Blue Luminescent Leaves

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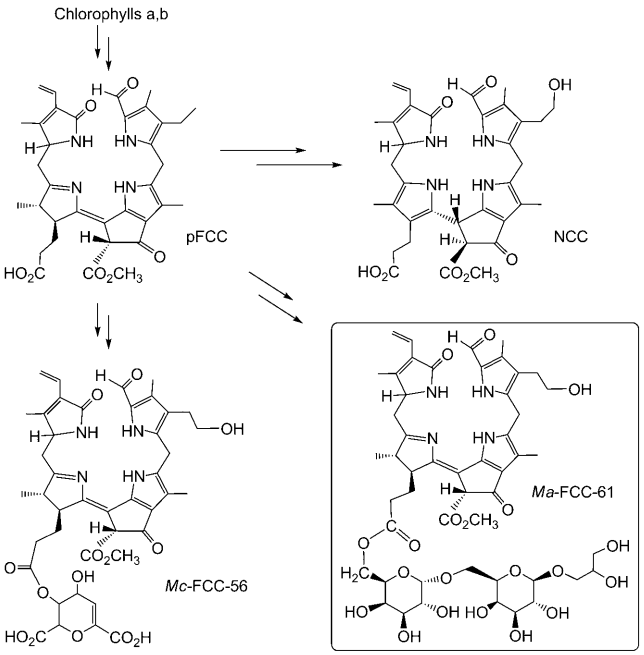
Hypermodified Fluorescent Chlorophyll Catabolites: Source of Blue Luminescence in Senescent Leaves**

*Srinivas Banala, Simone Moser, Thomas Müller, Christoph Kreutz, Andreas Holzinger, Cornelius Lütz, and Bernhard Kräutler**

Dedicated to Professor Albert Eschenmoser on the occasion of his 85th birthday

Breakdown of chlorophyll is the characteristic visual sign of leaf senescence.[1,2] In the past two decades it has been shown that the chlorophyll in degreening leaves is degraded to colorless and nonfluorescent chlorophyll catabolites (NCCs).[2–5] In apples and pears chlorophylls are also broken down to NCCs that are identical to those from senescent leaves of the corresponding fruit trees.[6] Chlorophyll breakdown was thus suggested to follow a common path in senescence and fruit ripening, and to yield NCCs as its "final" product (Scheme 1).[4,7]

In contrast, fluorescent chlorophyll catabolites (FCCs)[5] accumulate in senescent, yellow banana leaves, and NCCs occur only in traces in their extracts (Figure 1 and Figure S1 in the Supporting Information). Indeed, FCCs (such as pFCC) were observed earlier in senescent leaves, but only as short-lived precursors of NCCs.[8,9] In ripening bananas, "persistent" FCCs such as *Mc*-FCC-56 (Scheme 1)[10] have been discovered only recently; these are the compounds responsible for bananas' blue luminescence. The FCCs from banana leaves (*Ma*-FCCs) differ from their relatives in fruit peels (Figure 1), but appear to be "persistent" also and to feature complex propionyl ester functions.[10] The structure of one of them, *Ma*-FCC-61, was elucidated as a tetrapyrrole with an unprecedented digalactosylglyceryl moiety (Scheme 1).



Scheme 1. Structural formulae of representative chlorophyll catabolites from higher plants.[5] Hypermodified *Ma*-FCC-61 from leaves of banana (*Musa acuminata*) is highlighted.

HPLC analysis of an extract of senescent leaves of bananas (*Musa acuminata*, "cavendish" cultivar) indicated a variety of (*Ma*-)FCCs. An abundant and relatively polar FCC was observed at a retention time of 61 min, and was provisionally termed *Ma*-FCC-61. From a 42 g sample of a yellow banana leaf we isolated 4.2 mg of *Ma*-FCC-61 by HPLC which we characterized by UV/Vis and fluorescence spectra (see Figure S6 in the Supporting Information and Figure 1). The molecular formula of *Ma*-FCC-61 ($C_{50}H_{66}N_4O_{20}$) was deduced by HRMS from the observed molecular ion $[M+H]^+$ at m/z 1043.394 (calcd for $C_{50}H_{67}N_4O_{20}^+$: 1043.434).

