Kragujevac J. Sci. 34 (2012) 79-85. UDC 581.19:582.638.28

BEHAVIOUR OF *Fothergilla gardenii* CHLOROPHYLL CATABOLITE UNDER ACIDIC CONDITIONS

Nina Đapić

Technical Faculty "Mihajlo Pupin", Đure Đakovića b.b., Zrenjanin, Republic of Serbia
e-mail: ninadjapic@yahoo.com

(Received March 23, 2012)

ABSTRACT. The senescent leaves undergo the observable biochemical process – the lost of the intense green colour. The chlorophyll catabolites in the immature autumnal leaves of *Fothergilla gardenii*, Hamamelidaceae, were investigated. One chlorophyll catabolite was isolated from the *Fothergilla gardenii* immature autumnal leaves, the *Fothergilla gardenii* (*Fg*) chlorophyll catabolite or the thermodynamically less stable isomer of the yellow chlorophyll catabolite (*Cj*–YCC). The acid, present in traces, induced the formation of a thermodynamically more stable chlorophyll catabolite isomer, the *Cj*–YCC, from the *Fg* chlorophyll catabolite. To gain insight into the mechanism of the *Cj*–YCC formation, the thermodynamically less stable isomer of *Cj*–YCC, the *Fg* chlorophyll catabolite was studied. Changes in ^{1}H NMR spectrum showed that the thermodynamically stable isomer, the *Cj*–YCC, was formed in the presence of the traces of the trifluoroacetic acid (TFA) from the *Fg* chlorophyll catabolite. A model, in which, under the acidic conditions, the *Cj*–YCC is formed, is described.

Key words: chlorophyll catabolite, *Fothergilla gardenia*.

INTRODUCTION

The chlorophyll catabolism is observable biochemical process visualized by the loss of the green leaf colour before the programmed cell death. Chlorophyll catabolism begins with the oxygenolytic ring cleavage of pheophorbides which are the intermediates upon the release of magnesium from the core of chlorophyllides ring. Chlorophylls, chlorophyllides and pheophorbides have chromophores with the absorption in the visible region. After the ring opening of pheophorbides, an intermediate is formed which was, up to now, not isolated from the autumnal leaves of higher plants. In the next step of the chlorophyll catabolism, the primary fluorescent chlorophyll catabolites (pFCCs) are formed after the reduction of the intermediate. After the tautomerisation of pFCCs the non – fluorescent chlorophyll

catabolites (NCCs) are formed (KRAEUTLER, 2003). In the next step the oxidation of NCCs occurs and urobilinogenic chlorophyll catabolites are formed (DJAPIC, 2009).

Non – glycosylated NCCs were isolated from autumnal leaves of: Altingiaceae (ITURRASPE, 1995), Amaranthaceae (BERGHOLD, 2002), Cercidiphyllaceae (OBERHUBER, 2002), Hamamelidaceae (DJAPIC, 2008) and Vitaceae plant families (DJAPIC, 2009). Up to now, several chlorophyll catabolites were isolated from the senescent leaves extract of *Cercidiphyllum japonicum*. One chlorophyll catabolite isolated from the autumnal leaves of *Cercidiphyllum japonicum* has the *m/z* 629 (*Cj*–2), the other has the *m/z* 645 (*Cj*–1) (OBERHUBER, 2002). The chlorophyll catabolite isolated from extracts of senescent leaves of *Cercidiphyllum japonicum* had the *m/z* 643 and was called the yellow chlorophyll catabolite or *Cj* – YCC (Moser, 2008). The thermodynamically less stable isomer of *Cj*–YCC was isolated from the methanol extract of the immature autumnal leaves of *Fothergilla gardenii*, the *Fg* chlorophyll catabolite. In this work, ^{1}H NMR analysis of the solution behaviour of the *Fg* chlorophyll catabolite, in deutero methanol, under the acidic conditions is presented.

MATERIALS AND METHODS

The analytical LC separation of the immature *Fothergilla gardenii* autumnal methanol leaf extract was carried on the RP EC 250x4 mm Nucleosil 100-5 C_8 column together with the RP CC 8x4 mm Nucleosil 100-5 C_8 precolumn (Macherey – Nagel, Oesingen, Switzerland). The temperature of the column oven was 22°C. The injection volume was 1 µl via autosampler injection. The mobile phase consisted of water (0.1% TFA) and methanol. The proportion of methanol was increased linearly from 10% to 100% in 70 minutes and in the next 20 minutes elution was continued with methanol. The flow rate was 0.2 ml/min. After each separation the column was reequlibrated linearly from 100% to 10% methanol in 10 minutes and additional 5 minutes at 10% of methanol.

