Structures of Chlorophyll Catabolites in Bananas (Musa acuminata) Reveal a Split Path of Chlorophyll Breakdown in a Ripening Fruit

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Dedicated to Professor Hugo Scheer on the occasion of his 70th birthday.

Abstract: The disappearance of chlorophyll is a visual sign of fruit ripening. Yet, chlorophyll breakdown in fruit has hardly been explored; its non-green degradation products are largely unknown. Here we report the analysis and structure elucidation of colorless tetrapyrrolic chlorophyll breakdown products in commercially available, ripening bananas (Musa acuminata, Cavendish cultivar). In banana peels, chlorophyll catabolites were found in an unprecedented structural richness: a variety of new fluorescent chlorophyll catabolites (FCCs) and nonfluorescent chlorophyll catabolites (NCCs) were detected. As a rule, FCCs exist only “fleetingly” and are hard to observe. However, in bananas several of the FCCs (named Mc-FCCs) were persistent and carried an ester function at the propionate side-chain. NCCs were less abundant, and exhibited a free propionic acid group, but functional modifications elsewhere. The modifications of NCCs in banana peels were similar to those found in NCCs from senescent leaves. They are presumed to be introduced by enzymatic transformations at the stage of the mostly unobserved, direct FCC-precursors. The observed divergent functional group characteristics of the Mc-FCCs versus those of the Mc-NCCs indicated two major “late” processing lines of chlorophyll breakdown in ripening bananas. The “last common precursor” at the branching point to either the persistent FCCs, or towards the NCCs, was identified as a temporarily abundant “secondary” FCC. The existence of two “downstream” branches of chlorophyll breakdown in banana peels, and the striking accumulation of persistent Mc-FCCs call for attention as to the still-elusive biological roles of the resulting colorless linear tetrapyrroles.

Keywords: catabolite · chlorophyll · fruit · luminescence · pigment · structure elucidation

Introduction

Breakdown of chlorophyll is a major contributor to the diagnostic color changes in fall leaves.[1] De-greening and the typical changes of the colors of leaves are considered, first of all, as visible symptoms of senescence.[2, 3] A form of programmed cell-death in plants.[4] Many fruit also de-green during ripening and degradation of chlorophyll is an easily observed sign of ripening.[5]

In spite of broad research efforts, the “disappearance” of chlorophyll was a biological puzzle for a long time,[6] since its products remained concealed.[7] Indeed, a breakthrough in this area was provided by the identification and structure elucidation of a colorless tetrapyrole in senescent leaves of barley (Hordeum vulgare), named Hv-NCC-L.[1, 4] Meanwhile, identification of the structures of relevant chlorophyll catabolites,[9, 10] of key enzymes and their genes,[11–13] has resulted in general insights into the basic processes involved in chlorophyll breakdown in higher plants.[13, 14] Colorless “non-fluorescent chlorophyll catabolites” (NCCs) are typically found (to accumulate) in senescent leaves and have been suggested to represent (with notable exceptions[15–19]) the “final stage” of chlorophyll breakdown (see Figure 1).[13] Likewise, ripening apples and pears contained known NCCs, suggesting a “common path” of chlorophyll breakdown in leaf senescence and fruit ripening.[5, 20] Surprisingly, in (the peels of) ripening bananas (Musa acuminata, Cavendish cultivar) chlorophylls fade to give fluorescent chlorophyll catabolites (FCCs) mainly, and yellow bananas glow blue, when analyzed under UV light.[15, 20]
The “disappearance” of chlorophyll in ripening bananas has attracted considerable interest, as expected in view of the important commercial role of bananas. In one such study, two independent degradation pathways of the enzymatic breakdown of chlorophyll in Cavendish bananas were suggested. One of them was proposed to proceed through the so-called “Type I” degradation path leading to modified-green chlorophylls, whereas non-green catabolites were not identified in that study. Other recent reports dealt with the gene expression in the peels of ripening bananas. So far, enzymes assigned to proper de-greening reactions have remained elusive. However, in one of these studies, an NCC was tentatively identified by a UV spectrum, and was named M-NCC-1. A closer look at the degradation of chlorophyll in ripening bananas revealed a stunning variety of colorless chlorophyll catabolites (FCCs), which accumulated in the peels of ripe bananas. They had unprecedented complex structures and were typified as “hyper-modified” FCCs (hm-FCCs). Part of chlorophyll catabolism in bananas was thus indicated to deviate from the suggested “common” path of chlorophyll breakdown, which furnishes nonfluorescent chlorophyll catabolites (NCCs) as its typical “final” tetrapyrrolic products. However, the structures of many of the presumed chlorophyll catabolites in bananas remains yet to be elucidated. We have identified most of these catabolites, as well as their structures and their changing occurrence during the ripening process, as reported here.

Results

Occurrence of chlorophyll catabolites in the peels of ripening bananas: Freshly prepared extracts of the peels of commercial ethylene-ripened bananas (Musa acuminata, Cavendish cultivar, short: Mc-bananas) were analyzed for their chlorophyll catabolites by high performance liquid chromatography (HPLC), with UV absorbance and fluorescence detection. At the early ripening stage, about a dozen Mc-FCCs were tentatively identified directly on the basis of their absorbance and fluorescence characteristics, in addition to ten Mc-NCCs (see Figure 2).

Three minor yellow fractions were also found, and typified as yellow chlorophyll catabolites (YCCs). To describe the major colorless chlorophyll catabolites from the banana peels, they were first classified as FCCs or as NCCs, and specified further as Mc-FCC-\( t_R \) or Mc-NCC-\( t_R \) (\( t_R \) is their retention time in minutes under the conditions of a standardized analytical HPLC experiment (see ref. [21, 22]).

The abundance of these chlorophyll catabolites varied according to the extent of the ripening of the bananas, as de-
scribed below. The distribution of the catabolites also varied locally, in particular near “senescence-associated” dark spots appearing at the stages of over-ripening.\[23\] From extracts of two batches of peels of freshly ripe bananas (65 and 99 bananas of Chiquita or Dole brands, weighing about 13 and 20 kg, respectively), the most abundant six FCC and seven NCCs fractions were collected and used for further spectroscopic characterization (see Figure 2, and Tables 1 and 2). The main FCC- and NCC-fractions were separated by two (sometimes by three) consecutive runs of preparative HPLC (see the Experimental Section), and their structures were analyzed, as described below.

