Chlorophyll Catabolites in Senescent Leaves of the Plum Tree
(Prunus domestica)

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Dedicated to Professor Ernst-Peter Kündig on the occasion of his 70th birthday

In cold extracts of senescent leaves of the plum tree (Prunus domestica ssp. domestica), six colorless non-
fluorescent chlorophyll catabolites (NCCs) were characterized, named Pd-NCCs. In addition, several minor NCC
fractions were tentatively classified. The structure of the most polar one of the NCCs, named Pd-NCC-32, featured an
unprecedented twofold glycosidation pattern. Three of the NCCs are also functionalized at their 3’-position by a
glucopyranosyl group. In addition, two of these glycosidated NCCs carry a dihydroxyethyl group at their 18-position.
In the polar Pd-NCC-32, the latter group is further glycosidated at the terminal 18’-position. Four other major Pd-NCCs
and one minor Pd-NCC were identified with five NCCs from higher plants known to belong to the ‘epf’-series. In
addition, tentative structures were derived for two minor fractions, classified as yellow chlorophyll catabolites, which
represented (formal) oxidation products of two of the observed Pd-NCCs. The chlorophyll catabolites in leaves of
plum feature the same basic structural pattern as those found in leaves of apple and pear trees.

Keywords: Chlorophyll, Fruit, Phyllobilins, Porphyrins, Plant senescence.

Introduction

About 25 years ago, chlorophyll (Chl) breakdown and the appearance of the fall colors were still a stunning
mystery. [1][2] In 1991, a first colorless Chl degradation product from a higher plant was described, the ‘non-
fluorescent’ Chl-catabolite (NCC) Hv-NCC-1 from senescent leaves of barley (Hordeum vulgare). [3][4] Structural
identification of Hv-NCC-1 as a 1-formyl-19-oxobilin-type linear tetrapyrrole [5] opened the door to the
structure-guided discovery of the ‘PaO/phyllobilin’ pathway of Chl-breakdown. [5 – 9] As we know now, oxidative
cleavage of the Chl macroring generates 1-formyl-19-oxobilins and sets the stage for the formation of various bilin-type catabolites of Chl, [10][11] or ‘phyllobilins’. [7 – 9] The ‘early’ stages of Chl-breakdown, which take place in the chloroplasts, furnish one of two epimeric primary ‘fluorescent’ Chl-catabolites (pFCCs), with species-dependent configuration of their formation. [11][12] pFCCs are rapidly hydroxylated to 3’-OH-pFCC (probably still in the chloroplast). [17] Once exported into the cytosol, FCCs are mostly modified further and imported into the acidic vacuoles, where they are thought to isomerize spontaneously to corresponding NCCs (see Fig. 1). [13]

In the meantime, NCCs have been found in extracts of senescent leaves of a range of plants, [5 – 9] [14][15] where they accumulate and were suggested earlier to represent ‘final stages’ of Chl-catabolism. [5][6] [16] NCCs were also identified as products of Chl breakdown in ripening fruit [17 – 20] and in de-greening vegetables. [21 – 23] In the last 25 years, more than 20 structurally different NCCs from higher plants were, thus, detected, and their structures were characterized (see Table 1). [8][9][14][24] Evidence for further oxidative transformation of NCCs in leaves was also provided recently by the observation of yellow Chl-catabolites (YCCs) [25 – 28] and pink Chl-catabolites (PiCCs) [29] in senescent leaves of a variety of higher plants. [9] These colored phyllobilins were identified as formal dehydrogenation products of corresponding tetrapyrrolic NCCs. All of these observations were consistent with an essentially ‘linear’ path of Chl-breakdown in higher plants. [5 – 7]

However, as was recognized recently, Chl-breakdown ‘branches out’, and furnishes ‘1,19-dioxobilin-

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type’ Chl-catabolites (DCCs)[38] as second major family of phyllobilins. The latter (‘type-II’) phyllobilins are mostly colorless, such as the 1,19-dioxobilin-type NCCs (DNCCs).[9][39][40] Originally, DNCCs were suggested to be oxidative deformylation products of NCCs.[38] In view of a surprising stereochemical diversity observed in natural DNCCs, we suggested an earlier branching-point in the ‘PaO/phyllobilin’ pathway of Chl-breakdown.[7–9] Indeed, a P450-enzyme catalyzing deformylation of FCCs was identified,[40] which converts FCCs (1-formyl-19-oxobilin-type or ‘type-I’ phyllobilins) to corresponding 1,19-dioxobilin-type FCCs (DFCCs), hence, opening the pathway to ‘type-II’ phyllobilins.[9][40][41] Under weakly acidic conditions, the latter are indicated to isomerize stereoselectively to corresponding DNCCs (see Fig. 1).[41]

In the context of investigations of Chl-catabolites in domestic agricultural plants, we have studied the nature of such phyllobilins in stone fruit and report here our work on the Chl-catabolites in leaves of the plum tree (Prunus domestica ssp. domestica). As shown below, Chl-breakdown in senescent leaves of this fruit tree follows the ‘PaO/phyllobilin’ pathway of Chl-breakdown.[7–9] It produces 1-formyl-19-oxobilin-type catabolites, or ‘type-I’ phyllobilins, identified as NCCs of the ‘epi-type’. In addition, in the extracts several YCCs were also found.

