Urobilinogenic chlorophyll catabolite behavior in oxygen-containing moiety

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Abstract

The urobilinogenic chlorophyll catabolite being exposed to oxygen-containing moiety, after three months, forms the C-8^2-hydroxy urobilinogenic chlorophyll catabolite. The chromatographic and spectroscopic methods have been used to study the hydroxylated urobilinogenic chlorophyll catabolite product formed. Using liquid chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy C-8^2 hydroxylated urobilinogenic chlorophyll catabolite was identified. Analysis of the results obtained enables the propositions of the reaction mechanism.

Keywords: urobilinogenic chlorophyll catabolite, C-8^2 hydroxylation.

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The oxidation of chlorophylls by molecular triplet oxygen in aqueous methanol solutions happens to all chlorophylls that have an intact β-ketoester structure at the isocyclic ring [1]. The first oxidation product formed is the hydroxylated chlorophylls' derivative. The oxidation process continues further and the reaction was named the allomerization [2]. The allomerization occurs through chemical or enzymatic pathway and a complex mixture of products is formed [3]. An early stage reaction in chlorophyll breakdown, under natural conditions, is the allomerization [4,5]. The reversed--phase (RP) and normal phase (NP) high-performance liquid chromatography (HPLC) was utilized in the separation of chlorophyll allomers [6,7]. The HPLC coupled with mass spectrometry (HPLC–MS) in conjunction with UV–Vis absorption has been used for analysis of chlorophyll allomers [8–10]. The nuclear magnetic resonance (NMR) spectroscopy was used in assignment of several allomers [11–13]. The allomerization is initiated in aqueous methanol solution containing traces of bases, acids and metals. The base catalyzed allomerization mechanism comprises the removal of the acidic α-proton at chlorophylls' C-13^2 position and formation of an enolate. After keto-enol tautomerization of the C-13^1=C-13^2 double bond the allomer is formed. Free radical mechanism was proposed for the allomerization [14]. Further investigations support an idea of alternative allomerization pathway [10]. There is still not enough data to back up proposed acid catalyzed allomerization mechanism of chlorophylls. The urobilinogenic chlorophyll catabolite isolated from the *Parrotia persica* autumnal leaves differs from chlorophylls and chlorophyll catabolites by having one carbon atom less [15]. The *Parrotia persica* urobilinogenic chlorophyll catabolite structure (**1**) refers to urobilinogen and the carbon atom numeration is the same as in urobilinogen. The C-8^2 hydroxylation of *Parrotia persica* urobilinogenic chlorophyll catabolite in deutero methanol containing traces of water and acid is described in this paper. RP LC-MS and ^{1}H-NMR spectra were used in the identification of C-8^2-hydroxy urobilinogenic chlorophyll catabolite (**2**) and the mechanisms that can explain the formation of the C-8^2-hydroxy urobilinogenic chlorophyll catabolite are proposed.

MATERIALS AND METHODS

The urobilinogenic chlorophyll catabolite was isolated from *Parrotia persica* (*Pp*), Hamamelidaceae autumnal leaves according to the methods described previously [15]. The isolated *Pp* urobilinogenic chlorophyll catabolite was left for 3 months in the NMR tube, in the cold and dark place. After 3 months the RP LC––MS analysis was done. The LC chromatogram revealed the presence of two compounds. The final purification was done by RP HPLC using Waters 600 HPLC system coupled with Waters 2996 PDA UV–Vis detector (Waters Corp., Milford, USA) and RP EP 250 mm×16 mm Nucleosil 100-7 C_8 column along with RP EP 30 mm×16 mm Nucleosil 100-7 C_8 precolumn (Macharey--Nagel, Oesingen, Switzerland). The detection wavelength was set at 244 nm, temperature of the column was kept at 22 °C and the injection volume was 2 ml *via* loop injection. The mobile phase consisted of water (0.1% trifluoroacetic acid (TFA)):methanol, 1:1 (v/v) and operating an isocratic flow of 3.2 ml/min. The urobilinogenic chlorophyll catabolite was collected to obtain 1.92 mg and the C-8^2-hydroxy urobilinogenic chlorophyll catabolite was collected to obtain 0.20 mg. All solvents used were HPLC grade (Acros Organics, Geel,

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Belgium). The recording of the ^{1}H-NMR spectrum was done under the same conditions as described previously [15].