Isolation and purification of the *Fothergilla gardenii* chlorophyll catabolite was, as follows: the *Fothergilla gardenii* immature autumnal leaves (74.22 g dry weight, 100 g "fresh" weight) were frozen with liquid nitrogen, grinded and extracted with methanol. The methanol extract was filtered and partitioned between hexane and methanol. Water was added to the methanol phase. The chlorophyll catabolite was extracted with dichloromethane from the methanol – aqueous phase. After the evaporation of dichloromethane under the reduced pressure (<40^{0}C) *Fothergilla gardenii* crude extract was obtained. The yield was 87.46 mg. The obtained compounds were subjected to MPLC on lab – made column, size 310 x 25 mm, filled with LiChroprep® RP8, particle size 25 – 40 µm 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 320 nm) yielded 5.6 mg of the prepurified *Fg* chlorophyll catabolite. Final purification was done by reverse – phase (RP) 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 424 nm, temperature of the column oven was 22^{0} C and the injection volume was 2 mL via loop injection. The PDA detection was in range of 200 – 800 nm and the chromatogram was extracted at λ=424 nm. The eluent: water (0.1% TFA): methanol= 25:75 and the flow rate of 3.2 ml per minute was used. The compound eluting at the 20 min. was collected. The yield was 0.78mg. All solvents used were HPLC grade (Acros Organics, Geel, Belgium).

RESULTS AND DISCUSSION

The chromatogram of immature methanol autumnal leaf extract revealed the presence of three compounds and in the total ion chromatogram the presence of two compounds one with *m/z* 643 and the other with *m/z* 641. On the semi – preparative column, the compound eluting at the 20 minutes was collected. The compound collected had the UV bands at the following wavelengths: 244, 314.4 and 424.5 nm. The High Resolution ElectroSpray Ionisation Mass Spectrum (HRESIMS) determined the molecular ion of *m/z* 643.2782 for the molecular formula $C_{35}H_{39}N_4O_8$ $[M+H]^+$, calculated *m/z* 643.2762, Δ +3.10 ppm.

The proton NMR spectrum revealing the process taking place in deutero methanol under the acidic conditions is depicted in Figures 1, 2 and 3.

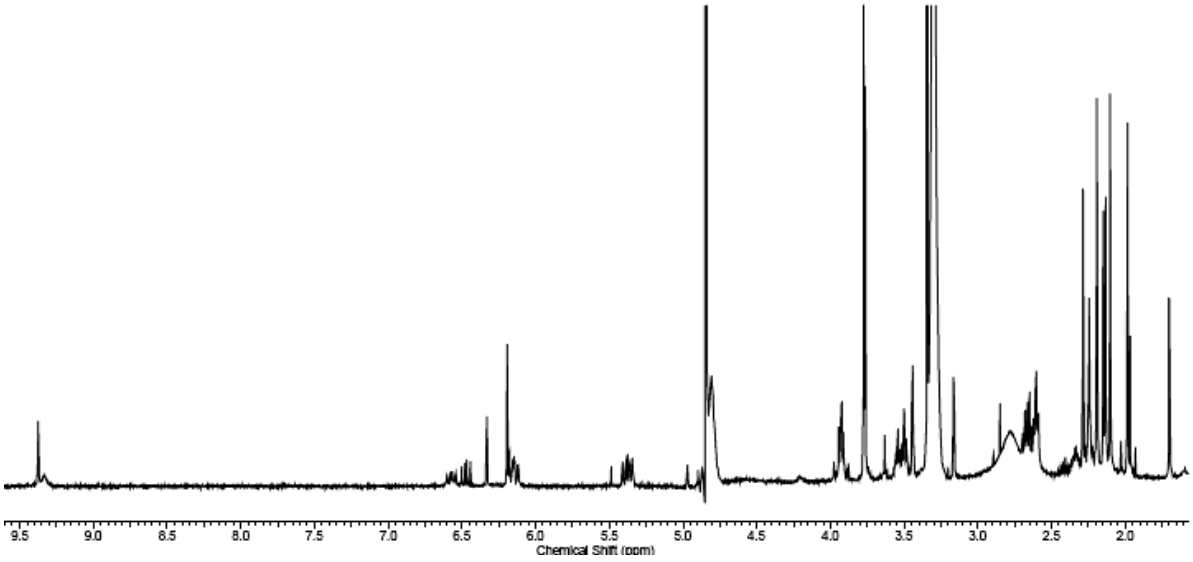


Figure 1. - The proton spectrum of the *Fg* chlorophyll catabolite.

