Phyllobilins from Senescence-Associated Chlorophyll Breakdown in the Leaves of Basil (Ocimum basilicum) Show Increased Abundance upon Herbivore Attack

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ABSTRACT: In view of the common use of the herb basil (Ocimum basilicum) in nutrition and in phytomedicine, the contents of its leaves are of obvious interest. In extracts of fresh yellowish-green basil leaves, phyllobilins (PBs), which are bilin-type catabolites of chlorophyll (Chl), were detected using high-performance liquid chromatography (HPLC). Two such PBs, provisionally named Ob-nonfluorescent chlorophyll catabolite (NCC)-40 and Ob-YCC-45, exhibited previously unknown structures that were delineated by a thorough spectroscopic characterization. When basil leaves were infested with aphids or thrips or underwent fungal infections, areas with chlorosis were observed. HPLC analyses of the infested parts of leaves compared to those of the healthy parts showed a significant accumulation of PBs in the infested areas, demonstrating that the senescence-associated phophorbid a oxygenase/phyllobilin (PAO/PB) pathway is activated by herbivore feeding and fungal infection.

KEYWORDS: herb, infestation, Ocimum basilicum, pigment, phyllobilin, tetrapyrrole

INTRODUCTION

The natural breakdown of the green plant pigment chlorophyll (Chl) in higher plants furnishes linear tetrpyrroles, classified as phyllobilins (PBs), which resemble bilins, the degradation products of heme.3−5 Chl breakdown is particularly visible in autumn, when deciduous trees and bushes display the fascinating “fall” colors or when fruit are ripening. Despite its visibility, Chl degradation had remained obscure until roughly three decades ago, when the first structure of a presumed colorless chlorophyll catabolite was elucidated.4 Nowadays, over 50 different colorless Chl catabolites from senescent leaves of a wide variety of higher plant species have been identified and their structures have been characterized.16−18 In the earlier investigations, only colorless compounds were identified as the breakdown products of chlorophyll, which were either blue fluorescent or nonluminous and were hence named “fluorescent” Chl catabolites (FCCs) or “nonfluorescent” Chl catabolites (NCCs), respectively.5 However, more recently, yellow pigments were discovered as further Chl degradation products, named yellow Chl catabolites (YCCs) originally,6 and were also classified as belonging to the phylloxanthobilins (PxBs).7 The PxBs have the same chromophore as the heme-catabolite bilirubin (BR) and possess some similar remarkable chemical and physical properties.7 PBs are readily oxidized to pink-colored Chl catabolites (PCC), the phylloroseobilins (PrBs), which have also been detected in the extracts of senescent leaves.7

As established in the past three decades, Chl degradation follows a basically similar “phophorbid a oxygenase/phyllobilin (PAO/PB) pathway” during “normal” leaf senescence and fruit ripening (see Scheme 1). The enzyme PAO achieves the crucial oxidative opening of the macrocycle of phophorbid a (Pheo a), generating the “red” Chl catabolite (RCC)8 as the first bilin-type precursor of the colorless phyllobilins (PBs),9,10 which is also related to a red Chl catabolite from the green alga Auxenochlorella protothecoides.11

In senescent leaves, Chl degradation starts by the enzymatic reduction of Chl b to Chl a,12 followed by the removal of the central Mg ion by “stay-green” enzymes SGR1/SGR2.3 A previously suggested formation of chlorophyllide by the action of chlorophyllase, however, seems to be unrelated to the senescence-induced Chl breakdown in the leaves.13 Pheophitin, the product of SGR-catalyzed Mg removal, is hydrolyzed by pheophytinase (PPH) to Pheo a,14 The red Chl catabolite (RCC), obtained by the oxidative cleavage of Pheo a by PAO, is directly converted by RCC reductase (RCCR)3 to a colorless “primary” fluorescent catabolite (pFCC).15 The pFCC is hydroxylated at its 3β-position by a Rieske-type oxygenase at the inner chloroplast membrane,16 which was originally named translocon at the inner chloroplast envelope 55 (TIC55). After export into the cytosol, FCCs are further modified and then imported into the vacuoles,17 where an acid-induced isomerization (ISO) of the FCCs to the corresponding NCCs takes place.18,19 This isomerization is inhibited in “hypermodified” FCCs (hmFCCs), which accumulate in the peels of ripening banana, making them glow with a blue fluorescence.18,19

The disappearance of Chl and the senescence of leaves are induced by developmental and abiotic causes, such as drought

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and lack of light, in addition to biotic stresses, such as pest infestation. The latter is of high economic importance in the agricultural sector. Methods for monitoring of chlorophyll disappearance by measuring the optical changes near 700 nm have received considerable interest. In recent years, methods were developed for remote sensing of crop infestation using satellite and airplane data to establish vegetation indices, to distinguish between healthy and infested plants using differences in leaf spectral transmittance or reflectance without the need for costly on-site surveillance and controls.