The 600 MHz ^{1}H NMR spectrum of *Ma*-FCC-61 showed the set of the characteristic signals of the tetrapyrrole moiety, among them signals at low field due to a formyl and a vinyl group, three singlets and one doublet of four methyl groups at high field, as well as a sharp singlet of a methyl ester at 3.71 ppm (see Figure S2 in the Supporting Information). Detailed information on the constitution of *Ma*-FCC-61 was gained from multidimensional NMR spectroscopy (^{1}H,^{1}H COSY and ROESY spectra (Figure 2), as well as ^{1}H,^{13}C

[*] Dr. S. Banala,[+] Dr. S. Moser,[#] Dr. T. Müller, Dr. C. Kreutz, Prof. Dr. B. Kräutler
Institute of Organic Chemistry and Centre of Molecular Biosciences
University of Innsbruck, 6020 Innsbruck (Austria)
Fax: (+43) 512-507-2892
E-mail: bernhard.kraeutler@uibk.ac.at
Priv.-Doz. Dr. A. Holzinger, Prof. Dr. C. Lütz
Institute of Botany, Department of Physiology and Cell Physiology of Alpine Plants
University of Innsbruck, 6020 Innsbruck (Austria)
[+] Present address:
Technical University of Berlin
Department of Chemistry, Organic Chemistry
Straße des 17. Juni 124/TC 2, 10623 Berlin (Germany)
[#] Present address:
Institute of Chemical Sciences and Engineering
École Polytechnique Fédérale de Lausanne
1015 Lausanne (Switzerland)

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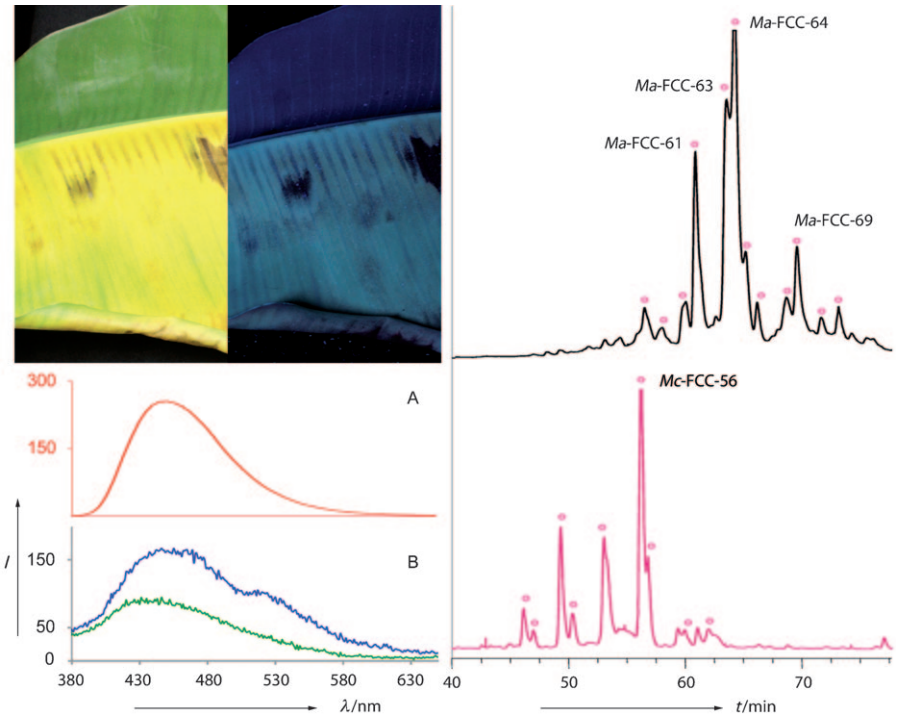

Figure 1. Top left: Photographs of a banana leaf with green and yellow sections, observed under day light (left) and under UV light (at 366 nm, right). Bottom left: Fluorescence spectra (with excitation at 366 nm) of a solution of *Ma*-FCC-61 in MeOH (panel A) and of green and yellow sections of the banana leaf (panel B, green and blue lines, respectively.). Right: HPLC analyses of FCCs in extracts from a yellow banana leaf (top) and from the peel of a yellow banana (bottom); excitation: 350 nm, detection: 450 nm, FCCs are labeled, major fractions specified.

