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Thermal Oxidation of 9′-*cis*-Neoxanthin in a Model System Containing Peroxyacetic Acid Leads to the Potent Odorant β-Damascenone

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The potent odorant β-damascenone was formed directly from 9′-*cis*-neoxanthin in a model system by peroxyacetic acid oxidation and two-phase thermal degradation without the involvement of enzymatic activity. β-Damascenone formation was heavily dependent on pH (optimum at 5.0) and temperature, occurring over the two sequential phases. The first was incubation with peroxyacetic acid at 60 °C for 90 min, and the second was at above 90 °C for 20 min. Only traces of β-damascenone were formed on application of only one of the two phases. Formate and citrate solutions produced a much better environment for β-damascenone formation than acetate and phosphate. About 7 μg/L β-damascenone was formed from 5.8 mg/L 9′-*cis*-neoxanthin under optimal experimental condition. The detailed pathway by which β-damascenone is formed remains to be elucidated.

KEYWORDS: β-Damascenone; 9′-*cis*-neoxanthin; peroxyacetic acid; epoxide; thermolysis

INTRODUCTION

β-Damascenone [1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one] is a potent odorant having an odor threshold of 2 ppt in water (*1*). It was first reported in the oil of the Bulgarian rose (*Rosa damascena*) (*2*) and later in many other fresh and processed foods and natural products such as coffee, black tea, tobacco, tomatoes, beer, apple products, grapes, wines, tropical fruits such as star fruit and passion fruit, and more (*3*−*16*).

Isoe et al. (*17*) suggested a possible mechanism for the biogeneration of β-damascenone from allenic carotenoids such as neoxanthin, via oxidative cleavage, resulting in the grasshopper ketone **(1)** as an intermediate which must then be reduced by an enzyme to the allenic triol **(2)** (**Figure 1**). The latter then may undergo the Meyer−Schuster rearrangement (*18*) with subsequent dehydrations under acidic conditions to yield β-damascenone. Evidence supporting such a biochemical pathway came from the discovery of both glycoconjugate and nonglycosidic polar precursors of β-damascenone in white wines and fruit juices (*14*, *19*, *20*) and later in a variety of food products (*6*, *10*, *13*, *21*, *22*). The work of Skouroumounis et al. (*19*), who studied the possible hydrolytic pathway of these precursors, suggested that compound **2** is a key intermediate in the formation of β-damascenone. These authors found 3-hydroxy-β-damascone **(3)** to be the major transformation product of **2**, and only about 5% yield of β-damascenone at elevated temperatures.

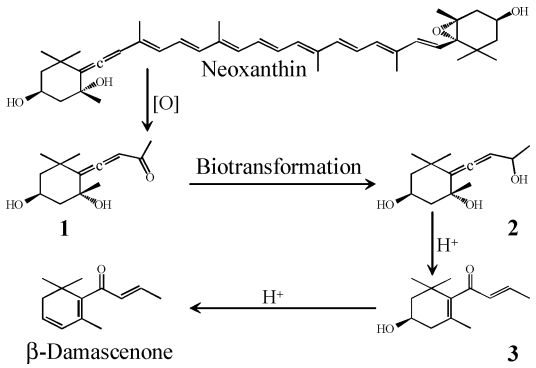

Figure 1. Proposed pathway for the biogeneration of β-damascenone from neoxanthin, (**1**) grasshopper ketone, (**2**) allenic triol, and (**3**) 3-hydroxy-β-damascone, according to Isoe et al. (*17*).

A significant elevation in the content of β-damascenone occurred when fresh food products are subjected to thermal processing (*1*, *6*, *10*, *13*); for example in roasting green Arabica coffee content may increase by 100-fold (*4*). The increased β-damascenone content was usually proposed to involve hydrolysis and transformations of glycoconjugates and polar precursors at the elevated temperatures during processing and under the acidic conditions occurring in many food systems. Interestingly, roasted coffee and beer wort (made of dark malt),

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although possess pH range of 5 or above, contain β-damascenone at levels between 255 and 450 ppb (*4*, *10*) (among the highest level in food products) under conditions where acid hydrolysis is not favored. On the other hand, these two products (green coffee and some barley malts) are subjected to roasting temperatures (e.g., 200 °C in the case of coffee) (*23*). In coffee, degradation of carotenoids to β-damascenone due to heating, oxidation, and isomerization was proposed (*24*) but no evidence for its formation from direct chemical degradation of neoxanthin was provided.

In this study we used a model system to explore conditions in which neoxanthin may be degraded to β-damascenone by thermal oxidation without involvement of enzymatic activity. Our working hypothesis (see Discussion for a hypothetical pathway) included the use of moderate temperatures (defined as phase 1) prior to applying high temperatures (e.g., phase 2).