Although improved methods are being developed for detecting leaf chlorosis as a consequence of pest infestation and applying those to distinguish between different infectants, the mechanisms behind Chl breakdown triggered by pathogens are still hardly understood. Whether the biochemical formation of phyllochlorophyllins in the course of senescence-associated chlorophyll degradation is triggered by herbivores or pathogens has remained obscure. In the leaves of apple and apricot trees infected with phytoplasmoses, the PAO/PB pathway of Chl degradation was indicated to be operating, and the Chl catabolites identified in healthy and infested plants showed no structural differences. This study provided the first evidence that pathogen-induced Chl breakdown produced NCCs via the PAO/PB pathway of senescence-induced Chl degradation.

However, studies on the expression of Chl catabolic enzymes involved in leaf chlorosis triggered by herbivore attack are still sparse. In fact, earlier work in *Diuraphis noxia*-infested wheat plants on a possible chlorophyllase activity turned out negative; therefore, the authors hypothesized that the disappearance of Chl elicited by herbivores is different from senescence-induced de-greening. They found, however, an increase in Mg-dechelatase activity upon aphid feeding on the plants. Another study in a different wheat line could detect Mg-dechelating as well as chlorophyllase activity in response to aphid infestation. Chlorophyllase, however, has been shown not to be involved in senescence-induced Chl degradation in the leaves. It has been proposed to play a role in plant defense by producing chlorophyllide as a protecting agent against herbivores chewing the leaves. In contrast, a correlation between the expression levels of SGR and a hypersensitive response-related cell death induced by *Pseudomonas syringae* was found in *Arabidopsis thaliana*, leading to accelerated kinetics of cell death with higher expression levels. Furthermore, the reduced levels of PAO were shown to lead to the accumulation of Pheo, a phototoxic Chl catabolite that can generate singlet oxygen, possibly contributing to the hypersensitive response.

The colorless NCCs typically accumulate in senescent leaves, and NCCs were considered earlier to represent the “final Chl-detoxification” products of the PAO/PB pathway. However, the discovery of yellow-colored phylloxanthobilins (PxBs) indicated the pathway to proceed further, beyond the colorless catabolites. YCCs are produced in leaves by an abundant, but still hardly characterized oxidative activity. The first studies on the possible bioactivities of PBs from ripe apples and pears have revealed the remarkable antioxidative activities of NCCs. YCCs were described very recently as more powerful antioxidants and were shown to protect cells from oxidative stress, suggesting PxBs to be of particular interest in terms of their physiological activities.

Here, we describe an analysis of the PBs in the leaves of (sweet) basil (*Ocimum basilicum* L. subsp. basilicum). Thanks to its various constituents, (sweet) basil is not only used as a culinary herb in many cuisines around the world but also relevant as a medicinal plant, being appreciated for its...
antioxidative, antimicrobial, antiviral, and larvicidal properties. Several studies have dealt with the phytochemical analysis of basil leaves, many of which focus on essential oils. In aqueous leaf extracts, compounds of further pharmacological relevance were identified, such as saponins, tannins, and alkaloids. The analysis of partially de-greened basil leaves led to the detection of several PBs, which were structurally identified as products of the PAO/PB pathway of Chl breakdown (see Figure 1).

Figure 1. (A) Photo of the two basil plants that were used for phyllobilin analysis. The green plant on the right was acquired fresh, and the naturally senescent yellowish-green plant on the left was kept for a further 15 days in partial shade (see Materials and Methods). (B) High-performance liquid chromatography (HPLC) analysis of an extract of a yellowish-green basil leaf with detection at 320 nm indicated the presence of four NCCs (Ob-NCC-35, Ob-NCC-36, Ob-NCC-40, and Ob-NCC-47) and of Ob-YCC-45 (labeled by full circles or by a triangle, respectively; analytical HPLC system I; see the Supporting Information (SI)).

Two PBs, a colorless NCC and a (yellow) YCC, were rather abundant in the naturally senescent leaves. Their detailed spectroscopic analysis revealed them to feature an unprecedented structure. We also investigated the spatial distribution and identity of the Chl catabolites upon infestation of the basil plant and showed that the PBs that were detected in the infested parts of a leaf were identical to those found in the healthy part of the leaf. In the infested parts, the amounts of the studied NCC and YCC were, however, significantly higher compared to those of the healthy parts.

**MATERIALS AND METHODS**

**Reagents and Chemicals.** HPLC-grade acetonitrile (MeCN) was obtained from VWR International GmbH (Ismaning, Germany), and ultrapure water (18 MΩ cm⁻¹) was obtained from a Millipore S.A.S. Milli-Q Academic system (18.2 MΩ cm⁻¹, Molsheim, France). The presence of four NCCs (Ob-NCC-35, Ob-NCC-36, Ob-NCC-40, and Ob-NCC-47) and of Ob-YCC-45 (labeled by full circles or by a triangle, respectively; analytical HPLC system I; see the Supporting Information (SI)).

**Analytical HPLC System I.** A Dionex UltiMate 3000 HPLC system, an UltiMate 3000 pump, an UltiMate 3000 diode array detector, and an RF2000 fluorescence detector with a 200 μL injection loop. Column: 1) Phenomenex Hyperclone ODS 5 μm 250 × 4.6 mm² i.d. column protected with a Phenomenex ODS 4 × 3 mm² i.d. precolumn; flow rate 0.5 mL min⁻¹. Solvent A: 50 mM aq potassium phosphate (pH 7), solvent B: methanol (MeOH); solvent composition A/B (v/v): 0–5 min: 80/20; 5–55 min: 80/20–30/70; 55–60 min: 30/70–0/100; 60–70 min: 0/100; 70–75 min: 0/100–80/20. Data were collected and processed with Chromeleon V6.80.

