How the Colourless ‘Nonfluorescent’ Chlorophyll Catabolites Rust

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Dedicated to Prof. Karl Rigger on the occasion of his 90th birthday

For a long time, the appearance of the fall colours has been associated with the enigmatic chlorophyll breakdown in higher plants.[10] However, the extensive earlier search for coloured chlorophyll breakdown products has remained unsuccessful.[9] When chlorophyll catabolites from higher plants were first tentatively identified, they were indicated to be colourless.[3,4] These colourless compounds readily decomposed to rust-coloured materials upon analysis by thin-layer chromatography and were thus named “rusty pigments”, originally.[3,4] The puzzling picture cleared up, when one of the presumed chlorophyll breakdown products was structurally characterized as a colourless linear tetrapyrrole,[4] the type of which is meanwhile classified as a “non-fluorescent” chlorophyll catabolite (NCC, see Scheme 1).[2,5] Indeed, the colourless NCCs are ubiquitous in various senescent leaves and have been considered to represent the major “final” products of chlorophyll breakdown in senescent plants.[9] However, Cj-NCC-1 (1), a colourless NCC isolated from senescent leaves of the deciduous tree Cercidiphyllum japonicum (Katsura tree) could be chemically oxidized to a yellow chlorophyll catabolite, named Cj-YCC, which has also been detected in fall leaves recently.[7] Here, we analysed the major coloured products, when 1 decomposed to ‘rust’ on silica gel.

Application of a solution of Cj-NCC-1 (1)[8,9] to a silica gel TLC plate first gave a nearly colourless “spot”, which acquired a brown colour (“rust”) within 2–5 min, when exposed to air and daylight. TLC analysis of such a “spot” of 1 separated off a yellow zone on the plate, as was observed earlier with “rusty pigments”,[3,4] and an additional pink-red spot developed eventually. Thin-layer re-chromatography in a second dimension of the TLC trace originating from the NCC 1 revealed the yellow fraction to directly form on the plate from the colourless 1, whereas the pink-red spot correlated with the yellow fraction (see Figure S1 in the Supporting Information).

In an analytical experiment, NCC 1 (13.8 mg, 21.4 µmol) was adsorbed on silica gel 60 (5 g). The slightly yellow powder was suspended in hexane (20 mL) and was exposed to daylight, while being stirred magnetically under air. After 90 min the powder had acquired an orange-red colour, and

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it was extracted with MeOH. The orange-red extract contained a colourless fraction of 1 ($t_{R}=17$ min), two yellow compounds (Cj-YCC-1 (2b), $t_{R}=15$ min; Cj-YCC-2 (2a), $t_{R}=22$ min) and a pink red fraction (Cj-PiCC (3), $t_{R}=35$ min), as seen by analytical reversed-phase HPLC (see Figure 1). In a preparative experiment, NCC 1 (192.9 mg) was adsorbed on silica gel 60 (19.3 g). The dried powder was stirred magnetically under air, while being illuminated with a 100-W tungsten lamp. After 14 h the powder had acquired an orange-red colour. The adsorbed organic compounds were eluted with MeOH. The crude product mixture was separated by RP-MPLC. A colourless fraction of the NCC 1 (33.5 mg, 17.4%), two main YCCs, 2b (29.2 mg, 15.2%) and 2a (30.3 mg, 15.8%), and the pink-red PiCC 3 (5.5 mg, 2.9%) were isolated and were obtained as dry powders (see Experimental Section).

Cj-YCC-2 (2a), the less polar of the two yellow compounds, was obtained earlier by direct oxidation of Cj-NCC-1 (1) and was characterized as (13(5S,15R,20Z)-3,3-didehydro-4,5,10,15-(22,24H)hexahydro-13-methoxycarbonyl-4,5-seco-4,5-dioxophytoporphyrinate (2a, then tentatively named Cj-YCC, see Figure 1).[7] The UV/Vis spectrum of this yellow tetrapyrrrole exhibited three characteristic maxima at 244, 310 and 426 nm (in methanol, relative intensities of 0.50:0.69:1.00, see Figure 2).[7] The molecular formula of Cj-YCC-1 (2b) was deduced as C_{35}H_{38}N_{4}O_{8} from the pseudo-molecular ion at $m/z$ 643.2. This indicated the yellow tetrapyrrrole 2b to be an isomer of 2a, and to have two H atoms less per molecule than the parent 1. The constitution of the yellow catabolite Cj-YCC-1 (2b) was deduced from $^1$H NMR spectra, as well as homo- and heteronuclear 2-D spectra (ROESY, $^1$H,$^1$C-HSQC and HMBC, see Figure 3 and Figure S2 in the Supporting Information): In the $^1$H NMR spectra of 2b (CD$_3$OD, at 25°C) the signals of all 34 carbon-bound hydrogen atoms could be observed.

