Identification of Epoxy- and Hydroxyphytoene from Norflurazon-Treated *Scenedesmus*

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In heterotrophically grown *Scenedesmus* kept in permanent darkness, treatment with the bleaching herbicide norflurazon resulted in the accumulation of five different compounds with a phytoene-like spectrum which could be separated and detected by HPLC. They were all purified by chromatographic procedures and identified by spectroscopic methods. The major compound detected was phytoene. Another was identified as 1,2-epoxy-1,2-dihydrophytoene by IR, NMR, and mass spectroscopies. Six different derivatives could be separated and identified as positional isomers of monohydroxyphytoene by their molecular ion and their fragmentation pattern in mass spectroscopy. The presence of a single hydroxy group was further substantiated by a positive acetylation and silylation reaction. The positions of the hydroxy groups in the different monohydroxy derivatives have been assigned by cochromatography with phytoene derivatives carrying hydroxy groups in defined positions.

INTRODUCTION

11,12-Desaturation of phytoene to ζ-carotene via phytofluene as an intermediate is the initial reaction in the sequence of carotene interconversion to α- and β-carotene. This reaction is dependent on NAD(P)$^+$ and is stimulated by oxygen (Sandman and Kowalczyk, 1989). A mechanism involving hydride transfer and a subsequent proton loss has been assumed (Goodwin, 1983; Sandmann and Kowalczyk, 1989). Recently, the gene of phytoene desaturase has been isolated and sequenced from *Rhodobacter capsulatus*. It encodes for a protein with 491 amino acids (Armstrong et al., 1989) which shows a final apparent molecular weight of 54 000 on SDS–polyacrylamide gels regardless of its origin from photoautotrophic prokaryotes, eukariotic algae, or higher plants (Schmidt et al., 1989). Several bleaching herbicides interfere with carotenogenesis by direct interaction with phytoene desaturase [see Sandman and Böger (1989) for a review]. As demonstrated for the phenylpyridazinone norflurazon, this inhibition is noncompetitive (Sandmann et al., 1989).

Phytoene desaturase is under negative control of subsequent carotenes, and the whole biosynthetic pathway is effectively regulated in a way that no carotene before α- and β-carotene is accumulated in detectable amounts (Bramley and Davies, 1976; Sandmann and Kowalczyk, 1989). However, treatment of plants or algae with herbicides like norflurazon results in a massive accumulation of phytoene. In addition, nonidentified polar phytoene derivatives have been reported (Ben-Aziz and Koren, 1974). In the present investigation we analyzed heterotrophically grown cultures of the green alga *Scenedesmus* for formation of phytoene and derivatives of this colorless carotene after treatment with norflurazon. By chromatographic procedures several compounds with a spectrum typical of phytoene could be detected. Different spectroscopic methods have been employed to identify several oxygenated phytoene derivatives. The nature of the oxygenated phytoene derivatives might possibly give an indication whether they are involved as intermediates in the desaturation reaction of phytoene.

MATERIALS AND METHODS

Scenedesmus acutus (strain 276-3a, Algal Culture Collection, University of Göttingen) was heterotrophically grown in a medium containing 0.5% glucose and 0.25% (w/v) yeast extract as previously described (Sandmann et al., 1989). Norflurazon was added from a 10^{-2} M methanol stock solution to a final concentration of 1 μM. After growth for 1 week in complete darkness, cells were harvested by centrifugation (4000g, 10 min) and the carotenoids extracted with methanol at 60 °C for 30 min in the dark. The filtered extract was partitioned against diethyl ether and the diethyl ether phase evaporated. For saponification of chlorophylls and acyl lipids the residue was redissolved in methanol containing 6% (w/v) KOH and incubated under N_2 overnight. The diethyl ether phase of a second partitioning step containing the carotenoids was employed for HPLC and TLC separation as well as for subsequent purification.