**Analytical HPLC System II.** An Agilent 1260 Infinity II LC system with a 1260 Infinity Degasser, an 1100 Series quaternary pump, and a 1100 Series diode array detector; Agilent Poroshell column 120EC-C18 4 μm 46 × 150 mm² at 15 °C protected by Phenomenex ODS 4 × 3 mm² i.d. precolumn; injection volume: 100 μL. Solvent system: mobile phase A = ammonium acetate buffer 10 mM pH 7, B = MeCN, flow 0.5 mL/min; solvent composition: 5 min to 40% B in 30 min. Data were processed with OpenLab CDS Data Analysis.

**Ultraviolet/Visible (UV/Vis) Spectroscopy.** An Agilent Technologies spectrophotometer (type: Cary 60 UV–Vis), in MeOH; λ_{max} [nm] (ε₆₅₀): 10 × 10 mm² UV cells (quartz). 1H- and 13C-nuclear magnetic spectroscopy (NMR): Bruker (Ultrashield 600 MHz) spectrometer, δ in ppm with δ (HDO) = 5.00 ppm at 5 °C, J_{HH} (Hz): 13C signal assignment from heteronuclear 1H- and 13C-hetero single quantum correlation (HQC) and HMBC experiments. LC-MS: a Shimadzu HPLC system, an LC-20AD pump, a DGU-20A5 online degasser unit, an SPD-M20A diode array detector, a Rheodyne injection valve with a 10μL injection loop. Phenomenex Hyperclone ODS 5 μm 250 × 4.6 mm² i.d. column protected with a Phenomenex ODS 4 × 3 mm² i.d. precolumn; flow rate 0.5 mL min⁻¹. Solvent A: 50 mM aq potassium phosphate (pH 7), solvent B: MeOH; solvent composition A/B (v/v): 0–5 min: 65/35; 5–28 min: 65/35–42/58; 28–33 min: 42/58–0/100; 33–38 min: 0/100; 38–43 min: 0/100–65/35. Data were collected and processed with Chromeleon V6.80.

**Isolation of Ob-NCC-40 and Ob-YCC-45.** A Dionex UltiMate 3000 HPLC system, an UltiMate 3000 pump, an UltiMate 3000 diode array detector, and an RF2000 fluorescence detector with a 200 μL injection loop. Phenomenex Hyperclone ODS 5 μm 250 × 4.6 mm² i.d. column protected with a Phenomenex ODS 4 × 3 mm² i.d. precolumn; flow rate 0.5 mL min⁻¹. Solvent A: 50 mM aq potassium phosphate (pH 7), solvent B: MeOH; solvent composition A/B (v/v): 0–5 min: 80/20; 5–55 min: 80/20–30/70; 55–60 min: 30/70–0/100; 60–70 min: 0/100; 70–75 min: 0/100–80/20. Data were collected and processed with Shimadzu LC Solution software (version 1.24 SP1).

**Liquid Chromatography—High-Resolution Mass Spectrometry (LC–HRMS).** A ThermoScientific LTQ Orbitrap XL mass spectrometer, equipped with an electrospray ionization (ESI) source (positive ion mode, spray voltage 4.5 kV, solvent: MeOH/4 mM ammonium acetate). Data were collected and processed with Xcalibur 2.2 software.

**Isolation of Ob-NCC-40 and Ob-YCC-45 for Spectroscopic Analysis.** Leaves weighing 24.5 g of two yellowish-green basil plants (Figure 1) were deep-frozen in liquid nitrogen and ground in a mortar and pestle. The resulting powder was extracted in 80 mL of MeOH, and the slurry was centrifuged for 10 min at 4000 rpm. The supernatant was collected, and the extraction was repeated using 70 mL of MeOH. The combined methanolic extracts were filtered through a Buchner funnel. After washing the extract two times with n-hexane (1 × 75 mL, 1 × 50 mL), the methanolic solution was concentrated to 30 mL using a rotary evaporator. The solution was diluted with 100 mL H₂O and centrifuged for 2 min at 4000 rpm. The supernatant was diluted with 120 mL H₂O, applied to a preconditioned SepPak-cartridge (5 g), and washed with 10 mL H₂O. After eluting the sample with 10 mL MeOH, the solution was...
concentrated to 3 mL using a rotary evaporator. The residue was isolated by semipreparative HPLC. Fifteen consecutive HPLC runs were performed, and the fractions containing Ob-NCC-40 or Ob-YCC-45 were collected. The combined Ob-NCC-40 fractions, diluted with 150 mL H2O, were applied to a preconditioned SepPak-cartridge (5 g). After washing with 20 mL H2O, the Ob-NCC-40 was eluted with 7 mL MeOH. The solvents were removed using a rotary evaporator and by drying under high vacuum, furnishing 2.9 μmol of Ob-NCC-40.

