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Bacterial Carotenoids XXVII*

C_{50}-Carotenoids. 3. Structure Determination of *Dehydrogenans*-P439

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Chemical and physical properties of *dehydrogenans*-P439, a novel C_{50}-carotenoid from *Flavobacterium dehydrogenans* Arnaudi, and seventeen derivatives thereof, are reported.

The oxygen functions comprise two allylic, primary hydroxyl groups separated from the polyene chain.

Electronic, infrared, proton magnetic resonance and mass spectra are discussed. The evidence obtained is compatible with P439 being 2,2′-di(3-hydroxymethyl-but-2-enyl)-ε-carotene (*1*).

The main carotenoid of *Flavobacterium dehydrogenans* Arnaudi,[1] isolated in the pure state and shown by mass spectrometry to represent the first C_{50}-carotenoid found in nature,[2] was given the preliminary designation *dehydrogenans*-P439.[2,3]

Dehydrogenans-P439 was claimed to be identical with sarcinaxanthin.[4] However, judged by further mass spectrometric data sarcinaxanthin was later suspected to be a mixture of several carotenoids.[5] A re-isolation of sarcinaxanthin from the original source will be attempted. In the meantime the name *dehydrogenans*-P439 (in the following abbreviated to P439) is maintained in the present paper, which reports on studies on the chemical structure of P439. A preliminary note on the present work has already been published.[6]

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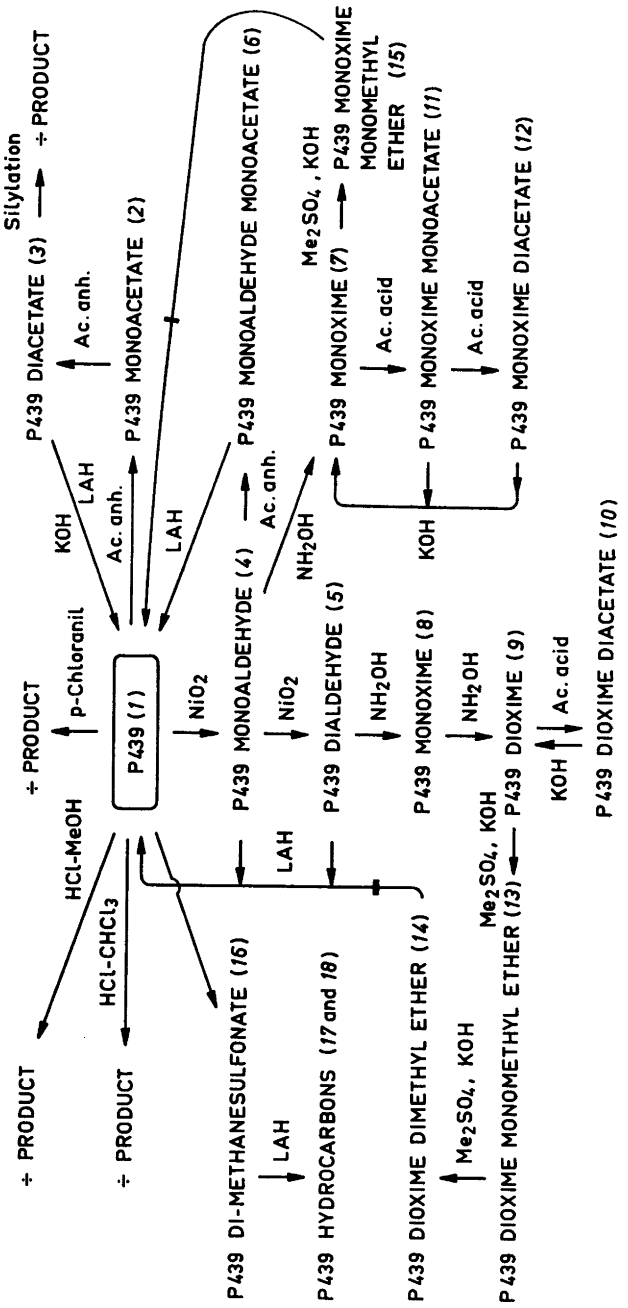

÷ PRODUCT
p-Chloranil
P439 (1)
NiO2
P439 MONOALDEHYDE (4)
NiO2
P439 DIALDEHYDE (5)
NH2OH
P439 MONOXIME (8)
NH2OH
P439 DIOXIME (9)
KOH
Ac. acid
P439 DIOXIME DIACETATE (10)
HCl-MeOH
HCl-CHCl3
÷ PRODUCT
÷ PRODUCT
LAH
P439 DI-METHANESULFONATE (16)
LAH
P439 HYDROCARBONS (17 and 18)
Me2SO4, KOH
P439 DIOXIME DIMETHYL ETHER (14)
Me2SO4, KOH
P439 DIOXIME MONOMETHYL ETHER (13)
KOH
LAH
Ac. anh.
P439 MONOACETATE (2)
LAH
P439 MONOALDEHYDE MONOACETATE (6)
Ac. anh.
NH2OH
P439 MONOXIME (7)
Ac. acid
P439 MONOXIME MONOACETATE (11)
KOH
Ac. acid
P439 MONOXIME DIACETATE (12)
Me2SO4, KOH
P439 MONOXIME MONOMETHYL ETHER (15)
KOH
LAH
Ac. anh.
P439 DIACETATE (3)
Silylation
÷ PRODUCT

