On the Nature of Isomeric Nonfluorescent Chlorophyll Catabolites in Leaves and Fruit – A Study with a Ubiquitous Phylloleucobilin and its Main Isomerization Product

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Dedicated to Professor Dieter Seebach on the occasion of his 80th birthday

The typical main products of chlorophyll (Chl) breakdown in higher plants are non-fluorescent, colorless phyllobilins, named phylloleucobilins. These long elusive Chl-catabolites are linear tetrapyrroles, whose structure elucidation has required thorough spectroscopic analyses. Interestingly, in recent LC/MS studies of leaf extracts, isomeric forms of phylloleucobilins were detected. The existence of isomeric phyllobilins may suggest incomplete stereo-selectivity of catabolic processes, or isomerization processes in plant cells or in the analytes. Here we report a study with the phylloleucobilin NCC-1, a basic Chl-catabolite in extracts of leaves and fruit. NCC-1 and its main isomerization product in aqueous solution were identified as 82-epimers. Formation of 82-epi-NCC-1 from NCC-1 implies an unstable enol(ate)-intermediate, which reverts to NCC-1 or converts to 82-epi-NCC-1. Such reversible epimerization reactions are a non-biological in vitro feature of typical phylloleucobilins, and probably also take place in vivo.

Keywords: Chlorophyll breakdown, Natural product, Phyllobilin, Structure elucidation, Tetrapyrrole.

Introduction

In senescent leaves and ripening fruit colorless phyllobilins often accumulate as typical main products of chlorophyll (Chl) breakdown.[1] Among them are the so-called non-fluorescent Chl catabolites (NCCs), which are direct products of the ‘PaO/phyllobilin’ pathway of Chl breakdown typical for higher plants (see Figure 1).[2][3]

The basic, chiral NCC structures exhibit three asymmetric carbon centers (C82, C10 and C16),[1][4] all of which appear to be generated in a plant via highly stereo-selective transformations. The original phyllobilin skeleton (a 1-formyl-19-oxobilin) is produced by a critical and characteristic enzyme-catalyzed ring opening of the porphyrinoid macrocycle of Chl a, generating red chlorophyll catabolite (RCC). Subsequent enzymatic reduction of RCC to a ‘primary’ fluorescent Chl-catabolite (pFCC) installs asymmetric C16 from saturating the C15 = C16 double bond.[2] Enzymatic functionalization of pFCC and stereo-selective isomerization of the resulting, short lived fluorescent FCCs furnishes the colorless NCCs, and establishes their configuration at C82 and C10.[1][2]

In a particular plant NCCs may typically be expected to be formed as single isomers. However, in a recent combined LC/MS study of extracts of ripening loquat fruit NCC isomers were detected.[5] One group of these was described as representing remarkably abundant and unprecedented enol tautomer of NCCs.[5] So far, enol tautomers of NCCs would be considered unstable isomers of their established β-keto-ester or β-keto-acid forms.[1][4] To help clarify the more general relevance of this structural issue, we have embarked on a study of the spontaneous isomerization in aqueous solution of NCC-1 (8S5,10R)-32-hydroxy-1-formyl-19-oxo-16-epi-16,19-dihydrophyllobilane), an abundant phylloleucobilin in higher plants (see e.g., Refs.[5 – 11]). As verified here, α ring E of phyllobilins is a site prone to isomerization in protic media, due to its characteristic β-keto-ester (or β-keto-acid) functions.[12][13] and ii) the main reversibly formed isomer of NCC-1 is the epimer 8S-epi-NCC-1 (see Figure 2).

Results and Discussion

NCC-1 is a typical main phyllobilin found in senescent leaves and in ripening fruit.[11] As it was first found as the main NCC in fall leaves of the Katsura tree (Cercidiphyllum japonicum),[6] NCC-1 is also called CJ-NCC-1.

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When isolated from such yellow leaves following an established laboratory procedure, NCC-1 is accompanied by a minor NCC fraction, in an roughly 10:1 ratio. In an RP-HPL-chromatogram the minor NCC exhibited a slightly shorter retention time compared to NCC-1. This minor fraction was classified as an NCC, as its UV-absorption spectrum was nearly superimposable on that of NCC-1, with maxima at around 315 nm, characteristic for an $\alpha$-formyl-pyrrole (ring A of NCCs). MS analyses established the unknown fraction as an isomer of NCC-1: both samples showed pseudo-molecular ions $[M + H]^+$ and $[M + K]^+$ at m/z 645 and m/z 683, respectively, when analyzed by ESI-MS. To further characterize their structural relationship, NCC-1 and its presumed isomer were isolated in pure state from pre-purified NCC-1, obtained from an extract of senescent C. japonicum leaves as a roughly 10:1 mixture of both substances. Hence, from an aliquot of 3 mg of the pre-purified NCC-1 pure samples of both NCCs, 1.9 mg of NCC-1 and 211 $\mu$g of the isomer, respectively, were obtained by semi-preparative HPLC (see Experimental Section). Both of these samples were > 95% pure according to analytical HPLC and were used for a thorough spectroscopic characterization. High resolution MS confirmed the two substances as isomers; the pseudo-molecular ion $[M + H]^+$ of both of them was observed at m/z 645.291, consistent with their common molecular formula C$_{35}$H$_{40}$O$_8$N$_4$. Similar ESI-MS fragmentation patterns typical for NCCs were, likewise, found for both compounds; a fragment at m/z 613.3 indicated loss of MeOH (from the ester group at C8$^2$), and a fragment at m/z 522.2 resulted from the characteristic loss of ring D.