RESULTS AND DISCUSSION

The *Parrotia persica* (*Pp*) urobilinogenic chlorophyll catabolite (**1**) was, after recording of the NMR spectra, left in the cold and dark place for 3 months. After 3 months the RP LC–MS chromatogram was recorded. The chromatogram revealed the presence of two compounds (Figure 1).

Methanol and acidified water eluent can influence the formation of hydrogen bonds in the solution and reduce the interactions between compounds. The stationary phase particle surface can form hydrogen bonds with the compounds being separated. The prolonged retention time of the C-8^2-hydroxy urobilinogenic chlorophyll catabolite (**2**) can be attributed to additional hydroxyl group present that can form hydrogen bond with the silanol sites of the column packing. The ESI-MS showed a molecular ion at *m*/*z* 633, an $[M+H]^+$ for the *Pp* urobilinogenic chlorophyll catabolite (**1**), Figure 2. C-8^2-hydroxy derivative showed a molecular ion at *m*/*z* 651, an $[M+H]^+$ for the C-8^2-hydroxy *Pp* urobilinogenic chlorophyll catabolite (**2**). The sample was subsequently purified by semi-preparative HPLC under the same conditions as described previously [15].

The ^{1}H-NMR spectra were recorded for all purified compounds. The ^{1}H-NMR spectra of the *Pp* urobilinogenic chlorophyll catabolite (**1**) and its C-8^2-hydroxy derivative (**2**) are depicted in Figures 3 and 4, respectively.

The difference between the two ^{1}H-NMR spectra is the absence of the signal for the C-8^2 proton in the C-8^2-hydroxy *Pp* urobilinogenic chlorophyll catabolite (Figure 4). The chemical shifts, multiplicity and coupling constants of the *Pp* urobilinogenic chlorophyll catabolite (**1**) were as published previously [15]. The chemical shifts, multiplicity and coupling constants of the isolated C-8^2-hydroxy *Pp* urobilinogenic chlorophyll catabolite (**2**) are depicted in Table 1.

In chlorophylls, the acidic catalyzed allomerization mechanism is comprised of protonation of the C-13^1 oxo group. This mechanism was not sufficient to explain the formation of the C-13^2-hydroxy and C-13^2--methoxy chlorophylls [10]. The other mechanism proposed for the allomerization of chlorophylls was a free

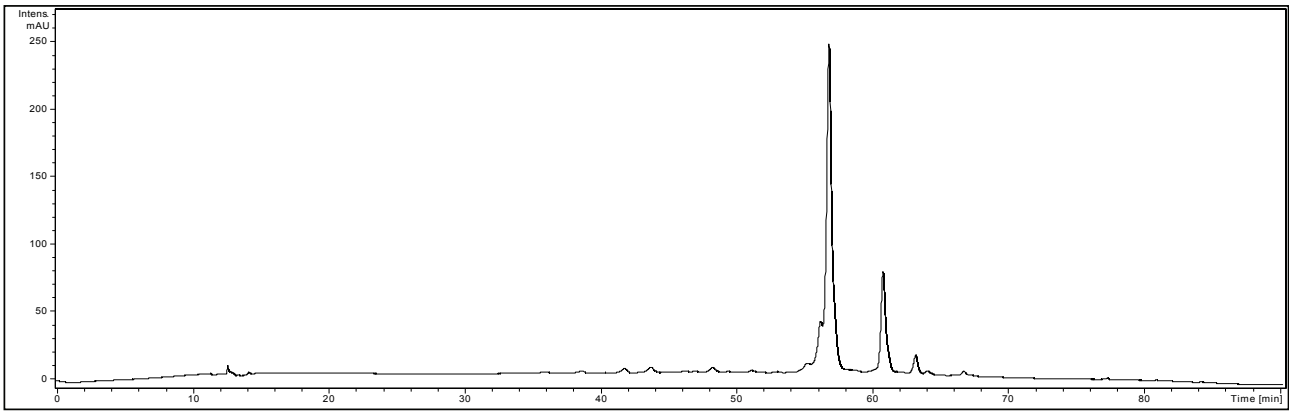

Figure 1. The chromatogram of the Pp urobilinogenic chlorophyll catabolite and its C-8^2-hydroxy derivative separated on the analytical RP C_8 column. Conditions: mobile phase: water (0.1 % TFA): methanol, gradient elution, the proportion of methanol was increased linearly from 10 to 100% in 70 min and in next 20 min elution was continued with methanol. The column temperature was 22 ℃, flow rate 0.2 ml/min, detection. UV 244 nm, injection: 10 µl via autosampler.