Among these, two singlets at lower field, of HCS=O and of HC20, the spin system for a peripheral vinyl group at an intermediate field, a singlet near $\delta = 3.7$ ppm (ester methyl) and the singlets of four methyl groups at high field stand out. From $^1$H,${^1}$C heteronuclear NMR correlations (HSQC,
g-HMBC and ROESY spectra\textsuperscript{[10]} of 2b, complete assignment of the \textsuperscript{1}H and \textsuperscript{13}C signals could be achieved. The constitution of 2b could thus be confirmed as that of an oxidation product of \textit{Cj}-NCC-1 (1), resulting from removal of two hydrogen atoms from the C1 and C20 positions (of 1). \textsuperscript{1}H-ROESY spectra helped to establish the \textit{E} configuration in 2b of the new double bond between C20 and C1. On this basis, and assuming that the stereostructure of 2b would correspond to that of \textit{Cj}-NCC-1 (1) elsewhere, the yellow oxidation product 2b was thus assigned the structure of a (15E,20Z)-3\textsuperscript{3},3\textsuperscript{2}-didehydro-4,5,10-(22\textsuperscript{2}H)-hexahydro-13\textsuperscript{2}-methoxycarbonyl-4,5-sec-4,5-dioxophytoporphyrinate (see Figure 2 and Scheme 2). The suggested retention of the configuration at the C13\textsuperscript{2} and C15 positions was supported by comparison of the basic sign-properties of the CD spectra of 1, 2a and 2b. The two isomeric yellow compounds (2a and 2b) are thus oxidation products of 1, from which they both arise by formal loss of two H atoms from the C1 and C20 positions. 

The significantly less polar pink-red compound \textit{Cj}-PiCC (3) was indicated to be a further oxidation product of the YCCs (2a, 2b). Its UV/Vis spectrum in MeOH exhibited strong absorbance bands at 312 and 522 nm, with relative intensities of 0.92 to 1.00 (see Figure 3). The molecular formula of 3 was deduced by mass spectrometry to be C\textsubscript{35}H\textsubscript{36}N\textsubscript{4}O\textsubscript{8}, indicating two H atoms less (per molecule) than were present in 2a or 2b. The constitution of the pink-red catabolite 3 was deduced from \textsuperscript{1}H NMR spectra, as well as homo- and heteronuclear 2D spectra (ROESY, \textsuperscript{1}H,\textsuperscript{13}C-HSQC and HMBC,\textsuperscript{[10]} see Scheme 2 and Figure S3 in the Supporting Information): In the \textsuperscript{1}H NMR spectra of \textit{Cj}-PiCC (3, in CD\textsubscript{3}CN, at 25\textdegree C) the signals of all 30 carbon-bound hydrogen atoms could be observed. Among these were two singlets at low and at an intermediate field, of HC\textsubscript{5}=O and of HC\textsubscript{20}, the spin system for a peripheral vinyl group and the singlets of four methyl groups at high field and of an ester methyl group (near \(\delta=3.7\) ppm). In addition, the signal of HN\textsubscript{21} could be observed. From \textsuperscript{1}H,\textsuperscript{13}C heteronuclear NMR correlations (HSQC, g-HMBC) and ROESY spectra of 3, the complete assignment of the \textsuperscript{1}H and \textsuperscript{13}C signals could be achieved and 3 was delineated to have the constitution of a 3\textsuperscript{3},3\textsuperscript{2}-didehydro-4,5,10-(22\textsuperscript{2}H)-tetrahydro-13\textsuperscript{2}-methoxycarbonyl-4,5-sec-4,5-dioxophytoporphyrinate (see Scheme 2). \textsuperscript{1}H-ROESY spectra of 3 helped to establish the \textit{Z} configuration of the double bond between C20 and C1: the observed NOEs (e.g. from the ester methyl group to HN\textsubscript{21} of ring A) were all consistent with an \textit{E} configuration of the “new” double bond between C15 and C16. A CD-spectrum of the isolated sample of 3 showed very weak signals only. Apparently, practically racemic 3 was isolated, due to equilibration at its single stereo-center, the exchange labile C13\textsuperscript{2}. The pink-red oxidation product 3 was thus assigned the structure of a (15E,20Z)-3\textsuperscript{3},3\textsuperscript{2}-didehydro-4,5,10-(22\textsuperscript{2}H)-tetrahydro-13\textsuperscript{2}-methoxycarbonyl-4,5-sec-4,5-dioxophytoporphyrinate.