Isomerization experiments were performed in order to verify the chemical relationship between the two compounds. For this purpose, samples of pure NCC-1 in dilute aqueous potassium phosphate buffer (pH 7) and of its isomer in aqueous ammonium acetate buffer (pH 7) were, both, stored at ambient temperature under argon atmosphere protected from light. Equilibration of the two samples was analyzed at different time points by HPLC (Figure 3).
The experiments showed that NCC-1 was formed from a pure sample of the isomer reaching an equilibrium ratio of about 10:1 (NCC-1/isomer). Likewise, formation of the same equilibrium ratio with about 10% of the isomer of NCC-1 occurred in reverse, when a pure sample of NCC-1 was stored at room temperature in the buffered aqueous solution. Noticeable fractions of other (isomeric) phyllobilins did not appear during these experiments.

Electronic absorption spectra of NCC-1 and of its isomer exhibited the typical features of an NCC (Figure 4). The observed UV spectra of NCC-1 in acetonitrile, with maxima 307 nm, as well as a shoulder at 243 nm, was insignificantly different from that of its isomer, which exhibited a maximum at 306 nm, and a shoulder near 240 nm. Merely small differences were noted in the CD spectra of NCC-1 and its isomer. Noticeable fractions of other (isomeric) phyllobilins did not appear during these experiments.

The structure of NCC-1 was deduced earlier from NMR-spectra in CD$_3$OD.[6] In order to clarify the structural nature of both of the two isomeric NCCs, thorough NMR investigations in CD$_3$CN were carried out here. For this purpose, 20 mg of pre-purified mixture of NCC-1 and its isomer were further separated by HPLC, yielding 490 l of the isomer of NCC-1, which was >95% pure according to HPLC. The samples of NCC-1 and of its isomer were dissolved in CD$_3$CN, and NMR measurements were performed at 0 °C.

The internally referenced$^{[1]}$ 600 MHz $^1$H-NMR spectra of NCC-1 in CD$_3$CN allowed for the assignment of all 34 carbon-bound hydrogens, as well as of the four H-atoms at nitrogen, from 2D spectroscopy evaluating homo- and hetero-nuclear correlations ($^1$H-$^1$H-COSY, $^1$H-$^3$H-ROESY, $^1$H-$^13$C-HSQC, and $^1$H-$^13$C-HMBC spectra, see Figure 5). A singlet at 9.31 ppm of the $\alpha$-formyl group (HC20) at ring A, an ABX spin system
at 5.32, 6.16, and 6.43 ppm of the vinyl group at C18, four singlets of the four methyl groups at C2, C7, C13, and C17 (2.20, 2.16, 1.80, and 1.99 ppm), a singlet of a methyl ester group at 3.69 ppm were all clearly observed. The protons H2C15 coupled with HC16, giving multiplets at 2.53 and 2.83 ppm, as well as at 3.97 ppm, respectively. The hydroxyethyl side chain gave rise to multiplets at 2.61 ppm (C31) and 3.47 ppm (C32), both of which coupled via NOE correlations with H2C5 (at 3.88 ppm). The propionic acid side chain showed multiplets at 2.49/2.59 ppm (C121) and 2.28/2.34 ppm (C122). The four methylene protons of the propionic side chain showed NOEs to the spin system of two doublets that were assigned to the proton at C82 (3.64 ppm) and the proton at C10 (4.77 ppm). These two methine protons interacted with each other via scalar coupling ($J = 3.3$ Hz) and via NOE correlations, furnishing proof of the saturated carbon centers C82 and C10 (see Figure 6).

Furthermore, four signals were found in the low field part of the $^1$H-NMR spectra of NCC-1 that showed no correlations in the HSQC spectrum and were identified as the four nitrogen-bound protons of the four pyrrole moieties. Two of the protons could be assigned as the pyrrole NH protons of ring C and ring D (chemical shifts of 8.43 and 6.89 ppm, respectively) due to heteronuclear long-distance correlations (HMBC spectra). The two remaining low field protons at 10.37 and 10.44 ppm showed an HMBC correlation to the carbon at position 5 and they were assigned as the pyrrole NH protons of rings A and B.