RESULTS AND DISCUSSION

P439 (*1*) crystallized as needles, m.p. 153—155°C. Adsorptive properties and partition behaviour were indicative of a diol, confirmed by standard acetylation, whereupon a monoacetate (*2*) and a diacetate (*3*) were formed. The diacetate (*3*) was completely epiphasic and provided no trimethylsilyl ether on silylation, thus demonstrating the presence of two primary or secondary and no tertiary hydroxyl groups in P439 (*1*). Judged by IR-absorption (Fig. 1) at 1002 cm^{-1} and NMR-absorption at 5.96 τ (4 protons Fig. 3) the two hydroxyl groups appeared to be primary and allylic.[7,8] Attempted oxidation with *p*-chloranil [9] was negative, whereas selective allylic oxidation with nickel peroxide [10,11] gave a monoaldehyde (*4*) and a dialdehyde (*5*). Both products (*4* and *5*) exhibited absorption spectra in visible light indistinguishable from that of P439 (*1*) itself (see Fig. 4). The dialdehyde (*5*) provided no acetate on acetylation and the monoaldehyde (*4*) gave a monoacetate (*6*). The aldehydic character of the oxidation products (*4* and *5*) was suggested by IR-absorption at 2850 and 1680 cm^{-1} (conj. aldehyde),[12] see Fig. 2, and the facile formation of a monoxime (*7*) from the monoaldehyde (*4*) and a monoxime (*8*) and a dioxime (*9*) from the dialdehyde (*5*). However, oxidation of the aldehydes (*4* and *5*) to their corresponding acids with silver oxide [13] failed. Treatment of P439 dioxime (*9*) with acetic acid gave the dioxime diacetate (*10*), and of the monoxime (*7*) the monoxime monoacetate (*11*) and the monoxime diacetate (*12*). Attempts to obtain the P439 mono- and di-acids in micro scale according to Kuhn and Grundmann [14] from *10* and *12* were unsuccessful. Alkali treatment of *10* and *12* resulted in hydrolysis to the dioxime (*9*) and monoxime (*7*), respectively, and not as previously assumed [2] in dehydration to the corresponding nitriles and subsequent saponification to the mono- and di-acids. The oximes (*9* and *7*) thus obtained exhibited weakly acidic properties (judged by their partition behaviour in neutral and alkaline medium) and were methylated with dimethyl sulphate and alkali to products (*13*, *14*, *15*) previously considered to be the expected methyl esters, but now shown to be the oxime methyl ethers (*13* and *14* from *9*, and *15* from *7*). Whereas the methyl esters should be reduced readily with lithium aluminium

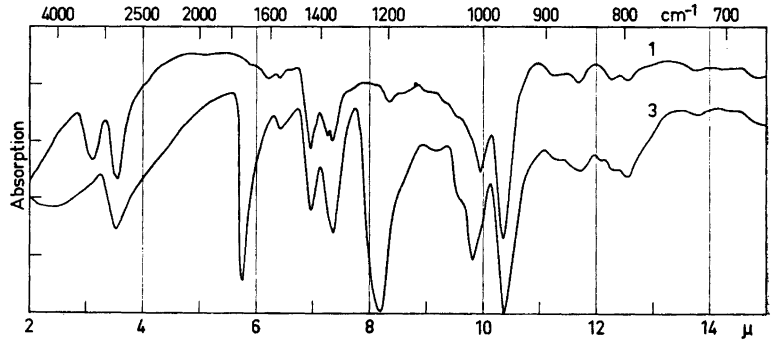


Fig. 1. Infrared spectra in KBr-pellet of P439 (*1*) and P439 diacetate (*3*).

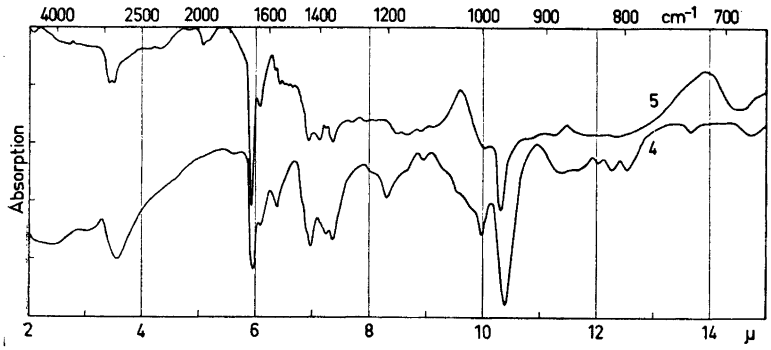


Fig. 2. Infrared spectra of P439 monoaldehyde (*4*) in KBr-pellet and of P439 dialdehyde (*5*) in chloroform.

hydride, the methyl ethers *14* and *15* were not reduced. Similar experience has been obtained and discussed for attempted elimination of aldoxime acetates in the rhodopinal series.[15]

Further proof of the aldehydic character of the oxidation products *4* and *5* was thus not obtained by further oxidation to the corresponding acids. However, the NMR-spectrum of *5* showed a typical aldehyde absorption (0.60 τ) in place of the 5.98 τ absorption from P439 (*1*).

No evidence for other oxygen functions was obtained from the IR-spectrum (Fig. 1) and mass spectral data discussed below confirmed the presence of only two oxygen functions in P439 (*1*). From these data it may be concluded that the oxygen functions of P439 (*1*) are two primary, allylic hydroxyl groups separated from the polyene chain. Primary hydroxyl groups are not common in natural carotenoids, and so far are known to occur only in rhodopinol,[14] lycopenol,[16] lycoxanthin, and lycophyll,[7] where they may formally be considered as having arisen by oxidation of allylic, lateral methyl groups.

P439 was resistant towards treatment with hydrochloric acid in methanol or chloroform, providing neither allylic methyl ethers[11,17] nor elimination products.[18]

Isopropylidene groups were shown to be absent by quantitative ozonolysis.[19] Attempted isomerization of isolated double bonds into conjugation using potassium hydroxide or potassium *tert*-butoxide [20] failed.

P439 (*1*) provided a di-methanesulfonate (*16*), which on small scale hydride reduction afforded two P439 hydrocarbons (*17* and *18*), one of which may be identical with sarcinene.[21,5]

Ozonolysis provided no α,α-dimethylsuccinic, α,α-dimethylglutaric, or α-cyclogeranic acid, but apparently one unidentified acid.

Further information about the chemical structure of P439 (*1*) was obtained from its spectral properties. Its electronic spectrum agrees with that of neurosporene [22] or 5,6,5′,6′-tetrahydrolycopene,[23] and is almost superimposable on that of ε-carotene (*19*, Fig. 4), demonstrating a chromophore of nine conjugated

double bonds in an aliphatic polyene chain. The IR-spectrum also supports the carotenoid nature of P439 and the absence of aryl end-groups.