Results and Discussion

Yellow senescent and green leaves were collected from plum trees (Prunus domestica) and frozen for storage. Five major and nine minor colorless NCCs were provisionally identified in extracts of senescent leaves of plum trees on the basis of their characteristic UV-absorbance properties, using analytical HPLC (Fig. 2). All NCC fractions showed UV/VIS spectra featuring a longest wavelength maximum near 314 nm, characteristic of an α-formyl-pyrrole moiety (ring A), as first reported in the spectrum of Hv-NCC-1.[3] Likewise, minor fractions of two YCCs and a trace of a pink Chl-catabolite (PiCC) were also tentatively identified.

For spectroscopic analysis of the most abundant NCCs in the leaves of P. domestica, 18.7 g of

Figure 1. Short outline of the main path of chlorophyll breakdown in higher plants, displaying structural formulas of chlorophylls α and β, of the primary fluorescent chlorophyll catabolites (pFCC/epi-pFCC), of non-fluorescent chlorophyll catabolites (NCCs), of yellow chlorophyll catabolites (YCCs), of dioxobilin-type FCCs (DFCCs) and of dioxobilin-type NCCs (DNCCs) (generalized formulas, see Table 1 for individual NCCs[8][9]).
Table 1. Structures of known natural nonfluorescent Chl-catabolites (NCCs).

| R¹ | R² | R³ | C(16)¹ | Provisional names² | Ref. |
|----|----|----|--------|-------------------|-----|
| H  | H  | CH=CH₂ | n     | Bo-NCC-2 (At-NCC-3)² | [23][30] |
| H  | Me | CH=CH₂ | epi   | Cj-NCC-2 (So-NCC-5/Pd-NCC-71)³ | [13][22] |
| OH | H  | CH=CH₂ | n     | Bn-NCC-3            | [31]  |
| OH | H  | CH=CH₂ | epi   | So-NCC-3 (Mc-NCC-49) | [18][22] |
| OH | H  | CH(OH)-CH₂OH | epi | So-NCC-1 (Mc-NCC-26) | [18][22] |
| OH | Me | CH=CH₂ | n     | Sw-NCC-58            | [32]  |
| OH | Me | CH=CH₂ | epi   | Cj-NCC-1 (So-NCC-4/Md-NCC-2/Pd-NCC-60) | [13][17][22][33] |
| OH | Me | CH(OH)-CH₂OH | n | Hv-NCC-1             | [3][4] |
| OH | Me | CH(OH)-CH₂OH | epi | So-NCC-2 (Md-NCC-42/Pd-NCC-40)⁴ | [18][21][22] |
| O-Glc | H | CH=CH₂ | n | Bn-NCC-2 (At-NCC-1)/Bo-NCC-1 | [15][23][31] |
| O-Glc | H | CH=CH₂ | epi | Cj-NCC-2⁵ | [20]  |
| O-Glc | Me | CH=CH₂ | n     | At-NCC-4⁶ | [15]  |
| O-Glc | Me | CH=CH₂ | epi   | Nr-NCC-2 (Md-NCC-1/Pd-NCC-56)⁷ | [17][34][35] |
| O-Glc | Me | CH(OH)-CH₂OH | epi | Zm-NCC-1 (Tc-NCC-1/Pd-NCC-35)⁸ | [26][35] |
| O-Glc | Me | CH(OH)-CH₂O-Glc | epi | Pd-NCC-32⁹ | [34]  |
| O-(6'-O-Mal)Glc | Me | CH=CH₂ | epi | Nr-NCC-1 | [34]  |
| O-Mal | H | CH=CH₂ | n     | Bn-NCC-1            | [31][36] |
| O-Mal | Me | CH=CH₂ | epi   | Ej-NCC-2⁵ | [19]  |
| O-Glc | Me | CH=CH₂ | epi   | Ug-NCC-53            | [37]  |