All carbons were assigned signals via heteronuclear correlations from $^1$H,$^13$C-HSQC and $^1$H,$^13$C-HMBC spectra (Figure 5); in the HSQC spectrum, all 14 saturated

![Figure 3. Isomerization experiments with NCC-1 and its main isomer, as monitored by analytical HPLC. Analyses a) of the formation of NCC-1 from its isomer in 50 mM potassium phosphate buffer pH 7 and b) of the isomer from NCC-1 in 10 mM ammonium acetate buffer pH 7.](image-url)
carbon centers showed a signal; in addition the three unsaturated hydrogen-bound carbons of the vinyl group at ring D (119.0, 127.3, and 128.1 ppm) and the formyl moiety of ring A (177.3 ppm) were visible. An HMBC spectrum allowed for the assignment of all remaining 18 carbon centers.

Thorough NMR investigations were also performed for the isomer of NCC-1. When the 1H-NMR spectra of 82-epi-NCC-1 were collected, a mixture of both isomers (roughly 1:1) was already present in the sample, which equilibrated further slowly during the measurement of the spectra at 0 °C. However, no other bilin-type species was observable in the sample. The presence of these two isomeric species did not hamper the interpretation of the spectrum of 82-epi-NCC-1, since the assignment of all signals of NCC-1 was available. Measurement of the mixture of NCC-1 and of its isomer allowed for quantitative determination of chemical shift differences between their spectra and unambiguous identification of their respective signals (see Figure 6 and Supporting Information). In the internally referenced 600 MHz 1H-NMR spectrum of the isomer of NCC-1 (i.e. of 82-epi-NCC-1) all 34 carbon-bound hydrogens could be assigned on the basis of homo- and hetero-nuclear 2D-spectra (1H,1H-COSY, 1H,1H-ROESY, 1H,13C-HSQC, and 1H,13C-HMBC spectra). Most importantly, two doublets of the isomer were found at 4.06 ppm (C82, J = 7.4 Hz) and 4.92 ppm (C10, J = 7.4 Hz), establishing it as 82-epi-NCC-1. These two doublets showed 0.44 ppm and 0.15 ppm shifts to lower field, respectively, when compared to the corresponding values of NCC-1 (C10: 4.77 ppm) and (C82: 3.64 ppm). Vicinal couplings between the two protons were larger (J = 7.4) for the epimer 82-epi-NCC-1, consistent with a cis arrangement. Based on the Karplus equation, J = 7.4 Hz indicates a dihedral angle of roughly 20° for the epimer, whereas J = 3.3 Hz found for the corresponding signals in NCC-1 suggests a larger dihedral angle, of about 50° (and a trans arrangement of the two methine protons), as found earlier.

The pair of vicinal protons in both isomers excludes the presence of an enol double bond at C82. In further support of the NMR-derived constitutional and stereochemical analysis of the ring E moiety of the two isomeric NCCs, clear NOE correlations from the proton at C82 to the propionic acid side chain are observed for NCC-1, but not for its isomer 82-epi-NCC-1. The structure of NCC-1 is consistent with short distances of both, HC82 and HC10, and methylene protons of the propionic acid side chain. In contrast, the inverted configuration at C82 in the isomer, results in HC82 pointing ‘upwards’, placing HC82 at a longer distance from the protons of the propionic acid side chain.

Furthermore, the signal of the pyrrole NH of ring C, which is adjacent to C10, was affected by the isomerization and showed a shift of 8.26 ppm for the isomer compared to 8.43 ppm observed for NCC-1. The NH signal of ring D showed no large shift difference compared to NCC-1. Both of the NH signals of the ‘eastern’ region (rings A and B) appear at slightly higher field in the isomer (82-epi-NCC-1), when compared to NCC-1. They could not be assigned individually; therefore, their shift differences could not be defined more precisely.

Most other of the prominent signals in the 1H-NMR spectrum of 82-epi-NCC-1 were found at (very) similar chemical shifts as those assigned to NCC-1.
Among these were the four singlets for the methyl groups at C2, C7, C13, and C17, the spin systems of the vinyl group at C18, of the hydroxyethyl side chain at C3, the methylene groups at the two meso positions, and the CH₂ groups of the propionic acid side chain, as well as the formyl group HC20.