The mass spectrum (Fig. 5) exhibited the parent peak at $m/e = 704$ ($C_{50}H_{72}O_2$). Other characteristic peaks appeared at M—16, M—18, M—79, M—92 (toluene),[24] M—140, and M—232 (= M—92—140). Since the two oxygen functions are shown to be primary hydroxyl groups, it follows that P439 has a C_{50}-skeleton. The mass spectra of the diacetate (*3*) and the dialdehyde (*5*) supported these conclusions. The molecular ion of the diacetate (*3*) occurred at $m/e = 788$ ($C_{54}H_{76}O_4$) and other significant peaks were at $m/e = 730$ (M—58), 728 (M—60, acetic acid), 696 (M—92, toluene), 606 ($C_{43}H_{58}O_2$ = M—182 = M—$C_{11}H_{18}O_2$), and 514 (M—182—92). The parent peak of the dialdehyde (*5*) was at $m/e = 700$ and prominent peaks occurred at $m/e = 685$ (M—15), 682 (M—18), 672 (M—28), 608 (M—92, toluene), and 562 (M—138). The peaks at M—(140 + 42) in the diacetate (*3*) and at M—(140—2) in the dialdehyde (*5*) corresponded to the characteristic loss of $C_9H_{16}O$ (M—140) in P439 (*1*). It is noteworthy that none of the P439 derivatives (*1*, *3*, and *5*) examined exhibited M—56 peaks characteristic of carotenoids with unsubstituted α-rings.[24]

Table 1. NMR-Properties of P439 (**1**).

Signal position in τ-value	Relative intensities [a]	Tentative assignment
2.76		$CHCl_3$
3.38—3.93	*ca.* 12	olefinic H
4.38—4.68	*ca.* 6	olefinic H
5.98	4	$=C-CH_2OH$
7.52	*ca.* 8	allylic CH_2 and allylic CH
7.62		
7.78		
7.83		
8.02	6	in-chain CH_3
8.06	6	in-chain/end-of-chain CH_3
8.32	*ca.* 6	CH_3 on double bond
8.47	*ca.* 6	CH_3 on double bond
8.75	(3)	
9.05	6	*gem.* CH_3 on α-ring
9.25	6	*gem.* CH_3 on α-ring

[a] Average of two samples, based on integrals and weight of cut-out areas.

The signals of the NMR-spectrum (Fig. 3) are compiled in Table 1, including tentative assignments. The NMR-spectrum is interpreted in support of a chromophore of type *1*a. Two in-chain (8.02 τ), two in-chain/end-of-chain (8.06 τ), and no true end-of-chain (8.19 τ) methyl groups are present in P439 (*1*). Double resonance experiments supported the presence of the two terminal, conjugated double bonds and two allylic methine groups (*1*a) or allylic methylene groups. The number of in-chain olefinic protons of *1*a (*12*) corresponds

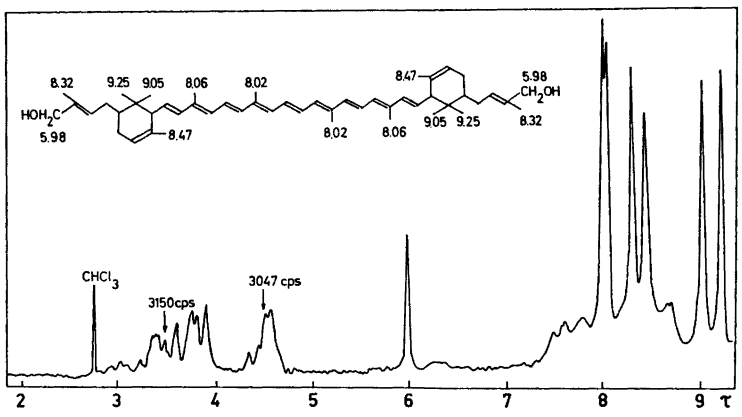
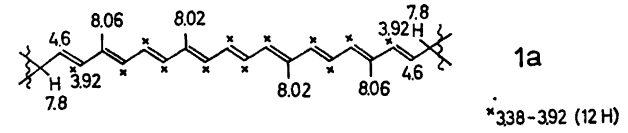
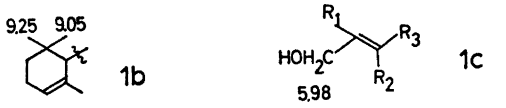
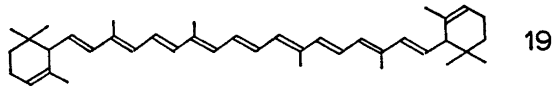


Fig. 3. Proton magnetic resonance spectrum at 100 Mc/sec of P439 (*1*) in deuterochloroform.

with the integration of the 3.38—3.92 τ absorption. The high-field 9.05, 9.25 τ signals are tentatively ascribed to the *gem.* methyl groups in two α-rings (*1*b).[24,25] The methylene protons of the allylic hydroxymethyl groups cause the 5.98 τ singlet, hence R_1 (*1*c) is not hydrogen.





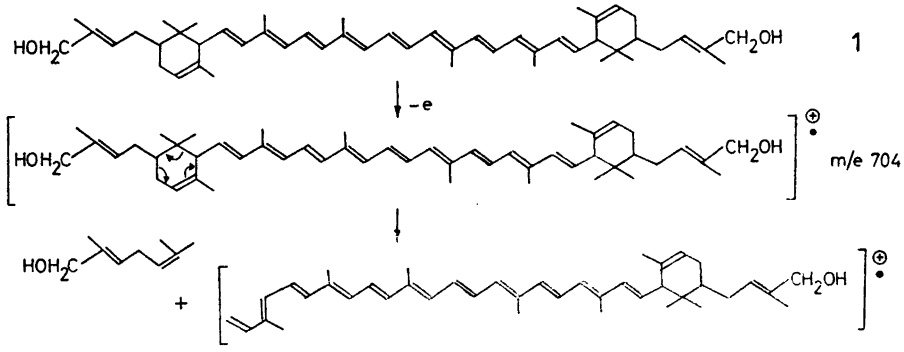


In addition four other tertiary methyl groups (8.32 and 8.47 τ) appeared to be present. Consideration of the molecular formula ($C_{50}H_{72}O_2$) for P439 (*1*), a chromophore of nine conjugated double bonds, presence of two isolated double bonds with hydroxymethyl substituents and two rings, revealed the presence of two additional saturated rings or double bonds. The chemical shifts of the so far unidentified methyl groups indicated that they were attached to double bonds, and the presence of two more olefinic protons in the 4.5 τ signal than required by the α-ring (*1*b) and the polyene chain (*1*a) both favoured the second alternative. Isopropylidene groups were already ruled

out, but the end-group *1*c, $R_1 = CH_3$ ane $R_2 = H$, recently established in lycoxanthin,[7] accommodated the primary allylic hydroxy-groups, the extra double bonds, the extra olefinic protons and two of the unassigned methyl groups (8.32 τ). End-group *1*c caused a methyl signal at 8.31 τ and an olefinic one at *ca.* 4.5 τ in lycoxanthin (acetate). The two remaining methyl signals (8.47 τ) were assigned to the methyl groups attached to the double bond in two α-rings (*1*b), *cf.* the 8.43 τ signal of the $C_{18,18'}$-methyl groups in ε-carotene (*19*), Fig. 6.

