Isolation and Characterization of a Urobilinogenoidic Chlorophyll Catabolite from *Hordeum vulgare* L.*

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A new type of chlorophyll catabolite was isolated from extracts of de-greened primary leaves of barley (*Hordeum vulgare* cv. Lambic). Its constitution was elucidated by one-dimensional and two-dimensional (1H,13C)-homonuclear and heteronuclear NMR spectroscopic techniques and by high resolution mass spectroscopy. The isolated catabolite, a water-soluble, colorless, and nonfluorescent linear tetrapyrrole, resembles urobilinogen in which one of the propionic side chains forms a five membered isocyclic ring system, indicating its origin from the chlorophylls.

Metabolic disappearance of the chlorophylls (Chls) in phototrophic organisms indicates programmed close down of photosynthesis. Although several linear tetrapyrrolic Chl catabolites were isolated during the last decade from green algae and higher plants, the metabolic pathway of tremendous amounts of the Chls is still under question. What products follow after the familiar formybilinones? What are the ultimate products of Chl degradation? Degradation of the Chls occurs in light as well as in darkness. During senescence cellular components are hydrolyzed and metabolized; liberated rare elements such as [N], [P], [S] and metal ions are relocated (1, 2).

The first structures of Chl catabolites isolated from a green alga and a higher plant were published in 1991 (3, 4). Several Chl catabolites have been isolated since then (Fig. 1). The similarity of the structures of the red Chl catabolites (3, 4) isolated from the green alga *Chlorella protothecoides* with the colorless catabolites isolated from higher plants (5–7) suggest a close relationship in the basic skeleton. Biologists regard members of the phyllum Chlorophyta as progenitor of the higher plant cell (9, 10). This information triggered research activities in several disciplines to elucidate the apparently unique catabolic pathway of the Chls in the green plant lineage. The studies range from the elucidation of chemical and enzymatic reaction mechanisms to molecular biological research (6, 11, 12). Previous labeling experiments with oxygen isotopes and reaction mechanisms to molecular biological research (6, 11, 12). Previous labeling experiments with oxygen isotopes and reaction mechanisms to molecular biological research (6, 11, 12).

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**EXPERIMENTAL PROCEDURES**

**General**—Chemicals were reagent grade; all solvents were distilled before use. HPLC solvents were supplied from Fluka (Buchs, Switzerland). HPLC columns and Nucleosil 100-7 C8 VP 250/10 were from Macherey-Nagel (Oensingen, Switzerland), and MPLC columns Lobar® LiChroprep® RP-18 (40–63 μm) were purchased from Merk (Darmstadt, Germany). 35cc Sep-Pack® Vac C-18, 10 g, were provided from Waters (Milford, MA).

Homo- and heteromagnetic resonance experiments were performed on a Bruker Avance DRX-500 spectrometer operating at the frequencies of 500.13 MHz for 1H and 125.75 MHz for 13C. Chemical shifts (δ) are given in parts/million downfield from the solvent used and coupling constants (J) in Hz. Mass spectra were obtained with a Bruker FTMS 4.7T BioAPEXII, using electrospray ionization technique in the positive mode. UV-visible spectra were recorded on a Perkin-Elmer Lambda 40 spectrometer, λmax (log e) in nanometers. CD spectra were measured on a Jobin-Yvon Auto Dichograph Mark V, Δε [l mol⁻¹ cm⁻¹].

**Plant Material**—Barley seeds (*Hordeum vulgare* L. cv. Lambic) were a gift from Florimond Desprez. The seeds were germinated in high density (5 seeds/cm²) in moist garden soil and grown under natural light conditions. The primary leaves were harvested when they reached about 10–15 cm in height. The greening and de-greening procedure of barley leaves was essentially as described previously (17).