Figure 5. Graphical analysis of the NMR-data for a) NCC-1 and b) its isomer 8²-epi-NCC-1. Left: NMR Correlations from ¹H,¹H-COSY and ¹H,¹H-ROESY spectra (bold or dotted lines, respect.); italics indicates tentative assignments of two NH’s. Right: Correlations from ¹H,¹³C-HSQC spectra (shaded boxes) and ¹H,¹³C-HMBC spectra (arrows); c) Comparison of NMR-data of NCC-1 and 8²-epi-NCC-1, highlighting positions with chemical shift differences larger than 0.1 ppm (¹H) and 1 ppm (¹³C) with grey boxes.
Likewise, evaluation of carbon signals from heteronuclear 2D spectroscopy ($^1$H,$^13$C-HSQC and $^1$H,$^13$C-HMBC spectra, Figure 5), allowed for the unambiguous assignment of all carbon atoms in the isomer. As already found in the $^1$H-NMR spectra, the $^{13}$C shifts of the isomer (8$^2$-epi-NCC-1) showed comparable values to NCC-1, except for the ‘southern’ region, in which $^{13}$C shift differences of > 1 ppm were observed. C8$^2$ and C10 carbon shifts were assigned from the HSQC spectrum at 65.2 and 36.1 ppm, respectively, indicating slight high field shifts of these two carbons, which appeared at 67.9 and 37.0 in the spectrum of NCC-1. The spectroscopic data allow an unambiguous characterization of the structure of the (reversibly formed main) isomer of NCC-1 as a (8$^2$R,10R)-1-formyl-3$^2$-hydroxy-19-oxo-16-epi-16,19-dihydrophyllobilane, i.e., as the 8$^2$-epimer of NCC-1. Hence, our equilibration experiments revealed a readily occurring, reversible isomerization in aqueous solution of NCC-1 with its 8$^2$-epimer, which takes place by inversion of the configuration at asymmetric C8$^2$ (see Figure 2).[1]

An enol-isomer remained unobserved during all of our experiments. In striking contrast, in recent mostly LC/MS based analyses of extracts of ripening loquat fruit, Rios et al. proposed the presence of sizeable fractions of enolic tautomers of NCCs as the main separable isomers of the also observed keto forms.[5] In apparent support of this claim, trimethylsilylation of such an isolated NCC isomer to a stable enol-ether was achieved, as deduced by mass spectrometric analysis.[5] Further spectroscopic analysis of the ‘enol-isomer’ or of the chemically prepared silylated enol-ether was not reported. O-trimethylsilylation, by itself, does not establish a sizeable equilibrium concentration of an enol(ate) in the respective analytes. Indeed, this method may help to trace hardly detectable and unstable enol-forms, as was shown by Hynninen and coworkers for enols of the Chls.[17] However, a fully characterized, stable enol-form of a Chl has become accessible after careful design and synthetic remodeling by Eschenmoser et al.[18] As a result of its enol-functionalization, the optical properties of the new Chl-derivative were modified extensively and this enol became non-fluorescent.[18] Interestingly, the same Chl-enol was (re)discovered recently in marine organisms, which exploit the non-fluorescent behavior of this natural Chl-enol to ‘detoxify’ their ingested Chls.[19] On this basis, keto- and enol-forms of phylloleucobilins would also be expected to have significantly different (chir)optical properties.

The issue of phyllobilin structure and stereochemistry are considered characteristic results of the specific PaO/phyllobilin pathway. Hence, the configuration of NCCs at C16 is derived from the action of a stereoselective RCC-reductase (RCCR),[20] which attaches two hydrogen atoms (one each) at the unsaturated carbons C15 and C16 in the course of the reduction of red chlorophyll catabolite (RCC) to ‘primary’ fluorescent Chl catabolites (pFCCs).[21][22] In fact, two epimeric lines of FCCs occur in higher plants.[21][22] due to the existence of two types of RCCRs that generate either one of two epimeric pFCCs in a species-specific way, with

![Figure 6](image_url)
unknown, but opposite absolute configurations at their chiral C16 (see Figure 1).\(^{[23]}\) Unfortunately, crystal structures of a natural NCC or of an FCC precursor are still unavailable. Likewise, a crystallographic analysis of the RCCR-1 type reductase (AtRCCR)\(^{[20]}\) from Arabidopsis thaliana, which catalyzes stereo-selective formation of pFCC, with (defined) ‘normal = n’ configuration, failed to derive stereo-chemical details of the reduction process catalyzed by this enzyme.\(^{[24],[25]}\) FCCs and NCCs from A. thaliana are, hence, classified as belonging to the ‘normal = n’ series.\(^{[21],[26]}\) Once installed by an RCCR, the absolute configuration of pFCCs at C16 is retained in the subsequent transformations to modified FCCs and to corresponding NCCs, and the colorless phyllobilins from a particular plant are considered to consistently exhibit only one of the possible two configurations at their asymmetric C16.\(^{[1]–[3]}\) Hence, the abundant NCC-1 studied here (see Figure 2) displays ‘epi’ configuration at C16, as a consequence of the specific formation of epi-pFCC by a RCCR-2 type reductase\(^{[22]}\) in C. japonicum.\(^{[12]}\) The natural n-epimer of NCC-1 is available from senescent leaves of the Peace Lily (Spatiphyllum wallisii).\(^{[1]}\)