Mal, malonyl; Glc, β-glucopyranosyl. ¹ Configuration at C(16): NCCs derived from pFCC (n, ’normal’) or from epi-pFCC (epi, ’epimeric’), the absolute configuration at C(16) is not determined. ² Bo-NCCs (from broccoli, Brassica oleracea var. italica), At-NCCs (from Arabidopsis thaliana), Cj-NCCs (from Katsura tree, Cercidiphyllum japonicum), Mc-NCCs (from spinach, Spinacia oleracea), NCCs (from oilseed rape, Brassica napus), Mt-NCCs (from banana peels, Musa acuminate, Cavendish cultivar), Sw-NCC-58 (from Peace Lily, Spathiphylum wallisii), Md-NCCs (from Malus domestica), Hv-NCC-1 (from barley, Hordeum vulgare), Co-NCC-2 (from quince fruits, Cydonia oblonga), Nr-NCCs (from tobacco, Nicotiana rustica), Zm-NCC-1 (from maize, Zea mays), Tc-NCC-1 (from Lime tree, Tilia cordata), Pd-NCCs are from this work and are shown in bold (from Plum tree, Prunus domestica), Ej-NCC-2 (from loquat fruits, Eriobotrya japonica) and Ug-NCC-53 (from Wych Elm tree, Ulmus glabra). ³ At-NCC-3 carries a HOCH₂ group at C(2) (see [30]; see [31]) (4.17 ppm) and H-1″ (4.33 ppm), as well as H-(2″) (3.17 ppm) and H-(2‴) (3.21 ppm), the chemical shifts of the pairs of signals differed significantly (for atom numbering: see Experimental Section, Fig. 9). ⁴ The β-glucopyranosyl group attached at C(3′) is also esterified with its primary OH group at the propionate function, giving a bicyclo[17.3.1]motif. ⁵ In a 600 MHz ¹H-NMR spectrum of Pd-NCC-32 (1) in CD₃OD at 10 °C (see Fig. 5) signals of 47 of the 48 C-bound H-atoms were observed. Among these signals there were a singlet for the formyl H-atom (H=C(20)) at low field, four Me group singlets at high field and a singlet for the methyl ester group at 3.75 ppm. The typical signals for a peripheral vinyl group were not observed. From ¹H,¹³C-heteronuclear (HSQC and HMBC) and ¹H,¹H-homonuclear NMR-correlations (COSY and ROESY) of Pd-NCC-32 (1) in CD₃OD, assignment of the signals of 47 H-atoms and 45 ¹³C-nuclei could be achieved (see Fig. 6). In addition to the signals of the NCC-core, those of 14 H-atoms were observed in the intermediate field of the ¹H-NMR spectrum. ¹H,¹H-COSY and ¹H,¹³C-HSQC correlations indicated two hexopyranose units, with closely similar ¹H- and ¹³C-shifts in both sugar moieties. Only for atoms at or close to the anomeric centre, H-C(1′) (4.17 ppm) and H-C(1″) (4.33 ppm), as well as H-C(2″) (3.17 ppm) and H-C(2‴) (3.21 ppm), the chemical shifts of the pairs of signals differed significantly (for atom numbering: see Experimental Section, Fig. 9).
Chemical shifts and doublet nature ($J = 7.8$ Hz) of H–C(10) and H–C(1″) indicated β-anomeric attachment of both sugar moieties, as observed earlier for the 32-glu-copyranoside moieties of NCCs. Indeed, both sugar units were identified as glucopyranosides by comparing the 1H- and 13C-chemical shifts of Pd-NCC-32 (1) with those of the known NCCs with a peripheral glucopyranosyl group at C(32). 1H,13C-HMBCs from H–C(10) with C(32) and from H–C(1″) with C(182) established the attachment of one sugar moiety at each one of the terminal C-atoms of the Et side chain at C(3) (ring A) and of the 1,2-dihydroxyethyl group at C(18) (ring D). The 1H- and 13C-chemical shifts at the positions C(182) and C(32) were also consistent with an attached peripheral sugar substituent. However, as with other 1,2-dihydroxyethyl substituted NCCs, in 1 the configuration at C(182) remains unknown.

Five other Pd-NCC fractions (see Fig. 2), i.e., Pd-NCC-35 (2), Pd-NCC-40 (3), Pd-NCC-56 (4), Pd-NCC-60 (5), and Pd-NCC-71 (6) were also isolated and purified by HPLC. A positive-ion-mode ESI-MS spectrum of Pd-NCC-60 (5) showed a pseudo-molecular ion [M + H]+ at m/z 645.2, consistent with the molecular formula of C35H40N4O8. Characteristic fragment ion peaks were visible at m/z 613.2 and 522.1, corresponding to the loss of MeOH and the loss of ring D from [M + H]+. The same molecular formula and fragmentation is known for the major NCC from Cercidiphyllum japonicum (Cj-NCC-1), an abundant NCC with ‘epi’-configuration at C(16). To test the probable identity of these two NCCs, their elution properties were compared in HPLC experiments. Thus, solutions of Pd-NCC-60 (5) and Cj-NCC-1 were separately analyzed by analytical HPLC, as well as a 1:1

Figure 2. HPLC Analysis of an extract of senescent plum tree (Prunus domestica) leaves (online detection at 320 nm). Main catabolites are highlighted by standard names of catabolites; minor fractions classified as ● non-fluorescent chlorophyll catabolites (NCC), ▲ yellow chlorophyll catabolites (YCC) and ■ pink chlorophyll catabolites (PiCC), based on their UV/VIS spectra (see text for details).

Figure 3. UV/VIS Spectrum of Pd-NCC-32 (1) in MeOH ($c = 4.3 \times 10^{-5}$M).
mixture of both in a co-injection (see Experimental Section, Fig. 10). Their common elution time and their common UV/VIS- and mass spectral data, suggest structural identity of the NCCs Cj-NCC-1 and Pd-NCC-60 (5) (see Fig. 7), implying 'epi'-configuration at C(16) of Pd-NCC-60 (5). Consistent with their origin from a common
primary FCC, now indicated to be epi-FCC, the other colorless Pd-NCCs were also deduced to belong to the epi-series.

The molecular formula of Pd-NCC-56 (4) was determined as C_{41}H_{50}N_{4}O_{13} by ESI mass spectrometry, which furnished a base peak [M + H]^+ at m/z 807.2. Fragment-ions at m/z 775.3, 684.2 and 645.2 corresponded to the loss, alternatively, of MeOH, of ring D and of a hexose moiety from [M + H]^+ . These data indicate the presence of one hexopyranose moiety at the HO–C(3^\text{r})
group of ring A of Pd-NCC-56 (4) and a vinyl group at C (18) of ring D. This indicates a common chemical constitution of 4 and of Nr-NCC-2[34] (see Fig. 7).