**Isolation of the Chlorophyll Catabolite**—150 g of de-greened yellow leaves of *H. vulgare* (fresh weight) were homogenized in a blender with 300 ml of a solution consisting of 0.1 M potassium phosphate (KP) buffer, pH 6.8:acetone:MeOH (1:1:1). Work-up was essentially as described previously (17). Aliquots of the aqueous phase were injected into a Lobar® LiChroprep® RP-18 (40–63 μm) column and eluted (4 bar, 22 ml/min) with a solution of 30 volume % MeOH in 0.01 M KP buffer, pH 6.8. Two fractions positive in the chromic acid degradation assay were sampled. MeOH was evaporated in vacuo. The polar fraction contained both urobinlinogens, the less polar fraction contained formybilinone 5. The resulting aqueous phases were again concentrated on a 35cc Sep-Pack® RP-18 cartridge. Separation and purification of the urobinlinogens were achieved on a HPLC Nucleosil 100–7 C8 VP 250/10 column eluted isocratically (flow rate: 5 ml/min) with a solution of MeOH in 0.01 M KP buffer, pH 6.8 (13 volume %). The fractions with a retention time of 13 min and of 14 min, respectively (DAD-UV detection at 250 nm) were collected at 0 °C and stored under argon. After removal of the volatile solvent in vacuo, each product was de-salted on a 35cc Sep-Pack® C-18 cartridge by first washing with 200 ml of distilled water and afterward eluted with aqueous acetone (50 ml of 50 volume %). The gross solvent was eliminated in vacuo, and the remaining aqueous solution was lyophilized giving 15 mg and 10 mg, respectively, of a slightly yellow powder.
RESULTS

A triad of tetrapyrrolic compounds was isolated by HPLC from yellow cotyledons of barley. After chemical degradation all showed on TLC plates the same characteristic maleimide fragments, namely 3-(2-hydroxyethyl)-4-methyl maleimide, 3-(2,3-dihydroxyethyl)-4-methyl maleimide and hematinic acid imide (cf. Ref. 17).

The less polar compound isolated in about 7 mg was spectroscopically identical in all aspects with catabolite 5 (Fig. 1) previously isolated from H. vulgare cv. Gerbel (4). The second product, which was isolated in about 15 mg, was spectroscopically analyzed. Mass spectrometric analysis at high resolution showed two molecular ions at \( m/z \) 705.2530 atomic mass units (100%) and \( m/z \) 743.2093 atomic mass units (10%). This corresponds exactly with a molecular ion \([C_{34}H_{41}KN_4O_{10} + H]^+\) (calculated: 705.2532; error: \( 2.5 \times 10^{-4} \)) and \([C_{34}H_{41}KN_4O_{10} + K]^+\) (calculated: 743.2091; error: \( 1.8 \times 10^{-4} \)), respectively, establishing the bulk molecular formula to be \( C_{34}H_{41}KN_4O_{10} \).

Details on the constitution of this metabolite were deduced from the analysis of the one-dimensional and two-dimensional \(^1H,^{13}C\)-homo- and heteronuclear NMR spectroscopy (Fig. 2).

The compound when dissolved in \( D_2O \) shows 33 carbon-bound hydrogen atoms and 33 carbon atoms. Seven protons are bound to hetero-atoms, which rapidly exchange for solvent. Attached proton test spectra show 5 primary, 7 secondary, 4 tertiary, and 17 quaternary carbon atoms. All protons resonate largely as isolated systems and are well resolved, except the signal group at about \( \delta_H 2.7 \) (\( H_a(5) \) and \( H_a(15) \)) and \( \delta_H 3.6 \) (\( H_a(3) \)) and \( H_a(18^2) \)) in which two and three protons overlap, respectively (Table I). Four separated methyl groups resonance at high field \( \delta_H 1.45 H_a(2^1), \delta_H 1.82 H_a(13^1), \delta_H 1.91 H_a(17^1), \) and \( \delta_H 1.97 H_a(7^2) \). The fifth methyl group, which absorbs at \( \delta_H 3.69 H_a(8^5) \), is assigned to a methyl ester group, because of its chemical shift, line sharpness, and intensity. The highest resonance frequency was observed at \( \delta_H 4.74 \) H(10); this excludes the presence of an aldehyde group. Two-dimensional \(^1H,^1H\)- and \(^1H,^{13}C\)-correlation spectra show the presence of two ethylene groups and three AMX systems, the X portions of which have resonances at \( \delta_H 4.16 H_a(16), \delta_H 4.32 H_a(4), \) and \( \delta_H 4.44 H_a(18^1) \), respectively. At \( \delta_H 3.55 \) a weak CH cross-peak was found in the \(^1H,^{13}C\)-correlation spectrum with the H(10) at \( \delta_H 4.74 \) ppm.