After evaporation of the solvent, the carotenoids were redissolved in hexane and fractioned on a column of neutral Al_2O_2 (Whoelm, Eschwege, Germany) Brockman Grade III (Britton and Goodwin, 1971). Elution was with 0–2% diethyl ether in hexane, 2–10%, 10–20%, and 20–40% diethyl ether in hexane, and 100% diethyl ether. Compound P5 was further purified from the 0–2% fraction by TLC on Al_2O_3 60 F_{254} plates (Merck, Darmstadt, Germany) in petroleum ether (bp 60–80 °C)/toluene (98:2 v/v) and the fluorescence-quenching band at an R_f of 0.75 scraped off and eluted with diethyl ether. This TLC step was repeated. The 2–10% Al_2O_3 column fraction was chromatographed by TLC on silica gel 60 F_{254} plates (Merck) with hexane/diethyl ether (95:5 v/v). The fluorescence-quenching band with an R_f of 0.4 containing P4 was collected. Then this TLC step was repeated. Finally P4 was absorbed on a Al_2O_3 Grade III column and eluted with hexane/diethyl ether (90:10 v/v).

Derivatives P1 to P3 were found in the 10–20% diethyl ether/hexane fraction of the initial Al_2O_3 column and were further purified by TLC on silica gel plates 60 F_{254} in hexane/diethyl ether (80:20 v/v). The three distinct fluorescence-quenching bands with R_f values of about 0.25, 0.3, and 0.35 were scraped off, eluted, and subsequently rechromatographed in the same TLC system followed by additional column chromatography on Al_2O_3 Grade III including elution with hexane/diethyl ether (75:25 v/v).

This separation was controlled at each stage by HPLC. The reversed-phase system employed a Spherisorb 5-μm ODS-1 column (250 × 4.6 mm) and a Philips Pye Unical PU 4021 multichannel diode array detector which records the optical spectra

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of the elution peaks. A linear gradient from 5% methanol/2-propanol (2:1 v/v) in acetonitrile was started, reaching 15% within 20 min, was continued to 20% after t = 25 min, and was kept at 20% to t = 30 min. The flow was 1 mL/min. When a complete separation of peaks P1 to P3 was anticipated, acetonitrile/methanol/2-propanol (95:3.5:1.5 v/v) was used for isocratic reversed-phase HPLC (see inset of Figure 1). Cochromatography of hydroxyphytoenes was carried out on a Spherisorb Apex 5-μm silica column with a linear gradient from hexane to 5% ethyl acetate in hexane over 30 min with a flow of 2 mL/min.

Infrared (IR) spectra were recorded in CS_2 in the range 1000–4000 cm^{-1} with a Mattson Polaris FT IR spectrometer. Mass spectra were obtained by electron impact mass spectroscopy in a MAT 112 S mass spectrometer with an ion source temperature of 240–280 °C and an ionization potential of 70 eV. NMR spectra were determined in $CDCl_3$ on a 400-MHz instrument.

For acetylation 100 μg of compound was dissolved in 0.5 mL of pyridine and added to 0.25 mL of acetic anhydride. The mixture was incubated under N_2 in the dark overnight. Formation of triethylsilyl ether was carried out with 25 μg of compound dissolved in 0.1 mL of a 1:1 mixture of bis(trimethylsilyl)trifluoroacetamide and pyridine and incubation for 20 min at 40 °C under N_2 in the dark. After evaporation, the residual reaction products were detected by mass spectroscopy.

Phytoene and phytoene epoxide present in tomatoes (Britton and Goodwin, 1969) were isolated as reference compounds for TLC and HPLC from ripe fruit bought from the local market. Isolation and purification procedures were the same as for compounds P5 and P4, respectively.