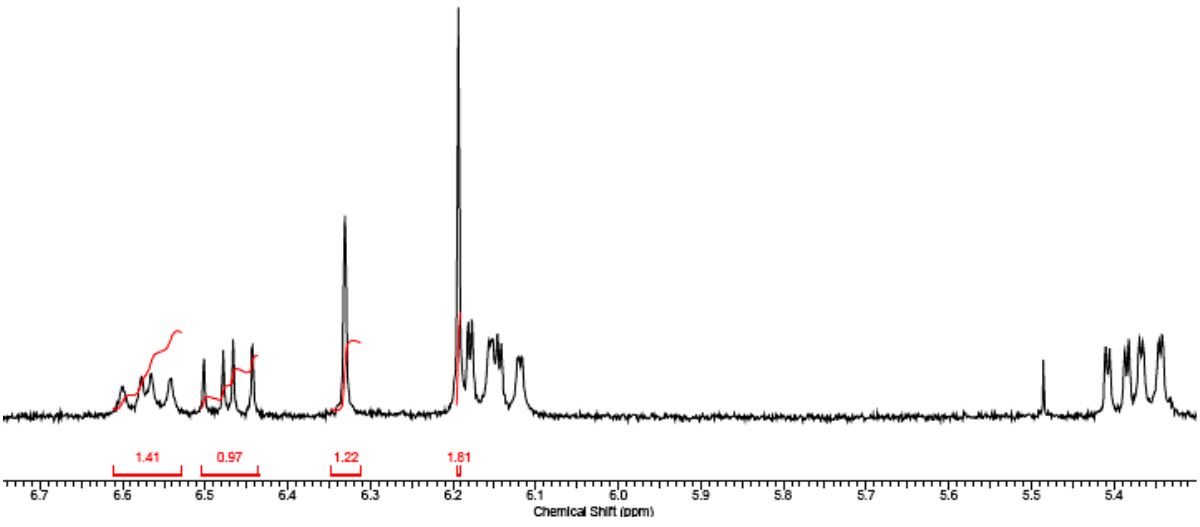


Figure 2. - The spectral magnification of the *Fg* chlorophyll catabolite proton spectrum in the low field during the tautomerization to the *Cj*–YCC.