MATERIALS AND METHODS

Chemicals. Tween-80 and EDTA were purchased from Sigma (St. Louis, MO). Ammonia solution (25%), formic acid, citric acid, sodium acetate, sodium dihydrogen phosphate, and disodium hydrogen phosphate were from Merck (Darmstadt, Germany). Methanol (HPLC grade), diethyl ether, and dichloromethane were from J. T. Baker (Deventer, Holland). Potassium hydroxide, sodium hydroxide, sodium chloride, sodium sulfate, acetic acid, ethanol, and petroleum ether 40−60 °C were purchased from Frutarom (Haifa, Israel). Methyl *tert*-butyl ether (MTBE) was from Aldrich (Milwaukee, WI), and peroxyacetic acid (39% in acetic acid) was from Fluka (Steinheim, Germany). β-Damascenone was kindly donated by Dr. Gary Takeoka of the USDA (WRRC, Albany, CA), neoxanthin marker was a gift of Prof. Naoharu Watanabe of Shizuoka University (Shizuoka, Japan), and 3-OH-β-damascone was a gift of Dr. George Skouroumounis of the Australian Wine Research Institute (Adelaide, Australia). Deionized water (Barnstead, Dubuque, IA) was used throughout the study.

Neoxanthin Isolation. Isolation followed the procedure of Baumeler et al. (*25*) with modifications. Briefly: 1 kg of fresh spinach leafs (purchased at a local supermarket) was washed with water, blanched in a microwave oven for 3 min at maximum power (1000 W), and manually squeezed to remove water as much as possible. Subsequently, the spinach was extracted with 4 L of methanol/diethyl ether/concentrated ammonia solution (8:2:0.25 v/v) for 24 h at ambient temperature. Following extraction, the solvent was paper filtered and evaporated under reduced pressure by a rotary evaporator (40 °C). The residue was dissolved in 280 mL of 15% (w/v) KOH in methanol and 140 mL of diethyl ether and left to stand overnight at dark. Following saponification, the carotenoid fraction was extracted in a separatory funnel (100 mL aliquots of the saponified extract, 100 mL of 10% NaCl solution, and 100 mL of diethyl ether), and the alkalinity was removed by repeatedly washing with water. Finally, the combined etheral phase was dried over anhydrous sodium sulfate and concentrated under reduced pressure. Column chromatography [2 × 40 cm, 60 g alumina (6% moisture) filled in as a slurry with petroleum ether (40−60 °C)] was used to separate the carotenoids. The column was first conditioned with 100 mL of 75/25 (v/v) petroleum ether 40−60 °C/diethyl ether prior to loading. Then step-gradient elution was applied with 100 mL of each: 75/25 petroleum ether 40−60 °C/diethyl ether, 50/50 petroleum ether 40−60 °C/diethyl ether, 25/75 petroleum ether 40−60 °C/diethyl ether, 100% diethyl ether, 5% ethanol in diethyl ether, and 20% ethanol in diethyl ether (all v/v). The neoxanthin fraction was eluted as the last band at 20% ethanol in diethyl ether. After removal of the solvent under reduced pressure (40 °C), the residue was redissolved in 3 mL of methanol and stored in a glass vial under nitrogen at −20 °C. The yield was calculated spectrophotometrically [EtOH, 437 nm, ϵ = 139 250 (*25*)] to be approximately 25 mg of neoxanthin. This fraction was used for identification and purity analyses as well as for the experiments throughout this study.

Spectroscopic Data. UV: a Unicam UV1 spectrophotometer (Unicam LTD, Cambridge, U.K.) was used. NMR: a Bruker DRX-500 NMR spectrometer, operating at 500 MHz for ^{1}H and at 125 MHz for ^{13}C, was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referring to the solvent peaks δ_H 3.34 and δ_X 49.0 for CD_3OD; coupling constants, *J*, are in hertz. DEPT, ^{13}C, ^{1}H−^{1}H COSY, ^{1}H−^{13}C HMQC, and HMBC NMR experiments were carried out using the conventional pulse sequences as described in the literature.

9′-cis-Neoxanthin: UV (EtOH) λ_{max} nm 324, 392 (s), 414, 437, 466; % (III/II) ratio = 86%; NMR parameters presented in Tables S1 and S2 (see Supporting Information).

High Performance Liquid Chromatography (HPLC). Analyses were performed on a Merck-Hitachi L-7100 pump equipped with L-7455 UV−vis photodiode array detector (set to scan from 350 to 550 nm at 2 nm spectral bandwidth) and D-7100 interface module. The data were recorded and analyzed with Merck-Hitachi D-7000 chromatography data-station software. An RP-18 column (ODS 3 μ, 100 × 4.6 mm) (Hypersil, Runcorn, U.K.) was used for carotenoids separation using a tertiary solvent gradient elution adopted from Rouseff and Raley (*26*) with the following modifications: Initial solvent composition consisted of 80% methanol, 19% water, and 1% MTBE (v/v) and then changed to 80% methanol, 12% water, and 8% MTBE in 15 min. Next, the proportion was modified to 85% methanol and 15% MTBE in 5 min. All solvent changes were made in a linear fashion. Initial conditions were reestablished in 5 min, and the column was reequilibrated for 10 min before the next injection. Flow rate was 0.85 mL/min, and injection volume was 20 μL.