**Fluorescent catabolites (Mc-FCCs) in extracts of the peels of freshly ripe bananas:** From our previous work on chlorophyll catabolites from the peels of ripe bananas, the surprising accumulation of four polar “hyper-modified” FCCs (hmFCCs) is known. These hmFCCs were provisionally named \(\text{Mc}-\text{FCC-46}, \text{Mc}-\text{FCC-49}, \text{Mc}-\text{FCC-53}\) and \(\text{Mc}-\text{FCC-56}\), and their structures were established in detailed spectroscopic studies (see Figure 3).\[21,22\] A less polar FCC, \(\text{Mc}-\text{FCC-62}\), was a significant further FCC-fraction in the peels of freshly bought bananas, which were still incompletely ripe (i.e., at \(r = 5\); see Figure 2). From the peels of 65 bananas (“Dole” brand), a pure sample of about 115 mg of \(\text{Mc}-\text{FCC-62}\) was obtained after three rounds of purification by preparative HPLC (see the Experimental Section). Mass spectrometric analysis of \(\text{Mc}-\text{FCC-62}\) revealed its molecular formula as \(\text{C}_{35}\text{H}_{40}\text{N}_{4}\text{O}_{8}\). \(\text{Mc}-\text{FCC-62}\) was thus indicated to contain only one oxygen atom more than a “primary” FCC (\(\text{pFCC}\))[29,30] and to be less complex than the more polar “hyper-modified” \(\text{Mc}-\text{FCCs}\) (e.g., \(\text{Mc}-\text{FCC-56}\)).\[21,22\] The structure of \(\text{Mc}-\text{FCC-62}\) was elucidated with the help of homo- and heteronuclear NMR spectroscopic investigations, which indicated a hydroxyl group at the terminal position of the ethyl substituent at C8 (see the Experimental Section, Figure 7). \(\text{Mc}-\text{FCC-62}\) was thus revealed to be a \(8\)-hydroxy-13\(^{-}\)-(methoxycarbonyl)-3\(^{-}\},3\(^{-}\},3\(^{-}\},3\(^{-}\},3\(^{-}\},3\(^{-}\},3\(^{-}\},3\(^{-}\})\text{-didehydro-1,4,5,17,18,20,22-octahydro-4,5-seco-(22H)}\)-phytoporphyrin (see Table 1 and Figure 3).

The least polar FCC named \(\text{Mc}-\text{FCC-71}\), which was detected in small amounts in fresh peel extracts (57 µg from 99 bananas), had a molecular formula of \(\text{C}_{36}\text{H}_{42}\text{N}_{4}\text{O}_{8}\). \(\text{Mc}-\text{FCC-71}\) was thus identified as the methyl ester of \(\text{Mc}-\text{FCC-62}\). As the natural occurrence of a methyl ester of an FCC was unprecedented, we examined the likelihood of the formation of \(\text{Mc}-\text{FCC-71}\) during work-up and spectral analysis. In fact, the same compound was formed (with partial deuteration) when \(\text{Mc}-\text{FCC-56}\), the major hmFCC, was dissolved...
in the NMR solvent CD$_3$OD and the sample was kept at room temperature for several days during spectral analysis. The NMR sample was analyzed and separated by HPLC: a less polar FCC formed, which had the characteristics of Mc-FCC-71. It exhibited, in particular, a $^1$H NMR spectrum similar to that of Mc-FCC-71 (from the original extract), except for the absence of the propionate methyl ester singlet at $\delta = 3.58$ ppm, as well as partial loss of the signals assigned to H$_2$C-20 and HC-1 (see Tables 1 and 2 for atom numbering). ESI-MS of the newly formed 

Nonfluorescent catabolites (Mc-NCCs) in extracts of the peels of ripe bananas: In fresh extracts of the peels of ripening Mc-bananas, ten NCC fractions were detected by analytical HPLC (solvent composition A1, see the Supporting Information) and classified tentatively, based on their UV spectra. Seven of the Mc-NCCs were isolated by preparative HPLC, and further analyzed by mass spectrometry and NMR spectroscopy (see the Experimental Section).

Mc-NCC-26 was the most polar NCC identified in extracts from yellow banana peels. From mass spectrometric analysis, the molecular formula of Mc-NCC-26 was deduced as C$_{36}$H$_{38}$N$_4$O$_{10}$ providing the basis for a tentative structure assignment. Mc-NCC-26 was indicated to have no ester function at C(13)$^b$, but a 3,3'-dihydroxyethyl group (at C3) and a 8'-hydroxyethyl group (at C8 of the tetrapyrrolic NCC core, see Table 2 and Figure 3).

A slightly less polar NCC, Mc-NCC-42, was a significant fraction in most extracts from peels of yellow bananas (0.89 $\mu$mol (600 $\mu$g) from 65 bananas; see Figure 2). Mass spectra indicated Mc-NCC-42 to have the molecular formula of C$_{38}$H$_{40}$N$_4$O$_{10}$. The molecular constitution of Mc-NCC-42 was elucidated with the help of homo- and heteronuclear NMR-correlations (see the Experimental Section, Figure 8). It was thus identified as a 3,3'-8'-trihydroxy-13,3'-methoxy-carbonyl-1,4,5,10,15,20,24-octahydro-4,5-seco-(22H,24H)-phytopyrophyrin, and was revealed to carry 3,3'-dihydroxyethyl and 8'-hydroxyethyl groups, respectively, as side chains at C-3 and C-8 of the NCCs tetrapyrrole core (see Table 2 and Figure 3). It thus had the same molecular constitution as Hv-NCC-1, the NCC originally isolated from senescent leaves of barley (Hordeum vulgare),[8,31] Mc-NCC-42 also had the same constitution as an NCC found in senescent leaves of spinach (Spinacia oleracea), called So-NCC-2, and described as the (C1)-epimer of Hv-NCC-1.[33] Indeed, the identity of Mc-NCC-42 with So-NCC-2 was deduced from a combination of its NMR data and by a HPL-chromatographic comparison (see below).

The next less polar NCC, Mc-NCC-49, a rather abundant fraction in most extracts, was isolated from peels of 99 yellow bananas (0.35 $\mu$mol (226 $\mu$g)). A molecular formula of C$_{38}$H$_{40}$N$_4$O$_{10}$ was deduced for Mc-NCC-49 by mass spectrometry. The molecular constitution of Mc-NCC-49 was again elucidated with the help of heteronuclear NMR spectroscopic investigations, which indicated it to be a 8'-hydroxy-13,3'-carboxyethyl-3,3'-didehydro-1,4,5,10,15,20,24-octahydro-4,5-seco-(22H,24H)-phytoporphyrin. The presence of one hydroxyl group at the side chain C8 (of the tetrapyrrolic NCC core), and of two carboxyl groups elsewhere, indicated Mc-NCC-49 (see Figure 3 and Table 2) to have the same molecular constitution as NCCs isolated from senescent leaves of oil seed rape (Brassica napus),[8,32] of Arabidopsis thaliana,[8,33] or of spinach (and then named So-NCC-3).[33]
A still less polar NCC, Mc-NCC-59, was a minor fraction in most extracts from peels of yellow bananas. Mass spectra revealed Mc-NCC-59 to have the molecular formula of \( \text{C}_{68}\text{H}_{68}\text{N}_{30}\text{O}_{3} \), that is, to be more complex than the other Mc-NCCs, listed above. The molecular constitution of Mc-NCC-59 was derived tentatively as a 8'-\beta-glucopyranosyl-13'-methoxy carbonyl-3',3'-didehydro-1,4,5,10,15,20,24-octahydro-4,5-seco-(22H,24H)-phophyrrin. Mc-NCC-59 would thus carry a hexose moiety (presumably a glucopyranose unit) at the hydroxyl group at the side-chain C8 of the tetrapyrrolic NCC core, as observed in other NCCs[9] (see Table 2). Mc-NCC-59 is thus suggested to have the same molecular constitution as NCCs isolated from senescent leaves of tobacco (Nicotiana rustica)[10] or of maize (Zea mais) (then named Zm-NCC-2)[17] (see Figure 3 and Table 2).

