4*). An aliquot (0.1 mg) was oxidized with air (solution shaken with air and left without nitrogen) in the presence of iodine [3] overnight. Paper chromatography revealed transformation to *1* (80 % of total).

4′-Acetyl-4′-dihydro-actinioerythrin (*5*). Acetylation of *4* (0.1 mg) gave *5* as a single product.

4,4′-Tetrahydro-actinioerythrin (*6*). After purification on a cellulose column some red needles crystallized from petroleum ether/methanol. The IR-spectrum in KBr (Fig. 2) had abs. max. at 2920, 2850 (CH); 1740 (saturated ester); 1624 (conj. double bonds); 1460 (CH_2); 1380, 1365 (methyl, *gem.* dimethyl); 1260; 1165 (ester C−O); 1115, 1100, 1040; 970 (*trans* disubst. double bonds) and 725 ($(CH_2)_4$) cm^{-1}.

6 was readily oxidized when stored in air. When *6* (0.1 mg) was oxidized as described above for one day, with air in benzene solution in the presence of iodine, paper chromatography and spectrometry revealed the formation of the mono-ol (*4*) and actinioerythrin (*1*).

6 (1 mg) was saponified in 5 % KOH-solution for 2 h and gave the tetraols *14a*, *14b*, and *14c* with identical absorption spectra (see below), the two latter as main products. Acetylation of *14a,b,c* thus produced gave the peracetate (*15*, single zone) see below.

6 (*ca.* 0.5 mg) was submitted to acetylation, resulting in the formation of a monoacetate and a diacetate (*7*).

4,4′-Diacetyl-4,4′-tetrahydro-actinioerythrin (*7*) had absorption properties like *6*.

3′-Desacyloxy-3′-hydroxy-4,4′-tetrahydro-actinioerythrin (*8*). *8* (0.1 mg) was saponified with 5 % KOH-solution overnight and gave *14a,b,c*. Acetylation of *14a,b,c* thus produced gave the peracetate (*15*, single zone), identical with *15* obtained by acetylation of *6* above.

Hydrogenolysis of *8* (0.1 mg) with lithium aluminium hydride gave *14a,b,c*, mainly *b* and *c*.

3'-Desacyloxy-3'-acetoxy-4,4'-dihydro-4,4'-diacetyl-actinioerythrin (*9*). The acetylation of *8* was followed by paper chromatography. On kieselguhr paper (10 % acetone in petroleum ether) three intermediary acetates were produced ($R_F = 0.40$, 0.60, and 0.70). *9* exhibited absorption properties like *7*.

3'-Desacyloxy-3'-hydroxy-4'-dihydro-actinioerythrin (10), 3'-desacyloxy-3'-hydroxy-4-dihydro-actinioerythrin (11), and 3'-desacyloxy-3'-hydroxy-actinioerythrin (12). The triol (*8*, 0.1 mg) was oxidized with air in the presence of iodine for 2 h as above. Paper chromatography revealed the formation of *10, 11* and *12*. Alkali treatment of *12* gave *18* below.

3'-Desacyloxy-3'-acetoxy-actinioerythrin (13). Acetylation of *12* gave a monoacetate (*13*) with the same absorption spectrum. No intermediary products were formed.

Lithium aluminium hydride reduction of 1. One of several experiments was as follows: *1* (3.0 mg) in dry, peroxide-free tetrahydrofuran (5 ml) was treated with $LiAlH_4$ (*ca.* 20 mg) for 3 min; pigment recovery was 74 %. The recovered pigment consisted of *14a,b,c* mainly *a* and *b*.

Violerythrol (14). *14a,b,c* were obtained by i) $NaBH_4$-reduction of *1* or alkaline hydrolysis of *6* and *8*, producing mainly *b* and *c*, ii) $LiAlH_4$-reduction of *1* giving mainly *a* and *b* or iii) $LiAlH_4$-reduction of violerythrin (*20*) below giving mainly *a* and *b*.

Components *a*, *b*, and *c* could not be further reduced with $LiAlH_4$. They were not interconvertible on iodine catalyzed stereomutation and had identical absorption spectra in visible light. Spectral shape and absorption maxima of the following compounds in acetone: γ-carotene (438), 463 and 492 nm, 4'-desoxo-okenone [56] (448), 472 and 502 nm, lycopene (448), 473 and 505 nm, and eschscholtzxanthin [57] (450), 476 and 505 nm, differed from those of *14*.