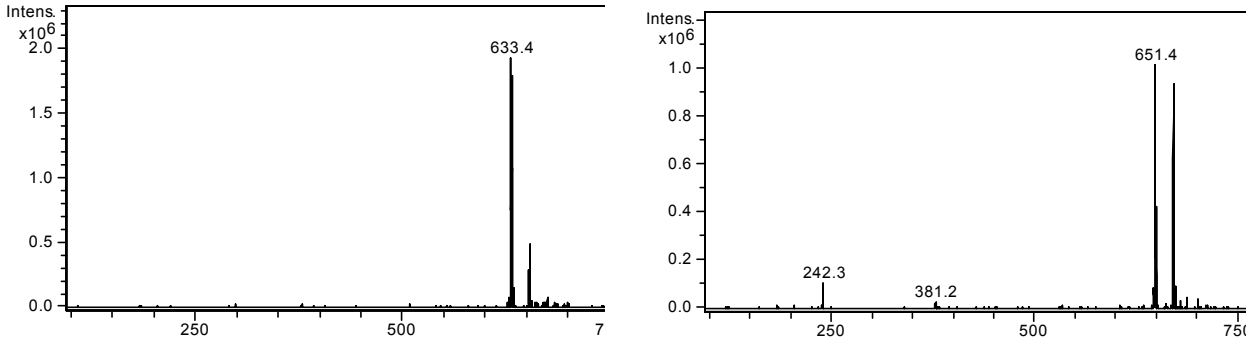

Figure 2. Electro-spray ionization (ESI) MS of Pp *urobilinogenic chlorophyll catabolite and its C-8^2-hydroxy derivative extracted at 57.0 and 60.9 min, respectively.*

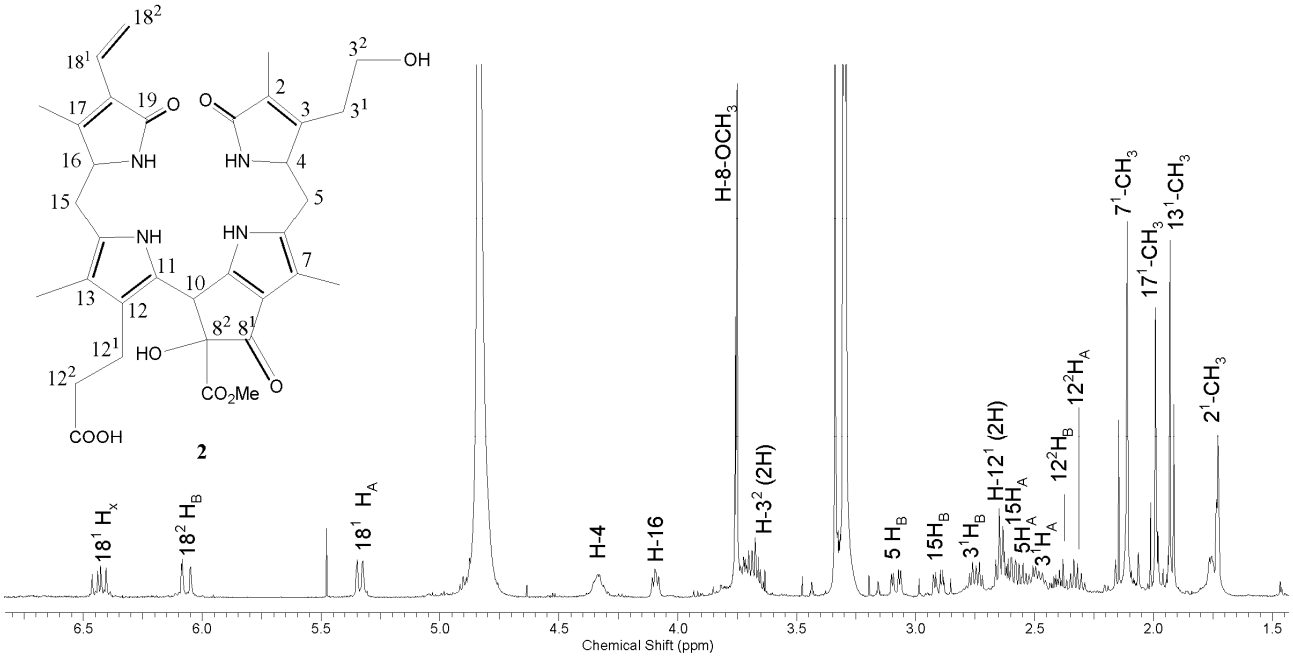

*Figure 3. The ^{1}H-NMR spectrum of Pp urobilinogenic chlorophyll catabolite (**1**).*