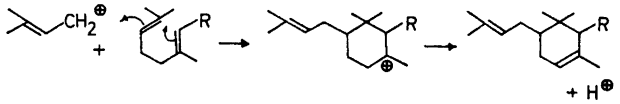
If P439 (*1*) indeed contained twelve methyl groups in addition to two hydroxymethyl groups and a carotenoid-like skeleton, it followed that the simplest hypothesis that P439 possessed a normal C_{40} carotenoid-skeleton extended linearly by two C_5-units, must be disregarded. Furthermore, the appearance of the NMR-spectrum (Fig. 3, Table 1) and the fact that only one monoacetate (*2*) and one monoaldehyde (*4*) were isolated, suggested a symmetrical molecule.

The NMR-features of P439 (*1*) have much in common with those of ε-carotene (*19*), see Fig. 6, and are best compatible with P439 (1) being ε-carotene (*19*) substituted by two C_5-units *1*c, $R_1 = CH_3$, $R_2 = H$ and $R_3 = CH_2$. The NMR-data rule out the possibility of the C_5-units being attached to the polyene chain. Moreover, a stable *cis*-configuration may be expected in such a case.[15] The NMR-spectrum is best accommodated with P439 (1) being ε-carotene (*19*) substituted by two *1*c units ($R_1 = CH_3$, $R_2 = H$ and $R_3 = CH_2$) in 2,2'- or 3,3'-positions. Preference for 2,2'-substitution is derived from the mass spectrum. The characteristic loss of M—140 for P439 (*1*) and corresponding losses for the diacetate (*3*, M—182) and the dialdehyde (*5*, M—138) may be interpreted as the result of a Retro-Diels-Alder fragmentation analogous to that observed by Schwieter *et al.*[24] for ε-carotene (*19*).



The allylic spin-spin coupling of the C_{18}-methyl group (τ 8.43, $J_{cis} = 2$ cps) of ε-carotene (*19*, Fig. 6) was present, but smaller in P439 (*1*). The stereochemistry of P439 (*1*) at C_6 and C_2 is not established. The stereochemistry around the terminal, isolated double bonds is also unknown, and the structure (*1*) is arbitrarily presented with a *trans* relationship between the two largest substituents.

Some biosynthetic studies on the formation of P439 (*1*) have already been reported by Weeks and Garner.[3] Structure *1* does not obey the isoprene rule, and it is tentatively suggested that the two supernumerary C_5-units are introduced after the formation of the C_{40}-skeleton. Analogies may be found for instance in the biosynthesis of irones [26] presumed to involve C-methylation in the corresponding 2-position, or in a large number of other natural products containing branched C_5 side-chains considered to be synthesized *in vivo* by alkylation with 3,3-dimethylallyl pyrophosphate.[27] The electrophilic alkylation reaction is less likely to occur after cyclization, but the derived isopentenyl cation could conceivably initiate the cyclization:



However, elucidation of the biosynthetic route leading to this C_{50}-carotenoid must await biosynthetic evidence.

EXPERIMENTAL

Materials and methods. When no special references or details are given these were as summarized in an earlier paper of this series.[28] Adsorptive properties are compiled in Tables 2 and 3. The terms in which spectral characteristics are described have been

Table 2. Composition of the carotenoid mixture extracted from *Flavobacterium dehydrogenans* grown under cultivation conditions referred to in the text.

Tentative identification	Required eluent from Woelm neutral alumina activity grade 3	Abs.max. in mμ in acetone	$E^{1\%}_{1\,cm}$ at λ_{max} used	% Of total
Lycopene-like	4 % acetone [a]	476	3100	0.2
OH-ζ-Carotene-like	8 % acetone	378, 400, 420	2000	0.4
P422 = OH-α-Zeacarotene-like	8 % acetone	(380), 400, 422, 451	2200	0.8
Di-OH-ζ-carotene-like	10–15 % acetone	378, 400, 420	2000	0.3
P439 (*1*)	15 % acetone	(400), 419, 443, 472	2300	98.3

[a] In petroleum ether.

defined elsewhere.[29a] Reactions were followed by periodical paper-chromatographic examination of aliquots. Pigment recoveries were determined by spectrophotometry in visible light. Mass spectra were recorded on an MS-9 mass spectrometer equipped with direct insertion probe. The ion source temperature was about 250°C. *Flavobacterium dehydrogenans* Arnaudi was used. The cultivation procedure has been described elsewhere.[3]

Pigment extraction. A small amount of water was added to the lyophilized cells in order to form a homogeneous slurry. The pigments were extracted with methanol for 20 h at room temperature and the cells removed by centrifugation.

Table 3. Adsorptive properties and partition ratios of P439 (*1*) and derivatives.

	R_F-value [a] on Schleicher & Schüll No. 287 paper				Partition ratio	
	Petroleum ether	2 % acetone [b]	5 % acetone	10 % acetone	Petroleum ether/ 95 % methanol	Petroleum ether/ 85 % methanol
P439 hydrocarbon (*17*)	0.63					
P439 hydrocarbon (*18*)	0.25					
P439 dioxime dimethyl ether (*14*)		0.88				
P439 diacetate (*3*)		0.65	0.98		96:4	
P439 monoaldehyde monoacetate (*6*)		0.58	0.98			
P439 monoxime methyl ether (*15*)			0.83			
P439 dialdehyde (*5*)		0.18	0.80	0.95	88:12	
P439 monoxime diacetate (*12*)			0.79			
P439 dioxime diacetate (*10*)			0.70	0.83		
P439 monoacetate (*2*)			0.66			
P439 monoaldehyde (*4*)			0.50		65:35	
P439 monoxime monoacetate (*11*)			0.40	0.76		
P439 dialdehyde monoxime (*8*)			0.32	0.62		
P439 dioxime monomethyl ether (*13*)			0.30			
P439 (*1*)			0.18	0.40	32:68	88:12
P439 monoxime (*7*)				0.36	31:69	88:12 76:24 [c]
P439 dioxime (*9*)				0.17	35:65 6:94 [c]	90:10 30:70 [c]
P439 di-methanesulfonate (*16*)			0			

[a] For *trans* isomer.
[b] In petroleum ether.
[c] Alkaline hypophase.