Likewise, the combined Ob-YCC-45 fractions were diluted with 150 mL H2O and applied to a preconditioned SepPak cartridge (5 g). After washing with 10 mL H2O, Ob-YCC-45 was eluted with 5 mL MeOH. The solvents were removed using a rotary evaporator and by drying under high vacuum, furnishing 0.98 μmol of Ob-YCC-45.

For a separate NMR spectroscopic analysis of Ob-YCC-45, 99 g (in total) of fresh or frozen yellowish-green basil leaves was extracted in four batches. The fresh leaf material was ground with liquid nitrogen in a mortar and pestle and suspended with a solvent mixture consisting of 40% MeOH and 60% potassium phosphate buffer 50 mM, pH 5.2. The frozen leaves were put into a beaker and mixed in a blender with a chilled solvent mixture (100 mL of the solvent mixture was used for 25 g of the leaf material). The mixtures were incubated at RT for 1 h and then centrifuged for 10 min at 5 krpm. The solid residues were washed with 50 mL of the chilled solvent mixture, and the pooled supernatants were filtrated. The clear solution was then acidiﬁed to pH 3.5 by the addition of chilled 50% acetic acid (3–5 mL). The solution was then stirred at RT in the dark for 3–5 h (the conversion of NCC to YCC was monitored by analytical HPLC). After that time, the mixture was filtered over a celite layer, and an SPE step was carried out (Phenomenex 5g RP 18 cartridge, elution with MeOH). The eluate was diluted with phosphate buffer 50 mM, pH 7, and puriﬁed by semipreparative HPLC (flow 2.5 mL/min, solvent composition 15% MeCN/85% of 10 mM NH4OAc, pH 7, t = 0–2 min, to 28% MeCN by t = 3 min, and to 38% MeCN by t = 23 min). For all four batches, a second round of semipreparative HPLC was carried out using the alternative solvent composition of 40% MeOH/60% of 50 mM potassium phosphate, pH 7, at t = 25 min. The purity of all four batches was controlled by analytical HPLC prior to pooling the batches containing Ob-YCC-45 and desalting the pooled solutions by SPE. The methanolic eluate (7 ml) was ﬁrst concentrated on a rotary evaporator at 25 °C and then lyophilized. Ob-YCC-45 of 0.70 μmol was obtained.

Spectral Data of PBs from O. basilicum. Ob-NCC-35. UV/vis (online, analytical HPLC system I, 50 mM aq potassium phosphate (pH 7)/ MeOH 47/53) λmax (εrel): 240 (1.05), 315 (1.00).

ESI-MS: m/z (%): 785.2 (45, [M + K]+); 749.3 (10), 748.3 (43), 747.3 (100, C38H43O12N4+,[ M+H ]+); 698.3 (45); 624.2 (40, M-C7H9NO+ H]+).

HRMS: [M + H]+ = 747.2877; C38H43O12N4, m/z calc = 747.2877 (δ = −0.8 ppm).

Presumed Stereoisomer of Ob-NCC-35. UV/vis (online, analytical HPLC system I, 50 mM aq potassium phosphate (pH 7)/MeOH 46/54) λmax (εrel): 248 sh (0.62), 312 (1.00).

Ob-NCC-36. UV/vis (online, analytical HPLC system I, 50 mM aqueous potassium phosphate (pH 7)/MeOH 46/54) λmax (εrel): 248 sh (0.62), 312 (1.00).
ESI-MS: \( m/z \) (%) = 799.3 (55, \([M+K]^+\)); 763.3 (10), 762.3 (43), 761.3 (98, \(\text{C}_{39}\text{H}_{45}\text{O}_{12}\text{N}_4^+\), \([M+H]^+\)); 729.3 (100, \(\text{M-CH}_4\text{O}^+\)); 725.3 (25), 638.2 (60, \(\text{M-C}_7\text{H}_9\text{NO}^+\)).

HRMS: \([M+H]^+\) = 761.3027; \(\text{C}_{39}\text{H}_{45}\text{O}_{12}\text{N}_4\), \(m/z_{\text{calc}} = 761.3034\) (\(\delta = -0.9\) ppm).

Ob-NCC-40. UV/vis (MeOH, \(c = 3.8 \times 10^{-5}\) M) \(\lambda_{\text{max}}\) (\(\varepsilon_{\text{rel}}\)): 245 sh (1.14), 313 (1.00).