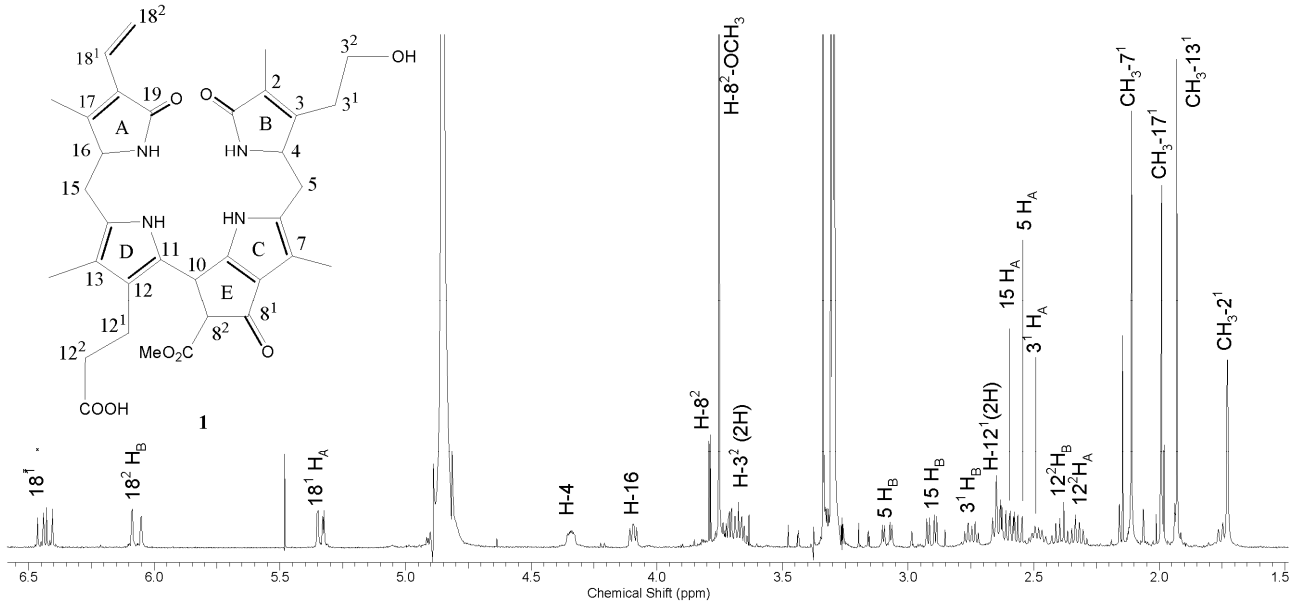

*Figure 4. The ^{1}H-NMR spectrum of the C-8^2-hydroxy Pp urobilinogenic chlorophyll catabolite (**2**).*

Table 1. ^{1}H-NMR (500 MHz) data in CD_3OD of the isolated C-8^2-hydroxy Pp *urobilinogenic chlorophyll catabolite (**2**)*

H/C	δ_H, multiplicity	J / Hz
1	1.73 *s*	
2		
2^1		
3	2.49 *dd* H_A,	6.9; 14.8,
3^1	2.75 *dd* H_B	6.6; 13.8
3^2	3.68 *m* H_A and H_B	
4	4.34 *m*	

Table 1. Continued

H/C	δ_H, multiplicity	*J* / Hz
5	2.57 *dd* H_A,	8.5; 14.9;
	3.08 *dd* H_B	13.8; 5.6
6		
7		
7^1	2.11 *s*	
8		
8^1		
8^2		
8^3		
8^4	3.75 *s*	
9		
10	Signal being located under residual HDO signal	
11		
12		
12^1	2.65 *dd* H_A and H_B	7.4; 15.4
12^2	2.33 *dd* H_A,	6.9; 14.8;
	2.41 *dd* H_B	7.8; 15.6
12^3		
13		
13^1	1.93 *s*	
14		
15	2.60 *dd* H_A,	5.9; 14.5;
	2.91 *ddd* H_B	2.4; 5.2; 14.6
16	4.09 *dt*	5.5; 2.0
17		
17^1	1.99 *s*	
18		
18^1	6.43 *dd*	17.8;12.3
18^2	5.34 *dd* H_A,	13.6; 2.2;
19	6.07 *dd* H_B	17.7; 2.3