RESULTS

The green alga *Scenedesmus* is a convenient model system for studies on the mode of action of herbicides that interfere with pigment biosynthesis (Sandmann et al. 1979, 1984). In contrast to higher plants the algae can be grown heterotrophically (i.e., in complete darkness) with a fully developed chloroplast and unrestricted pigment biosynthesis. We have employed this advantage to study the formation of oxygenated phytoene derivatives in the presence of norflurazon, avoiding peroxidation processes that occur in the light.

Five different compounds that all exhibit the optical absorption maxima of phytoene (276, 286, 297 nm) could be found by HPLC separation and simultaneous recording of their spectra with a diode array detector (Figure 1). They are designated P1 to P5 according to increasing retention time. P5 accumulated with the highest concentration followed by P4, P1, P3, and P2. In the routinely used HPLC runs, compounds P1 to P3 were not completely separated from each other. However, decreasing the polarity of the eluent resulted in an isocratic separation of P1 to P3, which was appropriate for integration of these peaks (inset of Figure 1).

Table I shows some characteristics of the five detected phytoene derivatives. Separation could be achieved not only by HPLC but also by TLC on silica gel plates with petrol (bp 60–80 °C)/diethyl ether (75:25 v/v). The molecular mass of all compounds is 560 with the exception of P5, which exhibited 544. A comparison with phytoene isolated as reference compound gave the same retention time in HPLC, the same R_f value by TLC, and the same molecular mass as for compound P5. All three parameters determined for phytoene epoxide from tomatoes coincided very well with those of compound P4.

The results of Table II allow for the final identification of derivative P4 as 1,2-epoxy-1,2-dihydrophytoene. Besides the molecular ion at 560, compound P4 as well as 1,2-epoxy-1,2-dihydrophytoene shows major fragment ions at 339 and 355 in mass spectroscopy. Furthermore, IR spectroscopy exhibits an absorption band at 1261 cm^{-1} which is characteristic for an epoxy group (Grob and Pflugshaupt, 1965). The position of this epoxy group can be assigned by NMR spectroscopy in comparison with the spectroscopy data of synthetic 1,2-epoxy-1,2-dihydrophytoene (Arm et al., 1986).

Although the molecular ion of compound P4 is identical with the values obtained for P1 to P3, the fragmentation pattern of the mass spectra are different (Figure 2A). In addition, for fragment ions 339 and 355, a strong peak at m/e 542 as well as characteristic weak ones at 405 and 473

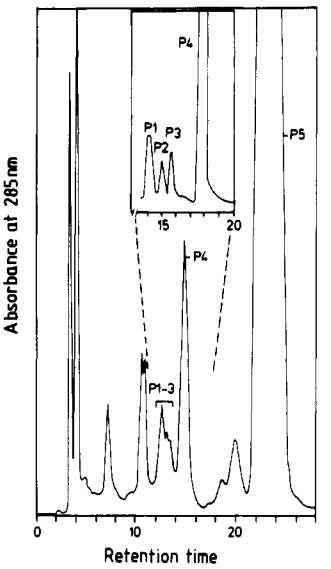

Figure 1. Separation of phytoene derivatives P1 to P5 by HPLC and detection by their phytoene-like spectra. The HPLC system employed consisted of a Spherisorb 5 ODS-1 column (250 × 4.6 mm) and an eluent gradient from 5% methanol/2-propanol (2:1) in acetonitrile to 20%. The separation in the inset was isocratic with acetonitrile/methanol/2-propanol (95.5:3:1.5).

Table I. Characteristics of Compounds with an Optical Spectrum of Phytoene Accumulated by Heterotrophic *Scenedesmus* in the Presence of Norflurazon

compd	R_f[a]	retention time,[b] min	molecular ion m/e
P1	0.28	14.2	560
P2	0.35	15.0	560
P3	0.39	15.9	560
P4	0.55	17.8	560
P5	0.70	32.4	544
phytoene[c]	0.70	32.4	544
phytoene epoxide	0.55	17.8	560

[a] TLC system: silica gel 60 F_{254} plates with 25% (v/v) diethyl ether in petroleum ether (bp 60–80 °C). [b] HPLC system: Spherisorb 5 ODS-1 column (250 × 4.6 mm) with acetonitrile/methanol/2-propanol (95.5:3:1.5) as isocratic eluent, flow 1 mL/min. [c] Isolated from tomato fruit.

