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Feeding deterrence properties of apo-fucoxanthinoids from marine diatoms. I. Chemical structures of apo-fucoxanthinoids produced by *Phaeodactylum tricornutum*

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Abstract Methanol extracts of freshly harvested cells of the marine diatom *Phaeodactylum tricornutum* were found to produce a feeding deterrent effect in the copepod *Tigriopus californicus*. Bioassay guided fractionation of the methanol extracts led to the isolation of four compounds possessing feeding deterrent activity. The compounds were identified as apo-10′-fucoxanthinal (1), apo-12′-fucoxanthinal (2), apo-12-fucoxanthinal (3), and apo-13′-fucoxanthinone (4) by detailed spectroscopic analysis and comparison with authentic compounds produced semi-synthetically from fucoxanthin. Compounds 1 to 4 exhibited feeding deterrent responses in *T. californicus* at concentrations of less than 20 ppm.

Introduction

Marine phytoplankton produce metabolites that cause a reduction in feeding (Targett and Ward 1991) by marine copepods (Huntley et al. 1986; Sykes and Huntley 1987; Uye and Takamatsu 1990), ciliates (Hansen 1989), rotifers (Egloff 1986), and bivalves (Ward and Targett 1989). Phytoplankton-produced chemical "feeding deterrents" are potentially of considerable ecological and commercial importance. In the natural environment, feeding deterrents may regulate herbivorous zooplankton grazing, thereby helping to determine which species of phytoplankton will bloom and how long the ensuing blooms will persist. This sort of regulatory influence on bloom dynamics would ultimately have a direct impact on the transfer of energy along some paths in the marine food web. In mariculture situations, feeding deterrent compounds produced by phytoplankton commonly utilized as "good food species" may lead to decreased growth rates or increased mortality in commercially reared mariculture species (e.g. oysters). Therefore, identification of phytoplankton species that produce feeding deterrent compounds and the characterization of the chemical structures and biological activities of the active compounds will improve our understanding of natural marine food web dynamics and facilitate the development of more efficient mariculture practices.

Although the general phenomenon of chemical feeding deterrent production by marine phytoplankton is well documented in the biological literature (Targett and Ward 1991), the chemical structures of the feeding deterrent compounds are almost completely unknown. To date, even though several studies have shown that some well known toxins produced by phytoplankton, such as saxitoxin, also affect feeding behaviour in zooplankton predators (Ives 1987; Hansen 1989), there are no literature examples of a rational bioassay guided approach successfully leading to the identification of the chemical structure of a phytoplankton-produced feeding deterrent. The recent development in our laboratory of a simple and rapid bioassay for detection of feeding deterrents produced by marine phytoplankton (Shaw et al. 1994) has facilitated the screening of phytoplankton species for feeding deterrent production and the subsequent bioassay guided isolation of the pure active metabolites. Using this bioassay, which is based on the rate of fecal pellet production by the copepod *Tigriopus californicus*, it was found that extracts of freshly harvested cells of the diatom *Phaeodactylum tricornutum* exhibited potent feeding deterrent activity. The present paper reports the isolation and chemical characterization of the apo-carotenoids 1 to 4 that are responsible for this feeding deterrent activity.

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Materials and methods

Phytoplankton cultures

Unialgal cultures of *Phaeodactylum tricornutum* Bohlin (NEPCC #640) were obtained from the Northeast Pacific Culture Collection, Department of Oceanography, University of British Columbia, Vancouver, British Columbia, Canada. Natural sea water (salinity ≈28‰) was collected from West Vancouver, British Columbia, at a site 100 m from shore and 15 m depth. Sea water used for culturing was filter sterilized using a 0.45 μm Millipore filter. Cultures were grown in 120 litre plastic bags using full ES enriched natural sea water (Harrison et al. 1980) at an irradiance of ≈100 $\mu mol\, m^{-2}\, s^{-1}$ with an 18 h light : 6 h dark cycle and at a temperature of 18 °C. The cultures were aerated vigorously with air filtered through a GF/F filter. Culture growth was measured by in vivo fluorescence. Cultures were harvested in late log or early senescence phase.

Copepod cultures

A culture of the harpacticoid copepod *Tigriopus californicus* was maintained to provide a continuous supply of organisms for the bioassay. *Tigriopus californicus* (Tig-1) was originally isolated from splash pools on the West Coast of Vancouver Island by Dr. A.G. Lewis, University of British Columbia, in 1966 (Sullivan and Bisalputra 1980). Copepods used for the bioassay were maintained in filtered natural sea water in 1 litre Pyrex flasks at 18 °C at an irradiance of ≈100 $\mu mol\, m^{-2}\, s^{-1}$ and an 18 h light : 6 h dark cycle. The copepods were fed either a diet of ground fish food (Wardley's basic fish food for tropical fish) or a diet of the diatom *Thalassiosira pseudonana*.