\(1^H\) NMR (600 MHz, \(\text{H}_2\text{O}/\text{D}_2\text{O} 9/1, 25^\circ\text{C}\)): \(\delta\) [ppm] = 1.85 (s, \(\text{H}_3\text{C}_{131}\)), 1.90 (s, \(\text{H}_3\text{C}_{171}\)), 2.10 (s, \(\text{H}_3\text{C}_{71}\)), 2.14 (s, \(\text{H}_3\text{C}_{21}\)), 2.29 (m, \(\text{H}_2\text{C}_{122}\)), 2.57 (m, \(\text{H}_2\text{C}_{31}\)), 2.61 (m, \(\text{H}_2\text{C}_{121}\)), 2.72 (m, \(\text{H}_2\text{C}_{121}\)), 3.21 (AB system, \(J_{\text{AB}} = 15.1\), \(\text{H}_2\text{C}_{35}\)), 3.74 (m, \(\text{H}_2\text{C}_{85}\)), 3.78 (s, \(\text{H}_3\text{C}_{85}\)), 3.80 (m, \(\text{HC}_{82}\)), 3.90 (m, \(\text{H}_3\text{C}_{85}\)), 3.91 (m, \(\text{H}_2\text{C}_{5}\)), 3.93 (m, \(\text{H}_2\text{C}_{32}\)), 3.96 (m, \(\text{H}_2\text{C}_{5}\)), 4.87 (HC10, superimposed by 5.00 (HDO)), 5.39 (d, \(J = 13.1\), \(\text{H}_3\text{C}_{182}\)), 5.83 (d, \(J = 19.3\), \(\text{H}_3\text{C}_{182}\)), 6.57 (dd, \(J = 11.5/17.4\), HC181), 7.70 (s, \(\text{HN}_{24}\)), 9.05 (s, HC20), 9.29 (s, HC20), 10.73 (s, HN21), 10.85 (s, HN22) (Table S1).

\(1^C\) NMR (\(\text{H}_2\text{O}/\text{D}_2\text{O} 9/1, 25^\circ\text{C}\), indirect detection): see Figure 3.

ESI-MS (Orbitrap): \(m/z\) (%) = 1457.6 (6, \([2\text{M}+\text{H}]^+\)); 843.1 (5, \([\text{M-2H}+3\text{K}]^+\)); 805.2 (10, \([\text{M-H}+2\text{K}]^+\)); 767.2 (38, \([\text{M+K}]^+\)); 731.3 (10), 730.3 (42), 729.3 (100, \(\text{C}_{38}\text{H}_{43}\text{O}_{11}\text{N}_4^+\), \([M+H]^+\)); 697.3 (92, \(\text{M-CH}_4\text{O}^+\)); 653.2 (6, \(\text{M-CH}_4\text{O}-\text{CO}_2^+\)); 458.2 (9); 443.1 (18); 411.1 (6).

HRMS: \([M+H]^+\) = 729.2759; \(\text{C}_{38}\text{H}_{43}\text{O}_{11}\text{N}_4\), \(m/z_{\text{calc}} = 729.2766\) (\(\delta = -1.0\) ppm).

Ob-NCC-47. UV/vis (online, analytical HPLC system I, 50 mM aq potassium phosphate (pH 7)/ MeOH 42/58) \(\lambda_{\text{max}}\) (\(\varepsilon_{\text{rel}}\)): 213 (1.69), 242 sh (1.10), 314 (1.00).

Online ESI-MS (Orbitrap): \(m/z\) (%) = 683.2 (32, \([\text{M}+\text{K}]^+\)); 662.3 (15); 647.3 (8), 646.3 (39), 645.3 (100, \(\text{C}_{35}\text{H}_{41}\text{O}_{8}\text{N}_4^+\), \([\text{M}+\text{H}]^+\)); 625.3 (33); 595.2 (11); 568.2 (7).

HRMS: \([M+H]^+\) = 645.2912; \(\text{C}_{35}\text{H}_{41}\text{O}_{8}\text{N}_4\), \(m/z_{\text{calc}} = 645.2920\) (\(\delta = -1.2\) ppm).

Figure 3. (A) Graphical analysis of the NMR data of Ob-YCC-45 and assignment of signals from 1D and 2D NMR spectra. Left: \(^1^H\) signal assignments from COSY (bold lines) and ROESY spectra (dashed lines); right: \(^1^C\) signal assignment from \(^{1}^H-^{1}^H\)-HSQC (shadowed boxes) and \(^{1}^H-^{1}^C\)-HMBC spectra (normal boxes). Spectra were recorded in DMSO-\(d_6\) correlations to the malonyl moiety were not detected. B. UV–vis spectrum of Ob-YCC-45 in MeOH (\(d = 1\) cm). C. \(^1^H\) NMR spectrum of Ob-YCC-45 (in DMSO-\(d_6\) 600 MHz, 25°C).
Phyllobilin Determination in Aphid-Infested Basil Leaves.

Green aphids were collected from an infested orchid (courtesy of Gärtnerl. Zanker, Buchendorf, Germany) and transferred to an organic basil. After approximately 1 week, the infestation had spread. Six leaves from three different plants were used for analysis (the infestation was transferred by placing the new plant next to the infested one).

Each of the yellow and green patches of a leaf (Figure 5) was cut out with a scalpel, transferred to Eppendorf vials, and weighed (around 30 mg of the leaf material was used per sample). Fifty microliters of methanol and 150 μL of phosphate buffer 100 mM, pH 7, were added per sample, followed by approximately 50 μL of acid-washed glass beads (0.5 mm diameter, Sigma-Aldrich). The mixtures were vortexed for 1 min. After centrifugation on a table-top centrifuge at the maximum speed for 3 min at 4 °C, the supernatants were directly applied to analytical HPLC (analytical HPLC System I). To measure the total NCC and YCC contents from the HPLC peak areas, NCC and YCC signals were identified by their UV/vis traces from the diode array detector, and sums of the peak areas of all identified NCCs at 320 nm and YCCs at 420 nm were calculated, corrected for the differences in the weight of the extracted leaf material, and expressed as the ratio between the green and yellow leaves.