Our experiments showed the “rust” colour of the NCCs to develop from oxidative decomposition of the colourless NCCs mainly, as suspected earlier\textsuperscript{[2]} (see Scheme 3). NCCs, the colourless linear tetrapyrroles from breakdown of chlorophyll were revealed to be rather strong antioxidants\textsuperscript{[13]}, nearly as effective as bilirubin.\textsuperscript{[14]} Oxidation of the colourless
Cj-NCC-1 (1) with DDQ provided YCC 2a, by dehydrogenation at the C20 meso bridge. In YCCs electronic conjugation via the “western” C20 meso position extends the tetrapyrrolic chromophore into the visible range. Indeed, YCCs (2a and 2b) have a chromophore that is remarkably similar to that of the heme breakdown product bilirubin. Likewise similar to bilirubin (which undergoes light-induced double bond (Z-E) isomerisations), YCCs 2a and 2b were observed in analytical experiments to interconvert in solution (e.g. in MeCl2), when they are exposed to daylight (see Figure S4 in the Supporting Information). Photo-isomerisation of 2a is thus a path for its preparative isomerisation to 2b. Further oxidation of the YCCs occurs at the saturated C15 meso position, and the extension of the conjugated system via C15 results in the red-shifted absorption properties of Cj-PiCC (3). This pink-red tetrapyrrole was also prepared in about 40% yield by direct oxidation of the YCC 2a with dichlorodicyanobenzoquinone (DDQ).

The ubiquitous colourless NCCs were suggested to represent the “final stage” of chlorophyll breakdown in senescent leaves (see Scheme 1) and were found to accumulate in the vacuoles. Until recently, NCCs were primarily looked at as products of a crucial detoxification process. However, NCCs were recently shown to be effective antioxidants, and they were suggested to possibly play a (still unknown) physiological role in senescent leaves and fruit. Indeed, a compound identified with the YCC 2a was observed in small quantities in fresh extracts of senescent leaves of C. japonicum. In the leaf, NCC 1 thus appears to be oxidized (by non-enzymic processes) to the yellow YCC 2a, from which the isomer 2b may possibly be produced by light-induced isomerisation. Further oxidation may result in the pink-red PiCC 3. A related oxidation process has been proposed to be responsible for the observation of colourless urobilinogenoidic tetrapyroles in senescent barley leaves, which were suggested to be the result of an alternative oxidation of the Hv-NCC-1, the main NCC from degreened leaves of barley.

Having identified the yellow tetrapyroles 2a-2b and the red-pink compound 3 as oxidation products of the NCC 1, we have now set out to analyse degreened plant material (from leaves and ripe fruit) more thoroughly for the appearance of coloured chlorophyll catabolites under the conditions of natural senescence and ripening. Indeed, ongoing studies in our laboratories hint at a more general significance in senescent leaves, not only of yellow chlorophyll breakdown products, but also of their red-pink oxidation products. Red and yellow chlorophyll catabolites may thus prove to be active contributors, after all, to the appealing yellow, orange and red colours of fall leaves.

Coloured tetrapyroles from chlorophyll breakdown may be of interest as a new class of nature-derived pigments. The yellow and red chlorophyll breakdown products are pigments related to important heme-derived tetrapyroles, such as biliverdine and its natural reduction products, such as bilirubin and phytocromobilin. Together with the fascinating ‘hypermodified’ fluorescent chlorophyll catabolites (hFCCs), that give ripe bananas an intriguing blue luminescence, YCCs and PiCCs represent noteworthy expansions of Nature’s repertoire of plant pigments. In view of the important biological roles played by the heme-derived linear tetrapyroles, for example, as chromophores in light-sensing enzymes and light-harvesting assemblies in photosynthetic organisms, possible physiological effects of the tetrapyrolic chlorophyll catabolites in plants and in higher animals continue to call for attention.

