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Occurrence of Benzoic Acid Esters as Putative Catabolites of Prunasin in Senescent Leaves of *Prunus laurocerasus*

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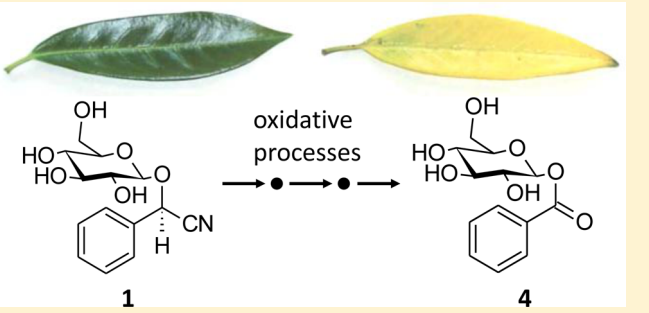
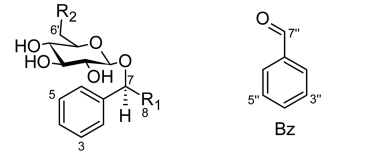
Supporting Information

ABSTRACT: *Prunus laurocerasus* is an evergreen shrub containing large quantities of the cyanogenic glycoside prunasin (**1**) in its leaves, which decomposes to prunasin amide (**2**) or glucose-1-benzoate (**4**) when the leaves become chlorotic as a result of senescence or pseudosenescence. This study was aimed at the systematic identification of senescence-associated metabolites to contribute further insight into the catabolism of **1**. LC-ESIMS profiles of senescent and green leaves were analyzed by principal component analysis. In senescent leaves, the concentrations of 36 compounds were increased significantly including several benzoic acid derivatives, of which prunasin amide-6′-benzoate (**5**) and prunasin acid-6′-benzoate (**6**) were isolated and identified. The observed metabolic changes were also induced by treatment of *P. laurocerasus* shrubs with exogenous ethylene. The data presented support an oxidative catabolism of **1** without release of hydrogen cyanide and the remobilization of its nitrogen in the course of senescence. The results are discussed in the context of functional diversification and drug discovery, where senescent plant material represents a widely unexplored source for the discovery of natural products.



Cherry laurel (*Prunus laurocerasus* L., Rosaceae) is an evergreen shrub originating from Asia Minor, where its fruits are a popular food and both the fruits and leaves are utilized as traditional herbal medicines.[1] More than 200 μmol/g of the cyanogenic glycoside prunasin (**1**) occurs in its freeze-dried or fresh green leaves, whereas similar molar amounts of prunasin amide (**2**) and glucose-1-benzoate (**4**) replace **1** in naturally occurring chlorotic leaves (Figure 1). It has been shown that **2** results from decomposition of **1** in the nonenzymatic, hydrogen-peroxide-consuming Radziszewski reaction, especially when strong illumination has bleached fresh green leaves during air drying.[2,3] In contrast, **4** has been suggested to accumulate under conditions of senescence, as leaves containing this compound as the main constituent are very easily detachable, thus indicating the formation of a petiolar abscission layer, which is a well-known indication for leaf senescence.[4]



	R_1	R_2	name
1	CN	OH	prunasin
2	$CONH_2$	OH	prunasin amide
3	COOH	OH	prunasin acid
4	=O	OH	β-D-glucose-1-benzoate
5	$CONH_2$	OBz	prunasin amide-6'-benzoate
6	COOH	OBz	prunasin acid-6'-benzoate

Figure 1. Structures of prunasin and related compounds identified in leaves of *Prunus laurocerasus*.

The present study aimed at providing further insight into the decomposition of **1** in the leaves of *P. laurocerasus* by identifying possible catabolites and providing additional evidence for the interrelation of leaf senescence with the accumulation of **4**.

