Chlorophyll Catabolite from *Parrotia Persica* Autumnal Leaves

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Biodegradation of chlorophyll is observable biochemical process visualized, in autumn, by the loss of the green leaf colour. A chlorophyll catabolite was isolated from scarlet – red, bright red and yellow Parrotia persica, Hamamelidaceae autumnal leaves. The structure of isolated chlorophyll catabolite was elucidated by UV – Vis – , CD – , HRESIMS – , 1D – and 2D – homonuclear and 2D – heteronuclear NMR – spectra. The full structure of the isolated chlorophyll catabolite was identified as the 3^1 – hydroxyl – 8^4 – methyl – 8^1 – oxo – 8^2, 10 – cyclo urobilinogen.

Keywords: chlorophyll catabolite, biodegradation, Parrotia persica, Hamamelidaceae

Biodegradation of chlorophyll begins with the oxygenolytic ring cleavage of pheophorbide *a* (Pheide *a*) which is the first intermediate upon the release of magnesium atom from the core of chlorophyllide *a* (Chlide *a*) ring by the enzyme dechelatase. The so – called "north" opening of Pheide *a* ring is mechanistically postulated. Once the Pheide *a* ring is opened, the subsequent tautomerization induces the loss of fluorescence. The chlorophyll catabolites with the lost fluorescence were named the non – fluorescent chlorophyll catabolites (NCC). Biodegradaton of chlorophyll further proceeds with the modifications on the side chain groups.

The NCCs have been isolated from the following higher plants: *Spinacia oleracea*, Amaranthaceae[1, 2], *Arabidopsos thaliana*, Brassicaceae[3], *Brassica napus*, Brassicaceae[4], *Cercidiphyllum japonicum*, Cercidiphyllaceae [5, 6], *Hordeum vulgare*, Gramineae [7], *Liquidambar orientalis*, Altingiaceae[8], *Liquidambar styraciflua*, Altingiaceae[8], *Capsicum annuum*, Solanaceae[9] and *Nicotiana rustica*, Solanaceae[10].

In this paper the structure elucidation of the chlorophyll catabolite isolated from scarlet – red, bright red and yellow *Parrotia persica*, Hamamelidaceae autumnal leaves is reported.

Experimental part

General

TLC was done on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany). The TLC plates were visualised by UV light (254 and 366 nm).

Medium-pressure liquid chromatography (MPLC) was carried out on Buechi Pump Manager C – 615, pump module C – 601 along with fraction collector C –660 and UV Photometer C – 635 (Buechi, Labortechnik AG, Flawil, Switzerland) set at 280 nm.

The semi – quantitative separations were performed using Waters 600 HPLC system coupled with Waters 2996 PDA UV – Vis detector (Waters Corp., Milford, USA) and Waters 2695 Separations Module (Milford, MA, USA) equipped with Waters 2996 PDA UV – Vis detector. The HPLC Empower Software (Waters Corp., Milford, USA) was employed for data acquisition and processing, run under Windows NT™ (Microsoft, Redmond, USA).

UV spectrum was obtained on a Perkin Elmer Lambda 40 UV – Vis spectrometer, Beaconsfield, Buckinghamshire, England, running under Windows NT™ (Microsoft, Redmond, USA).

CD spectra were recorded on CD/ORD Spectropolarimeter Jasco J – 715, Ishikawa – cho, Hachioji – city, Tokyo, Japan, running under Windows NT™ (Microsoft, Redmond, USA).

High resolution electrospray ionization mass spectrum (HRESIMS) was run on a Bruker Daltonics BioAPEX II FTMS 4.7 Tesla (Faellanden, Switzerland) with a direct insertion probe.