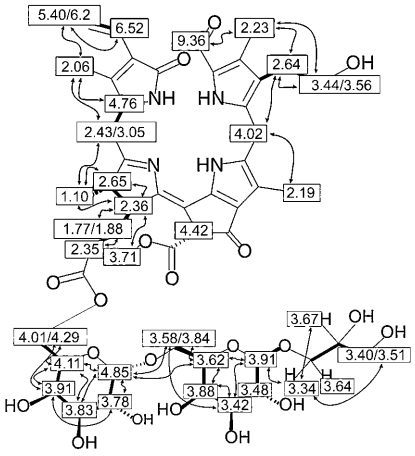

Figure 2. Chemical shift data of *Ma*-FCC-61 (CD_3OD, 10 °C) and a graphical representation of the derived molecular structure (see Scheme 1 for the structural formula of *Ma*-FCC-61). Structure elucidation with $^1H,^1H$ correlations; bold lines: bonds derived from COSY; arrows: correlations from ROESY. See Figure S3 in the Supporting Information for additional $^1H,^{13}C$ correlations.

HSQC and HMBC-spectra (see the Supporting Information).[11] The signals of all of the 35 non-exchangeable protons of the tetrapyrrole unit could be assigned, establishing the core structure of the functionalized tetrapyrrole (see, for example, Ref. [12]). Additional signals of 19 hydrogen atoms were observed in the intermediate field region of the ^1H NMR spectrum of *Ma*-FCC-61, and were also analyzed by extensive $^1H,^1H$ and $^1H,^{13}C$ correlations. In this way we could identify (two) pyranose units and a glycerol moiety as the intricate modification of *Ma*-FCC-61.

A $^1H,^{13}C$ correlation between the carbonyl carbon and one of the 6′-hydrogens of the directly bound pyranose unit indicated that the propionyl side chain of the tetrapyrrole core is the attachment site of the novel functionalized disaccharide unit. The assignments of both pyranose units as derived from galactose, as well as the relative configuration of their intermodular linkages at the anomeric centers (as 6′-α and 6″-β), were based on analysis of the values of the $^1H,^1H$ coupling constants within the two pyranose units, and comparison of the ^1H and ^{13}C chemical shift data with that of reference compounds (see the Supporting Information). The assignment was supported by data from $^1H,^1H$ ROESY spectra. *Ma*-FCC-61 was found to bear a propionyl ester function with a 6-α-galactopyranosyl-(1→6)-β-galactopyranosyl-(1→1)-glyceryl unit as well as a hydroxy group at the 8^2 position of its tetrapyrrolic FCC core. Thus, *Ma*-FCC-61 is a $3^1,3^2$-didehydro-8^2-hydroxy-13^2-(methoxycarbonyl)-17^3-[6′-α-galactopyranosyl-(1′→6″)-β-galactopyranosyl-(1″→1‴)-glyceryl)]-1,4,5,10,17,18,20,22-octahydro-4,5-seco-(22 H)-phytoporphyrin (Figure 2 and Scheme 1).

Ma-FCC-61 is a new type of multiply functionalized natural tetrapyrrole and a new representative of the "persistent" and "hypermodified" FCCs. These unique tetrapyrroles carry complex ester functions at the propionyl side chain and have turned up, so far, in ripening or senescent parts of the banana plant (for a second new case, see below).[5,10,13] The presence of ester functions at the propionyl side chain in the "persistent" *Ma*-FCCs explains the stunning lack of NCCs in the extracts of the banana leaves (see Figure S1 in the Supporting Information), since a free propionic acid function is required for the rapid formation of NCCs from FCCs by a stereoselective, acid-catalyzed isomerization.[9,12]

While there is no precedence for an esterification of a chlorophyll catabolite with a (di)galactosyl unit, as observed here for *Ma*-FCC-61, related chlorophyll analogues occur in the coccolithophore *Emiliana huxleyi*.[14] The light-harvesting porphyrinoids ("chlorophylls c") from this ubiquitous marine photo-autotroph display a galactosyldiacylglyceride ester, which appears to replace the phytol ester of the chlorophylls in a functional way.[14] In *Ma*-FCC-61 the 6-α-galactopyranosyl-(1→6)-β-galactopyranosyl-(1→1)-glyceryl moiety repre-

sents the core structure of digalactosyldiacylglycerides, ubiquitous membrane components of the thylakoids and elsewhere in plant leaves.[16] This digalactosylglyceryl unit is bound strongly by lipases that hydrolyze the ester functions of digalactosyldiacylglycerides with loss of the polar head.[17] *Ma*-FCC-61 may thus be a building block for further assembly (or an adventitious cleavage product) of more complex, so far unidentified pigments.