RESULTS AND DISCUSSION

In order to identify senescence-associated metabolites, leaves from six different *P. laurocerasus* shrubs were collected and immediately freeze-dried. The sequential mode of leaf senescence of this evergreen plant allowed for the collection of green and senescent leaves at the same time, and thereby leaf metabolomes could be compared unbiased by temporal variation. Chlorotic leaves considered as senescent rather than pseudosenescent[5] detached with a force of 6 newtons or less, whereas it always took more than 10 newtons to detach green leaves.

LC-MS fingerprints of all samples were analyzed by principal component analysis (PCA). The fingerprints of senescent leaves were discriminated readily from the fingerprints of green

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leaves along principal component 1 (PC1) in the score plot. Consequently, the phytochemical differences between senescent and green leaves clearly outclassed those among different shrubs. According to previous observations,[2] the molecular features with the most pronounced loadings on PC1 were attributed to **1** for green and to **4** for senescent leaves (Figure 2). For identifying further senescence-associated metabolites,

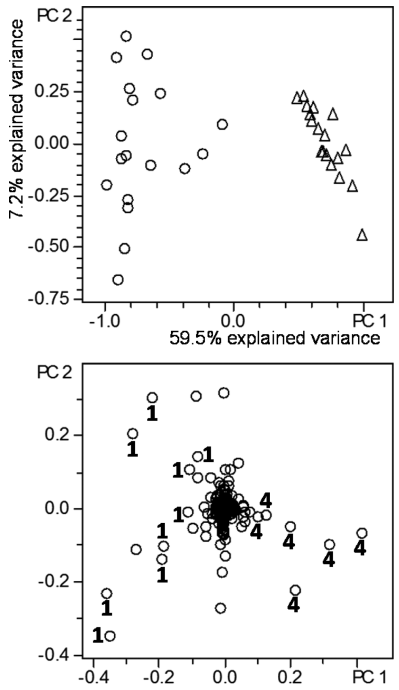

Figure 2. PCA score plot (upper panel) that discriminated LC-MS profiles of green (○) from those of senescent (△) leaf samples of *Prunus laurocerasus* along PC1. Labels in the PCA loadings plot (lower panel) refer to *m*/*z* data of prunasin (**1**) and glucose-1-benzoate (**4**).

molecular features with strong negative loadings on PC1 were selected and validated by tracing back to the original LC-MS data, comparing the extracted ion chromatogram (EIC) peak area of each compound's most abundant pseudomolecular ion with the Student's *t*-test. Senescent leaf samples featured 36 peaks that were increased significantly ($p \leq 0.05$) and at least 2-fold when compared with green leaf samples (Table S1, Supporting Information). High-resolution MS and, if available, MS/MS spectra of these peaks were obtained from a representative LC-MS data set that was analyzed using the Dissect Compounds algorithm of Data Analysis 4.0 (Bruker Daltonics). The algorithm detected 251 compounds and effectively subtracted background MS signals as well as MS signals of overlapping compounds from their mass spectra. Two prominent groups of senescence-related metabolites were identified tentatively on the basis of these data: (i) abscisic acid-related megastigmane glycosides; such compounds previously have been detected in several plant species, including *Prunus* spp. (Figure S1, Supporting Information);[6−8] (ii) the concentrations of compounds related to **1** [prunasin amide (**2**), prunasin acid (**3**), and especially **4**] were increased strongly in senescent leaves (Figure 1). The consistent detection of **2** and **3** also in freeze-dried green leaves is in accordance with a study on *Prunus dulcis* (Mill.) D. A. Webb.[9] These previous authors have suggested that these compounds along with their higher glycosylated derivatives and anitrile glycosides partake in a recycling pathway for the turnover of cyanogenic glycosides.

Further peaks were found with the exact masses of their respective sodium adduct ions matching those of prunasin anitrile (one peak, reduced in senescent leaves), prunasin pentoside (one peak, unchanged), or prunasin anitrile pentoside (three peaks, increased in senescent leaves), each described by Pičmanová et al.[9] Owing to the low abundance of these peaks, no mass spectrometric fragmentation nor UV data gave further evidence of their identity.