The configuration at C10 of NCCs arises by a non-enzymatic imine-pyrrole isomerization, by which C10 becomes saturated and asymmetric in NCCs that are directly formed from their immediate FCC precursors \(^{[12],[27]}\) in the course of this step the final configuration at C8\(^2\) is also established. The observed nearly stereo-uniform isomerization of a pFCC to the corresponding NCC-2 (see Figure 7) has been rationalized by the steric requirements of a rate-determining intramolecular protonation at C10 and by a subsequent thermodynamically driven net epimerization at C8\(^2\).\(^{[12]}\) This slower C8\(^2\)-epimerization of the intermediary 8\(^2\)-epi-NCC (see Figure 7) inverts its native (R)-configuration (inherited from its FCC precursor), to furnish a stable NCC with (S)-configuration at C8\(^2\).\(^{[27]}\) Indeed, epimerization of natural phyllobilins is considered to take place readily at C8\(^2\) of their characteristic Chl-derived ring E.\(^{[13],[28]}\) The neighboring keto- and ester- (or acid-) functions activate this position for exchange of its H-atom via enolization,\(^{[29]}\) a situation that is particularly relevant in aqueous media. H-exchange at C8\(^2\) and/or epimerization of an NCC under physiological conditions is expected to proceed via a de-protonation/re-protonation path and to involve the intermediate existence of a rather unstable enol tautom (see Figure 2) or of its deprotonated enolate form. Hence, the recently proposed identification of enol-isomers of NCCs\(^{[5]}\) is particularly intriguing, as enol tautomers of phylloleucobilins have not been considered isomerization intermediates that would accumulate and would be easily observable as separated isomers.\(^{[12]}\)

The absolute configuration at C10 of natural phylloleucobilins was deduced tentatively from the mechanism of the acid-induced isomerization of their direct FCC-precursors.\(^{[12]}\) According to this model, the methine group C10 of NCCs is generated with (R)-configuration. Published CD-spectra of natural NCCs are consistent with their common absolute configuration at C10. Natural ‘n’ and ‘epi’ phylloleucobilins show similar CD-spectra, due to a dominant chiroptical effect of C10 in both lines.\(^{[1]}\) The original assignment of the absolute configuration of phylloleucobilins at C10 and at C8\(^2\) was recently verified by the crystal structure of a yellow phyllobilin (a YCC), obtained as oxidation product of NCC-1.\(^{[30]}\) It also revealed the presence of two C8\(^2\)-epimeric forms and confirmed the respective relative and absolute configurations at C8\(^2\) of its major and minor epimeric forms.\(^{[30]}\) Crystals of the optically inactive, chiral oxidation product of the YCC were also analyzed, revealing this pink phyllobilin to be present as a (1:1) mixture of C8\(^2\)-isomers (enantiomers - in this case).\(^{[31]}\)

Figure 7. Isomerization of a pFCC in acidic environments generates the apolar NCC-2. This isomerization gives 8\(^2\)-epi-NCC-2, first, followed by epimerization at position 8\(^2\).\(^{[12],[27]}\) The label ‘n’ signifies an unspecified absolute configuration at C16 in the pFCCs, which is conserved in the conversion to the colorless NCCs, 8\(^2\)-epi-NCC-2 and NCC-2 (see Text).
Minor satellite type fractions are frequently observed in HPLC-analyses of purified samples of phylloleucobilins and their nature has occasionally been discussed. In the absence of further evidence, when minor isomer fractions were noticed, they were provisionally associated with the acidified position C8 of the isolated NCC-forms (see e.g. [12][28]). H/D-exchange at C8 of phylloleucobilins was observed early (in 1H-NMR spectra of Hv-NCC-1), when using deuterated, protic solvents. Epimerization at C8 was also implied in the proposed formation of NCCs by acid induced imine-pyrrole isomerization of FCCs (Figure 7).[12][27]

Here, the ‘model’ NCC-1 and its effectively slightly more polar major isomer (8-epi-NCC-1) were derived to be epimers, relating to each other by inversion of the configuration at C8 (see Figure 2). The major (more stable) isomer carries its larger groups at the adjacent asymmetric carbons C10 and C8 in a trans-arrangement, i.e., with (R) and (S) configuration, respectively. Indeed, we failed to find evidence for the proposed presence of significant fractions of NCC-enols in solution.[5] As established here for NCC-1 and its reversibly formed isomer, the existence of phylloleucobilin epimers at C8, can now be proposed to be a general feature of typical NCCs and DNCCs (or of the related phylloanthobilins, such as YCCs),[30] carrying a carboxylic acid or ester group at C8.[11][13]

Conclusions

The occurrence of isomeric NCCs in different plants is a natural consequence of the PaO/phyllobilin pathway of Chl breakdown and of its characteristically diverging stereochemical branches.[1 – 3] Indeed, the elucidation of the biochemical path of Chl breakdown would not have been achieved without far reaching structural characterization and classification of the phyllobilins isolated in extracts of leaves and fruit.[1][13] Besides the now known 1-formyl-19-oxo-type phyllobilins (or type-I phyllobilins) directly derived from RCC, such as FCCs, NCCs, and YCCs, corresponding representatives of a second branch of 1,19-dioxobilin-type (or type-II) phyllobilins, abbreviated as DFCCs, DNCCs, etc., have been discovered in recent work.[1][32][33] Thus, the stereochemical classification of NCCs and DNCCs as ‘n’ or ‘epi’ at C16, or of the DNCCs at their C4,[32][33] and the proper characterization of peripheral (sugar) modifications of natural phyllobilins,[9] are all relevant in this context.[1]