The molecular formula of Pd-NCC-40 (3) could be deduced tentatively as C_{35}H_{42}N_{4}O_{10} by ESI mass spectrometry, which showed the experimental base peak [M + H]^+ at m/z 679.2. In the mass spectra, characteristic fragment-ion peaks at m/z 647.2 and 522.1 were also detected, which corresponded to the loss of MeOH and ring D (from [M + H]^+). Accordingly, the catabolite Pd-NCC-40 (3) (see Fig. 7) was deduced to have the same chemical constitution as So-NCC-2 from spinach.[21][22]

A positive-ion-mode ESI-MS spectrum of Pd-NCC-35 (2) indicated a pseudo-molecular ion at m/z 841.2, consistent with the molecular formula of C_{41}H_{52}N_{4}O_{15}. The fragments at m/z 809.3, 684.2, 679.2 and 522.1 indicated the loss of MeOH, the loss of ring D, the loss of a sugar moiety and the loss of ring A (from [M + H]^+). Accordingly, the catabolite Pd-NCC-40 (3) (see Fig. 7) and of Pd-NCC-54 (see Fig. 7) were deduced to have the same chemical constitution.

The molecular formula of Pd-NCC-71 (6) was determined as C_{35}H_{36}N_{4}O_{7} with a pseudo-molecular ion at m/z 629.2. Fragments at m/z 597.2 and 506 indicate the loss of MeOH and ring D. Pseudo-molecular ion and fragment-ions are consistent with a chemical constitution of 6, as previously found for Cj-NCC-2 (Fig. 7).[13] Identity of Pd-NCC-71 (6) and of Cj-NCC-2 was supported by a common retention time of 6 and Cj-NCC-2 in a HPLC co-injection experiment.

Analysis of a minor NCC (tentatively named Pd-NCC-54) by LC/ESI-MS revealed a pseudo-molecular ion at m/z 661.2 ([M + H]^+), consistent with the molecular formula of C_{35}H_{40}N_{4}O_{9}. We suspected Pd-NCC-54 as product of the formal addition of an O-atom to Pd-NCC-60 (5) from an endogenous oxidation process. Indeed, as shown recently,[28] NCCs may undergo C (15) hydroxylation by endogenous, as well as by additional efficient adventitious oxidation during preparation of leaf homogenates and their extracts. From NCCs hydroxylated at their C(15) position, H_{2}O may eliminate easily, resulting in corresponding YCCs.[28] Indeed, a YCC was detected in the fresh plum leaf extracts, named Pd-YCC-67, which showed mass spectral data (pseudo-molecular ion with m/z 643.2) consistent with its formation as the formal product of an oxidative dehydrogenation of Pd-NCC-60 (5). A further minor fraction, classified as YCC from a prominent absorption maximum near 420 nm, was also subjected further to ESI-MS analysis. The latter data suggested Pd-YCC-61 (m/z 805.1) to represent a YCC derived from oxidation of the glucosylated Pd-NCC-56 (4). When extracts were prepared after storage of senescent leaves of the plum tree at room temperature for 7 min, an increase of the content of both YCCs (Pd-YCC-61 and Pd-YCC-67) was observed, as well as the formation of 15-OH-Pd-NCC-60, identified by comparison with its analogue from the established oxidation of Cj-NCC-1.[28] However, this hydroxylated NCC differed (in its retention time) from Pd-NCC-54. Clearly, work-up and preparation of extracts of cold senescent leaves need to be done swiftly, in order to avoid oxidation artefacts.

Conclusions

Extracts of naturally senescent leaves of the plum tree (Prunus domestica ssp. domestica) were shown to contain a range of NCCs, two YCCs, and, in traces, a PiCC, all members of the ‘type I’ phyllobilin family. In spite

Figure 7. Constitutional formulas of non-fluorescent chlorophyll catabolites (NCCs) found in senescent leaves of the plum tree (P. domestica ssp. domestica).
of the absence of DCCs, a remarkable structural diversity of Chl-catabolites was, thus, indicated. The polar NCC Pd-NCC-32 (1) showed a previously unknown structure and is functionalized with two glycopyranose moieties on the ‘distant’ pyrrole rings A and D. The structure of Pd-NCC-32 (1) also provided the first (indirect) evidence for enzymatic glycosidation of an FCC at the 18\(^2\)-position (a primary alcohol function resulting from dihydroxylation of the corresponding vinyl group of the precursor FCC). Five more NCCs were tentatively identified with known catabolites based on their matching UV/VIS- and mass spectroscopic features. Further identification by HPLC of Pd-NCC-60 (5) and Pd-NCC-71 (6) with corresponding Cj-NCCs, indicated the plum NCCs to belong to the C(16)-epi series, as well. Additional investigations will be required to secure the structures of several minor NCC- and of the YCC-containing fractions. Based on the deduced structures of the plum NCCs, a tentative pathway of their formation in the senescent leaves of the plum tree could be derived (see Fig. 8).