It was noted that several of those compounds displaying the largest differences between senescent and green leaf samples showed fragments at *m*/*z* 105 and *m*/*z* 123, which were attributed to benzoic acid derivatives (Figure 3).[10] Searching

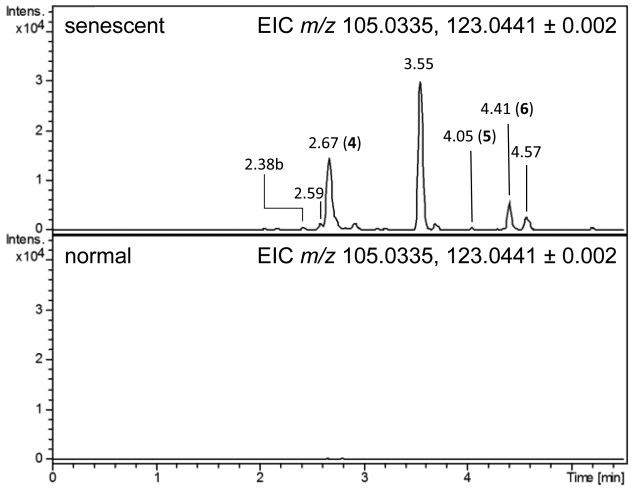

Figure 3. EICs indicative of benzoic acid derivatives of senescent and normal green leaf samples of *Prunus laurocerasus*. The peak labeling refers to Table S1, Supporting Information.

the LC-MS data for these fragments with EICs revealed that, despite small but consistent amounts of **4**, green leaves were free of benzoic acid derivatives. As benzoic acid has been suggested to be a catabolite of **1** in *P. laurocerasus* and *Olinia ventosa* (L.) Cuf.,[2] compounds **5** and **6** were selected for isolation. From their fragment signals, these compounds were also expected to comprise mandelic acid derivatives. The compounds were isolated from naturally occurring senescent leaves and readily identified as benzoyl derivatives of **2** and **3**. Both compounds showed in their ^{1}H NMR spectra distinct signals for two protons downfield of the anomeric proton (δ 4.44, 4.68 and 4.45, 4.67 ppm, respectively). From the HSQC spectrum of **6**, these signals were attributed to the protons at C-6 of the glucose moiety. The linkage of this position to the carbonyl carbon of benzoic acid was established using the HMBC spectrum. The presence of a mandelic acid moiety in **6** and a mandelic acid amide moiety in **5** was supported by the MS data; namely, an exact neutral fragment loss between $[M + H]^+$ and its respective fragment at *m*/*z* 267 was 152.0466 amu ($C_8H_8O_3$) for **6** and 151.0646 amu ($C_8H_9NO_2$) for **5**. Further support arose from characteristic C═O stretching and N−H deformation vibrations that occurred in their IR spectra (1719 cm^{-1} for **6** and 1670, 1602 cm^{-1} for **5**).[11] The sugar moiety was identified as D-glucose by capillary electrophoresis of a hydrolysate after derivatization with (*S*)-(−)-1-phenylethylamine.[12] Circular dichroism (CD) spectroscopy was applied to determine the absolute configuration of the mandelic acid parts,

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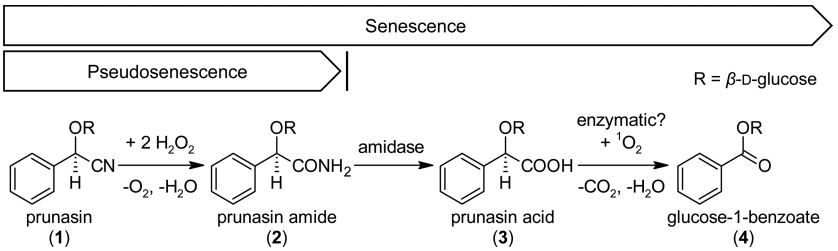