A stunning structural variety of Chl catabolites is available in plant extracts, including the here discussed isomers from (slow) spontaneous ‘chemical’ epimerization reactions.[1] On the basis of an unambiguous NMR spectral detection of a proton at both of the carbons C8 and C10 of two isomeric forms of a ubiquitous NCC, two C8-epimeric forms could be defined here as NCC-1 and 8-epi-NCC-1, and the (relative) configuration of their two asymmetric carbons C8 and C10. An enol(ate) form is, hence, excluded as essential component of the isomerization equilibria between NCC-1 and 8-epi-NCC-1, contrasting strikingly with the LC/MS-based suggestion of the occurrence of related phyllobilin-enols in extracts of the Loquat fruit.[5]

Without a doubt, the advent of modern LC/MS methodology is breaking new ground for routine work concerned with the identification of phyllobilins in plant extracts.[34][35] This approach is most promising in analyzing for known phyllobilins. LC/MS studies are also proliferative in the detection of phyllobilin isomers.[34][35] Furthermore, in favorable cases and on the basis of the documented fragmentation pattern of such isomers,[1][36][37] could the chemical constitution of new Chl-catabolites also be deduced in LC/MS-studies combined with detailed state-of-the-art ion fragmentation analysis.[35] However, profound structure analysis, including stereochemical issues characteristic of the structure of new typical phyllobilucobinoids generally require more structure sensitive methods, such as those provided by heteronuclear NMR spectroscopy and crystallography.

Chl-breakdown is observed as a most colorful fundamental biological phenomenon in the plants [3][38 – 40] and phyllobilins are molecular ‘biomarkers’ of fruit ripening [8][41][42] and leaf senescence.[1][2][39] Phyllobilins have, with few exceptions,[41 – 43] been analyzed in plant extracts, rather than directly in the original plant tissues. Significant new insights may be expected from in vivo analyses thanks to current methodological advances in the fields of mass spectrometry[43][44] and of fluorescence-spectroscopy.[41][42][45] The specific natural availability of phyllobilins and of natural phyllobilin isomers in plant cells may signal fundamental endogenous cellular processes and/or responses to pathogens[11] and external stress factors.[3][40]

The structures of phyllobilins are remarkably related to those of the heme-derived bilins,[1][13][48] in spite of reflecting widely different pathways of the specific formation in the plants. Similar to the important biological functions of the heme-derived bilins,[46][47] Chl-derived phyllobilins may also have important physiological roles. Chl-catabolites, such as the NCC-1, are actually ubiquitous components of our plant...
based daily food. This occurrence of phyllobilins in our nutrition, in particular, demands precise information on their structures. Indeed, phyllobilins have been identified, e.g., in fruit, in vegetables and in other agricultural produce and they may be qualified as among potentially health-supportive components of our daily food.

**Experimental Section**

**Materials**

HPLC grade acetonitrile (MeCN) and methanol (MeOH) were from WVR (West Chester, USA), ultrapure water (18 MΩ cm⁻¹) from a Millipore apparatus, dichloromethane (CH₂Cl₂) ≥ 99%, from Sigma-Aldrich, was freshly distilled and filtered over Alox prior to use. Potassium phosphate monobasic (K₂HPO₄), puriss. p.a., potassium phosphate dibasic (K₂HPO₄), puriss. p.a., and ammonium acetate, puriss. p.a. were from Fluka (Buchs, CH). 1 and 5 g SepPak C18 cartridges were from Waters Associates (Milford, USA).

**Chromatography**

**TLC. Macherey-Nagel Polygram SIL G UV254 silica with fluorescence indicator; solvent CH₂Cl₂/MeOH 9:1.**

Preparative HPLC: Agilent HPLC system with a 1260 Infinity Degasser, a 1100 Series quaternary pump and 1100 Series diode array detector; column, mobile phase A = ammonium acetate buffer 50 mM pH 7, B = MeOH, flow 5 ml/min; solvent composition: 0 – 1 min 45% B, 1 – 40 min 45–100% B. Data were processed with HP ChemStations. Semi-preparative HPLC: Dionex Summit HPLC system with manual sampler and online degasser, P680 HPLC pump, UVD340U diode array detector and a Rheodyne injection valve with 5 ml loop; column Phenomenex Luna 5μ C18, 100 A, with Phenomenex pre-column 10 × 10 mm (AU7220); mobile phase A = ammonium acetate buffer 50 mM pH 7, B = MeCN, flow 4 ml/min for injection, 5 ml/min for separation; solvent composition: 0 – 5 min 15% B, 5 – 35 min 15–40% B, 35 – 40 min 40% to 100% B. Data were processed with Chromelone 6.50. Analytical HPLC: Dionex Summit HPLC system with manual sampler and online degasser, P680 HPLC pump, UVD340U diode array detector and a Rheodyne injection valve with 20 μl loop; column Phenomenex Hyperclone ODS 5 μm 250 × 4.6 mm i.d., with Phenomenex ODS 4 × 3 mm i.d. pre-column; mobile phase A = ammonium acetate buffer 50 mM pH 7, B = MeCN, flow 0.5 ml/min; solvent composition: 0 – 32 min 45% B to 61% B, 32 – 37 min 61–100% B. Data were processed with Chromelone 6.50.