The variability in the bioassay due to differences in the sex or life stage of the copepods was reduced by using only the adult (C6) male copepods. Differences in grazing rates were determined by measuring fecal pellet production. The water in which the copepods were incubated was quantitatively transferred to a 10 ml cylindrical settling chamber, the contents were settled for 30 min, and fecal pellets were counted at low power (100×) using an inverted compound microscope.

Chromatography

Cell culture (360 litre) was harvested by gentle filtration through a 5 μm Nitex screen. The cells were rinsed from the screen with methanol and collected in a grinding tube. Cellular extracts of the phytoplankton cells were obtained by grinding the harvested cells in methanol and rinsing the cell debris repeatedly in methanol until all the pigments were removed. This methanolic extract was filtered through a GF/F glass fiber filter to remove remaining cell debris. The methanolic extract then underwent rotary evaporation to produce a dark green gummy oil (yield 20.4 g).

The dark green oil was divided into three portions, and each portion processed as follows. The oil was dissolved in 10 ml of 0.05 *M* H_2SO_4 and extracted with 50 ml of diethyl ether. The organic phase was dried, dissolved in 25 ml of 80% EtOH and extracted with 4 × 25 ml n-hexane. The ethanolic phase was dried, dissolved in 20 ml of 0.45 *M* NaOH and extracted with 50 ml diethyl ether. The organic phase was dried to give a brown gum. The three portions of brown gum were combined (1.88 g).

A portion (630 mg) of the brown gum was dissolved in 20% ethanol and applied to two C-18 sep-paks (in series). Sep-paks were rinsed with 20% ethanol, and the active material was eluted with ethyl acetate (yield 360 mg). This material was chromatographed on a silica gel short column (Taber 1982) with ethyl acetate/dichloromethane (1 : 1) to give four fractions, A (green), B (yellow), C (orange–red), D (blue–green). Fraction B (230 mg) was active. Further purification was carried out on a 1 g silica gel short column in chloroform giving fractions B1 (yellow, active) and B2 (orange–red). Final purification of B1 was carried out by high-performance liquid chromatography (HPLC) [Econosil C-18, 5 μm column, length = 250 mm, i.d. = 4.6 mm; gradient system #1: methanol/water/ethyl acetate (40 : 50 : 10 to 35 : 45 : 20), 0.7 ml min^{-1}, 90 min run]. Yield of pure apo-10′-fucoxanthinal (1) and apo-12′-fucoxanthinal (2) was ≈1 mg each.

A second portion (1.25 g) of the brown gum was chromatographed on a 10 g silica gel [thin-layer chromatography (TLC) grade] vacuum column packed in a buchner funnel using dichloromethane/ethyl acetate/acetic acid (25 : 25 : 1) to yield three fractions, X (green), Y (red–orange, active), Z (blue–green). Fraction Y was dried, pumped under vacuum to remove the acetic acid, applied to a 10 g silica gel short column, and eluted with dichloromethane/ethyl acetate (1 : 1) to give four fractions, Y1 (green), Y2 (yellow–orange, active), Y3 (red–orange), Y4 (blue–green). Y2 was further purified on an alumina (Grade IV) column using 5% acetone in hexane to give three fractions, Y21 (yellow, active), Y22 (orange), Y23 (orange–red). Final purification of Y21 was carried out by HPLC [Econosil C-18, 5 μm column, length = 250 mm, i.d. = 4.6 mm; gradient system #2 for apo-12-fucoxanthinal (3): methanol/water/ethyl acetate (65 : 25 : 10 to 65 : 0 : 35), 0.7 ml min^{-1}, 60 min run; gradient system #3 for apo-13′-fucoxanthinone (4): methanol/water/ethyl acetate (40 : 50 : 10 to 0 : 0 : 100), 0.7 ml min^{-1}, 80 min run]. Yield of pure apo-12-fucoxanthinal (3) and apo-13′-fucoxanthinone (4) was <1 mg each.