Figure 4. Proposed decomposition of **1** in *Prunus laurocerasus* under conditions of senescence and pseudosenescence.

using (*R*)- and (*S*)-mandelic acid and authenticated **1** and **2** (both *R*-configurated mandelic acid derivatives) as a reference (Figure S2, Supporting Information). In agreement with published data for different α-substituted phenylacetic acid derivatives,[13] only (*S*)-mandelic acid showed a positive Cotton effect at 222 nm, whereas (*R*)-mandelic acid as well as any other tested mandelic acid derivative showed a negative Cotton effect between 210 and 227 nm. Consequently, **6** was identified unambiguously as prunasin acid-6′-benzoate (prupersin B) and **5** as prunasin amide-6′-benzoate (persicaside) (Figure 1). Previously, both compounds have been isolated from the seeds of *Prunus persica* L.[14,15]

Further compounds with fragments indicative of benzoic acid were identified tentatively or characterized by their fragmentation patterns. In particular, these data strongly suggested the presence of prupersin D (1,6′-dibenzoyl gentiobioside), also known from *P. persica*,[15] and benzoylmalic acid, which was identified tentatively from a neutral loss of 134.0223 amu ($C_4H_6O_5$) between $[M + H]^+$ and the benzoic acid fragment at *m*/*z* 105.0331.[10] Notably, metabolic profiling of the seeds, roots, stems, or leaves of up to 12-week-old *P. dulcis* plants did not reveal any benzoic acid derivatives.[9] This difference may be attributed to the different *Prunus* species or to the developmental state of senescence. The latter hypothesis is supported by earlier work where benzoic acid released from senescent leaves has been suggested as an allelopathic mechanism of *Prunus serotina* Ehrh.[16,17]

A relevant question for the apparent metabolic relationship between the mandelic acid derivative glycosides and **4** was whether the glucose moiety had been maintained or instead cleaved at some point. The latter could be true if benzoic acid originated from benzaldehyde after cyanogenic decomposition of **1** and was glucosylated afterward. The presence of free mandelic acid, mandelic acid amide, benzaldehyde, or benzoic acid would support this hypothesis. These compounds were not detectable in this study. In order to test if **4** could result from some kind of oxidative reaction, compounds **1**−**3** were incubated with H_2O_2 at pH 6. Small amounts of **4** were detected by LC-MS when **3**, but not **1** or **2**, was incubated for 20 h.

Oxidative decarboxylation may have decomposed **3** to **4** under consumption of singlet oxygen that evolved spontaneously from the disproportionation of hydrogen peroxide.[18] Singlet oxygen evolution has been described to increase during leaf senescence and also to affect secondary metabolites.[19,20] A comparable decarboxylation reaction has been described to decompose indole-3-acetic acid to indole-3-carbaldehyde in the presence of singlet oxygen.[21] It may be suggested that under conditions of senescence, compound **1** decomposes to benzoic acid derivatives via an oxidative pathway, beginning with the formation of **2** by a nonenzymatic Radziszewski reaction,[2] followed by enzymatic hydrolysis of the amide group by an amidase to yield **3**. This step has also been discussed to occur in the recycling pathway proposed by Pičmanová et al. based on data from developing plants.[9] It seems reasonable to suppose that the putative amidase would be upregulated during senescence, as pseudosenescence caused by abiotic stress results in accumulation of **2**.[2] Compound **4**, and in turn other benzoic acid derivatives, may then derive from **3** without cleavage of the glucose moiety by oxidation with singlet oxygen, with or without enzymatic participation (Figure 4). Additionally, 1-*O*-acylglucose derivatives such as **4** have also been reported as acyl donor molecules for the acylation of plant secondary metabolites. Specifically, **4** has been described as an acyl donor for the benzoylation of glucosinolates in *Arabidopsis thaliana*.[10] The reaction is catalyzed by the serine-carboxypeptidase-like acyltransferase SCPL17, which also accepts 1-*O*-sinapoyl-β-glucose as an acyl donor.[22] Such an enzyme, using **4** as an acyl donor, might also catalyze the formation of **5**, **6**, and the tentatively identified molecules prupersin D and benzoylmalic acid in senescent leaves of *P. laurocerasus*. Acylation may modulate the transport and storage of plant secondary metabolites.[22] Therefore, 6′-*O*-benzoylation may prepare **2** for further breakdown, and compound **5** rather than **2** could be the substrate for a putative amidase. The observed presence of **3** with the simultaneous absence of free benzoic acid, however, supports a simpler breakdown, as shown in Figure 4.