**UV/VIS. Hitachi U-3000 spectrophotometer, concentrations of NCC-1 were determined gravimetrically; for 8²-epi-NCC-1 they were calculated using log ε = 4.25 at λmax = 307 nm (as determined for NCC-1). ε-Values were calculated using Lambert-Beer’s law; λmax (λrel) in nm: CD: Jasco J715 spectropolarimeter, solvent MeCN; λmin/max in cm⁻¹ (Δλ=1 mol⁻¹ cm⁻¹)). NMR Spectra: Bruker UltraShield Avance II+ 600 MHz spectrometer; solvent CD₂CN; δ in ppm rel. to CHD₂CN (δ(H) 1.96 ppm, δ(C) 1.79 ppm), J in Hz. ESI-MS: Thermo LTQ XL or LTQ Orbitrap (for high resolution MS) mass spectrometer, ESI source, positive ion mode, spray voltage 4.0 kV, data were processed with Xcalibur; in m/z (relative abundance, type of ion), signals due to isotopomers and their relative intensities are shown for [(M + H)⁺]²³²

**Separation, Purification, and Isolation of NCC-1 and 8²-epi-NCC-1**

Prepurified NCC-1 was obtained from senescent Cercidiphyllum japonicum leaves as a roughly 10:1 mixture of NCC-1 and 8²-epi-NCC-1, essentially as described. 3 mg of a pre-purified NCC-1 were dissolved in 85 ml of ammonium acetate 10 mM pH 7/MeCN 85:15 and left for 3 h under argon atmosphere at ambient temperature protected from light. The mixture was separated by semi-preparative HPLC; fractions were collected and immediately frozen (–80 °C). The fractions containing NCC-1 or 8²-epi-NCC-1 were diluted with MilliQ water and desalted via SPE (Waters Sep-Pak, 1 g C18 WAT023635, elution with MeCN). The eluates were further concentrated on a rotary evaporator (bath temperature at 25 °C) and then lyophilized. Pure samples of 8²-epi-NCC-1 (328 nmol = 211 μg, used for CD- and UV/VIS-spectroscopic analyses) and of NCC-1 (2.95 μmol = \(1.9\) mg, used for NMR-, CD- and UV/VIS-spectroscopy) were obtained (see Figures S1 and S2 for HPLC analyses).

**Purification of 8²-epi-NCC-1 for NMR-Spectroscopic Measurements.** 20 mg of pre-purified NCC were dissolved in 10 ml of ammonium acetate 10 mM pH 7/MeOH (55:45, v/v) and were stored for 4 h under argon atmosphere at ambient temperature protected from light. The mixture was separated by preparative HPLC; fractions were collected and immediately frozen.
The fraction containing 8²-epi-NCC-1 was diluted with MilliQ water and desalted via SPE (Waters Sep-Pak, 1 g C18 WAT023635, elution with MeOH). The eluate was further concentrated on a rotary evaporator (bath temperature at 25 °C) and the remaining solution was lyophilized. 761 nmol (490 μg) of pure 8²-epi-NCC-1 were obtained (see Figure S3), used for NMR analysis.

Isomerization Experiments with NCC-1 and 8²-epi-NCC-1. 1.0 mg of pre-purified NCC was dissolved in 15% MeCN and 85% ammonium acetate 10 mM pH 7 (v/v) and separated by semi-preparative HPLC. An aliquot of 8²-epi-NCC-1 was collected from semi-preparative HPLC and was diluted 1:1 with chilled, de-gassed ammonium acetate buffer 10 mM pH 7. The subsequent isomerization reaction was performed under argon atmosphere at ambient temperature protected from light. Samples were pulled at t = 0, 0.5, 1, 3, 4, and 20 h and analyzed by HPLC (see Figure 3, a). An aliquot of NCC-1 was collected and was diluted 1:1 with chilled, de-gassed potassium phosphate buffer 50 mM pH 7. The subsequent isomerization (at pH 7) was performed under argon atmosphere at ambient temperature protected from light. Samples were pulled at t = 0, 0.5, 1, 2, 3, 4, 5, 25, 48, 71, and 144 h and analyzed by HPLC (see Figure 3,b).