NMR spectra were recorded at 500.13 MHz for ^1H and 125.75 MHz for ^{13}C on a Bruker Avance DRX 500 NMR spectrometer using standard Bruker pulse sequence[11]. The sample temperature during measurements was 298.0 ± 0.5 K and was controlled by Bruker B – VT 1000 temperature control unit. ^1H NMR, ^1H – ^1H COSY, 1D NOE difference experiments, ^{13}C NMR, DEPT, HETCOR and COLOC were recorded in a 5.0 mm inverse detection microprobe head. Chemical shifts were referenced to residual proto – deuteron solvent signals in CD_3OD – d_4.

Plant material

Parrotia persica, Hamamelidaceae, leaves have been collected during the autumn season (2004) from the Botanical Garden of Fribourg, Switzerland.

Extraction and Isolation

Parrotia persica, Hamamelidaceae leaves (160.08 g dry weight, 220.00 g "fresh" weight) were chilled by liquid nitrogen, grinded and homogenized in a blender with 1 dm^3 methanol, at room temperature, for 10 minutes. The methanol extract was filtered and partitioned between hexane and methanol. Water was added to the methanol phase. Chlorophyll catabolite was extracted with dichloromethane from the methanol – aqueous phase. Evaporation of dichloromethane ($t < 40^0C$) yielded 310 mg of moderately polar compounds. Moderately polar compounds were subjected to MPLC on lab – made column, size 310 x 25 mm, filled with LiChroprep® RP8, particle size 25 – 40μm silica gel (Merck, Darmstadt, Germany). All solvents used for MPLC were distilled prior to use. Elution with water: methanol, 1:1→0:1 in 30 min. (flow rate 7 mL/min; UV detection 280 nm) yielded 18.6 mg of the prepurified *Parrotia persica* chlorophyll catabolite. Final purification was done by reverse –

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phase HPLC using Waters 600 HPLC system coupled with Waters 2996 PDA UV – Vis detector (Waters Corp., Milford, USA) and RP EP 250 x 16 mm Nucleosil 100 – 7 C_8 column together with the RP EP 30 x 16 mm Nucleosil 100 – 7 C_8 precolumn (Macherey – Nagel, Oesingen, Switzerland). The detection wavelength was set at 244 nm, temperature of the column oven was 22^0 C and the injection volume was 2 mL via loop injection. The mobile phase consisted of water (0.1% TFA) : methanol, 1:1 (v/v) and ran isocratically at a constant flow rate of 3.2 mL/min. PDA detection was in range of 200 – 800 nm, the chromatogram was extracted at λ=244 nm. Compound eluted at 52.8 minute was collected to obtain 2.55 mg of the pure *Parrotia persica* chlorophyll catabolite. The HPLC Empower Software (Waters Corp., Milford, USA) was employed for data acquisition and processing, running under Windows NT™ (Microsoft, Redmond, USA). All solvents used were HPLC grade (Acros Organics, Geel, Belgium).

TLC was done on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany), using dichloromethane : methanol : water, 83 : 15 : 2 solvent system for development. The compounds on TLC were detected from their visible. A spot at R_f = 0.48 revealed the presence of a non – fluorescent chlorophyll catabolite.

Results and Discussion

The *Parrotia persica* chlorophyll catabolite **1** (fig. 1) was obtained as yellow amorphous solid. The High Resolution ElectroSpray Ionisation Mass Spectrum (HRESIMS) showed a molecular ion at *m/z* 655.2736 for the molecular formula $C_{34}H_{40}N_4O_8Na$ $[M+Na]^+$, calculated *m/z* 655.2738, Δ + 0.31 ppm.

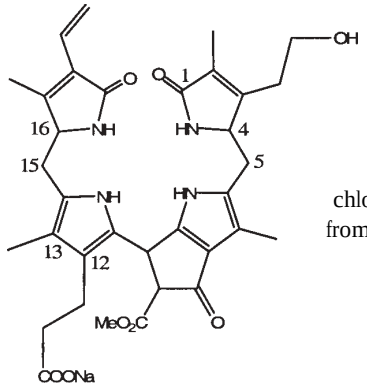

Fig. 1. Structure of the chlorophyll catabolite isolated from Parrotia persica autumnal leaves

The structure elucidation of the side chain substituents and the tetrapyrrolic ring system was determined by high resolution NMR spectra (500 MHz). The integration of the proton NMR spectrum revealed the presence of 33 protons. The proton overlapped with the HDO signal was not observed. In the ^{1}H NMR spectrum, the proton resonances were assigned starting from the downfield signals (fig. 2).