Table II. Assignment of Compound P4 as 1,2-Epoxy-1,2-dihydrophytoene by IR, NMR, and Mass Spectroscopies

	compd P4	ref values for 1,2-epoxy-1,2-dihydrophytoene
major peaks in mass spectroscopy	m/e 560, 355, 339	m/e 560, 355, 339
IR absorption bands in the epoxy region	1261 cm^{-1}	1245 cm^{-1}
endgroup ^1H NMR signals	C(2) H 2.72	C(2) H 2.711
	C(3) H_2 1.61–1.65	C(3) H_2 1.61–1.68
	C(4) H_2 1.9–2.4	C(4) H_2 1.96–2.21

[a] The IR and NMR reference values were from Arm et al. (1986) and the values for mass spectroscopy from Britton and Goodwin (1969).

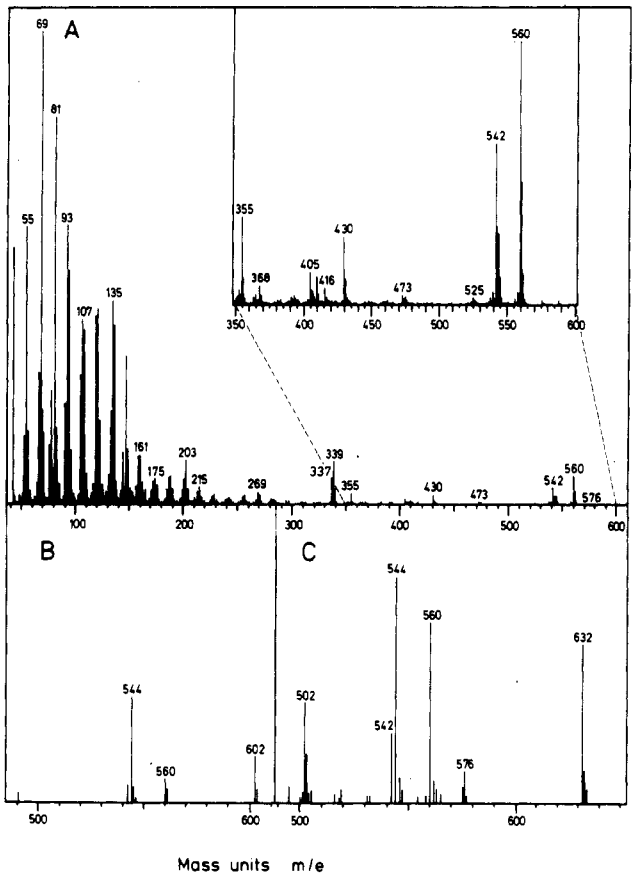

Figure 2. Identification of P2 (A) and its acetylation (B) and silylation products (C) by mass spectroscopy.

Table III. Separation of Positional Hydroxyphytoene Isomers by Normal Phase HPLC

compd	retention time, min	cochromatographs with[a]
P1-1	23.7	1-hydroxy-2,3-didehydrophytoene
P2-1	19.8	5-hydroxy-6,7-didehydrophytoene
P2-2	19.0	6-hydroxy-5,18-didehydrophytoene
P2-3	18.8	no ref compd available
P3-1	17.7	9-hydroxy-10,11-didehydrophytoene
P3-2	17.2	10-hydroxy-9,19-didehydrophytoene

[a] Hydroxyphytoenes assigned by ^{13}C NMR spectroscopy from Musker et al. (unpublished results).

was evident. Compounds P1 and P3 gave qualitatively the same picture (mass spectra not shown). In contrast to compounds P4 and P5, acetylation or silylation reactions occurred. Acetylated P2 showed a mass of 602 (Figure 2B), whereas silylation resulted in an increase of the molecular ion to m/e 632 (Figure 2C). These characteristics indicate that all compounds P1 to P3 are hydroxylated products of phytoene. Nevertheless, they show different intensities of their fragment ions in the mass spectra. Especially P1 showed 2.5–3.5-fold stronger fragment ions m/e 542 and 339 resulting from loss of water compared to the molecular ion (data not shown).