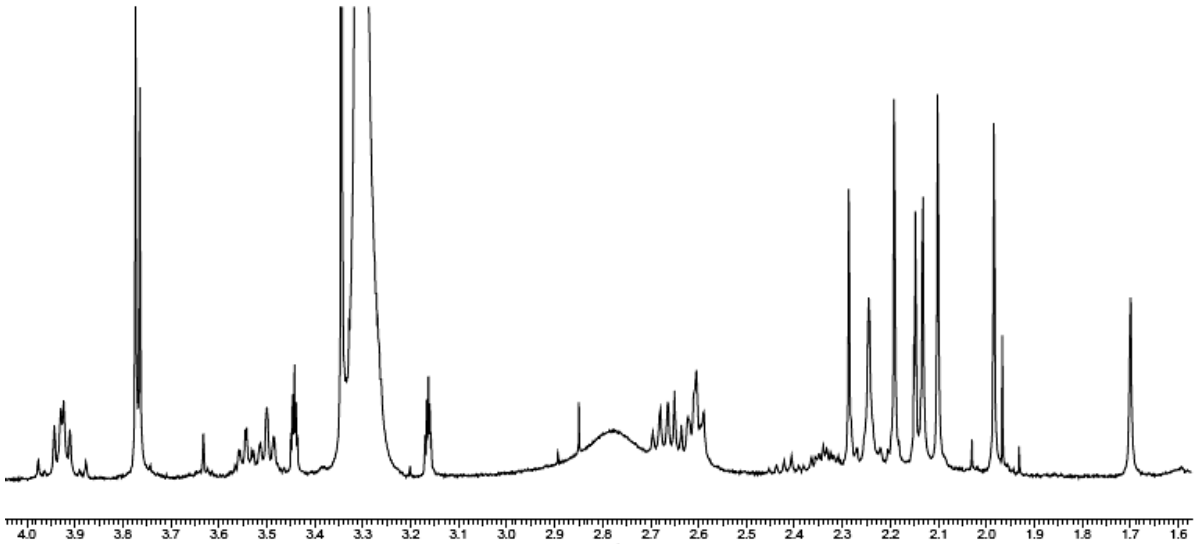


Figure 3. - The proton spectrum of the *Fg* chlorophyll catabolite in the high field during the tautomerization to *Cj*–YCC.

The structure of the *Fg* chlorophyll catabolite (**1**) and the transformation taking place in the presence of the TFA is depicted in Figure 4. The thermodynamically more stable isomer is formed, the *Cj* – YCC (**2**), in the presence of an acid in the NMR tube during the time. The signals of the H – 17 and H – 18 protons were present in the proton spectrum with an integral of one (Figure 2) indicating that the isolated chlorophyll catabolite had the structure **1**. The signal of the proton H – 13^2 has not been observed in the proton NMR spectrum therefore the deuterium exchange has occurred (Figure 5).

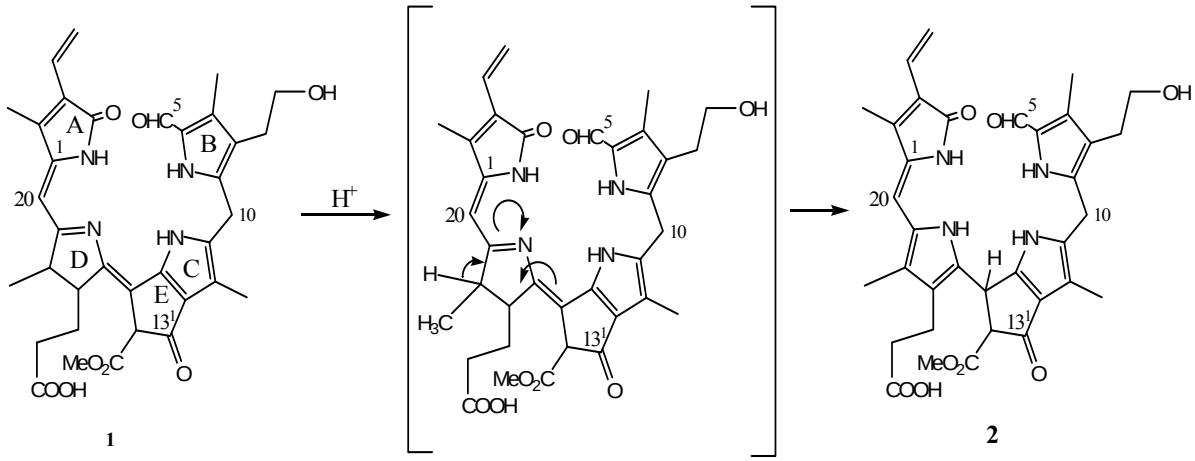
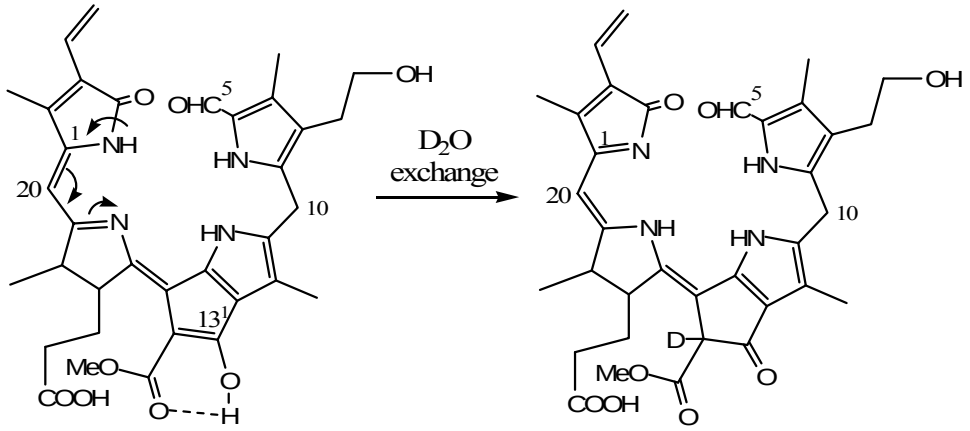


Figure 4. - The structure of the chlorophyll catabolite found in the *Fothergilla gardenii* immature autumnal leaves (**1**) and the formation of the *Cj* – YCC (**2**).