Synthesis

Fucoxanthin was isolated by the method described by Haugan and Liaaen-Jensen (1989) from *Fucus distichus* collected from Copper Cove, West Vancouver, British Columbia, on 6 March 1993. Zinc permanganate was prepared based on the methodology by Lux (1965) and Wolfe and Ingold (1983). Fucoxanthin (1.2 g) was oxidized with zinc permanganate based on the method described by Bonnett et al. (1969). The reaction mixture was chromatographed on an alumina (Grade IV) column using 5% acetone in hexane to give four fractions, E (pale yellow), F (orange), G (dark orange), H (red–orange). Fraction G (12.9 mg) was purified on HPLC system #1 to give 1.6 mg apo-10′-fucoxanthinal (1) and 1.8 mg apo-12′-fucoxanthinal (2). Fraction F (18.3 mg) was purified on HPLC systems #2 and #3 to give 0.2 mg each of apo-12-fucoxanthinal (3) and apo-13′-fucoxanthinone (4).

Feeding deterrent bioassays for bioassay-guided chemical fractionation

A weighed amount of dried material to be bioassayed was dissolved in 5 ml of autoclaved sea water and sonicated (1 min in bath sonicator at 60 Hz) to facilitate mixing. If the material to be tested was not soluble in sea water, it was dispersed using four drops of dimethylsulfoxide (DMSO). When DMSO was used to dissolve the extract, it was also added to the control. Five ml of an exponentially growing culture of the diatom *Thalassiosira pseudonana* was added to the dissolved cell extract. If the cell cultures were clumped, cell clumps were broken up using mild sonification (1 min in bath sonicator at 60 Hz) and filtration (forced through a 200 μm screen at a rate of ≈50 ml min^{-1} using a syringe; repeated four times). This mixture was placed in one well (volume ≈15 ml) of a tissue culture plate and two male C6 copepods (*Tigriopus californicus*) were added. Copepods were preconditioned in autoclaved sea water without food for 24 h prior to the bioassay. Copepods were pretreated with antibiotics (penicillin-G, dihydrostreptomycin, and chloramphenical) if they were heavily contaminated with bacteria. The assay

was incubated for ≈20 h at 18 °C with an irradiance of ≈100 μmol $m^{-2} s^{-1}$ and an 18 h light : 6 h dark cycle. Degree of feeding inhibition was measured by counting fecal pellets. Six replicates were conducted. The control used a cell extract from *Th. pseudonana* (which has no feeding deterrent effects on *T. californicus*; Shaw et al. 1994). Data were analyzed by analysis of variance (ANOVA) to determine statistical significance.

Spectroscopy

Proton nuclear magnetic resonance experiments and homonuclear correlation spectroscopy (1-D NMR and 2-D COSY) were carried out on either a WH-400 or a AMX-500 Bruker instrument using ambient probe temperature. All proton NMR spectra were referenced to trimethyl silane (TMS). Mass spectra were obtained by desorption chemical ionization using either a Nermag R10–10C (for low resolution spectra) or a Kratos MS80RFA (for high resolution spectra) mass spectrometer with NH_3 as the reagent gas. Infrared (IR) spectroscopy was done on a Perkin-Elmer 1600 Series Fourier Transform IR spectrometer. Ultraviolet-visible (UV) spectroscopy was done on an LKB Ultraspec II spectrophotometer (Berges and Virtanen 1993).

Results

Chromatography

The crude methanol extracts of freshly harvested cells of the diatom *Phaeodactylum tricornutum* were found to have feeding deterrent activity against adult C6 males of *Trigriopus californicus* at a concentration of 0.2 mg ml^{-1}. A bioassay guided fractionation scheme was used to identify the active components present in the cell extracts.

The crude cell extracts were concentrated to dryness, dissolved in dilute aqueous acid, and the resulting acidic solution was extracted with ether. The ether phase, which showed the only feeding deterrent activity, was concentrated to dryness and partitioned between hexane and 80% ethanol. Feeding deterrent activity tracked with the aqueous ethanol phase, which was concentrated to dryness in vacuo, dissolved in dilute aqueous base, and the resulting solution was extracted with ether. The ether phase, containing large amounts of orange–yellow xanthophyll pigments, was found to contain the biological activity. Repeated fractionation of the bioactive ether soluble materials via open column chromatography on normal and reversed phase silica-gel and alumina, followed by reversed phase HPLC, yielded four active compounds in small (<1 mg) amounts.