The hydroxyphytoenes P2 and P3 could be separated further into three and two positional isomers, respectively, by normal phase HPLC on a silica column (Table III). P1 gave only one peak in this system. Furthermore, it was possible to determine the position of the hydroxy groups by cochromatography with reference compounds characterized by ^{13}C NMR (D. Musker, G. Mitchell, and G. Britton, unpublished results). P1 cochromatographed with 1-hydroxy-2,3-didehydrophytoene, P2-1 with 5-hydroxy-6,7-didehydrophytoene, P2-2 with 6-hydroxy-5,18-didehydrophytoene, P3-1 with 9-hydroxy-10,11-didehydrophytoene, and P3-2 with 10-hydroxy-9,19-didehydrophytoene. Hydroxyphytoene P2-3 could not be identified with the standards available.

DISCUSSION

The carotenogenic pathway is regulated in a way that no precursors of α- and β-carotene are detectable (Bramley and Davies, 1976; Sandmann and Kowalczyk, 1989). Herbicidal inhibitors have been used to accumulate intermediates of this pathway. It has been known for a long time that inhibitors of phytoene desaturase accumulate the substrate phytoene (Bartels and McCullough, 1972). Recently, the detection of hydroxy derivatives of phytoene in diflufenican-treated radish has been reported (Britton et al., 1987). The identification of various phytoene derivatives might lead to a better understanding of the desaturation mechanism and the interaction of herbicides with this enzyme.

In heterotrophically grown *Scenedesmus* cultures treated with norflurazon the least polar compound with a phytoene-like spectrum (P5) accumulated most (Figure 1). As expected, it could be identified as phytoene by cochromatography and by its mass spectrum (Table I). It showed not only the molecular ion of m/e 544 but also the fragment of m/e 339 ($M^+ - 205$; data not shown), which is typical for phytoene (Moss and Weedon, 1976). Finally, we have established the identity of phytoene by its NMR spectrum (data not shown). The phytoene accumulated in the presence of norflurazon showed an isomer composition of mainly 15-cis together with up to 5% all-trans.

The phytoene derivative P4 differed from P1 to P3 by its chromatographic behavior on Al_2O_3. It cochromatographs by HPLC and TLC with phytoene epoxide isolated from tomatoes (Table I) and shows an IR absorbance band (Table II) typical for an epoxide (Grob and Pflugshaupt, 1965). As for P1 to P3, the molecular ion in mass spectroscopy is the one of phytoene plus an oxygen ($C_{40}H_{64}O$). The fragment m/e 355 ($M^+ - 205$) results from loss of three isoprenic units from the molecule, whereas m/e 339 [$M^+ - (205 \pm 16)$] consists of three isoprenic units carrying one oxygen (Moss and Weedon, 1976). As for epoxyphytoene isolated by Britton and Goodwin (1969), no peak at m/e 542 ($M^+ - H_2O$) was observed (compare Figure 2A for P2). The signals of the endgroup proton in the NMR spectrum correspond well with the ones obtained for synthetic 1,2-epoxy-1,2-dihydrophytoene (Arm et al., 1986). The most prominent one is the signal of H–C(2), which appeared as a triplet at δ 2.72. The influence of the epoxy group on H–C(2) can be understood by comparison with the high-field signal δ 5.07–5.25 of this proton in the unsubstituted phytoene. All the spectroscopic results obtained univocally identify P4 as 1,2-epoxy-1,2-dihydrophytoene. This phytoene derivative is not present in light-grown higher plants treated with another bleaching herbicide, diflufenican (Britton et al., 1987).