The formation of the deutero *Cj* – YCC is depicted in Figure 6.

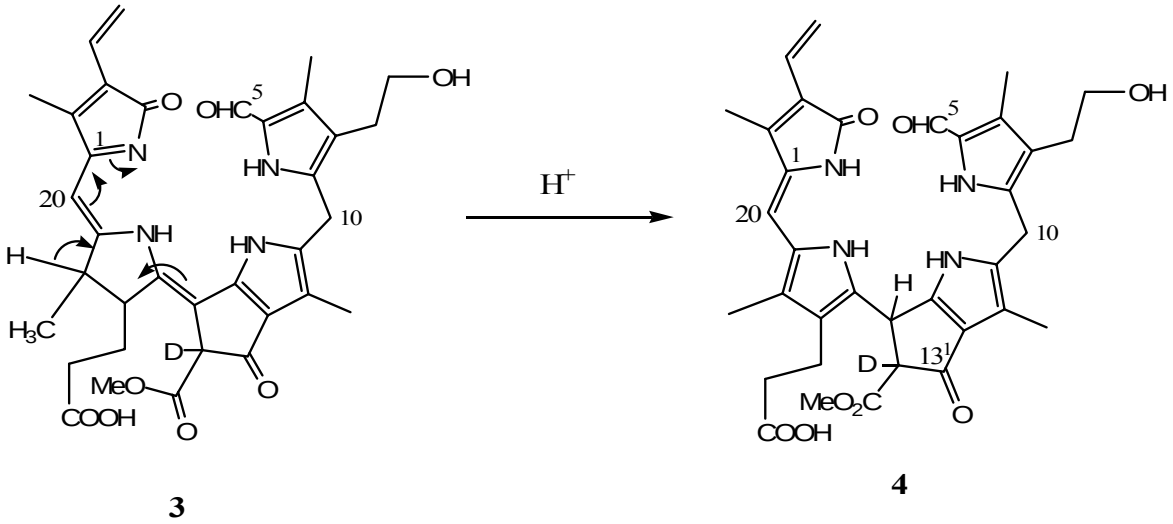
For the complete assignation and determination of the *Cj* – YCC see in (Moser, 2008). The proton chemical shifts and scalar couplings of the *Fg* chlorophyll catabolite (**1**) are depicted in Table 1.



1 **3**

Figure 5. - The enol tautomeric structures of the *Fg* chlorophyll catabolite.

The tautomer **2** is thermodynamically more stable and has three aromatic pyrrol rings while in the case of the isomer **1**, two rings are entropically favoured. The observed mixture had one thermodynamically favoured **2** and one unfavoured isomer **1**. The thermodynamically unfavoured isomer, the *Fg* chlorophyll catabolite **1**, interconverted to the thermodynamically favoured one, the *Cj*–YCC **2**. The tautomerization was induced in the acidic moiety. The presence of TFA has induced the migration of the *Fg* chlorophyll catabolite's π – conjugation system. The C – 13^2 methyl – carboxyl group and the carboxyl group in the propionyl side chain were intact under the acidic conditions. As deutero methanol was used for recording the proton NMR spectrum, the deutoro – exchange has occurred (Figure 6). The deutero – exchange occured before the acid had induced the tautomerization. It can be concluded that after the deutero exchange the *Fg* chlorophyll catabolite (**3**) tautomerized to the *Cj* – YCC (**4**). The *Fg* chlorophyll catabolite forms the thermodynamically more stable chlorophyll catabolite, the *Cj*–YCC under the acidic condition. All carboxyl groups in both isomers are intact under the acidic condition.



3 **4**

Figure 6. - The formation of the deutero *Cj* – YCC (**4**).

Table 1. - The ^{1}H (500 MHz) data in CD_3OD of the isomer **1** (left) and **2** (right).