Neoxanthin Oxidation in Aqueous Solutions. A 60 μL volume of 5% (v/v) Tween-80 in ethanol and 58 μg of neoxanthin were mixed with 1 mL of dichloromethane in a 4 mL glass vial (Fisherbrand, 15 × 45 mm). The solvent was evaporated under a nitrogen stream, and the residue (prereaction mixture of neoxanthin, PRMN) was dissolved in 1 mL of sodium formate solution [0.4 M, pH 5.0, 0.005% EDTA (w/v)] while gently mixing to the point where the solution was clear (final concentration of neoxanthin approximately 10 μM). Next, the oxidant was introduced [75 μL of 0.78% (v/v) peroxyacetic acid in water] to a final concentration of 9 mM in the reaction mixture, mixed, and sealed with a Teflon screw-cap (National Scientific Co.). The reaction mixture was then treated as described below. All experiments involving carotenoids were handled at dark or at dim light conditions, and treatments were executed in duplicates. Treated samples were stored at −80 °C before analyzed.

Oxidation of Neoxanthin at Different pH Levels. PRMN was dissolved in 1 mL of formic acid [0.4 M, 0.005% (w/v) EDTA] which was preadjusted to pH 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, and 7.0 by NaOH granules, and the oxidant was introduced as described above. Vials were then incubated in an oven set to 60 °C for 90 min (e.g., moderated conditions, phase 1), and then the temperature rose to 110 °C for additional 20 min (phase 2).

Varying Incubation Periods during Phase 1. Aqueous solutions containing neoxanthin (0.4 M formate, pH 5.0, 0.005% EDTA, 9 mM peroxyacetic acid) were incubated at 60 °C for 1, 3, 20, 90, and 300 min. Then the temperature was immediately elevated to 110 °C (rate = 50 °C/min) for an additional 20 min.

Effect of Temperatures during Phase 1. Aqueous solutions containing neoxanthin (0.4 M formate, pH 5.0, 0.005% EDTA, 9 mM peroxyacetic acid) were incubated for 90 min at 4, 20, 40, 60, 75, and 85 °C. Then each treatment was further heated to 110 °C for an additional 20 min.

Effect of Temperatures during Phase 2. Solutions containing neoxanthin (0.4 M formate, pH 5.0, 0.005% EDTA, 9 mM peroxyacetic acid) were incubated for 90 min at 60 °C (i.e. phase 1). Then temperature was set to 60, 80, 90, 100, 110, 120, or 130 °C for an additional 20 min in the oven (i.e., phase 2).

Excluding Molecular Oxygen. Formate solution (0.4 M, pH 5.0, 0.005% EDTA) was bubbled with argon for 10 min in a septum-sealed bottle. The oxidant solution [(0.78% (v/v) peroxyacetic acid in water)] was also treated with argon in the same manner. A 1 mL volume of the argon-treated formate solution and 75 μL of the treated oxidant solution were added to the vial containing PRMN while simultaneously purging argon and sealed immediately. Control samples were prepared in the presence of air with peroxyacetic acid or with air as the only oxidant.

Neoxanthin Oxidation in Different Buffer Solutions. A 1 mL volume of formate, citrate, acetate, or phosphate buffer (0.4 M, pH 5.0, 0.005% EDTA) and 75 μL of 0.78% peroxyacetic acid solution were added to vials containing PRMN. The vials were incubated at 60 °C for 90 min, and then the temperature was increased to 110 °C for an additional 20 min.

Solid-Phase Microextraction (SPME). Into each vial containing the incubated samples were added 0.25 g of NaCl and a small stirring bar, and vials were sealed with a Teflon septum cap. The vial content was continuously agitated in a water bath at 50 ± 0.5 °C. Samples were allowed to equilibrate for 5 min prior to SPME and maintained at 50 °C throughout the 95-min assay. SPME of the sample's volatiles was conducted by inserting a 2-cm fiber coated with 50/30 μm DVB/Carboxen/PDMS (Supelco, Bellefonte, PA). The full length of the coated fiber was exposed to the headspace for 90 min. The fiber was then removed from the headspace and immediately inserted into the gas-chromatograph (GC) injector.

Quantitative Determination Using SPME. Stock solution of β-damascenone (10 000 mg/L) was prepared in methanol. Five calibration solutions, ranging from 0.3 to 8 μg/L β-damascenone, were made by serial dilutions of the stock in formate solution [0.4 M, pH 5.0, 0.005% (v/v) EDTA]. The desired concentration was prepared in a vial containing dried Tween-80 as previously mentioned (final volume 1 mL). Quantification was performed by gas-chromatography mass spectrometry (GC-MS) according to peak areas of ion *m*/*z* 121 on the basis of the calibration curve of the same mass fragment.

GC-MS. β-Damascenone was identified and monitored using a Saturn 2000 mass spectrometer (Varian, Walnut Creek, CA) connected to a Varian 3800 gas chromatograph, equipped with a SPB-5 capillary column (30 m × 0.25 mm i.d., df = 0.25 μm) (Supelco, Bellefonte, PA). Following the aforementioned SPME procedure, samples were inserted into the injector (held at 250 °C) for 5 min, in splitless mode. The oven program was set to 40 °C for 3 min and then 4°/min to 185 °C and held for 15 min. Helium was used as the carrier gas with a flow rate of 1.5 mL/min. A 0.75-mm i.d. SPME injector liner was used. The transfer line was held at 250 °C, and the source, at 180 °C. Mass spectra in the electron impact (EI) mode were generated at 70 eV. A series of *n*-alkanes was used to generate a linear retention index (RI) with the above conditions. Chromatograms and spectra were recorded with Saturn GC/MS Workstation software, version 5.41 (Varian).