Although the derivatives P1 to P3 show qualitatively the same mass spectra (Figure 2; Table III), they can be separated from each other by HPLC and TLC (Figure 1; Table I). The striking difference to the mass spectrum of P4 is the fragment ion m/e 542 ($M^+ - 18$), which results from loss of water (Moss and Weedon, 1976). Furthermore, the fragment m/e 337 ($M^+ - 18 - 205$) is formed after water loss by scission of three isoprenic units. This fragmentation pattern demonstrates the existence of a monohydroxy substituent in the phytoene molecule. If the isoprenic units split off with the hydroxy group, fragment ions of m/e 339, 407, and 475 can be expected.

As only the m/e 339 fragment was evident, which results from the cleavage of the C-11,12 bond, we can exclude that any of the hydroxy groups are positioned at C-12–15. The positive acetylation and silylation reactions with molecular ions of the reaction products at m/e 602 and 632 are further indications for the identity of the hydroxy derivatives. As an acetylation product of m/e 602 was detectable in the case of P2 (Figure 2B), the hydroxy group has to be a primary or secondary one (McCormick and Liaaen-Jensen, 1966). This further excludes localization of the hydroxy group at C-1, C-5, and C-9 in a phytoene molecule with retained position of the double bonds. These isomers could only be formed by addition to H_2O to the corresponding double bonds as in the case of 1-hydroxy-1,2-dihydrophytoene (Malhotra et al., 1970). However, then the molecular ion would be found at m/e of 562, which is not the case (Figure 2A). The hydroxyphytoenes P1 to P3 only differ in the position of the hydroxy group. Especially for P1, the location of the hydroxy group favors the loss of water as evidenced by the comparably high intensity of the fragments m/e 337 and 542. This behavior is typical for an allylic hydroxy group as exemplified for β,β-carotene-4,4′-diol versus β,β-carotene-2,2′-diol (Kayser, 1976).

Six positional hydroxy isomers could be separated from the fractions P1 to P3 by normal phase HPLC (Table III). For five of these compounds the position of the hydroxy group could be determined by cochromatography with reference compounds (Musker et al., unpublished results) to be located at positions 1, 5, 6, 9, and 10 in the phytoene molecule. In herbicide-treated barley Musker et al. (unpublished results) found the same hydroxy isomers identified in Table III together with 2-hydroxy-1,16-didehydrophytoene, which was absent in *Scenedesmus*. The assignment of the hydroxyphytoenes from *Scenedesmus* treated with norflurazon corresponds well with the mass spectroscopy data (Figure 2). Furthermore, this result demonstrates that the hydroxyphytoenes are no direct intermediates of the phytoene desaturation process. Otherwise, 11- or 12-hydroxyphytoenes had to be expected.

As phytodynamic oxidation reactions can be excluded, we suggest that the phytoene accumulated is enzymatically converted to the 1,2-epoxide (e.g., by squalene oxidase). The hydroxyphytoenes identified might result from opening of the epoxy ring followed by rearrangement of the conjugated system. Then, however, formation of the epoxy and hydroxy derivatives is independent of the chemical nature of a phytoene desaturase inhibitor and should increase with the amount of phytoene produced. Further investigations on the effect of other bleaching herbicides and on oxidative conversion of phytoene and phytoene epoxide to their hydroxy derivatives are currently under way to support the proposed origin of the oxygenated phytoene derivatives.

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Registry No. P1-1, 130932-91-1; P2-1, 130905-27-0; P2-2, 130905-28-1; P3-1, 130905-29-2; P3-2, 130932-92-2; phytoene, 540-04-5; 1,2-epoxy-1,2-dihydrophytoene, 26107-96-0; norflurazon, 27314-13-2.