14a,b crystallized as red needles from acetone-petroleum ether; m.p. 192—194°C, yield 0.5 mg. In petroleum ether $E(1\ \%, 1\ cm) \geq 1700$ at λ_{max}. Partition ratio in petroleum ether/70 % methanol was 5:95. The IR-spectrum (Fig. 2) had abs. max. at 3350 (OH); 2900 (CH), 1670; 1440 (CH_2); 1380, 1360 (methyl, *gem.* dimethyl); 1064 (OH); 1025 (allylic OH); 960 (*trans* disubst. double bonds) and 880 cm^{-1}, and had striking similarity with that of 3,4,3',4'-tetrahydroxy-β-carotene (*28*) produced by $NaBH_4$-reduction of astacene.

The upper mass region of the mass spectrum is given in Fig. 4; M = 572 ($C_{38}H_{52}O_4$).

Violerythrol tetraacetate (15). The acetylation of *14a,b,c* was followed by paper chromatography. Four intermediary acetates were observed in the formation of the peracetate (*15*), which comprised a single zone on the paper chromatogram. *15*, precipitated after column chromatography, had IR-absorption (KBr) for acetate at 1740, 1240, and 1025 cm^{-1}. The NMR-spectrum (60 Mc/sec, $CDCl_3$) had methyl signals at τ 7.92 (4 acetate Me), 8.02 (4 in-chain Me), 8.28 (2 Me in 18,18'-position) and 8.88 (4 Me; *gem.* dimethyl). A signal at τ 8.72 was ascribed to a lipid contaminant, but may also contribute to the *gem.* dimethyl resonances.

Treatment of *14* (0.38 mg) with 0.03 N HCl in chloroform for 10 min caused a bathochromic shift; pigment recovery was 81 %. The product was irreversibly adsorbed on alumina.

Acetonide formation [58] of *14a,b,c* (0.2 mg) in dry acetone (2 ml) and anhydrous $CuSO_4$ (10 mg) for 2 days was attempted. *Ca.* 10 % of the recovered pigment was converted to two presumed acetonides *a* and *b* (Tables 2, 3). In a parallel experiment 3,4,3',4'-tetrahydroxy-β-carotene (*27*, 0.5 mg) produced by $NaBH_4$-reduction of astacene (*25*), was treated similarly. Only decomposed pigment was recovered.

Actinioerythrol (16). The tetraol (*14*) was rapidly bleached by treatment with 2,3-dichloro-5,6-dicyano-benzoquinone. Oxidation with *p*-chloranil was always negative. However, *14* was readily oxidized by air. *14a*, *14b*, and *14c* were separately oxidized as above, with air in benzene solution in the presence of iodine, to the same product (*16*). In a separate experiment *14a,b,c* (1 mg) was similarly oxidized overnight; pigment recovery was 60 %. The recovered pigment consisted of *16* only, for properties see Tables 2 and 3.

16 was also obtained by reduction of violerythrin (*20*, 0.9 mg) in ethanol (5 ml) with $NaBH_4$ (5 mg) for 3 min; pigment recovery was 84 %. The product comprised more than 90 % of *16*, judged by absorption spectrum, R_F-value and lack of formation of a quinoxaline derivative with *o*-phenylenediamine under conditions where *20* gave *23*. Acetyla-

tion of the product (*16*) gave a monoacetate and a diacetate (*17*) examined by mass spectrometry below.

16 (0.2 mg) in acetic acid (1 ml) was reacted with *o*-phenylenediamine (2 mg). No reaction occurred during 20 min at room temperature. *o*-Phenylenediamine (5 mg) was added and the mixture heated to 100°C for 2 h; pigment recovery was 60 %. Besides unreacted *16 ca.* 15 % of two less polar products (R_F=0.47 and 0.68 on kieselguhr paper; 10 % acetone in petroleum ether) were formed, both with abs. max. 516 and (550) nm in acetone; *cf.* results for echinenone.[14]

Actinioerythrol diacetate (17). During acetylation of *16* one intermediary acetate was observed in the formation of *17*. Silylation of *17* failed. The mass spectrum of *17*, derived from *20* above, had prominent peaks in the upper mass region at *m/e* 652 ($C_{38}H_{46}O_2(CH_3COO)_2$=M), 594 (M−58), 592 (M−60), 560 (M−92), 546 (M−106), 536 (M−116), 494 (M−158), 478 (M−174), 444 (M−92−146) and 430 (M−116−106).