The three low field *doublet of doublets* sets in the spectrum indicated the presence of an AMX spin – splitting pattern. The coupling constant of 17.9 Hz suggested *trans* – coplanar arrangement for H_M and H_X. The protons H_A and H_X had the coupling constant 11.7 Hz, thus lower coupling constant than the protons H_M and H_X ($J_{MX}>J_{AX}$). The coupling constant of 2.3 and 2.4 Hz indicated geminal positions of the protons H_A and H_M. The *E/Z* geometry of the vinyl protons was confirmed by "1D difference" nuclear Overhauser effect (NOE) spectrum. The 18^2 – H_A proton changed its signal intensity when 18^1 – H_X was irradiated. The signal intensity of the 18^2 – H_A proton increased due to NOE. By "1D difference" NOE experiment one of the four methyl groups' singlets in the upfield region was found to be in the vicinity of the vinyl group. The singlet at δ 1.99 had the enhancement after the irradiation of the 18^1 – H_X proton. Irradiation of the 15 H_B proton at δ 2.91 showed in the "1D difference" NOE spectrum altered intensity of the 16 H and 15 H_A proton signals. The correlations found by "1D difference" NOE are depicted in table **2**.

The propionyl side chain 12^1 protons came in a narrow δ range along with methylene bridge protons. The 12^2 protons, next to the electron withdrawing carboxyl group, had chemical shifts in the upfield region compared to 12^1 protons. The proton signal H – 10 was overlapped with the water signal. In the ^{1}H NMR spectrum of the isolated compound, the water signal was the most intense one as water is a physiological solvent as well as an eluent in the reverse phase chromatography. The proton's H – 8^2 *doublet of doublets* disappeared within 48 h. The most prominent peak in the proton spectrum was the signal assigned to the methoxycarbonyl group at the position 8^2. The irradiation of the well resolved $5H_B$ proton signal at δ 3.08 revealed: the neighboring $5H_A$ proton at δ 2.57, 7^1 methyl group at δ 2.11 and H – 4 proton at δ 4.34. The protons in the space vicinity of the H – 4 were identified