Two specific control experiments were performed regarding the use of SPME technology in these experiments. The first was designed to explore the possibility that β-damascenone was formed from a putative precursor such as compound **3** (3-OH-β-damascone) during SPME desorption at the GC injection port. For this purpose, a standard of compound **3** (over 95% purity) was exposed to a SPME fiber and then inserted into the GC injector for GC-MS analysis under the same conditions described above. A large peak of compound **3** (RI 1620) and traces of β-damascenone (RI 1390) (less than 0.02% of that of compound **3**) were observed in the GC-MS chromatogram (not shown). A GC-MS chromatogram obtained from the incubated PRMN showed the presence of β-damascenone peak (RI 1390) without any traces of compound **3** (expected RI 1620) under the experimental conditions (not shown). Therefore, as previously suggested (*27*) conversion of 3-OH-β-damascone to β-damascenone is very unlikely and certainly did not occur under the experimental conditions described here. The second control experiment was aimed at exploring if varying the pH of the PRMN solution may affect the volatility of β-damascenone (matrix effect) and thus the resulting quantitative determination by SPME. A 20 ppb concentration of the β-damascenone standard was inserted into each of six vials containing formate solutions (as in PRMN) adjusted to pHs of either 2, 3, 4, 5, 6, or 7. SPME was conducted, and β-damascenone content was determined by GC-MS under conditions described above. There were no differences (three replicates for each pH) in β-damascenone content across pHs, indicating no pH matrix effect under the experimental conditions (data not shown).

Data Processing. Statistical analysis (two-way ANOVA) of the resulting data was performed using JMP statistical software (SAS Institute Inc., Cary, NC).

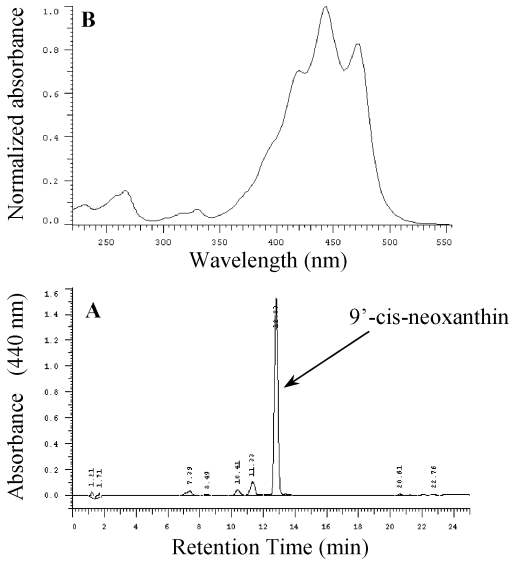

Figure 2. HPLC chromatogram at 440 nm (A) and UV–vis spectrum (B) for the fraction eluted from the alumina column with 20% ethanol in diethyl ether. (See Methods.)

RESULTS AND DISCUSSION

Neoxanthin Isolation. Since this xanthophyll is one of the principal carotenoids of green leafs, spinach was chosen as the plant material source for its extraction according to the relatively simple isolation procedure of Baumeler et al. (*25*). Approximately 25 mg of the supposed 9′-*cis*-neoxanthin was prepared from 1 kg (fresh weight) of spinach (80% pure, determined by HPLC with comparison to a marker and spectral data from literature (*28*)). A sample chromatogram and UV−visible spectrum of the isolated fraction is shown in **Figure 2**. The structure of the isolated compound was confirmed in an unequivocal manner by NMR technique (see Supporting Information). The ^{13}C NMR spectrum of 9′-*cis*-neoxanthin showed 38 different signals, 17 of which belong to the polyene moiety (C_{14} and $C_{14'}$ with the same chemical shift), 12 belong to the cyclohexane moieties, and 9 others to methyl groups ($C_{17'}$ and C_{18} with the same chemical shift) (Table S1; see Supporting Information**)**. The ^{1}H NMR spectrum can be divided in two regions. One includes signals from protons that belong to the polyene moiety. This part of the spectrum is well resolved and could be easily analyzed. The second region includes signals from the two cyclohexane rings and methyl groups. This part is characterized by substantial overlap between signals from different protons.