Spectroscopic Data

**NCC-1.** UV/VIS (c = 70.2 μM): 243 (sh, 4.34), 307 (4.25). CD (c = 62 μM): 222 (7.9), 245 (--3.3), 259 (--2.2), 281 (--4.9), 313 (1.4). ¹H-NMR (600 MHz, 0 °C, CD₃CN): 1.80 (s, H₂C13¹); 1.99 (s, H₂C17¹); 2.16 (s, H₂C7¹); 2.20 (s, H₂C2²), superimposed by 2.2 – 2.5 (m: 2.28 (m, H₆C12²), 2.34 (m, H₆C12²), 2.49 (m, H₆C12²), in total ca. 6 H); 2.5 – 2.8 (m: 2.53 (m, H₆C15); 2.59 (m, H₆C12²), 2.61 (m, H₆C3¹), in total ca. 4 H); 2.83 (dd, J = 4.8/14.8, H₆C15); 3.47 (m, H₂C3²); 3.64 (d, J = 3.4, H₆C8²); 3.69 (s, H₂C8²); 3.88 (br. s, H₂C5); 3.97 (dd, J = 6.6, HC16); 4.77 (d, J = 3.3, HC10); 5.32 (dd, J = 2.2/11.7, H₆C18²); 6.16 (dd, J = 1.5/17.4, H₆C18²); 6.43 (dd, J = 11.5/17.6, HC18¹); 6.89 (br. s, HN24); 8.43 (s, HN23); 9.31 (s, HC20); 10.37 (br. s, HN22); 10.44 (br. s, HN21). ¹³C-NMR (600 MHz, 0 °C, CD₃CN, signal assignment from HSQC and HMBC spectra): 8.8 (2¹); 9.7 (13¹); 10.0 (7¹); 13.1 (17¹); 20.4 (12¹); 22.9 (5); 27.8 (3¹); 29.0 (15); 35.8 (12¹); 37.0 (10); 53.1 (8¹); 61.0 (16); 62.2 (3¹); 67.9 (8¹); 111.0 (7); 115.5 (13); 119.0 (18¹); 119.6 (12); 120.6 (3); 123.8 (14); 124.9 (11); 126.1 (8); 127.3 (18¹); 128.1 (18); 129.6 (1); 133.7 (2); 134.8 (6); 138.1 (4); 156.7 (17); 159.0 (9); 171.6 (8¹); 174.3 (19); 176.1 (12¹); 177.3 (20); 189.8 (8¹).

**8²-epi-NCC-1.** UV/VIS (c = 60 μM): 240 (sh, 1.02), 306 (1.00). CD (c = 60 μM): 222 (9.6), 251 (–2.9), 257 (–2.6), 278 (–4.8), 312 (2.8). ¹H-NMR (600 MHz, 0 °C, CD₃CN): 1.81 (s, H₂C13¹); 2.04 (s, H₂C17¹); 2.18 (s, H₂C7¹); 2.22 (s, H₂C2²); 2.28 (m, H₆C12²); 2.34 (m, H₆C12²); 2.53 (m, H₆C12²); 2.55 (m, H₆C15); 2.65 (m, H₆C12²); 2.65 (m, H₂C3¹); 2.87 (dd, J = 4.3/14.8, H₆C15); 3.54 (m, H₂C3²); 3.69 (s, H₂C8²); 3.92 (H₂C5); 3.97 (m, HC16); 4.06 (d, J = 7.5, HC8²); 4.92 (d, J = 7.3, HC10); 5.32 (H₆C18²); 6.16 (H₆C18²); 6.43 (HC18¹); 6.80 (br. s, HN24); 8.26 (s, HN23); 9.41 (s, HC20); 10.31 (br. s, HN); 10.34 (br. s, HN). ¹³C-NMR (600 MHz, 0 °C, CD₃CN, signal assignment from HSQC and HMBC spectra): 9.0 (2¹); 9.9 (13¹); 10.30 (7¹); 13.2 (17¹); 20.7 (12¹); 23.3 (5); 28.0 (3¹); 29.1 (15); 35.8 (12¹); 36.1 (10); 53.3 (8¹); 61.3 (16); 62.7 (3¹); 65.2 (8¹); 111.5 (7); 116.4 (3); 119.1 (18¹); 120.2 (12); 120.6 (3); 124.4 (14); 122.0 (11); 127.8 (8); 127.3 (18¹); 129.0 (18); 129.7 (1); 132.7 (2); 135.2 (6); 137.9 (4); 157.2 (17); 158.9 (9); 171.7 (8¹); 175.0 (19); 177.0 (12¹); 177.4 (20); 191.0 (8¹). HR-ESI-MS: 645.29117, [C₅₅H₄₁N₄O₈]⁻; calc. 645.29189. ESI-MS: 1289.6 (8, [2M + H]⁺), 689.3 (6, [M – H + 2 Na]⁺), 683.3 (17, [M + K]⁺), 667.3 (35, [M + Na]⁺), 647.3 (8), 646.3 (40), 645.3 (100, [M + H]⁺), 613.3 (19, [M – CH₂OH+H]⁺), 522.2 (7, [M – ring D + H]⁺).

Supplementary Material

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.201700368.

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Author Contribution Statement

S. M. and G. S. carried out the experiments under the supervision of B. K., and the manuscript was written by S. M. and B. K.

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