Saponification. Preliminary experiments revealed that P439 (*1*) was unchanged on alkali treatment, and standard saponification was therefore included in the purification procedure.

The total carotenoid content obtained from 30 l of culture was spectrophotometrically estimated to 16.7 mg corresponding to 0.55 mg/l culture.

Column chromatography was carried out on neutral alumina, activity grade 3. The eluents required for the various components and the composition of the carotenoid mixture are given in Table 2. The main carotenoid only is further characterized below.

P439 (*1*)

Crystallization was effected from dry acetone-petroleum ether. *1* crystallized as shiny needles, m.p. 153—155°C, after two recrystallizations, yield *ca.* 9 mg. Another *ca.* 24 mg were obtained from other batches.

Paper-chromatographic purity test. Crystalline *1* contained the sterically homogeneous *trans* isomer as revealed by paper chromatography.

Absorption spectrum in visible light of *trans 1* in acetone had abs. max. at (400), 419, 443 ($E^{1\,\%}_{1\,cm}$ = 2340, ε = 165 000), and 472 mμ, % III/II = 101, % D_B/D_{II} = *ca.* 5. In benzene abs. max. were found at (405), 428, 452, and 482 mμ, % III/II = 98, % D_B/D_{II} = 6, and in petroleum ether (Fig. 3) at (394), 416, 439, and 470 mμ. For synthetic

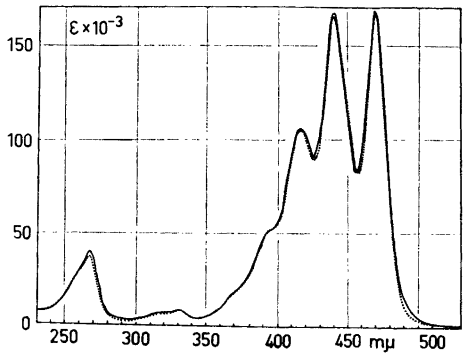


Fig. 4. Electronic spectra of ——— P439 (*1*) and ..... ε-carotene (*19*) in petroleum ether.

ε-carotene [29b] was found 416, 439 (ε = 167 200), and 470 mμ in petroleum ether (see Fig. 3).

Infrared spectrum in KBr-pellet is given in Fig. 1, abs. max. 3500 (OH); 3000 (CH); 1610, 1560 (conj. double bonds); 1450 (CH_2); 1385, 1365 (CH_3, *gem.* CH_3); 1200; 1050 infl.; 1005 (*prim.* allylic OH); 968 (*trans* disubst. double bonds); 850, 815, 798 (trisubst. double bonds) cm^{-1}.

Proton magnetic resonance spectrum in $CDCl_3$ at 100 Mc/sec is given in Fig. 3 and Table 1. When the spectrum was recorded with large expansion the 8.47 τ signal of the C_{18}-methyl group of P439 (1) had W_H^{29c} = 6 cps and the uncoupled methyl singlet at 9.28 τ W_H = 3 cps, as compared with W_H = 5 cps and 2 cps for the corresponding signals in ε-carotene (*19*). The position of hydroxyl proton signals could not be definitely established by shaking with D_2O, but is possibly masked in the 8.4—8.7 τ region. Irradiation with 3047 cps resulted in decoupling of the 3.92 τ (J = 17 cps) doublet and in some sharpening of the 7.82 (see *19*a) and 8.32 τ signals. Irradiation with 3150 or 3047 cps caused no decoupling of the 8.32, 8.47 τ signals.

The NMR-spectrum of synthetic ε-carotene (*19*) in $CDCl_3$ at 100 Mc/sec was recorded for comparison (Fig. 6). The chemical shifts of the protons at C_4 (4.58 τ), C_6 (*ca.* 8.8 τ), C_7 (4.48 τ), and C_8 (3.88 τ, J = 15.5 cps) were established by double resonance. The C_{18}-methyl group appeared as a doublet (8.43 τ, J = 2 cps).

Mass spectrum. The high mass region is shown in Fig. 5. Exact mass measurements were carried out for peaks at m/e = 704 and 612.

Found	704.5515	calc.	704.5531	$C_{50}H_{72}O_2$
	612.4910		612.4906	$C_{43}H_{64}O_2$

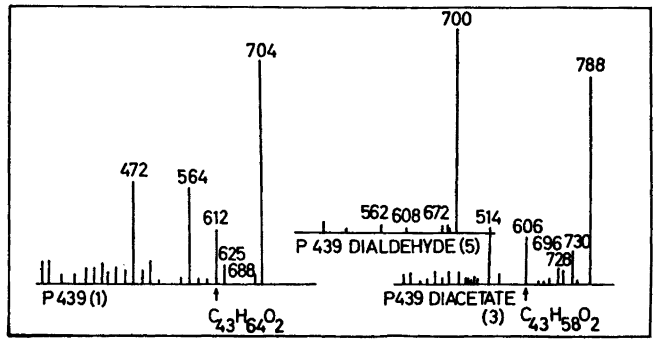


Fig. 5. High mass-end part of mass spectra of P439 (*1*), P439 diacetate (*3*) and P439 dialdehyde (*5*).

Partition ratios for P439 (*1*) are compiled in Table 3.

Stereochemical studies. Iodine catalyzed stereoisomerization in daylight of *trans* P439 (*1*) in benzene solution resulted in a hypsochromic shift to 335, (405), 425, 451 and 482 mμ, % III/II = 91, % D_B/D_{II} = 14. The equilibrium was reached after 1.5 h and the spectral shift was accompanied by a 14 % drop in extinction coefficient at the middle main maximum.

The composition of the iodine catalyzed equilibrium mixture is given in Table 4. The true nature of the neo U isomer as a member of the P439 stereoisomeric set was demonstrated by reversible isomerization in light.

Table 4. Composition of the iodine catalyzed equilibrium mixture of P439 (*1*) in benzene solution.