While the first Chl-catabolites in Rosaceae crops were found in leaves and fruits of apple and pear trees, which belong to the Pyreae tribus, here a stone fruit (that is part of the Amygdaleae tribus) was studied for the first time. The findings with senescent leaves of the plum tree are consistent with the related earlier studies with leaves of apple and pear trees. With members like apples, pears, peaches, strawberries, raspberries and many others, the Rosaceae family belongs to the six most economically important crop families worldwide. Thus, this study suggests the conserved PaO/phyllobilin pathway of Chl breakdown to NCCs to operate in senescent leaves of the Spiraeoideae subfamily of the Rosaceae.

**Experimental Section**

**General**

HPLC grade MeOH was purchased from HiPerSolv Chromanorm (Fontenay-sous-Bois, F), LC/MS gradient grade MeOH from or VWR (Milan, Italy), and AcONH\(_4\), puriss. p.a., from Fluka (Buchs, CH). KH\(_2\)PO\(_4\), puriss. p.a., K\(_3\)PO\(_4\) dibasic-anh., puriss. p.a., and hexane were from Sigma–Aldrich (St. Louis, USA). Sand was from J. T. Baker (Avantor, PA, USA), Sep-Pak\textsuperscript{\textregistered} C\(_{18}\) cartridges (1 and 5 g) were from Waters Associates. pH Values were measured with a WTW SenTix 21 electrode connected to a WTW pH525 digital pH meter.

**HPLC.** Dionex Summit HPLC system with manual sampler, P680 pump, online degasser and diode array detector, 1.35 ml or 200 \(\mu\)l injection loop. Data were collected and processed with Chromeleon V6.70.

![Figure 8](image-url)

**Figure 8.** Hypothetical steps of chlorophyll (Chl) breakdown in senescent leaves of the plum tree (*Prunus domestica* ssp. *domestica*) in a topographical model, highlighting the major catabolic steps with abridged short names of (hypothetical) fluorescent chlorophyll catabolites (FCC) intermediates and of non-fluorescent chlorophyll catabolites (NCCs), characterized in the present work.
i) Anal. HPLC. Kinetex 00G-4601-E0-5u-C18-100A 250 × 4.6 mm i.d. column at 20 °C protected with a Phenomenex AJ0-4287 C18 4 × 3.0 mm i.d. pre-column was used with a flow rate of 0.5 ml min⁻¹. Solvent A: 50 mM aq. potassium phosphate buffer (pH 7.0), solvent B: MeOH, solvent C: H₂O; solvent composition (A/B/C) as a function of time (0 – 90 min): 0 – 5, 80:20:0; 5 – 60, 80:20:0 to 40:60:0; 60 – 80, 40:60:0 to 0:100:0; 80 – 85, 0:100:0; 85 – 87, 0:100:0 to 0:20:80; 87 – 90, 0:20:80 to 80:20.

ii) Semi-prep. HPLC (90 min run). 0.0G-4252-NO Luna 5u C18(2) 100A 250 × 10 mm i.d. column at 20 °C protected with a Phenomenex AJ0-7220 250 × 10 mm i.d. pre-column was used with a flow rate as a function of time: 0 – 5 min: 1 – 4 ml min⁻¹; 5 – 90 min: 4 ml min⁻¹. Solvent A: 4 mM aq. AcONH₄, solvent B: MeOH with AcONH₄ (c = 4 mM), solvent C: H₂O; solvent composition (A/B/C) as a function of time (0 – 90 min): 0 – 5, 80:20:0; 5 – 60, 80:20:0 to 40:60:0; 60 – 80, 40:60:0 to 0:100:0; 80 – 85, 0:100:0; 85 – 87, 0:100:0 to 0:20:80; 87 – 90, 0:20:80 to 80:20.

iii) Semi-prep. HPLC (70 min run). 0.0G-4252-NO Luna 5u C18(2) 100A 250 × 10 mm i.d. column at 20 °C protected with a Phenomenex AJ0-7220/1 C18 250 × 10 mm i.d. pre-column was used with a flow rate as a function of time: 0 – 5 min: 1 – 4 ml min⁻¹; 5 – 70 min: 4 ml min⁻¹. Solvent A: 50 mM aq. potassium phosphate buffer (pH 7.0), solvent B: MeOH, solvent C: H₂O; solvent composition (A/B/C) as a function of time (0 – 70 min): 0 – 5, 80:20:0; 5 – 50, 80:20:0 to 47:3:52:7.0; 50 – 55, 47:3:52:7.0 to 0:52:7:47:3; 55 – 60, 0:52:7:47:3 to 1:00:0; 60 – 65, 0:100:0; 65 – 67, 1:00:0 to 0:20:80; 67 – 70, 0:20:80 to 80:20.

LC/MS. i) Pre-Purification of Minor Fractions Pd-NCC-54 and Pd-NCC-71 on an anal. HPLC. Minor catabolite fractions were first purified by HPLC (Agilent 1260 Infinity; Agilent Technologies, Santa Clara, California, USA) according to following procedure: 3 – 4 g of leaf material were ground in mortar and pestle under liquid N₂ with addition of ca. 1 g of sand, a tip of a spatula of CaCO₃ and 4 – 5 ml of MeOH. The mixture was centrifuged (6 min, 7200 g, 4 °C) and the supernatants were stored at −80 °C until use. An aliquot of the supernatant was centrifuged (1 min at 7200 g), diluted (1:1 v/v) with aq. potassium phosphate buffer (50 mM, pH 7.0) and centrifuged again (1 min at 7200 g, 4 °C). In total 300 µl (3 × 100 µl) of the supernatant were purified on the anal. HPLC (pre-column: Phenomenex SecurityGuard Cartridge C18, 4 × 3 mm; column: Phenomenex HyperClone 5 µm, ODS C18 120A; 250 × 4.6 mm; column temp., 20 °C) at a flow rate of 0.5 ml min⁻¹ using 50 mM aq. K₂PO₄ as solvent A and MeOH as solvent B (0 – 110 min: 0 – 5, 80:20; 5 – 80, 80:20 to 30:70; 30:70 to 0:100; 85 – 95, 0:100; 95 – 100, 0:100 to 80:20; 100 – 110, 80:20), and desired fractions were collected and combined.