To strengthen the hypothesis that the observed changes in the metabolic profiles were factually attributable to senescence and not to chlorosis by nonphysiological bleaching referred to as pseudosenescence, two small shrubs of *P. laurocerasus* were fumigated with ethylene in a commercial banana-ripening room, and a third shrub was kept as a control. Ethylene-treated shrubs showed a 4-fold to 8-fold increased number of senescing leaves after 7 days when compared with the control (Figure 5). LC-MS analysis of representative leaves sampled after 7 days showed that, with regard to their contents of benzoic acid derivatives, (i) senescent leaves differed strongly from young green leaves and therefore mirrored the aforementioned phytochemical characteristics. However, there was no difference between the senescent leaves of control and ethylene-treated shrubs, which supported the link of **4** with senescence. (ii) Young green leaves showed considerably smaller amounts of **4** when compared with full-grown green leaves, and (iii) in contrast to the control, older green leaves from the ethylene-treated shrubs resembled senescent leaves in their significant amounts of **5** and **6** and in their detachment force of less than 10 newtons (Figure 6). The latter observation suggests that the phytochemical changes had begun before chlorosis became conspicuous.

A simple but important conclusion from these results is that senescent leaves may yield significant amounts of natural products that are hardly detectable in green leaves. With regard

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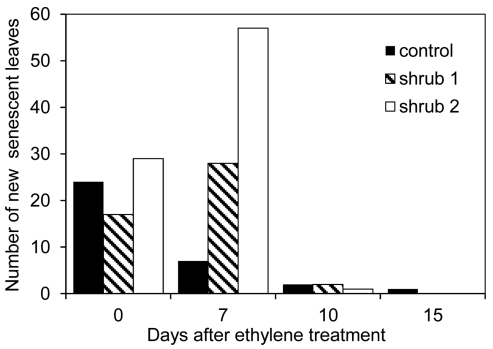

Figure 5. Development of new senescent leaves after fumigation of *Prunus laurocerasus* shrubs with ethylene. At days 0, 7, and 10, any chlorotic leaves present were counted and removed: (solid bars) control shrub; (hatched bars) treated shrub 1; (white bars) treated shrub 2.

to *P. laurocerasus*, this applies for **5**, **6**, and several tentatively identified benzoic acid and megastigmane derivatives (Figure S1, Supporting Information), which also occur after ethylene treatment of cherry laurel leaves in an industrial-scale fruit-ripening chamber. Although different plant parts vary strongly in sensitivity, the induction of senescence by exogenously applied ethylene is regarded as a general feature of higher plants.[23] Consequently, new natural products discovered in senescent leaves can be expected to be accessible biotechnologically by a well-established technology,[23] also in larger scale. Currently, senescent plant material remains a widely unexplored source for drug discovery. Whether or not senescence-associated plant metabolites are likely to show biological activities remains to be demonstrated. Studies on *Artemisia annua* L. provide promising evidence, since the antimalarial constituent artemisinin is present in senescent leaves in nearly 2-fold amounts when compared with the green leaves.[24] Comparable to the formation of **2** and possibly **4** in *P. laurocerasus*, the formation of artemisinin from its precursor dihydroartemisinic acid depends on nonenzymatic oxidation. This is obviously related to an oxidative burst, which is a prominent and well-known feature of leaf senescence.[19,20,24]