At present, a physiological function of the ubiquitous linear tetrapyrroles that arise from breakdown of chlorophyll is unknown. This is remarkable, as the structurally related tetrapyrroles from heme breakdown have important roles (e.g. biliverdin and phycobilins in plants and algae, biliverdin, and bilirubin in higher animals).[18–20] Chlorophyll breakdown may, first of all, be a detoxification process.[21] However, the ubiquitous, but invisible NCCs have been shown to be effective antioxidants of possible physiological benefit in fruit.[6] For the structurally similar FCCs, beneficial effects on the viability of the banana peels have also been taken into consideration.[10] The biosynthetic esterification of typical FCCs of banana plants and the intriguing disaccharide moiety in *Ma*-FCC-61, in particular, now draw attention to possible physiological roles of FCCs and to their further endogenous use. Clearly, as FCCs absorb UV irradiation efficiently and emit blue light instead, they are "optical brighteners" to the human eye,[22] and they may also have a role as endogenous "sun screens"[23] for UV light.

In senescent banana leaves chlorophyll breakdown deviates from the apparently "common" path in higher plants[4] and appears to stop at the stage of the blue-luminescent FCCs. FCCs were found to accumulate likewise and to induce blue luminescence in bright yellow bananas[10] and in senescent sections of the peels of overripe bananas.[13] However, banana leaf FCCs (*Ma*-FCCs) differ from their analogues in banana peels, and (most) *Ma*-FCCs are less polar.[10] Mass spectrometric analysis of several *Ma*-FCCs also suggested complex modifications that involve the propionyl ester functions (see the Supporting Information). New variants of the natural "persistent" FCCs thus accumulate in senescent banana leaves and make them luminesce blue. This remarkable discovery with banana leaves is not unique: accumulation of FCCs was also found (recent preliminary observation) in yellow leaves of the peace lily (*Spathiphyllum wallisii*),[13] a subject of ongoing work in our labs.

To learn about the spatial distribution of *Ma*-FCCs in senescent banana leaves, studies by light and fluorescence microscopy were carried out. Analysis of a leaf cross section revealed blue-luminescent material in senescent palisade parenchyma cells (Figure 3c). These cells thus appear to be the most likely location for the accumulation of the FCCs. Thicker cell walls, for example, of the epidermis, xylem tracheids, and fibers, also show blue fluorescence, which is generally ascribed to cell wall components.[23,24] Likewise, green banana leaves showed blue fluorescence in cell walls, but they lacked the marked blue luminescence in the lumen of the green cells (Figure S4 in the Supporting Information).

In the yellow banana leaf, red chlorophyll fluorescence was found in the largely intact chloroplasts of guard cells (of the stomata)[25] as well as in some parenchyma cells (Figure 3b). Additional yellow spots are assumed to be due to putative lipid droplets, as also depicted in Figure 3b. Indeed, osmiophilic globules were seen in senescent yellow banana leaves in transmission electron microscopic images, supporting this assumption (Figure S5 in the Supporting Information). In senescent leaves, chloroplasts transform into gerontoplasts in a process characterized by loss of thylakoid membranes and by massive accumulation of lipid-containing plastoglobules.[26] Eventual rupture of the gerontoplast envelope leads to a release of plastoglobules to the cytoplasm,[27] causing lipid droplets, as observed in this study. A similar process may occur in peels of ripe bananas during chromoplast differentiation.[28]