The full assignment of the signals in ^{13}C and ^{1}H NMR spectra was achieved taking into account the data obtained from the DEPT and HMQC spectra on the C atoms bonded to H atoms and on the number of the hydrogen atoms bonded to the same carbon. The splitting patterns of signals in the ^{1}H NMR spectrum, ^{1}H−^{1}H COSY, and HMBC spectra (Table S2; see Supporting Information) allowed understanding of the connectivity of carbon atoms. The attribution of the hydrogen atoms bonded to C_2 ($C_{2'}$) or to C_4 ($C_{4'}$) to pseudoequatorial or pseudoaxial positions is based on the values of *J*: $^1H_{4eq}-^1H_{3ax}$, $^1H_{4'eq}-^1H_{3'ax}$, $^1H_{2eq}-^1H_{3ax}$, and $^1H_{2'eq}-^1H_{3'ax}$ hyperfine interactions are relatively small, $J \sim 5$ Hz, and $^1H_{4ax}-^1H_{3ax}$, $^1H_{4'ax}-^1H_{3'ax}$, $^1H_{2ax}-^1H_{3ax}$, and $^1H_{2'ax}-^1H_{3'ax}$ hyperfine interactions are twice as large, $J \sim 10$ Hz. The spectral data are in full agreement with the suggestion that the isolated compound has the structure 9′-*cis*-neoxanthin and fit well with the previously published data for this compound (*25*, *29*, *30*).

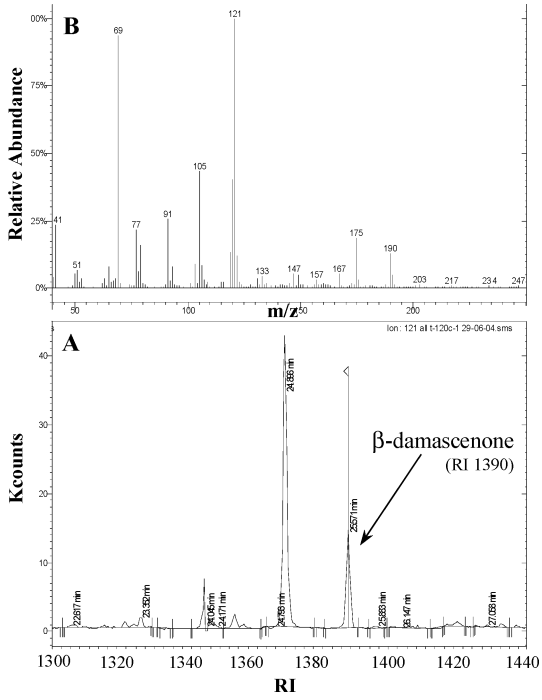

Figure 3. MS of a putative β-damascenone peak at RT = 25.6 (RI = 1390) (A) derived from GC-MS chromatogram (*m*/*z* 121) of 9′-*cis*-neoxanthin oxidation treatment (B).

The relatively scarce amounts of neoxanthin in plants made it hard to obtain large quantities of this pigment. Hence, a microscale experimental approach was used throughout the study.

Formation of β-Damascenone from Neoxanthin. This study provides for the first time the evidence that thermal oxidation of neoxanthin without the presence of an enzyme leads to formation of the potent odorant β-damascenone and indicates the experimental conditions for its occurrence. This is in line with the notion that for some food products the formation of β-damascenone during food processing cannot be explained alone by acid hydrolysis of glycoconjugates of this odorant. Our initial experiments investigating neoxanthin oxidation at pH 3.5, using citrate buffer and peroxyacetic acid as the oxidant, resulted in a clear detection of a β-damascenone odor signal in GC-olfactometer aromagram as evidenced by 3 trained panelists (data are not shown). This odor signal matched that of a β-damascenone marker in the GC-FID chromatogram, but only traces were detected in the FID and the GC-MS chromatograms of the putative β-damascenone extracted from the incubated sample. Subsequent optimization (in particular elevation of the pH; see below) clearly indicated direct chemical formation of β-damascenone (RI 1390) from neoxanthin by GC-MS analysis (**Figure 3**). The presented chromatogram is only a fraction of the full analysis range, monitored at *m*/*z* 121 (the base mass fragment of β-damascenone). Spectral data and blanks (oxidation system without the carotenoids) of the largest peak in this section (left to β-damascenone) suggest that it is part of the oxidation system and not a degradation product of 9′-*cis*-neoxanthin. Maximal levels were approximately 7 μg/L β-damascenone under the experimental conditions, which corresponds to a molar yield of about 0.04%. This yield indicates that the pathway is not chemically preferred. Nevertheless, even very low yields (ppb range) of β-damascenone produce strong olfactory sensation in the human nose due to the very low odor threshold of this odorant (*1*) and may be highly significant if occurs during food processing. Since the main focus was to extract β-damascenone from the aqueous medium, the use of SPME-headspace was selected as a rapid and efficient extraction tool.

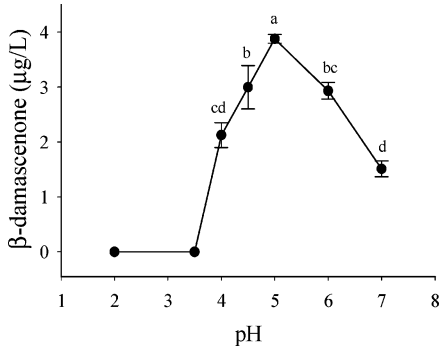

Figure 4. Effect of pH on β-damascenone formation. Samples adjusted to different pH values were incubated for 90 min at 60 °C (phase 1) and then for an additional 20 min at 110 °C (phase 2). Results are of GC-MS analyses monitored at *m*/*z* 121. Values are the means ± SEM of duplicates of an experiment performed at least twice. Values not sharing the same superscript letter are different ($p < 0.05$) in β-damascenone yield across different pH treatments.