Phyllobilin Determination in Thrips-Infested Basil Leaves.

An infested basil plant was bought at a local supermarket, and the identity of the pest as thrips (order Thysanoptera) was determined by microscopy (Institute of Microbiology, University of Innsbruck). A second, visibly healthy plant was placed next to the infested one. From the second plant, three leaves were harvested after 1 month and extracted as described above for the aphid-infested basil (analytical HPLC system II).

Statistical Analysis. Bar chart: results represent the mean of six independent experiments (means ± standard deviation). Statistical significance was determined by an unpaired t test (two-tailed p < 0.05).

RESULTS AND DISCUSSION

Basil (O. basilicum) is known for the pleasant scent and taste of its intensely green leaves. Since this flowering plant is a member of the family of the Lamiaceae, our analysis of the phyllobilins in basil may represent the first investigation of Chl catabolites generated in a member of this family. Loss of greenness was seen in commercial basil plants when the whole plants were either kept inside a room in a semi-shaded area (see Figure 1A) or kept outside at moderate summer temperatures and in a semi-shaded place. Analysis of the contents of visually senescent basil leaves by HPLC allowed the detection of four fractions with the (online) UV/vis characteristics of colorless Chl catabolites (identified as Ob-NCCs) and one fraction of a (yellow) Ob-YCC, as depicted in Figure 1B. HPLC and online UV/vis of Ob-NCC-40 and YCC (Ob-YCC-45).

Combined HPLC–MS analysis of an extract of a de-greened basil leaf (Figures S2 and S3) indicated the presence of three polar NCCs (Ob-NCC-35, its tentatively identified, more polar stereoisomer, and Ob-NCC-36) with assigned pseudo-molecular ions at m/z = 747.3, 747.3, and 761.3, respectively, of the main NCC of intermediate polarity (Ob-NCC-40, m/z = 731.3), of the main YCC (Ob-YCC-45, m/z = 729.3), and of a less polar minor NCC (Ob-NCC-47, m/z = 645.3). For the purpose of elucidating the structures of the two most abundant Ob-PBs, Ob-NCC-40 (molecular formula C_{38}H_{42}O_{11}N_{4}) from HRMS: m/z =
Figures S1 and S3) and Ob-YCC-45 (molecular formula C_{38}H_{40}O_{11}N_{4}, from HRMS: m/z = 729.276; Figure S3), these two Ob-PBs were collected from an extract of 24.5 g of yellowish-green basil leaves, furnishing samples of 2.9 μmol of Ob-NCC-40 and 0.98 μmol of Ob-YCC-45.

The structure of Ob-NCC-40 was established as 1-formyl-O_{33}-malonyl-19-oxo-16,19-dihydrophyllobilane by 1H,1H-homonuclear and 1H,13C-heteronuclear NMR spectroscopy at 600 MHz. The NMR data collected from an aqueous solution (H_{2}O/D_{2}O 9/1) of Ob-NCC-40 at 5 °C furnished the signals of all 40 carbon- and nitrogen-bound H-atoms, as well as of all of its 38 C-atoms. All signals could be assigned together with a consistent set of internuclear correlations, based on which the chemical constitution of Ob-NCC-40 could be deduced unambiguously (see Figure 2). In particular, an AB system at δ = 3.21/3.22 ppm was detected, which gave rise to a cross peak (at δ = 3.21 ppm) with a carbon at δ = 44 ppm in a 1H,13C-HSQC spectrum. It was assigned to the methylene group (H_{2}C_{35}) of a malonyl substituent (for atom numbering, see SI, Figure S4), as these methylene protons showed NOE correlations with the methyl groups H_{3}C_{21} and H_{3}C_{71}, as well as 1H,13C-HMBC correlations with their two neighboring carbonyl groups C_{34} and C_{36}. Attachment of the malonyl group at the side chain extending from C_{3} was secured by a further 1H,13C-HMBC correlation of the carbonyl-C_{34} with the methylene group H_{2}C_{32} of the PB ethyl side chain at C_{3}. The consistent set of assigned signals and their through-space or through-bond interactions allowed the unambiguous delineation of the structure of Ob-NCC-40 as an O_{33}-malonyl-16,19-dihydro-1-formyl-19-oxophyllobilane. The three polar NCCs (isomer of Ob-NCC-35, Ob-NCC-35 = 15-hydroxy-Ob-NCC-40, and Ob-NCC-36 = 15-methoxy-Ob-NCC-40) with assigned pseudo-molecular ions at m/z = 747.3, 747.3, and 761.3, respectively, would be tentatively assigned the structures of the endogenous oxidation products\(^{\text{31}}\) of Ob-NCC-40 (15-hydroxy- and 15-methoxy-Ob-NCC-40), the inferred precursors of the PxB Ob-YCC-45.