ii) LC/MS Analysis of minor fractions of the collected HPLC fractions were analyzed on an LC/MS system (Thermo Fisher, Accela 1250 pump, Accela PDA detector, TSQ Quantum Access Max) using AcONH₄ buffer (4 mM, solvent A) and MeOH (LC/MS gradient grade, solvent B) as eluents (pre-column: Phenomenex Security Guard Cartridge C18, 4 × 3 mm; Column: Phenomenex HyperClone column, 5 µm, ODS C18 120A; 250 × 4.6 mm; column temp., 25 °C). Twenty microliter of the collected catabolite fraction were injected and analyzed at a flow rate of 0.5 ml min⁻¹ (0 – 57 min: 0 – 5, 80:20; 5 – 30, 80:20 to 30:70; 30 – 35, 30:70 to 0:100; 35 – 50, 0:100; 50 – 51, 0:100 to 80:20; 51 – 57, 80:20).

Spectroscopy. UV/VIS Spectra: Agilent Technologies Cary 60 spectrophotometer, λmax (nm) (rel. c). CD Spectra: Jasco J715, λmax and λmin (nm), Δε. 1H and 13C-NMR: Bruker 600 MHz Avance II+, δ(C/H2O) (δ(C/H2O) 3.31 ppm, and δ(C/H2O) 49.0 ppm, δ in ppm), J in Hz. Mass Spectrometry: Finnigan LCQ Classic, electrospray ionization (ESI) source, positive-ion mode, 4.5 kV spray voltage (rel. abundance).

Analysis of Chl-Catabolites in Senescent Leaves by HPLC

Senescent plum tree leaves were harvested in November 2013 from a commercial orchard in Aldino (South Tyrol). They were immediately frozen in a freezer (−80 °C) and transported in a cold box (−20 °C) to Innsbruck, where they were stored cold (−80 °C).

A leaf segment (with the area of about 20 cm²) was frozen in liquid N₂, ground in a mortar and extracted with 1 ml of MeOH. The resulting suspension was centrifuged for 3 min at 13,000 g. Five hundred microliter of the MeOH supernatant were diluted with 2 ml of 50 mM aq. potassium phosphate buffer (pH 7.0). After centrifugation for 3 min at 13,000 g, 200 µl of the extract was analyzed by HPLC (see Fig. 2).

Isolation and Structure Elucidation of Pd-NCC-32 (1). Yellow-greenish senescent plum tree leaves (18.7 g) were frozen in liquid N₂, pulverized to a fine powder and extracted with 60 ml of MeOH. The suspension was centrifuged for 5 min at 4000 g. Forty-two milliliters of the supernatant were diluted with 168 ml of 50 mM aq. potassium phosphate buffer (pH 7.0). After centrifugation for 5 min at 4000 g, the soln. was extracted two times with hexane. The MeOH extract was diluted with 300 ml of 50 mM potassium phosphate buffer (pH 7.0) and applied to a pre-conditioned
5 g SepPak cartridge. This was washed with 35 ml of H₂O and the NCC-containing fraction was eluted with 30 ml of MeOH. The solvents were removed by using a rotary evaporator. The residue was dissolved in 1 ml of MeOH and 4 ml of 50mM aq. potassium phosphate buffer (pH 7.0) using an ultrasonic bath. After centrifugation for 3 min at 13,000 g, the sample was divided in four aliquots and applied to semi-prep. HPLC; injection volume, 1.25 ml; flow rate, 0 – 5 min: 1 – 4 ml min⁻¹, 5 – 90 min: 4 ml min⁻¹; solvent A: 4mM aq. AcONH₄, solvent B: MeOH with AcONH₄ (c = 4mM), solvent C: H₂O; solvent composition (A/B/C) as a function of time (0 – 90 min): 0 – 5, 80:20; 5 – 60, 80:20 to 40:60; 60 – 80, 40:60 to 0:100; 80 – 85, 0:100 to 0:20; 87 – 90, 0:20 to 80:20. Fractions containing Pd-NCC-32 (1), Pd-NCC-35 (2), Pd-NCC-40 (3), Pd-NCC-56 (4) and Pd-NCC-60 (5) were collected and to obtain pure samples from all fractions an anal. HPLC run with AcONH₄ had to be performed; injection volume, 200 μl; flow rate, 0.5 ml min⁻¹; solvent A: 4mM aq. AcONH₄, solvent B: MeOH with AcONH₄ (c = 4mM), solvent C: H₂O; solvent composition (A/B/C) as a function of time (0 – 90 min): 0 – 5, 80:20; 5 – 60, 80:20 to 40:60; 60 – 80, 40:60 to 0:100; 80 – 85, 0:100 to 0:20; 87 – 90, 0:20 to 80:20. In each anal. HPLC run, the desired catabolite was collected.