The least polar NCC, Mc-NCC-61, was a minor fraction in the extracts of yellow banana peels (0.47 \( \mu \text{mol} \) (302 \( \mu \text{g} \)) from 99 bananas). Mass spectrometry revealed Mc-NCC-61 to have the molecular formula \( \text{C}_{56}\text{H}_{56}\text{N}_{28}\text{O}_{28} \). The molecular constitution of Mc-NCC-61 was elucidated with the help of heteronuclear NMR spectroscopic investigations, which revealed it as a 8'-hydroxyl-13'-methoxycarbonyl-3',3'-didehydro-1,4,5,10,15,20,24-octahydro-4,5-seco-(22H,24H)-phophyrrin. Mc-NCC-61 is thus an isomer of the fluorescent catabolite Mc-FCC-62 (see above), and it has the same molecular constitution as NCCs analyzed previously,[15] for example, \( \text{C}_{57}\text{N}_{31} \)-1 from senescent leaves of the Katsura tree (Cercidiphyllum japonicum),[38,39] or of So-NCC-4 from spinach (Spinacia oleracea)[38] (see Figure 3 and Table 2).

Two further (very minor) polar components having the UV-spectral signature of NCCs were collected, which were named Mc-NCC-55 and Mc-NCC-58 (see Figure 2). These NCCs were identified by HPLC with the two main NCCs obtained from acid-induced isomerization of the \( \text{hmFCC} \) Mc-FCC-56 (see below and the Supporting Information, Figure S2–S5).

Slow acid-induced-isomerization of Mc-FCC-56 to Mc-NCC-55 and Mc-NCC-58: In an analytical experiment, a sample of raw Mc-FCC-56 (containing about 30% of its isomer Mc-FCC-53) was stored in an aqueous solution acidified to a pH of about 4, and was analyzed by HPLC. After 24 h about half of the \( \text{hmFCC} \) had converted; after 70 h complete conversion to two major NCCs was observed (see the Supporting Information, Figure S2). In a preparative experiment, a solution of Mc-FCC-56 (31 \( \mu \text{mol} \); containing about 30% of its isomer, Mc-FCC-53) in aqueous citric acid (0.02 mol) was stored for 159 h under an argon atmosphere in the dark at 15°C. Analysis of the resulting solution by HPLC indicated disappearance of FCCs and appearance of two major NCCs, which were isolated by preparative HPLC. The UV spectra of the two compounds confirmed them to be NCCs (see the Supporting Information, Figure S3), which were characterized as isomers of Mc-FCC-56 by their ESI-MS. They were further identified with Mc-NCC-58 and Mc-NCC-55, two fractions occurring naturally in extracts of banana peels (see Figure 2 and the Supporting Information, Figures S4 and S5). The less polar and more abundant Mc-NCC-58 exhibited a CD spectrum typical of the known natural NCCs.[39,40] The CD -spectrum of Mc-NCC-55 had nearly mirror-image properties to that of Mc-NCC-58 (see Figure 4 and the Experimental Section), indicating these two isomeric NCC to be C15 epimers.[40]

Identification of Mc-NCC-42 with an NCC from spinach leaves: Mc-NCC-42 was deduced to have the same molecular constitution as \( \text{Hv-NCC-1} \) and as So-NCC-2.[32] The latter two NCCs were assigned earlier to be (C1)-epimers, derived from \( \text{pFCC} \)[29] or from \( \text{epi-pFCC} \), respectively.[30,41] The pertinent NMR-data of Mc-NCC-42 were practically identical to those of So-NCC-2.[32] Nevertheless, the identity of Mc-NCC-42 with either \( \text{Hv-NCC-1} \) or So-NCC-2 was further tested by HPLC. Solutions of the three NCCs were separately analyzed by HPLC, as were 1:1 (v/v) mixtures of the solutions of Mc-NCC-42 with \( \text{Hv-NCC-1} \) and with So-NCC-2, respectively (see the Supporting Information, Figure S6). The HPL-chromatographic behavior of Mc-NCC-42 and of So-NCC-2 was the same, whereas that of Mc-NCC-42 and of \( \text{Hv-NCC-1} \) proved to be different.

Profile of abundance of natural FCCs during ripening of Mc-bananas: When bananas were bought at the early ripening stage (rs) 5 Mc-FCC-62 and Mc-FCC-56 represented the major FCC fractions observed in their peels, whereas the more polar Mc-FCC-53 was less abundant. On the following day, quantification of the major Mc-FCCs in the peels of the now fully yellow ripe bananas (i.e., at rs = 6–7)[25] indicated a higher total amount of the FCCs, but a significant decrease of the amount of Mc-FCC-62 (see Figure 5). In parallel, both of the esterified \( \text{hmFCCs} \) (Mc-FCC-56 and Mc-FCC-53) became more abundant. Further ripening through days two to four led to practically a complete disappearance.
of Mc-FCC-62. During the same time, the amount of Mc-FCC-56 also decreased, whereas, on day 2 and 3 (at rs = 7–8, when the formation of “senescence-associated” dark spots set in), the more polar and more highly functionalized Mc-FCC-53 underwent an increase, to be followed by a decrease on days three and four.