3'-Dihydro-violerythrin (18). *16* (0.2 mg) in petroleum ether was shaken carefully with 2.5 % methanolic KOH-solution until all pigment was transferred to the hypophase. After neutralization of the hypophase and extraction with ether, violerythrin (*20*) and *18* were isolated from the ether extract. *18* was also obtained from *12* above. *18*, on acetylation gave a monoacetate (no intermediates formed).

Isolation of the fatty acids of 1. *1* (3.8 mg crystalline) in ethanol-benzene was reduced with $NaBH_4$ as described above until the diol *6* was the main component. The products were saponified in 5 % methanolic KOH-solution for 4 h and the carotenoids transferred to ether. The hypophase was acidified to *ca.* pH 5 and the acids extracted with ether and reacted with diazomethane at −20°C overnight. The oily residue was submitted to gas chromatography on a polyethylene glycoladipate column at 165°C. Methyl esters of saturated unbranched C_8, C_{10}, C_{12}, C_{14}, and C_{16} fatty acids were used as reference. Hereby methyl capricoate (C_{10}) and an ester with slightly shorter retention time than methyl laurate (C_{12}) were found to be the main components together with methyl ester(s) with lower retention time than methyl caprylate (C_8), tentatively methyl caproate (C_6). The remaining sample was submitted to combined gas-chromatographic mass-spectrometric examination. Saturated C_{10}, C_{11} and C_{12} acids were identified unambiguously by this method.

Alkali treatment of 1 under high vacuum. To *1* (0.3 mg) in dry pyridine (1.5 ml) under vacuum (0.08 mm Hg) was added a saturated KOH-solution in butanol (1 ml).[49a] The red solution immediately turned dark blue-black. On access to oxygen the solution turned red again. The pigment, transferred to ether, comprised some violerythrin (*20*) and yellow, strongly adsorbed pigment.

Hydrolysis of 1. The colour changes, phase behaviour and influence of oxygen during hydrolysis of *1* in a two-phase system in principle according to Heilbron *et al.*[5] were studied in several experiments. A summary is given in Scheme 3. The hydrolysis was also followed manometrically in a one-phase system in a Warburg apparatus at 12°C in butanol, using cups with two side-arms. Oxygen uptake took place subsequent to the addition of the KOH-solution to the carotenoid (*1*) solution, but not after acidification with acetic acid in butanol.

Violerythrin (*20*)

20 was not present as such in *A. equina*, but is treated separately for convenience.

Preparation.[5] Solutions of *1* containing oily contaminants were generally used; yield 40−60 % of *20*. In one of several experiments *1* (30 mg) in petroleum ether (100 ml) was shaken carefully with 2.5 % methanolic KOH-solution (100 ml) for 30 min whereupon *ca.* 90 % of the pigment had turned hypophasic. The hypophase was made slightly acidic with acetic acid and the pigments extracted with ether on further dilution with water; pigment recovery was 56 %. The product was purified by chromatography on cellulose columns. In addition to violerythrin (*20*) two yellow products A and B were isolated (Tables 2, 3). On standing product A was partly converted to *20*.

Crystallization. Blue-black needles of *20* were obtained from chloroform-petroleum ether, m.p. 236°C, yield *ca.* 10 mg.

Solubility. *20* is readily soluble in chloroform and carbon tetrachloride, less soluble in carbon disulphide, ethanol, pyridine and only slightly soluble in acetone, methanol, ether, benzene, and petroleum ether.

Absorption spectrum in visible light. Data are given in Fig. 1 and Table 3. In chloroform $E(1\ \%,\ 1\ cm) \geq 1000$ at λ_{max}.

Infrared spectrum. In KBr abs. max. (Fig. 2) were located at 2910, (2850) (CH); 1750 (carbonyl in 3,3′-positions); 1675 (conj. carbonyl in five-ring); 1580, 1520 (double bonds); 1460, 1440 (CH_2); 1390, 1360 (methyl, *gem.* dimethyl); 1220, 1195, 1180, 1160, 1080, 1000, (980), 970, 950 (*trans* disubst. double bonds); 860 and 830 (*trans* trisubst. double bonds) cm^{-1}.

NMR-spectrum. Due to its low solubility in $CDCl_3$ no satisfactory spectrum was obtained. Signals at τ 7.95 (methyl groups attached to the polyene chain) and 8.59 (*gem.* dimethyl) were tentatively assigned.

Mass spectrum. The upper mass region of the spectrum is given in Fig. 4, M=564 ($C_{38}H_{44}O_4$).