A solution of Ob-YCC-45 in DMSO-d_{6} was analogously subjected to analysis at 500 MHz by 1H,1H-homonuclear and 1H,13C-heteronuclear NMR spectroscopy. The signals of 33 H-atoms (32 carbon and one nitrogen-bound) as well as of 33 of its 38 C-atoms were detected and could be assigned. From the available homo- and heteronuclear correlations, the basic chemical constitution of the YCC could be deduced as 1-formyl-O_{33}-malonyl-19-oxo-15Z-16,19-dihydrophyllobilene (see Figure 3). The C_{15}═C_{16} double bond of Ob-YCC-45 was deduced to feature a Z-configuration, on the basis of the strong NOE correlations of HC_{15} with H_{3}C_{131} and H_{3}C_{171}. This assignment was further supported by the characteristically similar 1H- and 13C-chemical shift data for C_{15} and C_{16} and their neighborhood atoms in the spectra of Ob-YCC-45 and in several YCCs.\(^{6,31}\) Unfortunately, no clear signals could be assigned to the hypothetical malonyl substituent, and the site of its attachment could not be delineated. However, based on the similarity of the chemical shift data for the critical ring A section of Ob-YCC-45 with those of Ob-NCC-40 (see above) and its UV/vis and HRMS data, the structure of Ob-YCC-45 was deduced as 1-formyl-O_{33}-malonyl-19-oxo-15Z-16,19-dihydro-
phytolobilene-c, i.e., as the product of a formal dehydrogenation of Ob-NCC-40 (see Figure 4).

O3\(^3\)malonylated NCCs\(^5,41\) and an O3\(^3\)-malonylated YCC\(^29\) with further modifications elsewhere in these molecules have been described earlier. A purified malonyl transferase (MAL) from oilseed rape (Brassica napus) was tested successfully in vitro, with several NCCs carrying a hydroxyl group at C3\(^2\). However, a corresponding set of FCCs carrying a hydroxyl group at C3\(^5\), such as 3\(^2\)-OH-pFCC\(^6\), the likely substrate of MAL in basil leaves (see Figure 4), were not available and were not tested at that time. Hence, the here-discussed malonylation reaction may probably occur in the cytosol at the stage of the 3\(^2\)-hydroxy-pFCC\(^6\). An alternative malonylation of 3\(^2\)-OH-NCC as substrate was expected to take place in the vacuoles, where precedence for such an enzyme activity is not available. Indeed, the least polar of the detected Ob-PBs, Ob-NCC-47, showed the same characteristic UV/vis spectra and also exhibited the MS-derived molecular formula \((C_{35}H_{40}O_{8}N_4)\) of a 3\(^2\)-hydroxylated NCC, an abundant NCC in flowering plants, discovered by Curty and Engel\(^73\) in senescent leaves of Ceridiphyllum japonicum. Hence, Ob-NCC-47 was tentatively assigned the structure of 1-formyl-3\(^2\)-hydroxy-19-oxo-16,19-dihydrophytolobilane (see Figure 4).

Having identified the products of senescence-associated chlorophyll breakdown in basil leaves, we investigated their occurrence in basil plants that showed signs of chlorosis as a consequence of infestation with herbivores. Insect feeding elicited diverse responses from the host plant. There were, however, minor differences in the signaling pattern associated with different pests—aphids, for example, induced responses mediated via the plant hormones jasmonic acid, ethylene, and salicylic acid, whereas the response to thrips seems to be rather related to wound signaling through jasmonic acid.\(^44,45\) Thrips is a common pest found in greenhouses, but also gardens. Thrips causes mechanical damage to the plant tissue to suck the plant saps,\(^44\) a procedure that is different from aphids, whose feeding mechanism is much more precise and relies on the injection of salivary proteins into the plant.\(^46\)

We analyzed the relative contents of Ob-NCCs and Ob-YCCs in aphid and thrips-infested basil plants, thereby differentiating between zones on the leaves that were visibly green and “unaffected”, and sections that showed symptoms of the pest infestations.

For the aphid infestation, aphids were transferred to a basil plant, and symptoms of the infestation, such as chlorosis, could be observed approximately 1 week later. The leaves of the aphid-infested plants showed de-coloration in small zones around the localizations of the aphids on the leaf (Figure 5A). When analyzing the extracts of the de-colored spots and of the surrounding still visibly green tissue, an increased amount in PBs becomes evident (Figure 5B). Indeed, the statistical analysis of six independent experiments of the green versus yellow section of six leaves from three different basil plants showed clearly that YCCs and NCCs occurred in elevated quantities in yellow sections compared to those in the surrounding green areas, i.e., 10 times more YCC and 6 times more NCCs as analyzed by the peak areas of the signals (Figure 6).

In comparison to the damage caused by aphids, symptoms of infestation were observed later for the thrips-infected basil plants, and de-colored zones were less defined and larger and appeared black-silvery on the leaf, as is typical for thrips. To investigate the relative amounts of PBs in the visibly affected areas and surrounding green tissue, the leaves were collected about 1 month after the infection of the plant; extracts were prepared of the different sections and analyzed by HPLC (see Figure S7). Three independent experiments were performed, and every single experiment showed elevated levels for each of the analyzed types of PBs and at least more than 2 times higher amount of total PBs compared to the green sections (Figure 7); however, a very large variance in the relative abundance was observed. This could be due to the diffuse nature of the affected zones on the leaves, which made cutting out defined sections for extraction difficult.