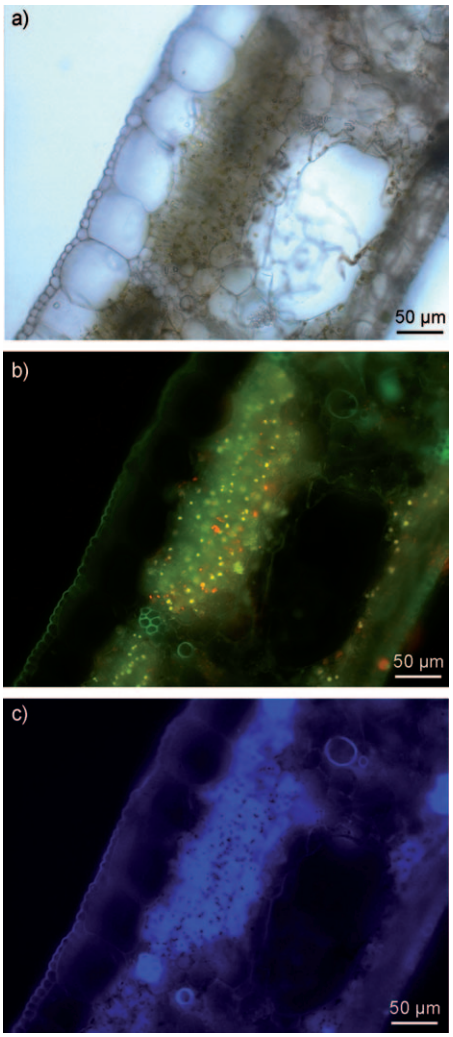

Figure 3. Light and fluorescence microscopy visualization of a cross-section of a yellow, senescent banana leaf. Bright-field (a) and fluorescence microscopic (b,c) images are shown; b) excitation at (470 ± 20) nm and emission at $\lambda > 515$ nm; c) excitation at (365 ± 12) nm and emission at $\lambda > 397$ nm.

When leaves of plants de-green and when fruits ripen, they develop fascinating colors (fall colors of deciduous trees and bushes[29]), provoking basic biological[21] and ecological questions.[30] However, strongly luminescent senescent leaves are known in only a few plants, one of which is *Ginkgo*

biloba.[31] Luminescence of its leaves is due to an unsaturated alkaloid.[31] Bright colors of fruit are believed to have evolved as valuable signals to attract frugivores, needed for seed dispersal.[32,33] Indeed, the blue luminescence of ripe bananas may fulfill such a role.[10] "Fruit flagging"[34] by colorful (and possibly by luminescent) leaves may be an additional optical signal of fruit-bearing plants. Perception of the bright and distinctive colors of both, leaves and fruit, may thus help guide the crucial interactions of plants with insects, birds, and other animals.[32,33]

We describe the observation of blue luminescence of senescent banana leaves. Its main contributors were identified as new types of "persistent" FCCs and uniquely "hypermodified" tetrapyrroles. This discovery, first of all, contrasts the earlier notion of a "common" basic path of chlorophyll breakdown in senescent leaves, in which NCCs were seen as the final products of a rapid and directed metabolic detoxification process.[4,21] It also draws attention to possible physiological roles of FCCs and other chlorophyll catabolites. Furthermore, as endogenous products and luminescent signals of cells undergoing programmed death, "persistent" FCCs commend themselves as natural molecular markers of senescence, which open a new noninvasive view into cellular processes in leaves and fruit.

Experimental Section

Isolation and spectroscopic characterization of *Ma*-FCC-61: Fresh yellow sections of de-greened banana leaves were collected (sample size about 42 g) and frozen in liquid nitrogen. Extraction and further purification by semipreparative HPLC (as described in the Supporting Information) gave crude *Ma*-FCC-61, which was desalted and purified further by semipreparative HPLC. The fraction containing *Ma*-FCC-61 was again desalted and dried in vacuo. A pure sample of *Ma*-FCC-61 (4.2 mg) was obtained, which was used for recording spectra: UV/Vis (MeOH): λ_{max} ($I_{rel.}$): 379 sh (0.482), 358.5 (0.692), 316.5 (1.0). Fluorescence (MeOH, excitation at 360 nm): λ_{max} at 446 nm (see Figure 1). High-resolution mass (HRMS, see the Supporting Information for details).

Photographs were taken with a mounted digital camera (Canon EOS 450D) and irradiation with day light (exposure time 0.167 s) or UV light (UV lamp Benda NU-15 KL, 15 W, nominal emission at 366 nm, exposure time 10 s). Light and fluorescence microscopy: Cross sections of a senescent yellow part of a banana leaf were cut with a razor blade. Sections were viewed with a Zeiss Axiovert 200 M microscope equipped with a Zeiss Axiocam MRc5 (Carl Zeiss AG, Jena, Germany). For fluorescence images either filter set 01 (excitation BP 365/12 nm, emission LP 397 nm) or filter set 09 (excitation BP 450–490 nm, emission LP 515 nm) was used.

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