Besides their well-established deterrent properties against herbivores, there is increasing evidence for multiple functions of cyanogenic glycosides.[25,26] Multifunctionality of **1** in leaves of *P. laurocerasus* seems reasonable, as its high concentration levels clearly outweigh the needs for defense. The suggested catabolism of **1** during senescence supports this concept with regard to nitrogen storage, allelopathy, and the regulation of abiotic stress and reactive oxygen species. Accumulation of **4** in senescent leaves indicates that the nitrile nitrogen of **1** is remobilized before leaf abscission, which is in accordance with the nutrient recycling described for senescent leaves.[27] Benzoic acid released from **4** has an allelopathic effect on red maple seedlings.[17] The apparent consumption of reactive oxygen species during the breakdown of **1** indicates a possible protection of plant tissue from reactive oxygen species that evolve excessively from senescing and stressed plant parts.[19,25,28] Further studies support a stress-protective effect of cyanogenic glycosides: (i) Lotaustralin in leaves of *Hevea brasiliensis* (Willd. ex. Juss.) Müll. Arg. follows a circadian rhythm with decreasing concentrations under illumination and recovery in darkness;[29] (ii) the so-called stay-green cultivars of sorghum contain 3 to 6 times more cyanogenic glycosides than medium stay-green and senescent types (N. Bodapati, personal communication), and (iii) drought-stressed sorghum plants show an increased HCN potential.[30]

Future studies should elucidate the interesting difference in catabolism of **1** to benzoic acid derivatives in senescent leaves of *P. laurocerasus* or to anitrile glycosides in germinating seeds

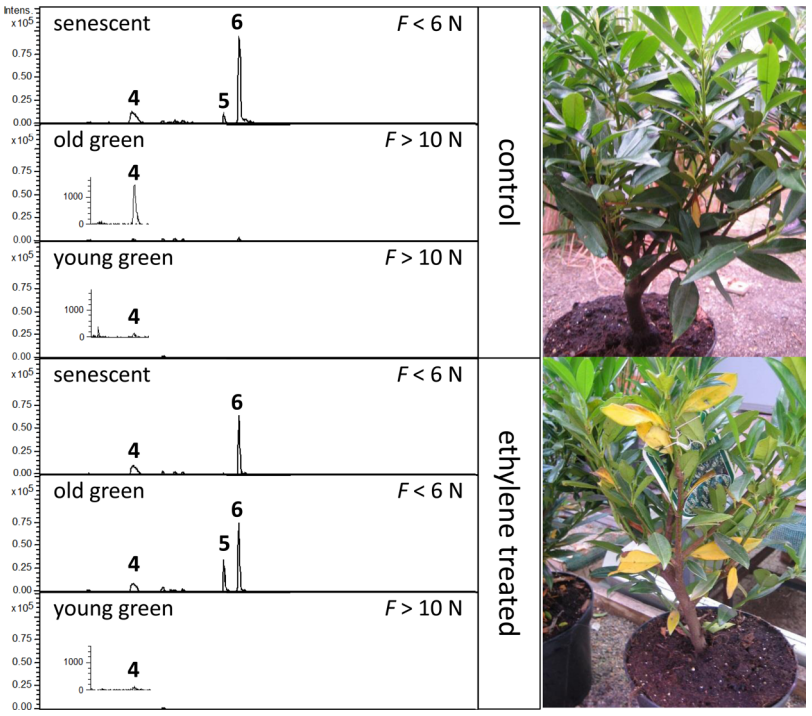

Figure 6. Overlayed EICs for compounds **4**–**6** in different leaves from ethylene-treated shrubs and control (overlay of EICs *m*/*z* 285.0969, 440.1324, 441.1165 ± 0.005). The forces [newtons] needed to detach the leaves are given in the upper right corner of each chromatogram.