Spectroscopic data (for atom numbering)

**Pd-NCC-32 (1)**, ₜᵣ = 32.6 min. UV/VIS (MeOH, c = 4.3 × 10⁻⁵M): 244sh (0.83), 314 (1.00) (see Fig. 9).

CD (MeOH, c = 4.3 × 10⁻⁵M): 226 (8), 249 (3), 263 (–3), 283 (–8), 319 (1). ¹H-NMR (600 MHz, CD₂OD, 10 °C): 1.92 (s, Me(13)); 2.08 (s, Me(17)); 2.12 (s, Me(7)); 2.23 (s, Me(1)); 2.26 – 2.30 (m, H₆-C(12)); 2.31 – 2.35 (m, H₆-C(12)); 2.37 – 2.41 (m, H₆-C(15)); 2.60 – 2.65 (m, CH₃(13)); 2.71 – 2.77 (m, H₆-C(12)); 2.91 (dd, J = 4.0, 14.6, H₆-C(15)); 3.17 (dd, J = 7.8, 9.2, H-C(2)); 3.21 (dd, J = 7.8, 9.2, H-C(2)); 3.24 – 3.28 (m, H-C(4′), H-C(4′)); 3.33 – 3.39 (m, H₆-C(3′), H-C(4′), H-C(5′), H-C(5′)); 3.62 – 3.69 (m, H₆-C(3′)); 3.71 (dd, J = 5.8, 12.0, H₆-C(18)); 3.75 (s, Me(8)); 3.83 – 3.87 (m, H₆-C(6)); 3.96 – 4.01 (m, H₆-C(15), H₆-C(18)); 4.02 – 4.09 (m, H-C(16), H₆-C(5)); 4.17 (dd, J = 7.8, H-C(1′)); 4.33 (dd, J = 7.8, H-C(1′)); 4.77 (dd, J = 3.5, 7.6, H-C(18)); 4.87 (s, H-C(10)); 9.32 (s, H-C(20)). ¹³C-NMR (¹³C-signal assignment from ²H-¹³C-HSQC and ¹H-¹³C-HMBC experiments in CD₂OD, 10 °C): 8.4 (C(2′)); 9.1 (C(7′)); 9.1 (C(13′)); 12.5 (C(17′)); 22.0 (C(12′)); 23.6 (C(5)); 24.8 (C(3′)); 29.7 (C(15)); 36.9 (C(10)); 39.6 (C(12)); 52.7 (C(8)); 62.1 (C(6)); 62.1 (C(6)); 62.5 (C(16)); 66.6 (C(18)); 67.7 (C(8′)); 70.3 (C(3′)); 71.1 (C(4′)); 71.1 (C(4′)); 73.2 (C(18′)); 74.8 (C(2′)); 74.8 (C(2′)); 77.7 (C(3′)); 77.7 (C(5′)); 77.7 (C(5′)); 103.9 (C(1′)); 103.9 (C(1′)); 112.2 (C(7)); 114.8 (C(13)); 120.0 (C(3)); 120.0 (C(12)); 124.2 (C(11)); 124.2 (C(14)); 125.6 (C(8)); 128.5 (C(11)); 133.9 (C(6)); 134.0 (C(2)); 139.6 (C(4)); 159.3 (C(17)); 130.2 (C(18)); 161.5 (C(9)); 171.4 (C(18)); 174.8 (C(19)); 177.2 (C(20)). ESI-MS: 1079.2 (6, [M − H + 2K⁺]); 1063.3 (12, [M − H + K + Na⁺]); 1047.3 (6, [M − H + 2Na⁺]); 1041.3 (57, [M + K⁺]); 1025.3 (73, [M + Na⁺]); 1005.1 (20, 1004.1 (53), 1003.1 (100, C₄₋₇H₆₋₈N₄O₊₂₀⁺, [M + H⁺]); 987.3 (7); 879.3 (7,
[M – C₈H₁₀O₅ + K⁺]; 863.4 (8, [M – C₈H₁₀O₅ + Na⁺]); 841.2 (36, [M – C₈H₁₀O₅ + H⁺]); 827.3 (4); 684.1 (3, [M – C₇H₁₁NO₃ – C₄H₁₀O₅ + H⁺]); 679.2 (4, [M – 2 C₈H₁₀O₅ + H⁺]).

**Pd-NCC-35 (2).** τᵣ = 35.5 min. UV/VIS (4mm aq. AcONH₄/MeOH 63:37): 284 (0.75), 316 (1.00). ESI-MS: 879.3 (15, [M + K⁺]); 863.3 (37, [M + Na⁺]); 843.2 (15, [M + Na⁺]); 842.2 (47), 841.2 (100, C₄₁H₅₃N₄O₧⁺, [M + H⁺]); 825.3 (6); 809.3 (4, [M – CH₂O + H⁺]); 684.2 (8, [M – C₇H₁₁NO₃ + H⁺]); 679.2 (6, [M – C₈H₁₀O₅ + H⁺]); 522.1 (1, [M – C₇H₁₁NO₃ – C₄H₁₀O₅ + H⁺]).