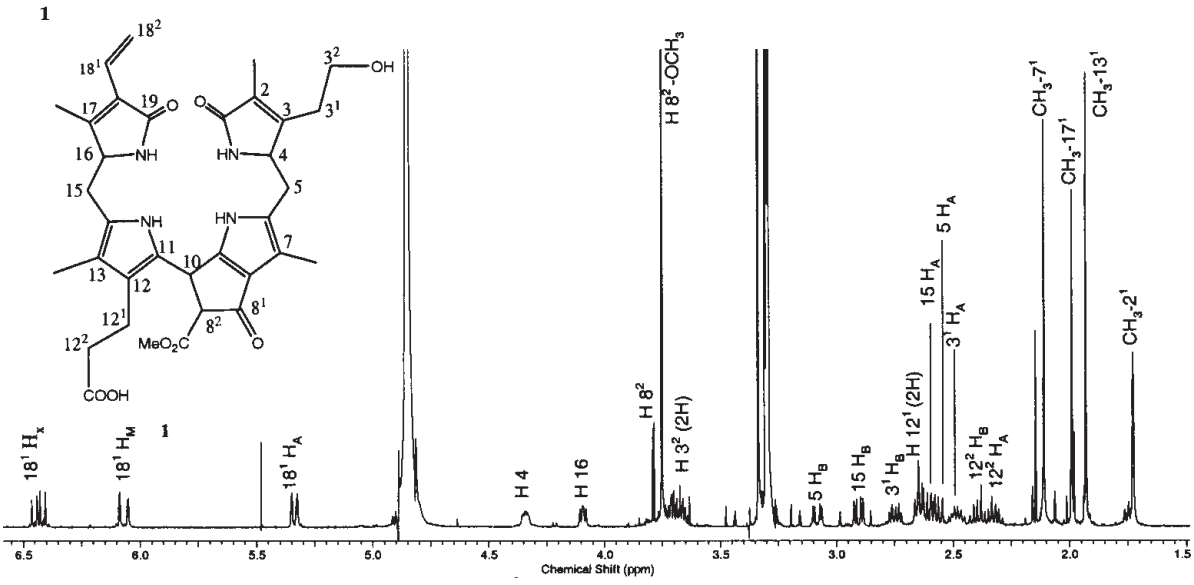

Fig. 2. Assigned signals of the ^{1}H NMR spectrum of the isolated chlorophyll catabolite from Parrotia persica autumnal leaves

Table 1

^{1}H (500 MHz) AND ^{13}C (125 MHz) NMR DATA in CD_3OD of **1** ISOLATED FROM *PARROTIA PERSICA* AUTUMNAL LEAVES

H/C	δ_H, multiplicity	J(Hz)	δ_C	DEPT	COSY (H→H)	HETCOR (H→C)
1			176.7			
2			131.0			
2^1	1.73 *s*		8.5	↑	3^1H_A, 4	C-1, 2, 3, 3^1, 3^2
3			156.0			
3^1	2.49 *td* H_A 2.75 *dd* H_B	6.4; 13.9 6.4; 13.3	31.0	↓	2^1, 3^1H_B, 3^2, 4, $5H_A$; 3^1H_A, 3^2	H_A: C-2, 3, 3^2 H_B: C-3^2, 3^1 H_A
3^2	3.68 *m* H_A and H_B	6.8; 13.4 7.3; 13.9	61.3	↓	3^1H_A, 3^1H_B	C-3, 3^1*
4	4.34 *ddd*	8.3; 4.5; 1.4	60.5	↑	2^1, 3^1H_A, $5H_A$, $5H_B$	C-3*
5	2.57 *dd* H_A 3.08 *dd* H_B	8.5; 14.9 14.9; 4.3	29.9	↓	4, $5H_A$, 7^1; 4, $5H_B$	H_A: C-3^1H_B, 4, 5 H_B: C-3, 4, 6, 7
6			134.4			
7			113.3			
7^1	2.11 *s*		9.5	↑	$5H_B$	C-3^1*, 4, 5*, 6, 7, 8, 8^1*
8			126.3			
8^1			191.6			
8^2	3.79 *d*	3.2	68.6			C-8^1, 9, 11, 14
8^3			171.8			
8^4	3.75 *s*		52.9	↑		C-8^3
9			160.4			
10	Signal being located under residual HDO signal		37.4	↑		C-8*, 8^1*, 8^2, 8^3, 9, 11, 14
11			120.1			
12			124.5			
12^1	2.65 *t* H_A and H_B	7.7	36.8	↓	12^1H_B; 12^1H_A	C-11, 12^2, 12^3, 13, 14
12^2	2.33 *dt* H_A 2.41 *dt* H_B	7.5; 15.6 7.8; 15.5	20.9	↓	12^1H_B, 12^2; 12^1H_A, 12^2	H_A: C-11, 12^1, 12^3 H_B: C-11, 12^1, 12^3
12^3			177.2			
13			115.7			
13^1	1.93 *s*		9.4	↑		C-11, 13, 14, 15*, 16*
14			124.4			
15	2.60 *dd* H_A 2.91 *dd* H_B	5.9; 14.5 5.2; 14.5	30.2	↓	$15H_B$, 16; $15H_A$, 16	H_A: C-13, 14, 16, 17 H_B: C-13, 14, 16, 17
16	4.09 *dd*	7.9; 5.6	61.7	↑	$15H_A$, $15H_B$, 17^1	C-14, 15, 17, 18, 19*
17			156.8			
17^1	1.99 *s*		12.7	↑	16	C-16, 18, 18^1, 18^2, 19
18			129.1			
18^1	6.43 *dd*	17.9; 11.7	127.1	↑	18^2H_A, 18^2H_M	C-17, 18, 18^2*, 19
18^2	5.34 *dd* H_A 6.07 *dd* H_M	11.7; 2.3 17.9; 2.4	119.3	↓	18^2H_M, 18^1; 18^2H_A, 18^1	C-18
19			175.0			