Spectroscopy

Spectroscopic analysis of the feeding deterrent compounds tentatively identified them as apo-10′-fucoxanthinal (1), apo-12′-fucoxanthinal (2), apo-12-fucoxanthinal (3) and apo-13′-fucoxanthinone (4) (Fig. 1). The apo-fucoxanthinoids 1 to 3 have been previously reported as products of semi-synthesis (Bonnett et al. 1969). However, they have not been reported as natural products, nor have they been tested for biological activity. In order to confirm the structural assignments of 1 to 4, authentic samples of the four apo-fucoxanthinoids were prepared semisynthetically from fucoxanthin (5) (Fig. 1), isolated from fresh *Fucus distichus* (Haugan and Liaaen-Jensen 1989), by the method described by Bonnett et al. (1969). The authentic semi-synthetic compounds 1 to 4 were found to be identical to the compounds isolated from *Phaeodactylum tricornutum* cell extracts by chromatographic, high resolution mass spectrometry (HRMS), UV, and NMR comparison. Compound 4, although not previously reported, was another compound in the apo-fucoxanthinoid series, and was readily identified by HRMS and NMR. Proton (1-D) NMR experiments

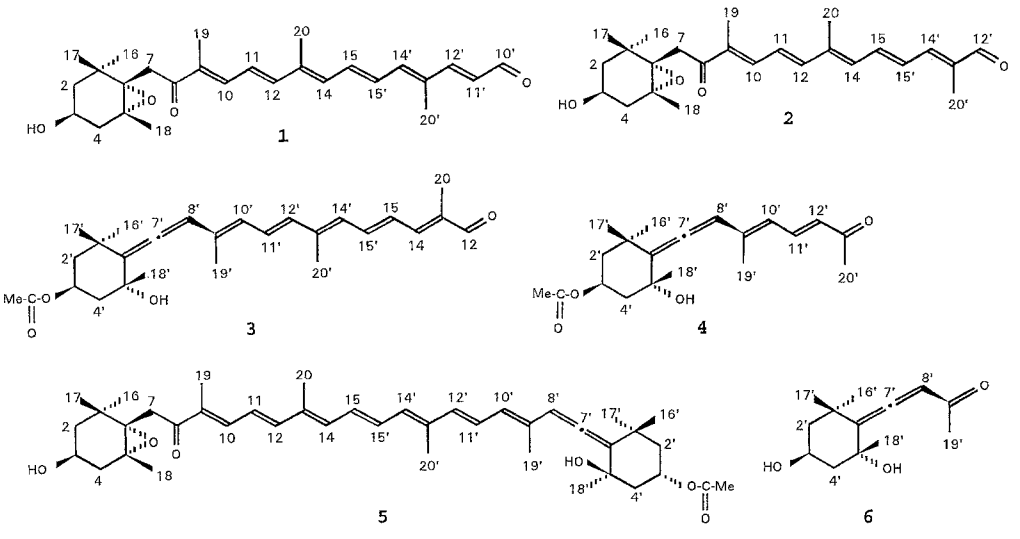

Fig. 1 Structures of apo-fucoxanthinoids isolated from *Phaeodactylum tricornutum*: **1** apo-10′-fucoxanthinal, **2** apo-12′-fucoxanthinal, **3** apo-12-fucoxanthinal, and **4** apo-13′-fucoxanthinone. **5** Fucoxanthin. **6** Grasshopper ketone isolated from *Romalea microptera*

were performed on all four compounds, but due to the low yields for the semi-synthetic preparation of the apo-fucoxanthinoids, and thus the limited amounts of material available for NMR experiments, COSY (2-D) NMR experiments were only performed on Compounds 1 and 4. The spectral data for these compounds is as follows.

Table 1 ^{1}H NMR (500 MHz, C_6D_6) data for apo-10′-fucoxanthinal (1). Peak multiplicity abbreviations: *s* singlet; *d* doublet; *dd* doublet of doublets; *m* multiplet

Position	Chemical shift (ppm)	Number of protons	Peak multiplicity	Coupling constant (Hz)
H-10′	9.53	1	d	7.6
H-14′	7.01	1	d	12
H-12′	6.61	1	d	16
H-11	6.60	1	dd	7.4, 13
H-15′	6.51	1	dd	12, 13
H-12	6.44	1	d	7.4
H-15	6.43	1	dd	12, 13
H-10	6.24	1	d	13
H-14	6.11	1	d	12
H-11′	6.10	1	dd	7.6, 16
H-3	3.80	1	m	–
H-7a	3.53	1	d	19
H-7b	2.64	1	d	19
H-4a	2.22	1	dd	4.1, 14
H-20′	1.92	3	s	–
H-4b	1.71	1	dd	8.8, 14
H-19	1.71	3	s	–
H-20	1.49	3	s	–
H-2b	1.36	1	dd	8.8, 19
H-18	1.25	3	s	–
H-16	1.09	3	s	–
H-17	1.01	3	s	–
H-2a	0.91	1	dd	5.3, 19