### Experimental Section

**General and chromatography:** See details in Supporting Information.

**Spectroscopy:** UV/VIS: HITACHI U-3000 spectrophotometer; λ<sub>max</sub> in nm (rel. ε). CD: JASCO J-715 spectropolarimeter; λ<sub>max</sub> and λ<sub>ε</sub> in nm (relative Δε). 1H NMR: Varian UNITYplus 500; δ in ppm with δ-

**Scheme 3. Structural outline of the oxidative decomposition of Cj-NCC-1 (1).** a) Oxidation of 1 to Cj-YCC-2 (2a), b) reversible photo-isomerization of 2a and 2b (Cj-YCC-1), c) oxidation of 2a to Cj-PiCC (3).
Preparative oxidation of Cj-NCC-1 (1) to coloured tetrapyrrolic compounds on silica gel: A sample of NCC 1 (192 mg, 270 μmol) was dissolved in MeCl2 (150 mL) and silica gel (19.3 g) in dichloromethane (200 mL) was added. The resulting suspension was filtered and dried. The dried powder was stirred magnetically under air, and was illuminated with a 100-W tungsten lamp for 14 h. An orange-red powder was obtained. Analytical HPLC revealed the following fractions: yellow Cj-YCC-1 (2b, r1 = 15 min), yellow Cj-YCC-2 (2a, r2 = 22 min) and pink-red Cj-PcC (3, r3 = 35 min). Extraction of the coloured powder and work-up with MPLC (as described9) resulted in re-isolated starting material Cj-NCC-1 (1. 335 mg = 52.0 μmol = 17.4 %) and two yellow pigments, isolated as powders (29.2 mg of Cj-YCC-1 (2b, 45.4 μmol = 15.2 %) and 30.3 mg of Cj-YCC-2 (2a, 45.2 μmol =15.8 %)). A third (red) fraction was also collected and delisted on a Sep-Pak cartridge. Crude pink-red Cj-PcC (3) was eluted with CH2O, the solvent was evaporated under reduced pressure and the red residue was re-dissolved in CH2O:water 1:1 (v/v) (2 mL), to be re-submitted to semi-preparative HPLC. After separation, delisting, isolation and drying Cj-PcC (3, 5.5 mg, 8.5 μmol, 2.9 %) was obtained as a red powder, which was characterised, as described below.

Selected spectroscopic data: Cj-YCC-1 (2b): UV/Vis: (CH3OH, c = 2.80 x 10−3 mol l−1) λmax (log ε) = 247 (4.58), 313 (4.62), 440 nm (4.41); CD: (methanol, c = 2.80 x 10−3 mol l−1) δmax (Δε) = 229 (37.7), 251 (–9.7), 261 (–6.5), 286 (−25.1), 312 (–0.3), 326 (−2.7), 442 nm (8.1); 1H NMR: (500 MHz, CD3OD): δ = 1.67 (s, H-c2), 2.03 (s, H-c8), 2.15 (s, H-c12), 2.26 (s, H-c7), 2.37 (m, H-c17), 2.68 (m, H-c8), superimposed by 2.70 (m, H-c17), 2.80 (m, H-c17), 3.51 (m, H-c8), 3.76 (s, H-c13), 3.95 (s, H-c10), 4.98 (s, H-c15), 5.38 (dd, J = 2.5/11.5 Hz, H-c3), 6.15 (dd br, J = 2.5/11.5 Hz, H-c3), 6.37 (s, H-c20), 6.51 (dd, J = 12/17.5 Hz, H-c3), 9.42 ppm (s, H-c5); MS (ESI positive-ion mode, m/z = 247 (4.58), 313 (4.62), 440 nm (4.41). CD: (methanol, c = 2.80 x 10−3 mol l−1) λmax (log ε) = 244 (4.22), 310 (4.35), 426 nm (4.51); CD: (CH3OH, c = 3.74 x 10−3 mol l−1) λmax (Δε) = 229 (7.9), 248 (−5.6), 263 (−2.2), 287 (−9.2), 311 (−1.1), 325 (−1.8), 345 (0.2), 357 (−0.2), 429 nm (3.6).

Cj-PcC (3): UV/Vis (CH3OH, c = 4.69 x 10−3 mol l−1) λmax (log ε) = 312 (4.27), 495 (4.25), 522 nm (4.31); 1H NMR: (500 MHz, CD3OD): δ = 2.12 (s, H-c12), 2.14 (s, H-c8), 2.22 (s, H-c7), 2.29 (s, H-c7), 2.42 (m, H-c7), 2.69 (m, H-c8), 3.13 (m, H-c17), 3.50 (m, H-c8), 3.76 (m, H-c17), 4.24 (s, H-c10), 5.50 (dd, J = 2.2/12.0 Hz, H-c3), 6.14 (H-CO), 6.37 (dd, J = 2.5/11.5 Hz, H-c17), 6.65 (dd, J = 12/17.5 Hz, H-c3), 9.45 ppm (s, H-c5); MS: (HR-FAB, pos.): m/z = 641.2626 (exptl): m/z = 641.2606 (calcld) [M+H]+ C24H30N4O4;

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