a: DEPT experiment: CH and CH3 are phased up, CH2 is phased down; * cross peak of low intensity

from the "1D difference" NOE spectrum. The H – 4 had the space proximity to the protons 3^1 H_A and 3^1 H_B. The protons at 3^2 position were deshielded with the electron withdrawing hydroxyl group, therefore had chemical shift in the lower magnetic field at δ 3.68. The methyl group left, at δ 1.73 was assigned to the 2^1.

The interpretation of the COSY spectrum explained the arrangement of the proton – proton connectivities. The well resolved downfield 18^2 H_B vinyl proton was assigned to the geminal 18^2 H_A tracing further to the vicinal 18^1 proton. This vinyl isolated spin system terminated at the 18^1 proton. The following connectivity network started from the furthest downfield aliphatic proton H – 16 resonating at δ 4.09 with the strong off diagonal correlating signals with the 15 H_A and 15 H_B spin system and the weak response with the methyl group 17^1. The next strongly coupled spin system being analyzed was the propionyl side chain with the geminal coupling constants of 15.5 Hz and the aliphatic vicinal couplings of 7.5 Hz. The H – 12^2 protons had off diagonal response to their neighboring H – 12^1 protons. This connectivity terminated at the 12^1 H_A and 12^1 H_B protons and could not be traced any further. The connectivity of the propionyl side chain was established from the COLOC spectrum. The starting point for the next connectivity network was from the isolated H – 4 proton having off diagonal response from the methyl group 2^1, 3^1 H_A, 5 H_A and $5H_B$ protons. The 5 H_B proton had a weak off – diagonal response with the methyl group at 7^1.

Table 2

^{1}H (500 MHz) AND "1D DIFFERENCE" NOE NMR DATA IN CD_3OD OF **1** ISOLATED FROM *PARROTIA PERSICA* AUTUMNAL LEAVES

Proton irradiated	δ_H, multiplicity	NOE, multiplicity
2^1	1.73 *s*	3^1H_B *td*
3^1	2.49 *td* H_A 2.75 *dd* H_B	H_A: 3^1H_B*, 3^2 H_A and H_B*, 4 *m*, $5H_A$ *dt*, $5H_B$ *t*
3^2	3.68 *m* H_A and H_B	
4	4.34 *ddd*	3^1H_A*, $5H_A$*, $5H_B$*
5	2.57 *dd* H_A 3.08 *dd* H_B	H_B: 4 *s*, $5H_A$ *dd*, 7^1 *s*, 3^1H_A*
7^1	2.11 *s*	4 *m*, $5H_A$ *dd*, $5H_B$*
8^2	3.79 *d*	
8^4	3.75 *s*	
10	Overlapped by the water peak	
12^1	2.65 *t* H_A and H_B	
12^2	2.33 *dt* H_A 2.41 *dt* H_B	12^1H *d*, 13^1 *s*
13^1	1.93 *s*	12^1H_A and H_B*, 12^2H_A *t*, 12^2H_B *t*, $15H_A$*, $15H_B$ *dd*, 16 *t*
15	2.60 *dd* H_A 2.91 *dd* H_B	H_B: $15H_A$ *dd*, 16 *t*
16	4.09 *dd*	$15H_A$*, $15H_B$*, 17^1 *s*
17^1	1.99 *s*	
18^1	6.43 *dd*	18^2H_A *d*, 17^1 *s*
18^2	5.34 *dd* H_A 6.07 *dd* H_M	

* multiplicity not identified

The initially low availability of the isolated compound has resulted in the low concentration of the sample, hence the poor ^{13}C NMR resolution and prolonged acquisition time.