Basil is known to be highly susceptible to gray mold caused by Botrytis cinerea, which often invokes the need for fungicide treatment.\(^47\) In a preliminary test, B. cinerea and two other important fungal pathogens, Rhizoctonia solani and Fusarium graminearum, were used to infect the visibly healthy basil plants. Three weeks after the infection, all plants showed symptoms of chlorosis. Symptomatic leaf samples were analyzed by HPLC...
analogous to the aphid and thrips experiments. Again, compared to an uninfected control plant, the signals of Ob-YCC-45 were much more prominent in all of the infected plants (see SI). Especially necrotrophic pathogens, such as B. cinerea, R. solani, and F. graminearum, cause wilting of their host, which is preceded by chlorosis of green plant parts, and utilize mycoxotins to induce a fast programmed cell death and hypersensitive response. Similar to insect pests, hormonal pathways are induced (shikimic acid/jasmonic acid) in plantae. In Arabidopsis thaliana infected with B. cinerea, cotranscriptional networks linked to chloroplast genes and genes specific for chlorotic leaves were consistently induced.48 The malonylated PBs Ob-NCC-40 and Ob-YCC-45 appear to be metabolites that are part of the general, hypersensitive response of O. basilicum to pathogens and pests.

Whether increased amounts of PBs are formed as a response to the herbivore attack to protect the surrounding tissue from oxidative stress or play a completely different role, such as involvement in signaling for the plant, has yet to be elucidated. In particular, the O33-malonylation, revealed by the detailed structural analysis of Ob-NCC-40 and Ob-YCC-45, raises interest in regard to defense-related mechanism(s), since introducing malonyl groups to secondary metabolites has been observed as a response to insect feeding. For example, the tagging of 17-hydroxygeranyl-linalool diterpene glycosides (DTGs) with malonyl groups was shown to occur upon herbivore attack in Nicotiana rustica, although this modification seems not to be relevant for plant defense, since the malonylated metabolites did not appear to be stable once taken up by the insects.49 Therefore, a role in signaling for the plant for malonylated DTGs may be more likely.49

Our investigations with basil leaves have revealed the presence of several phyllophobins from the senescence-associated PAO/PB pathway of Chl breakdown, which accumulated in green-yellow, in herbivore-infested, and in fungal-infected leaves, in particular. Four novel PBs were discovered, three (colorless) NCCs and a (yellow-colored) phylloxanthobilin (PxB). They displayed a specifically malonylated structure and were related to each other by modifications delineating their hypothetical metabolic interrelation. Recent studies with PxBs, e.g., of YCCs from Echinacea purpurea and of a dioxobilin-type analogue (DYCC) from savoy cabbage showed strong antioxidative activities for these PxBs, exceeding those of ascorbic acid and trolox, a water-soluble vitamin E derivative.33,35 The locally defined increase of PxBs from the PAO/PB pathway upon pest infestation could indicate that PxBs play a role as strong antioxidants in protecting the surrounding tissue from the oxidative stress elicited by the plant’s response to herbivores and fungi. Elevated levels of PBs appear to be independent of the nature of the plant pest, pointing toward a yet unexplored plant response on the molecular level. In this context, the malonylated PBs Ob-NCC-40 and Ob-YCC-45, which were newly identified in the basil leaves by full spectroscopic characterization, are particularly interesting since malonylation of secondary metabolites has been described as a response to insect feeding.49 The pronounced formation of PBs in the affected areas of the basil leaves also suggests an involvement of the products of the PAO/PB pathway of Chl breakdown in plant defense or signaling.

ASSOCIATED CONTENT

Supporting Information
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ABBREVIATIONS

Chl, chlorophyll; COSY, correlation spectroscopy; DTGs, 17-hydroxygeranyl-linalool diterpene glycosides; DYCC, 1,19-dioxobilin-type yellow chlorophyll catabolite; ESI-MS, electrospray ionization mass spectrometry; FCC, fluorescent chlorophyll catabolite; HCAR, 7′-hydroxy-Chl a reductase; HMBC, hetero multiple bond correlation; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; HSQC, hetero single quantum correlation; ISO, chemical isomerization; LC–MS, liquid chromatography–mass spectrometry; MAL, malonyl transferase; MeCN, acetonitrile; MeOH, methanol; NCC, nonfluorescent chlorophyll catabolite; NMR, nuclear magnetic resonance; NY1C/NOL, nonyellow coloring 1/ NY1C-like; PaO, pheophorbide a oxygenase; PB, phyllobilin; PheOA, pheophorbide a; PPH, pheophytinase; PiCC, pink chlorophyll catabolite; PrB, phylloxanthobilin; RCC, red chlorophyll catabolite; RCCR, RCC reductase; ROESY, rotated frame nuclear Overhauser
effect spectroscopy; SGR1/SGR2, stay-green 1/2; SI, Supporting Information; SPE, solid-phase extraction; TIC, total ion current; TIC55, transposon at the inner chloroplast envelope S5; UV/vis, ultraviolet–visible; YCC, yellow chlorophyll catabolite

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