**Pd-NCC-40 (3).** τᵣ = 40.6 min. UV/VIS (4mm aq. AcONH₄/MeOH 59:41): 278 (0.78), 316 (1.00). ESI-MS: 755.1 (8, [M – H + 2K⁺]); 739.2 (7, [M – H + K + Na⁺]); 717.3 (71, [M + K⁺]); 701.3 (46, [M + Na⁺]); 681.2 (10), 680.1 (40); 679.2 (100, C₃₅H₄₃N₄O₁₅⁺, [M + H⁺]); 647.2 (12, [M – CH₂O + H⁺]); 522.1 (5, [M – C₁₇H₁₁NO₃ + H⁺]).

**Pd-NCC-54.** UV/VIS (4mm aq. AcONH₄/MeOH 40:60): 316 nm. ESI-MS: 699.2 (14, [M + K⁺]); 678.2 (16, [M + NH₄⁺]); 663 (4), 662 (32), 661.2 (100, C₃₅H₄₁N₄O₁₆⁺, [M + H⁺]); 629.3 (3, [M – CH₂O + H⁺]).

**Pd-NCC-56 (4).** τᵣ = 56.4 min. UV/VIS (4mm aq. AcONH₄/MeOH 47:53): 316 nm. ESI-MS: 845.3 (23, [M + K⁺]); 829.3 (26, [M + Na⁺]); 809.2 (15), 808.2 (46), 807.2 (100, C₄₁H₅₃N₄O₁₅⁺, [M + H⁺]); 775.3 (10, [M – CH₂O + H⁺]); 684.2 (5, [M – C₇H₉NO + H⁺]); 645.2 (13, [M – C₈H₁₀O₅ + H⁺]).

**Pd-NCC-60 (5).** τᵣ = 60.0 min. UV/VIS (4mm aq. AcONH₄/MeOH 44:56): 315 (1.00). ESI-MS: 683.2 (15, [M + K⁺]); 667.3 (22, [M + Na⁺]); 647.2 (11), 646.2 (39), 645.2 (100, C₃₅H₄₁N₄O₁₆⁺, [M + H⁺]); 613.2 (19, [M – CH₂O + H⁺]); 522.1 (8, [M – C₇H₉NO + H⁺]).

Identification of Pd-NCC-60 (5) and Cj-NCC-1[33] by HPLC co-injection experiment; separate samples of purified Pd-NCC-60 (5), of Cj-NCC-1, as well as a 1:1 mixture of both were analyzed by anal. HPLC (see Fig. 10).

**Pd-NCC-71 (6).** τᵣ = 70.7 min. UV/VIS (50mm aq. potassium phosphate buffer (pH 7.0)/MeOH 20:80): 239sh (1.00), 316 (0.87). ESI-MS: 881.1 (18, [M + K⁺]); 865.3 (14, [M + Na⁺]); 827.3 (37, [M + Na⁺]); 810.2 (50, [M – CH₂O + H⁺]); 506 (5, [M – C₇H₉NO + H⁺]). Provisional identification of Pd-NCC-71 (6) with Cj-NCC-2[13] by HPLC co-injection experiment; an extract of a plum tree leaf containing Pd-NCC-71 (6), a separate sample of purified Cj-NCC-2, as well as a mixture of both were analyzed by anal. HPLC.

**Pd-YCC-61.** τᵣ = 61.3 min. UV/VIS (50mm aq. potassium phosphate buffer (pH 7.0)/MeOH 40:60): 246 (0.73), 313 (1.00), 429 (1.77). ESI-MS: 881.1 (18, [M – H + 2K⁺]); 865.3 (14, [M – H + K + Na⁺]); 843.1 (52, [M + K⁺]); 827.3 (37, [M + Na⁺]); 807.2 (17), 806.1 (48), 805.1 (100, C₄₁H₄₉N₄O₁₅⁺, [M + H⁺]); 796.6 (16), 774.4 (26), 756.3 (17), 700.3 (15), 643.1 (17, [M – C₆H₁₀O₅ + H⁺]); 611.3 (5, [M – C₈H₁₀O₅ – CH₂O + H⁺]).

**Pd-YCC-67.** τᵣ = 67.1 min. UV/VIS (50mm aq. potassium phosphate buffer (pH 7.0)/MeOH 25:75): 247
Figure 10. Identification of Pd-NCC-60 (5) with Cj-NCC-1 by HPLC. Samples a): of Cj-NCC-1; b): of Pd-NCC-60 (5); c): 1:1 mixture of Pd-NCC-60 (5) and Cj-NCC-1.

(0.74), 316 (1.00), 428 (1.29). ESI-MS: 719.1 (2, [M – H + 2K]+); 703.2 (8, [M – H + K + Na]+); 681.1 (12, [M + K]+); 665.3 (26, [M + Na]+); 645.2 (11), 644.2 (40), 643.2 (100, C35H39N4O8, [M + H]+); 611.2 (19, [M – CH3O + H]+).

Pd-PICC-75. tR = 75.0 min. UV/VIS (50mm aq. potassium phosphate buffer (pH 7.0)/MeOH 1:9): 314 (0.75), 525 (1.00).

Provisional identification of Pd-PICC-75 in an extract of a plum tree leaf and of a purified sample of Cj-PICC[29] by HPLC (separate runs and co-injection experiment).

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