**Light- and fluorescence-microscopic analyses of green and de-greened banana peels:** Surface sections of banana peels of different stages of ripeness (see the Supporting Information, Figure S8) showed that green bananas (Figure S8A), bright yellow bananas (rs = 5–6) [20] (Figure S8B), ripe bananas with few brown spots (rs = 7–8) (Figure S8C), and overripe bananas (rs = 10–11) (Figure S8D) still retained intact chloroplasts in the guard cells of the stomata (Figure S8E–L), as visualized by their red fluorescence seen in the confocal laser-scanning microscopic images (Figure S8I–L). Remarkably, in the yellow sections of banana peels at the late-ripening stages (rs = 6–9), the chloroplasts in the guard cells appeared to be still intact. Only in the dark brown areas the epidermal and respective guard cells were dead, and red fluorescence was much less pronounced or absent, as observed earlier. [22]

When investigating sub-epidermal parenchyma cells in surface sections in a similar way, a marked difference between the green and yellow peels was found (see Supporting Information, Figure S9). Whereas green peels exhibited intact green chloroplasts (Figure S9A), which showed bright red fluorescence (Figure S9B), only small, yellowish gerontoplasts were observed in de-greened sub-epidermal parenchyma cells of yellow banana peels (at rs = 6) (Figure S9D). The red fluorescence was totally lost in these cells (Figure S9E). Instead, blue fluorescence increased in the cell lumen of parenchyma cells in de-greened peels (Figure S9F), which is typical for FCCs. In contrast, green-banana peels showed less blue fluorescence in the cell lumen of sub-epidermal parenchyma cells, whereas the cell walls retained their fluorescence (Figure S9C). The cells from peels of overripe bananas had a similar appearance as those of yellow ones (not shown). However, their brown areas contained dead cells. [22]

**Discussion**

The change of color of sweet bananas (*Musa acuminata*; short: *M*-bananas) from green to yellow is a well-known visual sign of their ripening. Indeed, color is an important criterion for fruit ripeness and quality. [22] and the molecular mechanisms responsible for its generation are a subject of obvious interest (see, for example, ref. [43]). Surprisingly, the ease of the observation of the color change, due to disappearance of chlorophyll in bananas (and in many other important fruits), contrasts with a lack of knowledge of the chlorophyll catabolites produced in fruits.

Studies of chlorophyll breakdown in senescent leaves have suggested a common, metabolically controlled and directed path that yields colorless NCCs as its typical tetrapyrrolic (end) products (see Figure 1). [1-3,13,31] This pathway of chlorophyll catabolism passes through the stage of pheophorbide a to give the colorless “primary” FCCs (either pFCC, or its epimer, epi-pFCC) in the chloroplasts (“gerontoplasts”) as products of the “early” phase of chlorophyll breakdown. [1-3,31] The original structural analysis and identification of these short-lived (and often elusive) FCCs were only possible with the help of ex-vivo preparations using enzyme active extracts from senescent leaves. [29,30] Indeed, in weakly acidic aqueous solutions, FCCs with a free propionic acid substituent undergo a fast and thermodynamically favorable isomerization to the corresponding stable NCCs. [39,40] This type of chemical isomerization is now seen as accounting for the natural formation of the NCCs in the vacuoles, as the “last step” of chlorophyll breakdown. [13,39] Natural NCCs carry a variety of polar peripheral functional groups, [9] which are thus presumed to reflect the corresponding modifications of their elusive FCC-precursors. [2] Indeed, the structures of FCCs are mostly inferred from those of the corresponding stable NCCs. [39,40] The change of color of sweet bananas is a well-known visual sign of their ripening. Indeed, color is an important criterion for fruit ripeness and quality.

Chlorophyll breakdown in leaves has, primarily, been rationalized as a kind of detoxification process. [14] It rapidly, and directly, degrades the potentially phototoxic chlorophylls to colorless (and “photo-inactive”) tetrapyrollines [44] and, thus, removes chlorophylls from their protein partners, which are then made accessible to proteolysis. [45] Hence, chlorophyll breakdown contributes indirectly to the con-
trolled recuperation of nutrients, of reduced nitrogen, in particular,[40] and also helps extending the viability of senescent cells before the leaves of deciduous plants are shed.

In contrast, degradation of chlorophyll in ripening fruit has so far been less well studied, and remains a largely puzzling phenomenon. In some fruits, such as apples and pears, chlorophyll catabolism yields colorless NCCs, following the pathway found in senescent leaves.[9] Obviously, the natural disappearance of chlorophyll in ripening fruit eliminates their capacity for photosynthesis, with stringent consequences for metabolism.[42] However, chlorophyll breakdown may also play a role within the peel of the fruit, similar to that deduced in senescent leaves.[46] Indeed, chlorophyll breakdown in peels of ripening bananas does not only provide NCCs, it leads also to an unprecedented accumulation and structural variety of “persistent” hmFCCs. As such, the disappearance of the chlorophylls in ripening fruits induces an easily observable change of color, and provides an optical signal relevant for the interaction with frugivores.[47] Thus, chlorophyll breakdown in ripening fruits serves the communication of fruit-producing plants with (frugivorous) animals in their environment.

**Structures of chlorophyll catabolites in extracts of ripening banana peels:** Using HPLC, with on-line analysis by UV/Vis absorbance and fluorescence spectra, chlorophyll catabolites were systematically detected in the extracts, classified as FCCs and NCCs, and specified individually according to their polarity, for example, as Mc-NCC-1g. The structures of five Mc-FCCs and of seven Mc-NCCs were elucidated with mass spectrometric[48–50] and NMR spectroscopic analyses[51,52] mainly (see the Results section). A sixth FCC (tentatively named Mc-FCC-71) was shown to be a likely isolation artifact (see the Results section and the Supporting Information). The present analysis of chlorophyll catabolites in bananas establishes a firm structural basis for further detailed glimpses into the biochemistry of chlorophyll breakdown.

Recently, we described four of the fluorescent Mc-FCCs occurring in extracts of banana peels.[21,22] These were classified as the “hyper-modified” Mc-FCC-56 and Mc-FCC-49, as well as their less abundant isomers, named Mc-FCC-46 and Mc-FCC-53. In contrast to the (few) natural FCCs identified earlier,[13] “hyper-modified” FCCs (hmFCCs) carry an intriguing ester function at the propionate side chain.[21,22] Such ester functions stabilize hmFCCs against their spontaneous acid catalyzed isomerization to NCCs and render them “persistent.”[52,40] As shown here, a further natural FCC of medium polarity was present in Mc-bananas during early stages of ripening (at rs = 5),[28] in addition to the four “persistent” hmFCCs.[21,22] This FCC was named Mc-FCC-62, and its structure was established. Mc-FCC-62 carries a free propionic acid function, as is characteristic of most natural FCCs. It is prone to conversion to an isomeric NCC, which was identified in bananas as Mc-NCC-61. Compared with “primary” FCCs, Mc-FCC-62 exhibited an additional hydroxyl modification at the ethyl side-chain. This is the indirectly deduced earliest modification of FCCs in a variety of senescent leaves[13] and in fruit.[20,53] Mc-FCC-62 (8’-OH-epi-pFCC) (see Figure 3) may therefore be classified as an unprecedented example of a prototypical “secondary” FCC (sFCC). Inspection of the time dependence of the accumulation of the different major FCCs in the peels of ripening Mc-bananas indicated the amount of Mc-FCC-62 to be highest in freshly bought Mc-bananas, which were generally acquired at rs = 5 (see the Results section and Figure 5). The content of Mc-FCC-62 decreased rapidly during the first two days of storage at room temperature (i.e., at rs = 6–7), compensated, however, by a corresponding simultaneous increase of the amounts of the hmFCCs, Mc-FCC-53 and Mc-FCC-56. Indeed, the total content of FCCs was maximal in bright-yellow bananas (at rs = 6), that is, typically on “day two” after acquisition.[21] These findings and the structures of the hmFCCs suggested a biosynthetic sequence, in which Mc-FCC-62 would be their precursor.[22]