Effect of pH. There was a strong pH dependency of β-damascenone formation, with a maximal level of about 4 μg/L at pH 5.0 (**Figure 4**). This optimal pH is somewhat surprising, since lower pH values (i.e. 3.0−3.5) would be expected to favor acid-catalyzed conversion of compound **2** (**Figure 1**) via the Meyer−Schuster rearrangement, with subsequent dehydrations (*19*). This is also true for acid hydrolysis of glycoconjugate precursors of β-damascenone in some natural products. Perhaps a vicinal diol (see discussion later on), though its presence was not evidenced, was involved as an intermediate during neoxanthin degradation. If occurs, it would be expected to be sensitive to low pH values, since under such conditions it may undergo the Pinacol rearrangement (*31*), thus eliminating the possibility to generate **2**. Nevertheless, slightly acidic conditions are needed for the transformations of **2** into β-damascenone (*17*), which seems to be in line with the tendency for a decrease in the yield of β-damascenone at pH's higher than 5.

Time and Temperature during Phase 1. The objectives here were to study the effects of time and temperature on β-damascenone formation during the incubation period of the carotenoid with the oxidant (i.e, phase 1). Time effect was studied by varying incubation periods up to 300 min, under constant temperature of 60 °C, and with subsequent heating to 110 °C for an additional 20 min (i.e., phase 2). An optimal incubation period of 90 min at 60 °C yielding approximately 4 μg/L β-damascenone was found for phase 1 (**Figure 5A**). An interesting observation is the rather moderate decrease in β-damascenone formation during the period from 90 to 300 min. The effect of the temperature on the yield of β-damascenone during 90 min incubation is illustrated in **Figure 5B**. As mentioned above, the temperature was then elevated to 110 °C for additional 20 min. The optimal temperature for phase 1 under these experimental conditions was found to be 60 °C yielding approximately 4 μg/L of β-damascenone; even 4 °C during this phase facilitated the formation of β-damascenone. Heating to 70 and 85 °C adversely affected the yield, which might suggest that the effect of temperature may affect the degradation of the already formed intermediates.

Temperature Effect during Phase 2. All samples were treated equally during phase 1 (incubation for 90 min at 60 °C) but subsequently were treated at elevated temperatures (see

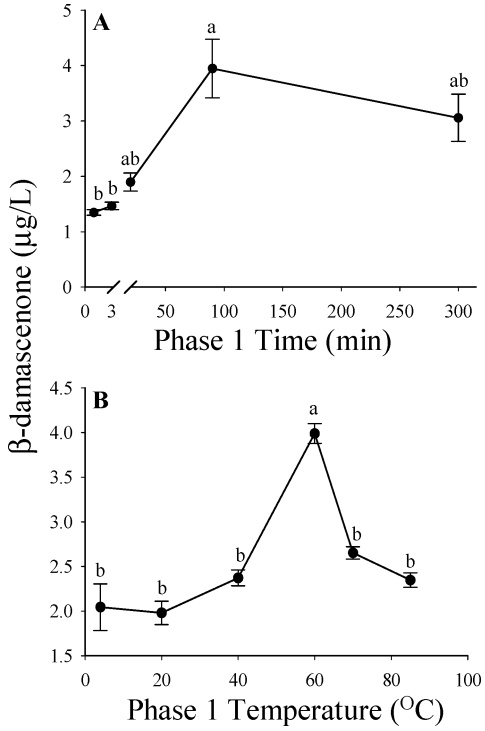

Figure 5. Effect of modifications in time (A) and temperature (B) during phase 1 incubation of 9′-*cis*-neoxanthin on β-damascenone formation. After phase 1 was completed, the temperature was immediately elevated to 110 °C (rate = 50 °C/min) for an additional 20 min (phase 2). Values are the means ± SEM of duplicates of an experiment performed at least twice. Values not sharing the same superscript letter are different ($p < 0.05$) in β-damascenone yields across different time (A) or among different temperature (B) treatments.

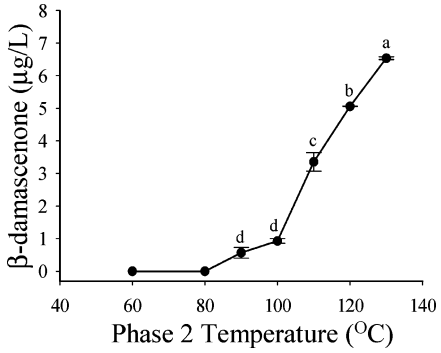

Figure 6. Temperature effect on β-damascenone formation during phase 2. Phase 1 was conducted for 90 min at 60 °C. Then samples were incubated for an additional 20 min (phase 2) at the indicated temperatures. Values are the means ± SEM of duplicates of an experiment performed at least twice. Values not sharing the same superscript letter are different ($p < 0.05$) in β-damascenone yields across different temperature treatments.