The DEPT spectrum gave, at the first glance, the number of the CH, CH_3 carbons and separated them from the CH_2 carbons..

The heteronuclear chemical shift correlation spectrum (HETCOR) established the molecular connectivity. The HETCOR spectrum facilitated the correlation of the 16 protons to its carbon. The nonequivalent geminal methylene protons in the propionic and the ethylene – hydroxyl side chain were easily observed. In the case of the methyl protons, although all 4 singlets were close to one another, the differentiation between them was possible in the ^{13}C DEPT spectrum. The difference in the chemical shifts between the "western" and "eastern" methylene bridge carbons was 0.3 ppm and made them nonequivalent. A higher range difference was in the case of the C4/H4 and C16/H16 as the recorded resonance was at δ 60.5 for the C4 and δ 61.7 for the C16. The heteronuclear correlation peak below the HDO peak was unequivocally assigned to the C10/H10. In the vinyl region, the $C18^2$ carbon had a cross peak with the 18^2 H_A and 18^2 H_M protons and the $C18^1$ had a weak correlation with the 18^1 H proton.

A long range heteronuclear multiple quantum chemical shift correlation map (COLOC) established the connectivities in the tetrapyrrole ring system. The most upfield methyl group (CH_3-2^1) resonating at δ 1.7 had the connectivities to five carbons resonating at β 176.7, 131.0, 156, 31.0 and 61.3 identifying three quaternary carbons: C1, C2 and C3. The connectivities in the ring A were established with the H – 3^1 proton being related to the C2, C3 and $C3^2$. Further on, the $5H_B$ proton's first next connectivity led to the C3, C4, C5 and C7 providing the next information on the C6 and C7, not gained with the previous NMR spectra. The connectivities of the $C7^1$ methyl group provided the information about the positions of the C8 and $C8^1$ withdrawn from their respective chemical shift. Continuing in the clockwise direction, the H – 10 proton revealed the connectivity with the four quaternary carbons $C8^2$, C9, C11 and C14 even though overlaid by the water peak. The protons H – 12^1 resonating at δ 2.65 established the key connectivity. These protons were related to the C11, 12^2, 12^3, 13

and 14 linking the pieces of the propinyl side chain together. The protons resonating at δ 2.33 and δ 2.41 determined no connectivity with the C13 and C14. The next information on the remaining part of the structure was established by the connectivity of the 15 H_A to the C16 and C17. The protons H – 16, H – 17^1 and H – 18^1 were assigned to the chemical shift of the remaining quaternary carbon C19.

The spectral data provided the means to assemble the complete structure of the chlorophyll catabolite isolated from *Parrotia Persica* autumnal leaves. The spectra described provided the strong arguments for the structure of the chlorophyll catabolite to be the **1** (fig. 1). The NMR data are systematized in table **1** and table **2**.

Spectroscopic data

UV – Vis in methanol, $C = 10^{-7}$ mol dm^{-3}; λ_{max}[nm] (log ε): 244 (6.78), 283 (6.57).

CD in methanol, $C = 10^{-7}$ mol dm^{-3}; λ_{max}[nm] (Δε): 244 (indeterminated), 283 (-32).

Conclusions

The chlorophyll catabolite was isolated from *Parrotia persica*, Hamamelidaceae autumnal leaves. Its structure was determined by UV – Vis, HRESIMS – and NMR – spectra. The isolated chlorophyll catabolite differs from other previously isolated NCCs in one lateral group. The lateral aldehyde group common in previously isolated NCCs is not present in the chlorophyll catabolite isolated from *Parrotia persica* autumnal leaves. The structure of the isolated chlorophyll catabolite from *Parrotia persica* autumnal leaves refers to the structure of urobilinogen and the isolated chlorophyll catabolite, described in this paper, can be named: urobilinogenic non – fluorescent chlorophyll catabolite (UNCC).

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