Stereoisomer	R_F-value S & S 287 10 % acetone in petroleum ether	In acetone			% Of total
		Abs.max. in mμ	% D_B/D_{II}	% III/II	
Trans	0.65	(322) (398) 419 443 472	6	95	73
Neo U	0.55	322 (398) 418 440 469	30	90	27

Attempted isomerization. i) Prolonged treatment of P439 (1) with 10 % KOH in methanol caused no isomerization of isolated double bonds into conjugation. ii) Treatment of *1* (0.3 mg) with K-*t*-butylate (0.1 N) in dioxane (2 ml) for 3 h in the absence of oxygen [20] gave no such isomerization products either.

General stability. Relative to carotenoids in general P439 (*1*) proved rather resistant towards *cis*-isomerization and was comparatively stable in the presence of air, base and acid.

Ozonolytic isopropylidene determination of P439 (1).[19] *1* (1.29 mg and 1.15 mg) dissolved in glacial acetic acid (3 ml) produced upon ozonolysis acetone equivalent to 0.81 ml and 0.89 ml 0.01 N thiosulphate, respectively, corresponding to 1.47 % and 2.25 % isopropylidene or 0.19 and 0.29 moles of acetone per mole of *1*.

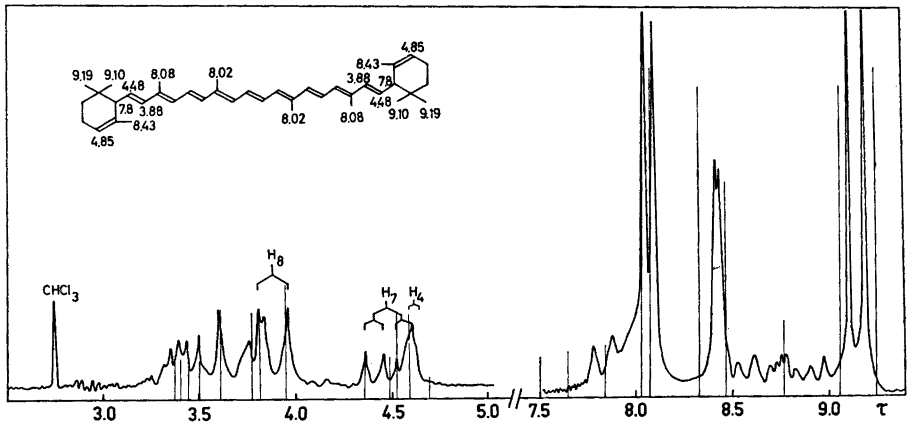


Fig. 6. Proton magnetic resonance spectrum of ε-carotene (*19*) at 100 Mc/sec in deuterochloroform. Lines indicate signal positions in P439 (*1*).

In a parallel experiment with lycopene (2.19 mg) an isopropylidene value of 18.6 % or 1.85 moles of actone per mole of lycopene was obtained.

In neither case were attempts made to characterize the acetone produced.

Ozonolysis of P439 (1). Through *1* (2 mg) in glacial acetic acid (4 mg) was bubbled ozone for 1.5 h at room temperature. Distilled water (3 ml) and hydrogen peroxide (0.2 ml, 35 %) was added and the mixture kept for 1 h at 90°C and 2 h at room temperature. The mixture was evaporated to dryness under vacuum and the residue extracted with ether. The ether extract was washed with water and aqueous bicarbonate, acidified with phosphoric acid and taken to dryness.[30] The residue was extracted with warm methanol for chromatography.

Thin layer chromatography was performed on kieselgel G using benzene-methanol-acetic acid (45:8:4) as developer.[31] The plate was dried at 120°C for 20 min and sprayed with bromocresol green No. 22 according to Stahl.[32] The acids formed yellow spots on a blue background. α,α-Dimethylsuccinic acid (R_F = 0.47), α,α-dimethylglutaric acid (R_F = 0.49), and α-cyclogeranic acid (R_F = 0.68) were used as reference compounds. Only one yellow spot (R_F = 0) appeared on the P439 chromatogram.

In ascending chromatography on kieselguhr paper with propanol-*conc.* ammonia (7:3) [33] for 15 h, followed by drying and spraying as above the P439 product again had R_F = 0. α,α-Dimethyl-succinic acid (R_F = 0.25) and α,α-dimethylglutaric acid (R_F = 0.35) were used for reference.

Neither of the above acids was found in the ozonization mixture left from the isopropylidene determination.

Acetylation. To P439 (*1*, 0.1 mg) in dry pyridine (1 ml) was added acetic anhydride (0.2 ml) at room temperature. The course of acetylation was followed quantitatively by paper chromatography and subsequent spectrophotometry, see Fig. 7. In a larger scale experiment *1* (4.1 mg) was quantitatively acetylated to the diacetate (*3*) in 18 h in the usual manner; pigment recovery was 96 %.

P439 monoacetate (2). The electronic spectrum corresponded to that of P439 (*1*). Adsorptive and partition properties are given in Table 3.

P439 diacetate (3) was purified by chromatography on deactivated alumina; the required eluent was 25—50 % ether in petroleum ether. *3* crystallized from benzene-methanol; yield 1.5 mg, m.p. 137—138°C. Another 4 mg was obtained from a separate experiment. The electronic spectrum corresponded to that of P439 (*1*). The IR-spectrum is presented in Fig. 1. Absorption at 1738, 1220, and 1020 cm^{-1} was caused by the acetoxy groups. The mass spectrum of *3* (Fig. 5) had prominent peaks at *m/e* = 788 (M), 730 (M—58), 728 (M—60), 696 (M—92), 606 (M—182), and 514 (M—92—182). The peak at *m/e* = 606 was matched with $C_{43}H_{58}O_2$.

Attempted silylation of P439 diacetate (3). *3* (0.1 mg) in dry pyridine (1 ml) proved resistant towards silylation with hexamethyldisilazane (0.2 ml) and trimethylchlorosilane (0.1 ml) as revealed by paper-chromatographic examination of the reaction mixture.

Saponification of P439 diacetate (3). *3* (3.8 mg) in ether-methanol (60 ml, 1:1) containing 5 % KOH was saponified for 4 h, pigment recovery was 100 % of P439 (*1*).

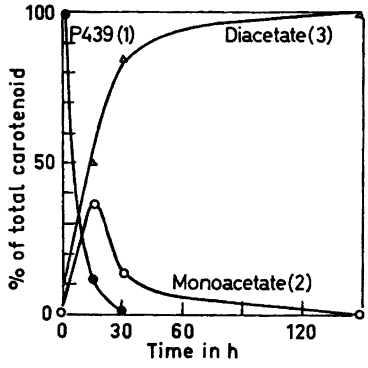


Fig. 7. The course of acetylation of P439 (*1*).