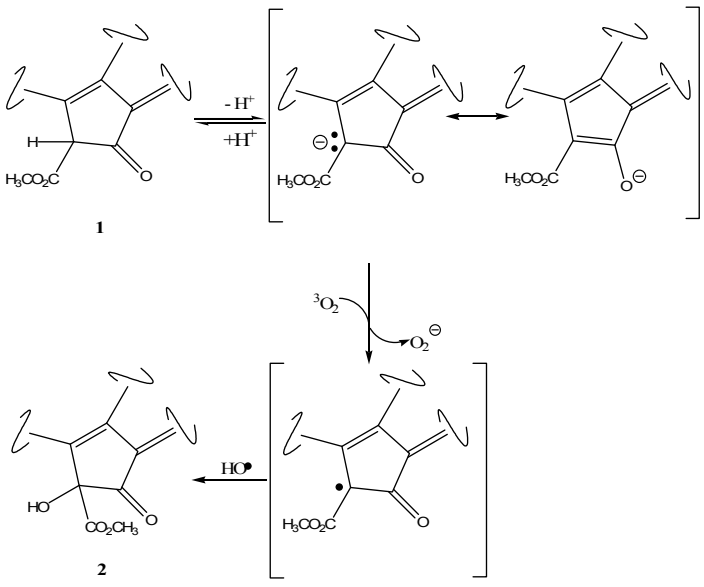

*Figure 5. The mechanism adopted from literature that can be proposed for the formation of the C-8^2-hydroxy Pp urobilinogenic chlorophyll catabolite (**2**) from the Pp urobilinogenic chlorophyll catabolite (**2**), for brevity only ring E is shown [14].*

radical one (Figure 5), which was able to explain the formation of the previously mentioned chlorophyll allomers [14].

CONCLUSIONS

The evidences described, clearly indicated that the *Pp* urobilinogenic chlorophyll catabolite (**1**), under the acidic conditions, in the presence of trifluoroacetic acid (TFA), which was used as a modifier during the chromatographic separation, in aqueous methanol solution upon standing for months induces the formation of the C-8^2-hydroxy *Pp* urobilinogenic chlorophyll catabolite (**2**). The mechanism proposed for the allomerization of chlorophylls can explain the formation of the C-8^2--hydroxy *Pp* urobilinogenic chlorophyll catabolite (**2**). The chromatographic and spectroscopic methods described can facilitate the identification of the C-8^2-hydroxy urobilinogenic chlorophyll catabolite (**2**).

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IZVOD

PONAŠANJE UROBILINOGENSKOG KATABOLITA HLOROFILA U ATMOSFERI KOJA SADRŽI KISEONIK

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(Naučni rad)

Urobilinogenski katabolit hlorofila stajanjem u atmosferi koja sadrži kiseonik, nakon tri meseca, formira C-8^2-hidroksi urobilinogenski katabolit hlorofila. Korišćene su hromatografske i spektroskopske metode za proučavanje formiranog hidroksi urobilinogenskog katabolita hlorofila. Upotrebom tečne hromatografije-masene spektrometrije i nuklearne magnetne rezonantne spektroskopije identifikovan je C-8^2-hidroksi urobilinogenski katabolit hlorofila. Dobijeni rezultati ukazuju da urobilinogenski katabolit hlorofila izolovan iz jesenjeg lišća biljke *Parrotia persica* u prisustvu tragova kiseline, koja je korišćena kao modifikator tokom hromatografskog razdvajanja, u vodenom rastvoru metanola i u atmosferi koja sadrži kiseonik nakon nekoliko meseci stajanja uzrokuje stvaranje C-8^2-hidroksi urobilinogenskog katabolita hlorofila. Analiza dobijenih rezultata omogućava predlog reakcionog mehanizma. Mehanizam kiselo katalizovane alomerizacije hlorofila podrazumeva protonovanje C-13^1 okso grupe. Ovaj mehanizam nije mogao da objasni stvaranje C-13^2-hidroksi i C-13^2-metoksi hlorofila. Drugi predložen mehanizam podrazumeva alomerizaciju hlorofila po slobodno radikalskom mehanizmu koji je mogao da objasni stvaranje prethodno navedenih alomera hlorofila.

Ključne reči: Urobilinogenski katabolit hlorofila • C-8^2 hidroksilacija