Although the proton part of the spectra of the compound when measured in \( Me_2SO-d_6 \) as solvent showed considerable lower resolution, the missing proton became clearly visible at \( \delta_H 3.88 \) H(8)\(^5\). This proton couples with a frequency of 3.35 Hz with a proton at \( \delta_H 4.65 \) H(10). The \(^1H,^{13}C\)-correlation spectrum showed in addition a cross-peak indicating a tertiary carbon atom at \( \delta_C 54.44 C(8) \), which correlates with the H(8)\(^5\) proton at \( \delta_H 3.88 \). This indicates that this carbon-bound proton is in dynamic equilibrium (keto-enol) and rapid exchange with deuterium from the solvent \( D_2O \). This “blind spot” has been previously observed when the plant catabolite 5 was measured in \( D_2O \) (20).

In the COSY spectrum (Fig. 2), particularly the appearance of six cross-peaks caused by long range couplings of the four methyl groups facilitated the constitutional assignment. Together with the information gathered from the heteronuclear correlation (HETCOR) spectrum, they provided the reference and indicated the starting points of the specific side chains and
the bridging network for each of the two dipyrrylmethanone units.

Nuclear Overhauser effect experiments corroborated the assignment of the fragments and showed, in addition, that both units are interconnected (Fig. 3 and Table I). Thus, when the proton at δH 4.74 H(10) was irradiated the signal group at δH 2.45–2.50 H2(121) was enhanced. When the latter signal was irradiated a methyl group H 3(132) and a methylene group H2(122) responded through space.

The overall bonding network and chemical shift assignments of the quaternary carbon atoms were deduced from correlation spectroscopy via long-range coupling (COLOC) spectra. The relaxation times used for the evolution of the long range couplings were equivalent to 1H,13C coupling constants of 5, 10, and 20 Hz, respectively. Although two quaternary carbon atoms at δC 191.77 and δC 160.19 showed no correlation, the former value was assigned for chemical shift reasoning to the carbonyl group C(81) and the latter consequently to the remaining aromatic carbon atom C(9). Thus, chemical degradation, mass, nuclear magnetic resonance, and UV spectra are consistent for the constitution of a potassium 31,181,182-trihydroxy-85-methyl-81-oxo-82,10-cyclourobilinogen (8) shown in Fig. 4.

The third tetrapyrrole was isolated in about 10 mg and showed in the mass spectrometer a molecular mass ion at m/z 705.2534 atomic mass units (100%), indicating the presence of a configurational isomer of the former. 1H,13C NMR spectroscopic investigation corroborates a diastereomeric relationship. A 1:1 mixture of diastereomers shows in 1H NMR only slight differences in chemical shifts. Two methyl groups (21) and (171) are shifted relative δ 0.079 ppm to lower field and δ 0.0134 ppm to higher field, respectively, the rest of the spectrum remains nearly identical. The CD spectrum shows that this compound too is optically active and that both diastereomers are chiroptically almost indistinguishable (Fig. 5).
**Discussion**

The new catabolites isolated from yellow cotyledons of barley are colorless, nonfluorescent, and optically active. The constitution resembles Urobiligen IXα, a common metabolite of heme catabolism in warm-blooded organisms. The similarities in the peripheral substitution pattern of both catabolites indicate that the new catabolite derives from catabolite 5 by an oxidative process.