Table 2 ^{1}H NMR (400 MHz, C_6D_6) data for apo-12′-fucoxanthinal (2). Peak multiplicity abbreviations: *s* singlet; *d* doublet; *dd* doublet of doublets; *m* multiplet

Position	Chemical shift (ppm)	Number of protons	Peak multiplicity
H-12′	9.44	1	s
H-15	7.01	1	dd
H-11	6.56	1	dd
H-14′	6.55	1	d
H-12	6.44	1	d
H-15′	6.43	1	dd
H-14	6.42	1	d
H-10	6.16	1	d
H-3	3.80	1	m
H-7a	3.53	1	d
H-7b	2.62	1	d
H-4a	2.23	1	dd
H-20′	1.91	3	s
H-19	1.78	3	s
H-20	1.68	3	s
H-18	1.24	3	s
H-16	1.10	3	s
H-17	1.01	3	s

Spectral data

Apo-10′-fucoxanthinal (1). Compound 1, isolated as 0.01% of the weight of the crude methanolic extract of the phytoplankton, showed the following spectral data: IR ν_{max}^{film} cm^{-1}: 3448, 1662, 1585, 1127, 971; UV λ_{max}^{MeOH} nm: 439 (log ε 4.60), 418 (log ε 4.62); UV λ_{max}^{ether} nm: 409; high resolution desorption chemical ionization mass spectrometry (HRDCIMS): $[M + 1]^+$ m/z observed = 425.2683 (deviation = −2.2) for $C_{27}H_{37}O_4$. ^{1}H NMR (500 MHz, C_6D_6) data are given in Table 1.

Apo-12′-fucoxanthinal (2). Compound 2, isolated as 0.01% of the weight of the crude methanolic extract of the phytoplankton, showed the following spectral data:

Table 3 ^{1}H NMR (500 MHz, CD_2Cl_2) data for apo-12-fucoxanthinal (3). Peak multiplicity abbreviations: *s* singlet; *d* doublet; *dd* doublet of doublets; *m* multiplet. Note: when exposed to light, there is an interconversion between the trans and 13-cis forms of apo-12-fucoxanthinal, complicating the ^{1}H NMR spectra

Position	Chemical shift (ppm)	Number of protons	Peak multiplicity
H-12	9.44	1	s
H-15′	7.06	1	dd
H-14	6.97	1	d
H-11′	≈ 6.74	1	dd
H-15	≈ 6.66	1	dd
H-12′	6.38	1	d
H-14′	6.32	1	d
H-10′	≈ 6.15	1	d
H-8′	6.08	1	s
H-20′	2.04	3	s
OAc	2.01	3	s
H-20	1.86	3	s
H-19′	1.84	3	s
H-18′	1.36	3	s
H-16′	1.34	3	s
H-17′	1.08	3	s

Table 4 ^{1}H NMR (500 MHz, CD_2Cl_2) data for apo-13′-fucoxanthinone (4). Peak multiplicity abbreviations: *s* singlet; *d* doublet; *dd* doublet of doublets; *m* multiplet

Position	Chemical shift, ppm	Number of protons	Peak multiplicity	Coupling constant (Hz)
H-11′	7.46	1	dd	12, 16
H-12′	6.17	1	d	12
H-10′	6.12	1	d	16
H-8′	6.10	1	s	–
H-3′	5.31	1	m	–
H-20′	2.25	3	s	–
H-4′a	2.21	1	dd	–
OAc	2.01	3	s	–
H-2′b	2.00	1	dd	–
H-19′	1.91	3	s	–
H-4′b	1.48	1	dd	–
H-2′a	1.42	1	dd	–
H-18′	1.37	3	s	–
H-16′	1.34	3	s	–
H-17′	1.08	3	s	–

IR ν_{max}^{film} cm^{-1}: 3453, 1660, 1604, 1015, 969; UV λ_{max}^{MeOH} nm: 410 ($\log\varepsilon$ 4.51), 393 ($\log\varepsilon$ 4.53); UV λ_{max}^{ether} nm: 383; HRDCIMS: $[M + 1]^+$ m/z observed = 399.2535 (deviation = −0.02) for $C_{25}H_{35}O_4$. ^1H NMR (400 MHz, C_6D_6) data are given in Table 2.