Partition ratio. In petroleum ether/70 % methanol the partition ratio was 18:82.

Stereoisomerization. The iodine catalyzed stereomutation mixture comprised neo U (traces, R_F=0.48, and *trans* (R_F=0.57, abs. max. 560 nm in acetone). R_F-values refer to kieselguhr paper (20 % acetone in petroleum ether). Neo U was reversibly isomerized to the *trans* isomer.

Acetylation. 20 (0.3 mg) was submitted to acetylation conditions, pigment recovery was 80 %. No acetate was formed.

Silylation. 20 (0.1 mg) gave only yellow decomposition products when submitted to standard silylation conditions.

Methylation. 20 (0.1 mg) in ether was treated with diazomethane over night; pigment recovery was 50 %, consisting of unreacted *20* only.

Hydride reduction. Short treatment of *20* with $NaBH_4$ gave *16* (see above). Longer treatment (30 min) resulted in complete reduction to *14*. Reduction with $LiAlH_4$, gave *14* (see above).

Treatment with alkali. When *20* in ether (abs. max. 550 nm) was treated with 2.5 % KOH in methanol-water the solution immediately turned yellow (abs. max. *ca.* 385, 410, 433, 460 nm). On acidification to pH *ca.* 6 the pigment could be transferred to ether and remained yellow (abs. max. *ca.* 380, 405, 430, 450 nm). On standing for some hours the ether solution gradually turned blue (abs. max. 555 nm). Paper chromatography revealed quantitative re-formation of *20*. The alkaline hypophase, when kept, never turned blue. Attempts to record IR-spectra of the yellow intermediates in neutral or alkaline medium did not give reproducible results. Absorption in the 1900 cm^{-1} region was not observed. Changes in the carbonyl absorption, with increased absorption in the 1600 cm^{-1} region, generally occurred.

Reaction with o-phenylenediamine. 20 (0.4 mg) in acetic acid (1 ml) reacted smoothly with *o*-phenylenediamine (10 mg). After 2 min paper chromatography revealed the formation of *21* and *23*. The pigment recovery was quantitative.

Violerythrin monoquinoxaline derivative (21). Reduction of *21* with $LiAlH_4$ gave *22*.

Violerythrin bisquinoxaline derivative (23). Chromatographically purified *23* was precipitated from petroleum ether. The entire yield was used for a mass spectrum. The upper mass region of the spectrum is given in Fig. 6, M=708 ($C_{50}H_{52}N_4$).

Treatment of *23* (0.1 mg) with $LiAlH_4$ gave 80 % pigment recovery and no reduced product.

Ester Y (*24*)

Isolation. 24 was eluted from the cellulose or kieselgel columns together with oily compounds and *cis*-actinioerythrin (*1*). Judged from the yield of *25* below it was roughly estimated to be *ca.* 2 % of the total carotenoid.

Paper chromatography allowed the determination of R_F-value and absorption spectrum in visible light.

Astacene (25). Alkaline hydrolysis of a mixture of *24* and *cis-1* provided a product inseparable from authentic astacene (*25*, of lobster origin) by co-chromatography tests and with identical absorption spectrum in visible light. The entire yield of crystalline *24* (*ca.* 0.2 mg from acetone-petroleum ether) was used for an IR-spectrum (KBr); characteristic abs. max. at 1620, 1550 (diosphenol), 1260 and 1060 cm^{-1} and general correspondence with the IR-spectrum of authentic astacene.[14]

Astacene diacetate (26). *25* derived from ester Y was acetylated. The product could not be chromatographically separated from authentic astacene diacetate (*26*).

3,4,3',4'-Tetrahydroxy-β-carotene (27). *25* derived from ester Y was reduced with $LiAlH_4$ in ether and gave a product paper-chromatographically and spectroscopically (visible light) indistinguishable from hydride reduced astacene. The absorption spectrum corresponded to that of *28* below.

3,4,3',4'-Tetraacetoxy-β-carotene (28). *27* derived from ester Y was acetylated. The product was chromatographically identical with authentic *28*.

Astacene bisphenazine derivative (29). *25* derived from ester Y was reacted with *o*-phenylenediamine for 5 h at 100°C, then at room temperature overnight. The product was spectroscopically (visible light) and chromatographically identical with authentic astacene bisphenazine derivative (*29*). Both compounds gave the same mass spectra; the spectrum of the upper mass region is presented in Fig. 6, M=736 ($C_{52}H_{56}N_4$).

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