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and newly developed leaves of bitter almonds.[9] However, the fate of nitrile nitrogen and the role of enzymes in the proposed catabolism need to be resolved.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured using methanol solutions with an Autopol automatic polarimeter (Rudolph Research Analytic). CD and UV spectra were recorded using methanol solutions on a JASCO J-815 CD spectrometer. IR spectra were recorded from pure substance on a Nicolet 4700 IR spectrometer with an FMIR insert. NMR experiments were performed on a Varian AS 400 Mercuryplus NMR spectrometer in deuterated methanol. The preparative HPLC system consisted of two Waters 515 pumps and a Latek A4080 UV detector (210 nm). Fast centrifugal partition chromatography (FCPC) was carried out with a Kromaton FCPC semipreparative instrument. The absolute configuration of D-glucose was established by capillary electrophoresis after hydrolysis (2 M TFA, 120 °C, 1 h) and derivatization of the hydrolysate with *S*-(−)-1-phenylethylamine.[12]

Plant Material. Green and senescent leaves of *P. laurocerasus* were collected from April to June 2010 from specified plants at 51.965, 7.605 ± 100 m (vouchers PBMS 237, 238, 239.1-6, plants identified by J.S.). Senescent and green leaves were collected from the plants at the same time. Senescent leaves were identified by their color and by the force needed for their detachment. Detachment force was characterized by a spring balance (1 to 10 N) that was attached to the petiole of a leaf using a wool string. The spring balance was then carefully pulled while monitoring the scale until the leaf detached or the scale ended. Green leaves could never be detached within the 10-newton scale, whereas senescent leaves never needed more than 6 newtons or even detached manually while trying to fix the wool string.

For PCA, both senescent and green leaves were collected from six individual shrubs in triplicate and freeze-dried immediately after harvest. Senescent leaves for the isolation of **5** and **6** were collected from the same plants and several further shrubs of *P. laurocerasus* to afford a sufficient amount of material.

Ethylene Treatment of Whole Shrubs. Three comparable shrubs of *P. laurocerasus* (about 50 cm height) were purchased in April 2011 in a local market garden. Two of these were treated with ethylene by placing them in a commercial banana-ripening room (Fruchtimport vanWylick GmbH) (two fumigation cycles were applied as used for bananas; details may be obtained on request from the senior author). The third shrub was used as an untreated control. The development of new senescent leaves was monitored for each shrub. Representative leaves were analyzed for their detachment force and the presence of compounds characteristic for naturally occurring senescent leaves.

UHPLC-ESI-qTOF-MS. Chromatographic separations were performed on a Dionex Ultimate 3000 RS liquid chromatography system on a Dionex Acclaim RSLC 120 C_{18} column (2.1 × 100 mm, 2.2 μm) with a binary gradient (A: water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid) at 0.8 mL/min: 0 to 9.5 min, linear from 5% B to 100%B; 9.5 to 12.5 min, isocratic 100% B; 12.5 to 12.6 min, linear from 100% B to 5% B; 12.6 to 15 min, isocratic 5% B. The injection volume was 2 μL. Eluted compounds were detected using a Dionex Ultimate DAD-3000 RS over a wavelength range of 200−400 nm and a Bruker Daltonics micrOTOF-QII time-of-flight mass spectrometer equipped with an Apollo electrospray ionization source in positive mode at 2 Hz over a mass range of m/z 50−1000 using the following instrument settings: nebulizer gas nitrogen, 5 bar; dry gas nitrogen, 9 L/min, 220 °C; capillary voltage 4500 V; end plate offset −500 V; transfer time 70 μs; collision gas nitrogen; collision energy 8 eV; collision RF settings were combined with each single spectrum of 2500 summations as follows: 1250 summations with 130 Vpp + 1250 summations with 500 Vpp. Internal data set calibration (HPC mode) was performed for each analysis using the mass spectrum of a 10 mM solution of sodium formiate in 50% 2-propanol that was infused during LC re-equilibration using a divert valve equipped with a 20 μL sample loop.