Hydride reduction of P439 diacetate (3). *3* (1 mg) in dry ether was reduced with lithium aluminium hydride in the usual manner; pigment recovery was 90 %, comprising only P439 (*1*).

Attempted allylic dehydration of P439 (1). i) When *1* (0.13 mg) was treated with 0.03 N HCl in chloroform, no less polar products were formed during 15 min in indirect daylight. ii) To *1* (0.59 mg) in dry pyridine (2 ml) was added tosylchloride (5 mg). After 22 h complete transformation to the tosylate (R_F = 0 on kieselguhr paper — 10 % acetone in petroleum ether) was observed. The tosylate was transferred to ether in the usual manner; pigment recovery was 71 %.

The tosylate in ether-methanol (1:7) containing 6 % KOH underwent no reaction during 23 h at room temperature. After refluxing for 3 h the pigments were transferred to ether; pigment recovery was 40 %. The reaction mixture contained P439 (*1*, 5 %) and unreacted tosylate (95 %).

Attempted formation of allylic ether of P439 (1). *1* (0.13 mg) was treated with 0.03 N HCl in methanol for 41 h; pigment recovery was 97 %. *Trans* and neo U P439 (*1*) exclusively were recovered.

Under similar conditions lutein gave 3′-methoxy-α-caroten-3-ol in quantitative yield after 1 h.[11]

Attempted oxidation with p-chloranil of P439 (1). *1* (0.15 mg) in benzene (2 ml) and methanol (0.5 ml), treated with *p*-chloranil (1 mg) and iodine (10 μg in 0.1 ml petroleum ether) for 43 h in artificial Na-light gave no keto-products under conditions [9] where isozeaxanthin gave canthaxanthin in high yield.

Nickel peroxide oxidation of P439 (1). Nickel peroxide was prepared and standardized according to the procedure of Nakagava *et al.*[10] *1* (6 mg) in dry ether (5 ml) and dry benzene (5 ml) was treated with nickel peroxide (50 mg, available oxygen 4.6×10^{-3} g atom/g nickel peroxide determined by titration) for 1.5 h; pigment recovery was 70 %. The reaction mixture contained P439 monoaldehyde (*4*; 25 %) and P439 dialdehyde (*5*; 75 %). Other experiments gave a similar result. The products were purified by chromatography on deactivated alumina.

P439 monoaldehyde (4) required 70 % ether in petroleum ether for elution from deactivated alumina and was crystallized from ether-petroleum ether or acetone-petroleum ether; total yield 2.8 mg, m.p. 142—143°C. The visible light absorption spectrum corresponded to that of P439 (1), $E^{1\,\%}_{1\,cm}$ = 2330 (ε = 164 000) at 441 mμ in acetone. The IR-spectrum (KBr-pellet) exhibited absorption at 3300 (OH); 2950 (CH); 1680 (conj. aldehyde); 1660, 1560 (conj. double bonds); 1430 (CH_2), 1380—1360 (CH_3, *gem.* CH_3); 1200, 1110; 1000 (*prim.* allylic OH); 960 (*trans* disubst. double bonds) 880; 830 (*trans* trisubst. double bonds), 815 and 800 cm^{-1} (see Fig. 2). The R_F-value and partition ratio are given in Table 3.

P439 monoaldehyde monoacetate (6). Acetylation of P439 monoaldehyde (*4*, 0.2 mg) in pyridine with acetic anhydride in the usual manner gave *6* in quantitative yield. The product (*6*) exhibited the same absorption spectrum in visible light as P439 (*1*).

Hydride reduction of P439 monoaldehyde monoacetate (6). Treatment of *6* (55 μg) in dry ether with a filtered suspension of lithium aluminium hydride resulted in complete conversion to P439 (*1*).

Attempted oxidation with silver oxide of P439 monoaldehyde (4). Treatment of aliquots (0.1 mg) of *4* in alcohol (2 ml) with aqueous silver nitrate and sodium hydroxide solutions (2 to 200 times molar excess) according to the method of Wahlbaum and Rosenthal [13] did not result in formation of the corresponding acid. Greater excess resulted in complete bleaching of the carotenoid.

P439 monoxime (7). P439 monoaldehyde (*4*, 0.8 mg) in benzene (2 ml) was treated with hydroxylamine hydrochloride (4.9 mg) in dry pyridine (0.7 ml) at 40°C. The reaction was complete after 35 min; pigment recovery was 100 %. *7* exhibited the same absorption spectrum as P439 (*1*). Partition ratio and R_F-value are given in Table 3.

In a parallel experiment oxime formation of 3′-keto-α-caroten-3-ol (0.4 mg) was attempted according to the same procedure. Less than 4 % of a product with properties compatible with the corresponding oxime was formed during 18 h.

P439 monoxime monoacetate (11) and P439 monoxime diacetate (12). P439 monoxime (*7*, 0.8 mg) in dry ether (0.7 ml) was treated with acetic anhydride (1 ml) at 50°C for 35 min according to the method of Kuhn and Grundmann;[14] pigment recovery was 23 %. After

transfer to ether the reaction mixture contained P439 monoxime monoacetate (*11*, 30 %) and P439 monoxime diacetate (*12*, 50 %) in addition to undefined decomposition products. *11* and *12* exhibited the same absorption spectrum in visible light as P439 (*1*).

Alkali treatment of P439 monoxime monoacetate (11) and P439 monoxime diacetate (12). The above mixture (0.18 mg) of *11* and *12* was treated with ether-methanol (20 ml, 1:1) containing 5 % KOH for 1.5 h when complete hydrolysis to P439 monoxime (*7*) occurred; pigment recovery was 72 %. The product (*7*) was readily transferred to petroleum ether from an alkaline hypophase, was indistinguishable in physical properties from *7* and could not be reduced to P439 (*1*) on standard treatment with lithium aluminium hydride.

P439 monoxime monomethyl ether (15). P439 monoxime (*7*, 65 μg) in ether (0.3 ml) and 0.1 N aqueous KOH-solution (2 ml) was treated with dimethyl sulphate (3 drops) for 3 h. Alkaline pH was maintained by periodic, small additions of *conc.* KOH-solution. After 3h the reaction mixture was transferred to ether in the usual manner; pigment recovery was 85 %. *15* constituted 80 % of the reaction mixture. Its absorption spectrum in visible light was identical with that of P439. Standard treatment of *15* in ether with lithium aluminium hydride caused no reduction to P439 (*1*).