The structures of the major five (of the seven) Mc-NCCs identified, so far (Mc-NCC-26, Mc-NCC-42, Mc-NCC-49, Mc-NCC-59 and Mc-NCC-61, see Table 2), suggested a second biosynthetic path, that also involved Mc-FCC-62 as their precursor. Indeed, these five NCCs carried a free propionic acid function and appear to be all formed through elusive mFCCs. Our earlier studies of chlorophyll breakdown in senescent leaves of other plants, such as spinach (Spinacia oleracea),[32,35] oil seed rape (Brassica napus),[33] or Arabidopsis thaliana[44] and so on, has provided precedence for NCCs with the molecular constitution of the Mc-NCCs. The structures of the Mc-NCCs suggest the intermediary existence of the corresponding mFCCs (Mc-FCCs, see Figure 3). In analogy to the related findings in Arabidopsis,[34] the lack of observation of the mFCCs is assumed to be due to their rapid spontaneous isomerization to the corresponding Mc-NCCs, once these FCCs have reached the vacuoles.[2]

Two further, minor NCC fractions (Mc-NCC-55 and Mc-NCC-58) were tentatively identified by HPLC-analysis. They co-eluted with “authentic” samples of Mc-NCC-55 and Mc-NCC-58 obtained by acid-induced isomerization of Mc-FCC-56. These two minor NCC fractions thus represent NCCs with an unprecedented propionate ester function. Remarkably, the CD spectrum of one of them (of Mc-NCC-55) was a near mirror image of the CD spectra of the known NCCs, indicating an opposite absolute configuration at C15,[45] which is consistent with their hypothetical formation from hmFCCs, such as Mc-FCC-56, by a stereo-unspecific isomerization.

**Chlorophyll breakdown in Mc-bananas involves epi-pFCC and the reductase RCCR-2:** Two stereo-divergent classes of the critical enzymes, called “red chlorophyll catabolite” reductases (RCCRs), exist in higher plants, typified as class-1 or class-2 RCCRs.[13] This surprising variability of chlorophyll breakdown appears to reflect a late outcome of their evolution,[41] and was discovered when the first structures of FCCs were elucidated. These turned out to be (C1)-epimeric
“primary” FCCs, named pFCC and epi-pFCC.[29,30] Indeed, in the context of a purely catabolic path, the stereo-structure of a presumed “detoxification” product appeared of lesser (biological) relevance.[90] A representative of the class-1 RCCRs was expressed from Arabidopsis [14] and its crystal structure was deduced recently.[55,56]

The type of RCC-reductase active in Mc-bananas was deduced as a class-2 RCCR,[31] based on the structural and chromatographic characteristics of the polar Mc-NCC-42. This banana NCC was identified with So-NCC-2, an NCC from spinach leaves (Spinacia oleracea)[32] (see the Supporting Information, Figure S5). By the same type of experiments, Hv-NCC-1 was shown to differ from Mc-NCC-42. Identification of Mc-NCC-42 with So-NCC-2 indicated the Mc-NCCs to originate from Mc-FCCs that belong to the stereo-lineage of epi-pFCC, which is formed by a “class-2” RCCR. The (stereochemical) identification of Mc-NCC-42 with So-NCC-2 implies also pairwise identities of Mc-NCC-26 and So-NCC-1, of Mc-NCC-49 and So-NCC-3, as well as of Mc-NCC-61 and So-NCC-4.

Structures of chlorophyll catabolites reveal a novel split path of chlorophyll breakdown in Mc-bananas and sFCC as the branching point and as a “last common precursor” of the downstream catabolites: Based on the information on the structures and on the abundance of colorless-chlorophyll catabolites, chlorophyll breakdown in the peels of ripening banana unfolds in a remarkable fashion. It is consistent with a “common earlier” path that provides epi-pFCC as the short-lived “primary” FCC.[13] However, no evidence was found, neither for (green) type I breakdown products[23] nor for catabolites derived from them.[17] The specific accumulation of Mc-FCC-62 at the stage of still incomplete ripening (classified as rs = 5)[29] is a remarkable feature of chlorophyll breakdown in the peels of Mc-bananas. The deduced structure of Mc-FCC-62 reflects the first modification of the (hypothetical) epi-pFCC[30] by hydroxylation at the terminal carbon of the ethyl group at C8. Indeed, the characteristic presence of such a hydroxylation among the peripheral modifications in most NCCs, found to date, suggest it to be a direct natural modification of “primary” FCCs.[13] All Mc-NCCs, and all Mc-FCCs, including the “hyper-modified” FCCs (hmFCCs), carry an oxygen-functionality at the terminal carbon of the substituent at C8, which may be modified by further functionalization. Mc-FCC-62 represents, therefore, the first example of a completely characterized “secondary” FCC (sFCC).

From the structures of the chlorophyll breakdown products in the peels of Mc-bananas, Mc-FCC-62 was indicated to be a rational biosynthetic precursor and (actually) the “last common precursor” of all Mc-FCCs and Mc-NCCs appearing further “downstream” in the path. The abundance profiles of the natural chlorophyll catabolites in ripening Mc-bananas were also compatible with this role of Mc-FCC-62, which was abundant only at the early ripening stages (up to rs = 5), and disappeared within about two days of further ripening. During this time the more polar hmFCCs, Mc-FCC-56 and Mc-FCC-53, became the dominant fractions, compatible with their suggested appearance as products of further functionalization of Mc-FCC-62 by catabolic enzymes[22] (see Figure 5). Mc-FCC-62 thus also marks the branching point of two major processing lines of chlorophyll breakdown in the banana peels, as derived on structure-based considerations (see Figure 3): In one branch, Mc-FCC-62 gives rise to the observed “hyper-modified” Mc-FCCs. In the other, this sFCC serves as the precursor of the mFCCs. The existence of these elusive catabolites is deduced from the detection of their presumed isomerization products, the correspondingly modified Mc-NCCs.