Apo-12-fucoxanthinal (3). Compound 3, isolated as 0.007% of the weight of the crude methanolic extract of the phytoplankton, showed the following spectral data: IR ν_{max}^{film} cm^{-1}: 3437, 2933, 1927, 1721, 1662, 1609, 1017, 966; UV λ_{max}^{MeOH} nm: 407 ($\log\varepsilon$ 4.20); HRDCIMS: $[M + 1]^+$ m/z observed = 425.2670 (deviation = −2.1) for $C_{27}H_{37}O_4$. ^1H NMR (500 MHz, CD_2Cl_2) data are given in Table 3.

Apo-13′-fucoxanthinone (4). Compound 4, isolated as 0.007% of the weight of the crude methanolic extract of the phytoplankton, showed the following spectral data: IR ν_{max}^{film} cm^{-1}: 3442, 2964, 2926, 1931, 1732, 1661, 1597; UV λ_{max}^{MeOH} nm: 329 ($\log\varepsilon$ 4.19); HRDCIMS: $[M + 1]^+$ m/z observed = 333.2056 (deviation = −1.0) for $C_{27}H_{37}O_4$. ^1H NMR (500 MHz, CD_2Cl_2) data are given in Table 4.

Bioassay results for semi-synthetic compounds

The four semi-synthetic apo-fucoxanthinoids were quantitatively bioassayed using the copepod *Tigriopus californicus*. The IC_{50} (concentration of compound which inhibits the fecal pellet production rate by 50%) values ranged from 20.2 to 2.2 ppm (Shaw et al. 1995).

Discussion

The marine diatom *Phaeodactylum tricornutum* was shown to elicit feeding deterrent activity in the copepod *Tigriopus californicus* (Shaw et al. 1994). Ianora et al. (1995) have shown that a diet of *Ph. tricornutum* also results in low egg production and viability in the copepod *Temora stylifera*. However, the compounds responsible for these activities were unknown. Using a feeding deterrent bioassay based on fecal pellet production by *T. californicus* (Shaw et al. 1994), the feeding deterrent activity was traced through a chemical isolation scheme, and four compounds were purified which had feeding deterrent activity.

Spectroscopic methods (UV-vis, IR, and NMR spectroscopy and mass spectrometry) were used to tentatively identify these compounds as apo-10′-fucoxanthinal, apo-12′-fucoxanthinal, apo-12-fucoxanthinal, and apo-13′-fucoxanthinone. These compounds appeared to be derived from the carotenoid pigment fucoxanthin by oxidative cleavage. Fucoxanthin (5) (Fig. 1) is a major pigment in both diatoms and macroscopic brown algae. In order to confirm the identification of the four feeding deterrent compounds, these compounds were prepared semi-synthetically (Bonnett et al. 1969) from fucoxanthin purified from the brown macroalga *Fucus distichus*. Spectroscopic analyses showed the semi-synthetic compounds to be identical to those isolated from *Phaeodactylum tricornutum*. Bioassays showed all four pure semi-synthetic compounds to be feeding deterrents at low levels (2.22 to 20.2 ppm) relative to the total intracellular concentration of apo-fucoxanthinoids within the diatom cell, which ranged up to 3.34 $g l^{-1}$ (Shaw et al. 1995). Thus, the feeding deterrent activity of *Ph. tricornutum* is due, at least in part, to apo-fucoxanthinoid compounds.

Although these apo-fucoxanthinoids have been made synthetically in the laboratory (Bonnett et al. 1969), they have neither been isolated as natural products, nor have they been tested for biological activity. These four compounds are the first feeding deterrent compounds to be isolated from marine phytoplankton using a bioassay specifically designed to detect chemicals responsible for feeding deterrent activity.

Apo-13′-fucoxanthinone is very closely related to the grasshopper ketone (6) (Fig. 1) isolated from the grasshopper *Romalea microptera* (Meinwald et al. 1968), differing only in having a somewhat longer olefinic chain and one acetylated hydroxyl group. Grasshopper ketone appeared to be produced by the degradation of fucoxanthin-like carotenoids in the grasshopper's diet. This compound had a repellent effect on ants and possibly other predators; thus there is a precedent for the feeding deterrent nature of apo-carotenoids. In terms of energetics, it would be quite logical to produce a deterrent compound from an abundant pre-existing material. Thus, it seems likely that the diatom *Phaeodactylum tricornutum* does produce apo-carotenoids from fucoxanthin, and these compounds function as feeding deterrents to grazers.

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