P439 dialdehyde (5) required 50 % ether (or 10 % acetone) in petroleum ether for elution from deactivated alumina. *5* crystallized as orange needles; m.p. 157—158°C, yield *ca.* 4 mg. The absorption spectrum in visible light was the same as for P439 (*1*). A single determination gave $E^{1\,\%}_{1\,cm} = 2590$ ($\varepsilon = 181\,000$) at 441 mμ in acetone. The IR-spectrum (in $CHCl_3$) had abs.max. at 2930 (CH); 2850 (CH stretching in aldehyde); 1680 (conj. aldehyde); 1640; 1432 (CH_2), 1400; 1358 (CH_3) and 968 (*trans* disubst. double bonds), see Fig. 2. The NMR-spectrum exhibited signals at 0.60, 8.04, 8.09, 8.26, 8.46, (8.76), and 9.06 τ (the spectrum was, unfortunately, not recorded at τ-values above 9.15) in addition to the absorption in the olefinic region, but had no signal near 5.98 τ as in P439 (*1*). The mass spectrum had prominent peaks at $m/e = 700$ (M), 685 (M−15), 682 (M−18), 672 (M−28), 608 (M−92), and 562 (M−138).

Attempts at acetylation of P439 dialdehyde (5). Standard treatment for acetylation of *5* (77 μg) gave 88 % pigment recovery and no acetylated products.

Hydride reduction of P439 dialdehyde (5). *5* (34 μg) was reduced in the same manner as *6* above; pigment recovery was 65 %. The reaction mixture contained P439 (*1*) exclusively.

Alkali treatment of P439 dialdehyde (5). *5* (20 μg) was dissolved in 5 % methanolic KOH-solution (3 ml), shaken with air and left for 20 h. No product with extended chromophore was produced.

Oximation of P439 dialdehyde (5). *5* (0.25 mg) in benzene (2 ml) was treated with hydroxylamine hydrochloride (5.1 mg) dissolved in dry pyridine (0.7 ml) at 40°C. Complete conversion to the dioxime (*9*) was observed after 1 h. The reaction was interrupted in the usual manner; pigment recovery was 100 %.

P439 dialdehyde monoxime (8) was formed as a transistory product. Its absorption spectrum was the same as for P439 (*1*).

P439 dioxime (9). *9* exhibited the same absorption spectrum in visible light as P439 (*1*). Partition ratio are included in Table 3.

P439 dioxime diacetate (10). P439 dialdehyde (*5*, 0.18 mg) dissolved in dry, peroxide-free ether (1 ml) was treated with acetic anhydride (1 ml) at 40°C for 20 min. The pigment was isolated in the usual manner; pigment recovery was 83 %. *10* had the same absorption spectrum in visible light as P439 (*1*).

Alkali treatment of P439 dioxime diacetate (10). *10* (0.15 mg) in ether (5 ml) was treated with 10 % methanolic KOH-solution (5 ml) for 1.5 h. The product was isolated in the usual manner; pigment recovery was 80 %. The reaction mixture contained P439 dioxime (*9*) exclusively, judged by its absorption spectrum in visible light, R_F-value (= 0.58 aluminium oxide paper in 50 % acetone in petroleum ether; see also Table 3) and partition behaviour (Table 3).

Attempt at hydride reduction of P439 dioxime (9). The above product (*9*, 60 μg) was submitted to standard reduction with lithium aluminium hydride; pigment recovery was 39 %. No transformation to P439 (*1*) was obtained.

Methylation of P439 dioxime (9). Another aliquot of the hydrolysis product (*9*, 60 μg) was treated for 6 h with dimethylsulphate and alkali in the same manner as 7 above;

pigment recovery was 78 %. The reaction mixture contained the monomethyl ether (*13*, 30 %) and the dimethyl ether (*14*, 40 %).

P439 dioxime monomethyl ether (*13*) *and P439 dioxime dimethyl ether* (*14*) exhibited the same absorption spectra in visible light as P439 (*1*).

Attempted hydride reduction of P439 dioxime dimethyl ether (*14*). Careful treatment with lithium aluminium hydride of 14 (13 μg) in dry ether (1 ml) did not result in any conversion to P439 (*1*).

P439 di-methanesulfonate (*16*). P439 (*1*, 0.8 mg) in dry pyridine (4 ml) was treated with excess methanesulfonyl chloride (0.1 ml) for 30 min at 0°C. The reaction mixture was transferred to benzene; pigment recovery was 100 %. The adsorptivity (Table 3) and unchanged absorption spectrum in visible light indicated complete transformation to *16*.

In a parallel experiment β-apo-2′-carotenol (C_{37}) (3.6 mg) was treated likewise for 3 h and gave 95 % pigment recovery and a strongly adsorbed product ($R_F = 0$ in 5 % acetone in petroleum ether on kieselguhr paper).

Hydride reduction of P439 di-methanesulfonate (*16*). *16* (0.8 mg) partly dissolved in tetrahydrofurane (100 ml) was reduced with a small amount of lithium aluminium hydride for 2 h; pigment recovery after transfer to ether was 50 %. The products were chromatographed on deactivated alumina.

In a parallel experiment the methanesulfonate of β-apo-2′-carotenol (C_{37}) (3.6 mg) above was reduced in the same manner; pigment recovery was 43 %. Chromatographic separation on deactivated alumina revealed the formation of non-polar products with unchanged absorption spectra compatible with the expected C_{37}-hydrocarbon.

P439 hydrocarbons (*17* and *18*). The least polar reduction products (*17*, 40 % of total) and *18* (50 % of total) required 1 % and 2 % acetone, respectively, for elution from deactivated alumina. *17* and *18* exhibited the same absorption spectra in visible light as P439 (*1*) and were completely epiphasic (Table 3). Separate iodine catalyzed isomerization revealed that *17* and *18* did not belong to the same stereoisomeric set. Neither *17*, nor *18* formed trimethylsilyl ethers under standard conditions for silylation. Treatment with 8 % KOH in methanol/ether (1:1) caused no isomerization of isolated double bonds into conjugation with the polyene chain.

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