Materials and Methods) for 20 min. As shown in **Figure 6**, a prominent effect of ascending temperatures on β-damascenone formation occurred, reaching about 7 μg/L β-damascenone at 130 °C. A threshold of 90 °C for phase 2 was evident, with only trace mass fragments detected by the MS at this temperature. These results strengthen the suggestion of a biphasic mechanism, since the lower temperatures such as those applied in phase 1 (i.e. 60 and 80 °C, **Figure 5B**) alone would not yield β-damascenone unless a subsequent step employing higher energy is applied.

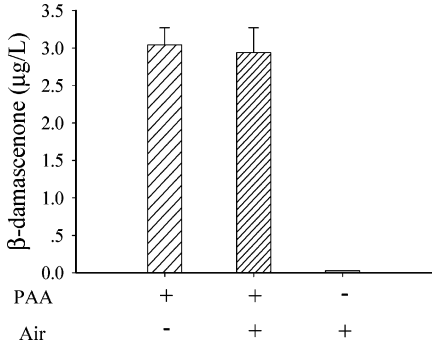

Figure 7. Molecular oxygen (air) not needed for peroxyacetic acid-induced neoxanthin degradation to β-damascenone. Peroxyacetic acid (PAA) was used as the oxidant. The involvement of molecular oxygen in β-damascenone formation was tested by the presence of air or after its removal by argon. Phase 1 was conducted by samples incubation for 90 min at 60 °C and then for an additional 20 min at 110 °C (phase 2). Values are the means ± SEM of duplicates.

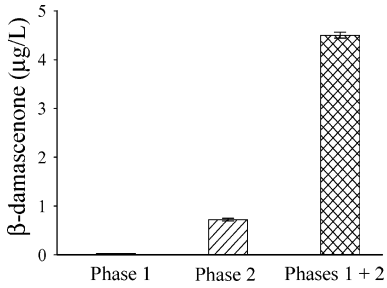

Figure 8. β-Damascenone formation during either phase 1 only (sample incubation for 90 min at 60 °C) or phase 2 only (sample incubation for 20 min at 110 °C) or when the two phases were sequentially conducted. Values are the means ± SEM of duplicates of an experiment performed at least twice.

It was technically impractical to use temperatures higher than 130 °C with this system, since the glass vials might burst under the steam pressure. However, it should be borne in mind that some food products, especially those containing low moisture, such as certain grains (e.g coffee beans and roasted malts), may be exposed to roasting temperatures of 180 °C and above (*23*).

Role of Molecular Oxygen. Samples containing or lacking oxygen (air) were studied to explore its involvement in the oxidation of neoxanthin to β-damascenone. Argon was introduced into the samples to ensure the removal of oxygen out of the solutions and the headspace of the treated samples. Results presented in **Figure 7** indicate no effect of oxygen presence on the yield of β-damascenone. Furthermore, when peroxyacetic acid was excluded and molecular oxygen was the only oxidant in the system, β-damascenone was not detected. Therefore, molecular oxygen did not play a role in the oxidation-induced β-damascenone formation from the degraded neoxanthin.

Two-Phase Pathway. Perhaps a direct way to verify the need for the proposed sequential two phases for β-damascenone formation from neoxanthin is to conduct experiments in which either phase 1 or phase 2 is omitted. When only phase 1 was conducted, no β-damascenone was formed during the 90 min incubation at 60 °C (see **Figure 8**, left). To study the role of phase 2 alone, we omitted phase 1 by preheating the neoxanthin solution to 100 °C prior to the addition of the oxidant, and then the oxidant was added and the temperature was

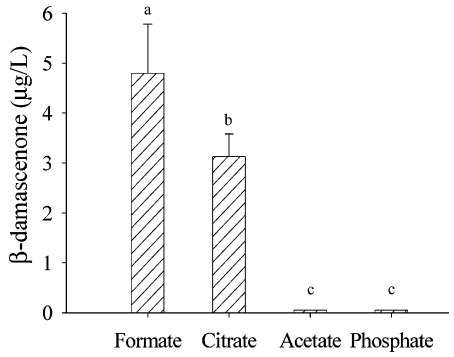

Figure 9. β-Damascenone formation during incubations conducted in different buffers. All buffer solutions were 0.4 M and adjusted to pH 5.0. Phase 1 was conducted by sample incubation for 90 min at 60 °C and then for an additional 20 min at 110 °C (phase 2). Values are the means ± SEM of duplicates of an experiment performed at least twice. Values not sharing the same superscript letter are different ($p < 0.05$) in β-damascenone yields across different buffer treatments.

immediately increased to 110 °C for 20 min. Results of these two series of experiments compared with that including both phases in a sequence are shown in **Figure 8**. A clear synergism of β-damascenone formation when both phases are conducted in a sequence is evident with phase 1 conducted at moderate temperatures and phase 2 at high temperatures.