The early steps of chlorophyll breakdown thus yield FCCs and still occur in the chloroplasts.[13,14] However, NCCs, the typical “final” catabolites, are found in the vacuoles[31,57] This is a consequence of transport processes that take place during chlorophyll breakdown. These processes, as well as the location of the catabolic enzymes and the corresponding spatial (re)distribution of chlorophyll catabolites during ripening and senescence are intriguing topics. Most studies in this area, if not all, have concentrated on chlorophyll breakdown in senescent leaves.[55,56] In analogy, the “earlier stages” of chlorophyll breakdown in ripening fruit are assumed to operate within the chloroplast (see Figure 6). They are presumed to follow the “common path” via phaeophorbid a and an enzyme-bound form of the red chlorophyll catabolite (RCC) to provide short-lived “primary” FCCs, as the first colorless catabolites.[13,14] In the banana peels, the “primary” FCC escaped its observation and was deduced, indirectly, to be epi-pFCC (see Figure 3). However, an unprecedented accumulation of a prototypical “secondary” FCC (sFCC), the hydroxylated Mc-FCC-62, was seen at an early stage of ripening of bananas (at rs = 5).[29] Its formation in ripening bananas represents the likely result of an as yet unidentified hydroxylating enzyme[44] that introduces the indicated first modification of epi-pFCC (see the Experimental Section, Figure 8).

Similar to the situation suggested for the well-studied chlorophyll breakdown in senescent leaves,[25] most, if not all of the further modifications of the FCCs, which give mFCCs in the banana peels, would be presumed to occur in the cytosol. This scenario requires export of Mc-FCC-62 (or of epi-pFCC) across the envelope of the chloroplasts, which is thought to occur by a catabolite transporter (cat-tr) and to be activated by ATP.[44] The various elusive mFCCs would be formed by one, or several, enzyme-catalyzed transformations of Mc-FCC-62, for example, by hydrolysis of its methyl ester function,[12] by oxidative dihydroxylation of its vinyl group, or by glucosylation of its hydroxyl function. These mFCCs would then be transported from the cytosol through the tonoplast by an apparently efficient, but rather substrate-unspecific ABC transporter (ABC-tr) into the (acidic) vacuoles, where a rapid FCC to NCC isomerization takes place (see Figure 6).[14]

A second and alternative branch of chlorophyll breakdown yields the “persistent” hmFCCs in a hypothetical parallel esterification of Mc-FCC-62. The accumulation of Mc-
Hyper-modified FCCs are a persistent source of blue fluorescence: Chlorophyll breakdown in the peels of ripening bananas provides “hyper-modified” FCCs (hmFCCs), in a newly revealed branch of the degradation pathway.[21,22] The naturally accumulating hmFCCs induce (the peels of) ripening bananas to fluoresce blue when excited by UV light. These luminescent products of chlorophyll breakdown may, thus, serve as a specific communication with the exterior or world. The luminescence of ripe bananas can be recognized by animals sensitive to that section of the light spectrum, and such bananas are likely to appear more attractive to them. Human eyes may also perceive FCCs in bananas, but, typically, only as optical brighteners.

hmFCCs appear to be generated in the cytosol from less polar precursor-FCCs. The known further fate of Mc-FCC-56 is associated with the formation of the more complex, and more polar, Mc-FCC-49 during later stages of fruit ripening, and accumulation of the latter in blue, luminescent rings[23] that surround the “senescence associated”[59] dark spots. The cytosol, the presumed predominant location of the formation of the hmFCCs, represents an extended compartment in the plant cell that may also function as a well-accessible site for their eventual intercellular transport. Indeed, the metabolic investment in the unique biosynthesis of these highly modified hmFCCs suggests the consideration of a physiological role (see below).

Accumulation of hmFCCs in commercially available Mc-bananas has been a stunning finding. It induced additional studies, which showed that the formation of hmFCCs is not a consequence of artificial ripening of this climacteric fruit by treatment with ethylene gas. Exploratory studies with naturally ripened Mc-bananas from a plantation on the Island of Tenerife, Spain, indicated these bananas to contain a distribution of the dominating hmFCCs, very similar to that of ethylene-ripened commercial ones (see the Supporting Information, Figure S7). However, differences in the content of NCCs, the minor type of chlorophyll catabolites in the banana peels, were noted, requiring further studies to test their significance.

In-vivo light- and fluorescence microscopic studies with Mc-bananas revealed the intense blue fluorescence of FCCs to predominantly occur in sub-epidermal parenchyma cells of bright yellow banana peels (see the Supporting Information, Figure S9). A temporarily marked increase of this type of fluorescence has been described in areas around the growing “senescence-associated dark spots”. [24] However, the fluorescence of cell-wall components (such as phenols, lignin, and coumaric acid derivatives) may also contribute to part of the overall “blue” luminescence.[59]
In de-greened sub-epidermal parenchyma cells, a characteristic rearrangement of the chloroplasts takes place. Whereas early studies described a “chromoplast” differentiation,[60] the remaining plastids are characterized by thylakoid membrane loss and occasional accumulation of lipid globules, and are now classified as gerontoplasts.[61,62] However, on the largely simultaneously de-greening peels of banana, chlorophyll breakdown does not occur (in a synchronized fashion) in all cells: in fully ripe bananas, the guard cells of stomata still contain intact chloroplasts and appear to be functional (see the Supporting Information, Figure S8). The observed “delay” of the senescence of stomatal guard cells of the banana peels is similar to the one in senescent leaves, in which the process of chloroplast aging in stomata has been shown to lag behind the one of the parenchyma cells.[63] This underscores the temporarily local relevance of photosynthesis at this developmental stage, which provides the metabolic support in the guard cells, to achieve the required continuous control of the exchange of oxygen, water, and other volatiles with the environment.[64] As one physiological aspect, oxygen-exchange remains important for respiration, because mitochondria are more stable than chloroplasts. When stomata eventually undergo a retarded form of chlorophyll breakdown in over-ripe bananas, their loss of function, and death, is marked by the appearance of “senescence-associated” dark spots,[22] which eventually coalesce to give a dark and leathery skin texture.

On the physiological roles of tetrapyrrolic chlorophyll catabolites in higher plants: In senescent leaves of deciduous trees chlorophyll breakdown occurs rapidly in the largely synchronized pattern characteristic of the fall colors, and appears mainly to yield a number of NCCs as the “final” tetrapyrrole products.[9,13] In senescing leaves of two evergreens (Peace Lily (Spathiphyllum wallisii) and banana (Musa acuminata))[66], chlorophyll breakdown has been revealed to be more complex. Only a few ripening fruits have been analyzed, so far, for their non-green chlorophyll catabolites.[67] In apples and pears, only known NCCs were detected.[5,20] As delineated here in the peels of ripening bananas, a diverse set of non-green chlorophyll catabolites were discovered: unique “persistent” hmFCCs, and a variety of typical NCCs. The structures of these chlorophyll catabolites indicated a forked pathway of chlorophyll breakdown in this fruit that splits into two separate “downstream” lines. Accumulation of hmFCCs and the existence of a split path of chlorophyll breakdown are, thus, not unique for ripening banana fruits, but are rather a more general outcome of chlorophyll catabolism in higher plants. The complex structures of the ubiquitous degradation products of chlorophyll, and the hypothetical metabolism that generates them, suggest such chlorophyll catabolites to be more than mere products of a detoxification process, and may point to a physiological role of tetrapyrrolic chlorophyll catabolites in senescent leaves and in ripening fruit. Indeed, the tetrapyrrolic chlorophyll catabolites are remarkably similar, structurally, to important plant pigments, such as the bilins,[58,60] which are products of the natural breakdown of heme.[70] As NCCs are effective antioxidants,[20] their availability may be helpful in extending the viability of the tissue during leaf senescence or fruit ripening. The structurally related FCCs may have a range of comparable chemical properties, which, as yet, have hardly been investigated. Therefore the abundance and the expected unusual properties of such tetrapyrrolic chlorophyll catabolites call for further investigations of their biologically relevant chemical properties, as well as of their possible physiological relevance in higher plants.