H/C	δ_H, multiplicity	J(Hz)
1	-	
2	-	
2^1	*	
3	-	
3^1	6.57 *dd*	17.7; 11.7
3^2	5.40 *dd* H_A 6.17 *dd* H_M	12.0; 2.6 2.1; 17.9
4	-	
5	9.37 *s*	
6	-	
7	-	
7^1	*	
8	-	
8^1	2.60 *t*	8.1
8^2	Overlapped by the HDO signal	
9	-	
10	3.93 *d*	6.8
11	-	
12	-	
12^1	*	
13	-	
13^1	-	
13^2	-	
13^3	-	
13^4	3.77 *s*	
14	-	
15	-	
16	-	
17	3.54 *dt*	6.9; 1.5
17^1	1.45-1.50 *m* H_A and H_B	15.4; 7.3
17^2	Overlapped by the methyl group signal	
17^3	-	
18	2.70-2.85 *br*	
18^1	2.14 *d*	7.7
19	-	
20	6.33 *s*	

H/C	δ_H, multiplicity	J(Hz)
1	-	
2	-	
2^1	**	
3	-	
3^1	6.47 *dd*	17.7; 11.7
3^2	5.36 *dd* H_A 6.14 *dd* H_M	11.9; 2.1 1.7; 17.5
4	-	
5	9.33 *br*	
6	-	
7	-	
7^1	**	
8	-	
8^1	2.60 *t*	8.1
8^2	Overlapped by the HDO signal	
9	-	
10	3.92 *d*	6.4
11	-	
12	-	
12^1	**	
13	-	
13^1	-	
13^2	-	
13^2	-	
13^4	3.76 *s*	
14	-	
15	-	
16	-	
17		
17^1	2.63-2.70 *m* H_A and H_B	15.7; 7.6
17^2	2.30-2.38 *m* H_A 2.38-2.44 *m* H_B	15.8; 8.1 14,9; 8.1
17^3	-	
18	-	
18^1	**	
19	-	
20	6.19 *s*	

* The chemical shifts for the methyl groups of the isomer (**1**): 2^1, 7^1 and 12^1 were not determined. The recorded δ_H were: 1.70, 2.19 and 2.29 and the assignation was not correlated in the ^{1}H NMR spectrum.

** For the isomer (**2**) see (Moser, 2008). The recorded chemical shifts for the methyl groups of the isomer (**2**) (2^1, 7^1, 12^1 and 18^1) were not correlated. The δ_H values were: 1.70, 1.98, 2.10 and 2.25.

This is an irreversible conversion at the 25^0C.

References:

[1] BERGHOLD, J., BREUKER, K., OBERHUBER, M., HOERTENSTEINER, S., KRAEUTLER, B. (2002): Chlorophyll breakdown in spinach: on the structure of five nonfluorescent chlorophyll catabolites. *Photosynthesis Research* **74**: 109–119.

[2] DJAPIC, N., PAVLOVIC, M. (2008): Chlorophyll catabolite from *Parrotia persica* autumnal leaves. *Rev. Chim.* (Bucuresti) **59**: 878 – 882.

[3] DJAPIC, N., DJURIC, A., PAVLOVIC, A., (2009): Chlorophyll biodegradation in *Vitis vinifera* var. Pinot noir autumnal leaves. *Research Journal of Agriculture Science*, **41**: 256 – 260.

[4] ITURRASPE, J., MOYANO, N., FRYDMAN, B. (1995): A new 5-formylbilinone as the major chlorophyll *a* catabolite in tree senescent leaves. *J. Org. Chem.* **60**: 6664–6665.

[5] KRAEUTLER, B. (2003) in *The Porphyrin Handbook*, **13**: 183.

[6] MOSER, S., ULRICH, M., MUELLER, T., KRAEUTLER, B. (2008): A yellow chlorophyll catabolite is a pigment of the fall colours, *Photochem Photobiol Sci.*, **7**(12): 1577–1581.

[7] OBERHUBER, M., BERGHOLD, J., BREUKER, K., HOERTENSTEINER, S., KRAEUTLER, B. (2003): Breakdown of chlorophyll: A nonenzymatic reaction accounts for the formation of the colorless “nonfluorescent” chlorophyll catabolites. *Proc. Natl. Acad. Sci. USA*, **100**: 6910- 6915.