The configurations of the five asymmetric centers cannot be deduced from the spectroscopic data. Suitable crystals are not available yet. Only one stereocenter at C(82) has its origin from the Chls, but due to the β-keto ester functional group, it is prone to epimerization. When measured in Me2SO-d6, a coupling of only 3.35 Hz between H(10) and H(82) indicates anticonfiguration.

As found previously, proton H(15) in formula 5 arrives from the protic solvent during acid-catalyzed pyrrole/pyrrole rearrangement of both geometric isomers of the red chlorophyll catabolite from C. protothecoides (11, 16). Intensive mechanistic-chemical studies showed a remarkable high stereoselectivity for this rearrangement. Because of the stereochemical consequence of these experiments, this position was tentatively assigned an R configuration. The proton at this position is highly resistant, even in boiling acetic acid-14C, as expected, only at position C(82). Moreover, a mirror experiment in which the leaves were bleached in the presence of heavy water (80 atom % D) showed by 2H NMR that a deuterium atom resides in position C(4) of compound 8 (data not shown). These results demonstrate that the catabolite is formed entirely in the plant cell and not during the work-up procedure and further that H(4) is tautomerization-stable under those conditions. Skeletal transformation between the two valence tautomers of urobilin/bilirubin was reported to be reversible only under basic conditions (21).

From the Chlorophyte Bryopsis maxima a red, water-soluble tetrpyrrole was isolated (22), and a portion of structural information was published (23). The proposed constitution, a biliviolin, lacks the formyl group too and contains a phytol group and a sugar moiety. Nevertheless, the UV-visible spectrum is in disagreement with the constitution, which remains to be determined.

In Cercidiphyllum japonicum almost quantitative amounts of the chls were isolated as catabolite 6 (8), whereas in this work only about 20% of the tetrapyrrole catabolites were recovered. As charged from a screening test of several autumnal plants using the chromic acid degradation method, (tetra)pyrroles are not always present. This raises the questions whether urobilinogenoids are peculiar or common intermediates in the catabolic pathway of the chls and whether they are finally degraded in the plant cell to nonpyrrolic, nitrogen-less compounds.

**Figure 4. Constitutions of the chlorophyll catabolites (5 and 8) isolated in this work from yellow leaves of H. vulgare.** The configurations of the chiral centers (dots) are not determined. The putative α-hydroxypyrrole derivative 9 formed by oxidative deformylation of 5 can be protonated on both diastereotopic faces, giving rise to the formation of diastereomers at C(4). Note that two different nomenclature systems are applied in parallel in this work. If all carbon atoms of the core macrocycle remain in the catabolite, then the compound is denominated as seco derivative of the original closed macrocycle in accordance with IUPAC rule and numbering system; if the methine carbon atom is lost from the former macrocycle, then the resulting tetrapyrrole is denominated and numbered in accordance with the IUPAC rules for the corresponding bile pigment (18, 19).

**Figure 5. Superimposed UV-visible and CD spectra of the major (solid line) and the minor (broken line) diastereomer of 8.** UV-visible and CD spectra were measured in 0.02 M potassium phosphate buffer, pH 6.8; c = 8 × 10⁻⁵ M.
Most recently, several maleimides were isolated from senescent cotyledones of barley (24). Therefore, it appears that phototrophic organisms are capable of degrading chls to maleimides derivatives (possibly via urobilinogenoids), a pathway that has already been discussed by Hendry et al. (25). Maleimides still contain the nitrogen atoms of the former Chls. Can this nitrogen be re-utilized by the plant? This adaptation would certainly be of evolutionary advantage for plants, which grow in environments in which nitrogen is in high demand. Nevertheless, the ultimate fate of the Chls is still a matter of speculation.

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