Fluorescent chlorophyll catabolites, such as the hyper-modified FCCs, also stand out on account of their optical properties.[22] The blue luminescence of “persistent” hmFCCs in fruits may signal ripeness to frugivorous animals. The luminescence of intact bananas can also be monitored non-invasively by humans: Sensitive (time-, space-, and wavelength-resolved) luminescence measurements may make use of the natural formation of FCCs as fluorescent markers.[53] In banana peels, hmFCCs accumulate in freshly ripening tissue and in still viable, senescent cells in the transition region from intact, ripened to dead peel tissue. Persistent fluorescent chlorophyll catabolites, which are endogenously provided by (one branch of the split path of) chlorophyll breakdown, may, hence, commend themselves as natural molecular in-vivo reporters of senescence and senescence-associated cell-death symptoms.

Experimental Section

Materials

Plant material: Freshly ripe bananas (Musa acuminata, Cavendish cultivar) were bought from different stores in Innsbruck at the early ripening stage *rs* = 5 (see[20,21]); bananas of the cultivar *Musa cavendish* at different stages of ripening (unripe (rs < 4), freshly ripe (rs = 5) and over-ripe (rs = 8–9))[29] were also harvested from the plantation Malpais Trece (Garachico, Island of Tenerife, Spain) and were transported at ambient temperature to Innsbruck, where they were directly analyzed.

Chemicals: Commercially available solvents (reagent-grade) were redis-tilled before use for extractions. HPLC grade methanol (MeOH) was from Merck (Darmstadt, Germany) and Acros Organics (Geel, Belgium); acetonitrile (ACN) from Acros Organics (Geel, Belgium); formic acid, purum (HCO2H), potassium dihydrogen phosphate puriss. p. a., and potassium phosphate dibasic-anhydrous puriss.p.a. were from Fluka (Buchs, Switzerland), Amberlite IRA-900, CI-form ion exchange resin was from Acros Organics (Geel, Belgium), Sep-Pak-C18 cartridges were from Waters Associates.

Methods

HPLC-Chromatographic methods: Solvent: A: 100 mM potassium phosphate (pH 7.0); B: MeOH; C: water (0.002 % HCO3H); D: ACN (0.002 % HCO3H).

Analytical HPLC: Dionex Summit HPLC system, used with manual sampler, P680 pump, online degasser and diode array detector and Jasco FP-920 fluorescence detector. Injection loop 1 mL. (Rhodamine injection valve), a Hypersil ODS 5 μm 250×4.6 mm i.d. column, used at room temperature, Phenomenex ODS4 mm x 3 mm i.d. pre-column; flow rate 0.5 mLmin⁻¹. Solvents: A, B; compositions: AIA-12 (for details, see the Supporting Information, Figure S1).

Nano-HPLC: LC Packings Ultimate Nano-HPLC System with Dionex UVD 340S diode array detector for LC-MS experiments. Injection loop
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HPLC-SPE: the HPLC system was coupled by a rheodyne injection valve without loop to a Gynkotek M300 Pump with a steady water flow of 2.5 mL min⁻¹ connected to a RP-18 cartridge (Thermo ODS 14 mm x 10 mm i.d. pre-column). Data were registered and processed with Chromelon V6.50.

Preparative HPLC: Gynkotek HPLC System with manual sampler, M300 pump, UVD 340 diode array detector and Jasco FP-920 fluorescence detector detection, a Hypersil ODS 5 µm 250 mm x 21.2 mm i.d. column, at room temperature, flow rate of 5 mL min⁻¹. Solvents: A, B; composition: iso-cratic solvent mixtures with 45 to 55% of MeOH.

pH values: Measured by using a WTWSentix 21 electrode connected to a WTWPH 535 digital pH meter.

Spectroscopy

UV/Vis: Hitachi U-3000, CD: Jasco J715.

Fluorescence: Varian Cary Eclipse.

NMR spectroscopy: 1H and 13C NMR: Bruker UltraShield 600 MHz or Varian Unity Inova 500 MHz spectrometers; 13C NMR (125 MHz, CD3OD, 1H SIGNAL assignments from HSQC and HMBC-correlations) [10, 11].

Mass spectroscopy: Finigan MAT 95-S, positive-ion mode, Jasco J715. Solvents: C, D; compositions: N1/N2 (for details, see the Supporting Information, Figure S1).

Isolation of Mc-FCCs and Mc-NCCs: Ninety-nine freshly ripe bananas (about 20 kg, Musa acuminate, Cavendish cultivar, Chiquita brand) from the supermarket in Innsbruck were worked up as follows: the outer parts of the peels were blended with liquid nitrogen in a beaker. MeOH (1600 mL) and potassium phosphate buffer pH 7 (500 mL of 100 molar solution) were added. The mixture was filtered over celite. The solid residue was again blended with MeOH (700 mL) and buffer (200 mL), the filtration was repeated. The filtrates were concentrated to 1 L on a rotary evaporator. The deeply colored solution was again filtered over celite; the residue was washed with water. The clear solution was applied to an RP18-column (6 g of material in water), washed with water (30 mL) and eluted with MeOH (200 mL). MeOH was evaporated on a rotary evaporator. The residue was dissolved in MeOH (2 mL) and the solution was neutralized again. The filtrate was collected on ice and concentrated to 20% of its volume at ambient temperature and filtered again. The filtrate was used for characterization by nano-HPLC-ESI-MS (see the Supporting Information, Figure S1).

For further mass spectrometric analysis with nano-HPLC-ESI-MS the outer parts of frozen peels of 9 yellow bananas were crushed. MeOH (150 mL) and potassium phosphate buffer pH 7.0 (50 mL of 100 molar solution) were added, and the mixture was subjected to filtration. The filtrate was evaporated to about 20% of its volume at ambient temperature and filtered again. The filtrate was used for characterization by nano-HPLC-ESI-MS of Mc-NCC-61, Mc-NCC-49 and Mc-NCC-42 (solvent composition N2) and Mc-NCC-59, (solvent composition N1).