Analysis of UHPLC-ESI-qTOF-MS Data. PCA was performed using Profile Analysis 2.0 with raw data that had been calibrated using Data Analysis 4.0. Chromatographic peaks were detected using the Find Molecular Features algorithm (s/n threshold 5, correlation coefficient threshold 0.7, minimum compound length 5 spectra, smoothing width 5) on line data after spectral background subtraction in the time range of 0.5 to 10 min and in the m/z range of 150 to 1000. A full cross validation was performed with the data set and resulted in a model containing no outliers. Molecular features were considered as senescence-associated if the average peak areas obtained from EICs were significantly different between green and senescent leaves ($p \leq 0.05$) and if the average peak area of senescent leaves was at least 2-fold greater than the average peak area of green leaves.

Full-scan MS- and MS^2 spectra containing the molecular features of interest were extracted by using the Dissect Compounds function of Compass DataAnalysis 4.0 SP1. Exact molecular weights were determined using an in-house VBA script. Fragment ions were obtained from MS^2 experiments of protonated molecules or ammonium adduct ions or from the Dissect spectra if no suitable MS^2 experiment was triggered. Sum formulas were determined using the Smart Formula tool of Data Analysis. MetFrag was applied to compare the observed fragments with fragments predicted for compounds with the same sum formula, using ChemSpider as upstream database.[31]

Extraction and Isolation. Freeze-dried senescent leaves (200 g) of *P. laurocerasus* were extracted three times with a 10-fold amount of acetone−water (7:3) in an ultrasonic bath. The extract was freeze-dried to yield 60 g of dry matter, of which 10 g was resuspended in 20 mL of water and poured into a glass column filled with 170 g of Extrelut. The column was successively eluted with 2 L of dichloromethane and 2 L of 1-butanol, yielding 1.1 and 7.6 g of dried material, respectively. The 1-butanol fraction was subjected to Sephadex LH-20 chromatography using ethanol as eluant to give 20 fractions. Compound **6** (26 mg) was isolated from fraction 9 (0.24 g) by preparative HPLC (Hypersil ODS, 5 μm, 250 × 16 mm eluted with 42% methanol in 0.1% TFA at 10 mL/min). Compound **5** was purified from fraction 8 (2.4 g) by FCPC using ethyl acetate−1-butanol−water (10:3:10; 4 mL/min) for separation in a 200 mL coil at 1000 rpm; the fraction eluting between 92 and 124 mL (0.042 g) was further subjected to preparative HPLC (see above, but with 26% acetonitrile in 0.1% TFA as eluant) to yield 3.2 mg of **5**.

Incubation of 1−3 with H_2O_2. Compound **1** was isolated from fresh green leaves of *P. laurocerasus* as described previously.[2] Compound **2** was obtained from **1** based on a prior investigation.[11] Compound **3** was obtained as a mixture of 2*S*- and 2*R*-epimers after boiling **2** for 1 h in 0.1 M sodium hydroxide, acidifying with 10% trifluoroacetic acid to pH 2−3, and repeatedly extracting this solution with a water-saturated mixture of two parts 1-butanol and one part 2-propanol. Compounds **1**−**3** were each dissolved in water to a concentration of 1 mg/mL (stock solutions). Of each stock solution, 100 μL was mixed in 1.5 mL Eppendorf tubes with 500 μL of phosphate buffer pH 6 and either 100 μL of 0.5% H_2O_2 (sample) or water (control). Each vial was filled up to a final volume of 1000 μL and incubated for 20 h at ambient temperature.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b01090.

Structural formulas of compounds identified tentatively by MS fragmentation data, CD spectra of compounds **5** and **6**, table with MS fragmentation data (PDF)

Tabulated LC-MS data: monoisotopic m/z signals and m/z signal intensities of all detected peaks of a senescent leaf sample, ordered by retention time, using tab as data separator and period as decimal separator (TXT)

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Notes

The authors declare no competing financial interest.

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