Buffer Effect. Formic acid was chosen as the medium for the experiments since it is a customary medium for alkene epoxidation in the presence of hydroperoxides (*31*). In addition, formic acid (as well as other natural products such as mannitol, glucose, and ethanol) is known to be an efficient hydroxyl-radical scavenger (*32*) and might help creating milder oxidation conditions. Nevertheless, we were interested to reveal whether the reaction still works under similar pH, in the presence of a more characteristic food systems buffers, e.g., citrate. Acetate and phosphate were also tested. Interestingly, β-damascenone was not formed in media containing acetate and phosphate buffers (**Figure 9**). We assume that the yield of this odorant in each buffer system was affected by the buffer ability to scavenge free radical products of the hydroperoxide such as hydroxyl and peroxyl radicals, thus inhibiting a more accelerated oxidative degradation of the carotenoids. Support for this notion comes from the bleaching degree of the treated samples: when acetate and phosphate media were used, a complete bleaching of the carotenoids was evident, whereas formate and citrate media retained much of the initial color intensity even at the end of the experiment (data not shown).

Hypothetical Pathway. The studies of Skouroumounis et al. (*19*, *33*) suggested that compound **2** is the key intermediate in the formation of β-damascenone, since it will spontaneously convert to the latter under acidic environment such as fruit juices and wines (**Figure 1**). Yet, oxidative cleavage of neoxanthin at the C9═C10 double bond, by radicals or carotenases, will only result in ketone **1** (*34*, *35*) rather than alcohol **2**, and the former is unlikely to be reduced in food systems without the involvement of an enzyme or some special reagents (e.g. metal hydrides). Although the detailed mechanism in which β-damascenone was produced from neoxanthin in the current study remains to be elucidated, the experimental approved two sequential phases allowing the following hypothesis (**Figure 10**). During phase 1, sporadic oxidation of the polyene backbone by hydroperoxides or peroxy acids may result in structures such as epoxide **4**, which might be further hydrolyzed by water

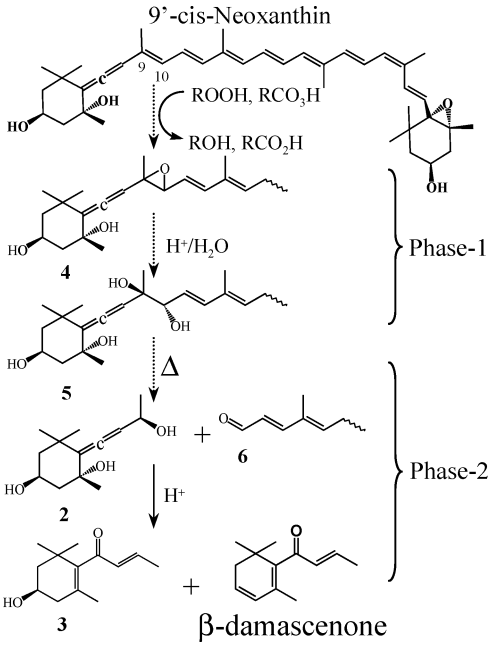

Figure 10. Hypothetical scheme indicating possible involvement of phase 1 and phase 2 in the thermal oxidation of neoxanthin to β-damascenone. Phase 1: direct oxidation of neoxanthin by hydroperoxide, leading to the formation of an epoxide (**4**), which hydrolyzed to a vicinal diol (**5**). Phase 2: at temperatures above 90 °C, the C9–C10 bond is thermolyzed with subsequent disproportionation to yield the allenic triol (**2**), which is further converted to β-damascenone and **(3)** under mild acidic conditions. Neoxanthin and β-damascenone were identified; dotted arrows indicate hypothetical stages along the pathway.

under the slight acidic environment to form the vicinal diol **5**. Phase 2 is carried out at relatively high temperatures to facilitate the thermal cleavage of the rather weak C9−C10 bond of **5**, with subsequent disproportionation, resulting in the key-compound **2** and the carbonyl residue **6** (of course, additionally expected products are ketone **1** and the respective alcohol of **6**). Finally, triol **2** may form β-damascenone as demonstrated by Skouroumounis et al. (*19*). Evidently, in view of the low yield of β-damascenone formation, the proposed mechanism is not a major pathway of neoxanthin degradation under these conditions. Peroxyacetic acid was selected as the oxidant since it is a common reagent for obtaining epoxides from alkenes and polyenes (*36*, *37*) and it is readily soluble in aqueous solutions compared with other peroxy acids such as *m*-chloroperbenzoic acid (*31*). Preliminary experiments indicated that hydrogen peroxide was inferior as 0.3 M of this hydroperoxide yielded approximately 1.0 μg/L β-damascenone (data not shown). Since such concentrations of hydroperoxides are unlikely to be found in any food product, and because of the low yields which were produced, hydrogen peroxide was not used further in this study. A possible source of hydroperoxides in food systems are the hydroperoxides of fatty acids resulting from lipoxygenase activity, mainly activated upon the first steps of food processing, e.g. maceration or grinding of the intact crop (*38*).

Conclusion. Since the original suggestion by Isoe et al. (*17*), this study provides the evidence for the first time that 9′-*cis*-neoxanthin can be oxidized and thermally degraded to β-damascenone. Furthermore, this degradation occurred under rather moderate pH and temperature values during phase 1 and a high temperature in phase 2, without the involvement of enzymatic activity under conditions which may occur in certain food systems.

ABBREVIATIONS USED

PRMN, prereaction mixture of neoxanthin; PAA, peroxyacetic acid.

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Supporting Information Available: Structure and NMR data for 9′-*cis*-neoxanthin presented in Figure S1 and Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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