Spectroanalytical data

Mc-FCC-62: 1H NMR (CD3OD, 0.3°C, 500 MHz): δ = 1.12 (d, J = 7.3 Hz, Hc(C18)), 1.75 (m, Hc(C17)), 1.95 (m, Hc(C13)), 2.05 (s, Hc(C2)), 2.17 (s, Hc(C12)), 2.24 (s, Hc(C3)), 2.34 (m, Hc(C17)), 2.41 (m, Hc(C17)), 2.57 (dd, J = 7.8/18.2 Hz, Hc(C20)), 2.65 (m, Hc(C8)), superimposed by 2.66 (m, Hc(C18)), 3.03 (dd, J = 4.4/18.2 Hz, Hc(C20)), 3.50 (m, Hc(C8)), 3.73 (s, Hc(C13)), 4.02 (s, Hc(C16)), 4.63 (m, Hc(C1)), 5.39 (dd, J = 2.2/ 11.7 Hz, Hc(C7)), 6.21 (broad, J = 17.0 Hz, Hc(C3)), 6.53 (dd, J = 11.8/ 17.6 Hz, Hc(C3)), 9.37 ppm (s, Hc(C5)). 13C NMR (CD3OD, 0.3°C, 500 MHz): δ = 9.07 (12), 9.33 (12), 12.7 (12), 18.4 (18), 23.3 (10), 27.8 (8), 47.7 (17), 52.4 (18), 53.0 (13), 55.7 (13), 58.2 (1), 62.5 (8), 113 (12), 119 (3), 122 (8), 123 (3), 127 (13), 130 (3), 136 (3), 137 (7), 139 (11), 157 (2), 171 ppm (19) (see the Supporting Information, Figure S1).

UV/Vis: λmax (λexc): solvents: MeOH/potassium phosphate buffer, pH 7, 55/45 (v/v), 100 molar solution = 248 (sh, 1.00), 314 (0.81), 362 (0.41); HR-FAB (matrix glycerol): m/z calecd for C30H39N3O5: 645.292 [M+H]⁺ found: 645.314. See Figure 7 for the molecular constitution of Mc-FCC-62.
Mc-CC-N55: \( t_{e} = 55 \text{ min} \); UV/Vis: \( \lambda_{	ext{max}} = 300 \text{nm} \) in MeOH; \( \lambda_{	ext{max}} = 230 \) (−61.1, 283 (6.9), 320 (−5.0)), MS (ESI): m/z (%): 891.32 (9, [M+K-Na]+ 4)), 891.31 (30, [M+K]+), 833.39 (19), 832.35 (55), 831.31 (100). \( [M+H]^+ \), 705.25 (13), 675.23 (16), 673.51 (21), [M-C8H-O]+, 4N+.

Mc-CC-N49: \( t_{e} = 49 \text{ min} \); \( [M-NCC-42] \text{ ppm} (\text{rel. abundance}) = 677.23 (2), 659.18 (83), 633.18 (6), 632.18 (17), 631.17 (100, [M+H]^+), 619.16 (6), 588.17 (20), 587.16 (39, [M-C8H-O]+), 508.06 (4, [M-ring A+H]+), 464.11 (3), 424.96 (2); HR-FAB matrix: glycerol: m/z: c/cd for C13H26N2O4, 631.276 [M+H]^+; found: 631.279.

Mc-CC-N42: \( t_{e} = 42 \text{ min} \); UV/Vis (online DAD spectrum in MeOH/100 mM potassium phosphate buffer, pH 7, about 2/3 (v/v)): 1H NMR (CDOD, 0°C, 500MHz): \( \delta = 1.93 \) (s, \( H_{C}(18) \)), 2.02 (s, \( H_{C}(17) \)), 2.08 (s, \( H_{C}(12) \)), 2.32 (J = 7 Hz, \( H_{C}(17) \)), 2.54 (dd, \( J = 8.5/14.6 \text{ Hz, } H_{C}(20) \)), 2.62 (m, \( H_{C}(17) \)), 2.63 (dd, \( J = 8.2/14.6 \text{ Hz, } H_{C}(20) \)), 2.79 (m, \( H_{C}(17) \)), 2.87 (J = 4.7/14.6 Hz, \( H_{C}(20) \)), 3.47 (m, \( H_{C}(10) \)), 3.62 (dd, \( J = 5.0/11.3 \text{ Hz, } H_{C}(3) \)), 3.69 (dd, \( J = 6.6/11.3 \text{ Hz, } H_{C}(3) \)), 3.74 (J = 4.9 Hz, \( H_{C}(16) \)), 3.77 (m, \( H_{C}(15) \)), 3.87 (m, \( H_{C}(10) \)), 4.01 (dd, \( J = 5.4/8.6 \text{ Hz, } H_{C}(11) \)), 4.56 (dd, \( H_{C}(3) \)), 9.36 ppm (s, \( H_{C}(5) \)). \( ^{13}C \) NMR (CDOD, 0°C, 500MHz): \( \delta = 8.93 (7), 9.16 (12), 9.31 (18), 12.4 (12), 19.1 (7.9), 23.8 (10.8), 28.1 (88), 29.7 (20), 37.2 (15), 39.1 (17), 52.7 (133), 62.4 (1.3), 76.9 (3.9), 76.8 (3.6) (rel. abundance) = 666.14 (40), 665.10 (100, [M+H]^+).

Citrnic acid induced isomeration of Mc-CC-56: Aqueous citric acid (1x, 60 \( \mu \)L) was added to a solution of Mc-CC-56 (3 mL of a 30.9 \( \mu \)L solution) and Mc-CC-53 (3.1 mM solution of 3 isoform mixers) in MeOH. The mixture was stored for 159 h under an Ar atmosphere in the dark at 15°C. Water (1 mL) then was added and the resulting solution was subjected to analytical HPLC. Complete disappearance of FCCs and appearance of two major NCCs were indicated (see the Supporting Information, Figure S2). The two most abundant NCCs (Mc-CC-55 and Mc-CC-58) were isolated by semi-preparative HPLC and their mass spectra, CD spectra (see Figure 4) and UV/Vis-spectra (see the Supporting Information, Figure S3) were recorded. They were identified by HPLC-analysis (co-elution experiments) with authentic Mc-CC-55 and Mc-CC-58 (see the Supporting Information, Figure S4 and S5).

Figure 8. Molecular constitution of Mc-CC-42 derived from 2D NMR data (500 MHz, CDOD). Left: graphical representation of homonuclear \( ^{1}H \)-\( ^{1}H \)-correlations, arrows with dotted lines represent correlations from COSY, bold arrows represent correlations from ROESY; right: heteronuclear \( ^{1}H \)-\( ^{13}C \)-correlations, shadowed boxes indicate assignment from direct correlations, obtained from HSQC-spectra, arrows indicate assign-ment from long-range coupling, obtained from HMBC-spectra.

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