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Chlorophyll Catabolites in Senescent Leaves of Lima Bean *(Phaseolus Lunatus)* and in the Frass of *Spodoptera Littoralis* After Defecation

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ABSTRACT
Chlorophylls (Chls), the green pigments responsible for photosynthesis in plants, algae and bacteria, are also part of the daily diet of herbivorous feeders. Chl degradation occurs during leaf senescence and fruit ripening but is also noticed as a response to biotic and abiotic stresses. Besides the programmed degradation during senescence different factors such as high temperature, extreme pH values, enzymatic actions, molecular oxygen, and light initiate the degradation of Chl. To gain more information on Chl degradation in the gut of plant-feeding insects, regurgitate and frass of *Spodoptera littoralis* caterpillars were analysed for late Chl catabolites by using LC-MS, UV, Fluorescence and NMR spectroscopy. The major metabolites were determined in fresh leaves of the food plant lima bean *(Phaseoluslunatus)*, and were compared with digestive products. The observed spectrum of metabolites can be attributed to the combined action of esterolytic gut enzymes and the strongly alkaline milieu in the digestive tract. Interestingly, linear Chl catabolites were not detected in the gut of the larvae of *S. littoralis*. Substantial amounts of Chl catabolites were found to be macrocyclic rings opened in the senescent food plants, but also in the aged frass. We studied two primary fluorescent chlorophyll catabolites in senescent leaves of lima bean. One of the primary fluorescent chlorophyll catabolites that have been found in the frass of *S. littoralis* might be generating opened tetrapyrroles of Chl, only after being exposed to the air and light.

Keywords: *Primary fluorescent chlorophyll catabolites, Lima bean, Spodoptera littoralis, Late chlorophyll catabolites, Opening macrocyclic tetrapyrroles*

1. INTRODUCTION

Chlorophyll (Chl) degradation is an important enzymatic process during leaf senescence and fruit ripening [1]. It allows the recycling of nitrogen and other nutrients and prevents the accumulation of phototoxic Chl intermediates causing cell death in plants [2]. More than one billion tons of Chls are biosynthesized and degraded every year on the land and in the ocean [3]. Chl is very stable in the chloroplast, but after disruption, it becomes susceptible to degradation [4]. Studies of the breakdown of Chl in the terrestrial environment have revealed a series of transformations, cleavage of the macrocycle, production of linear tetrapyrroles, and the loss of the corresponding cyclic tetrapyrrol echromophore [5]. In general, the enzyme-mediated Chl breakdown in senescent leaves proceeds in two main steps, first, the early processes of peripheral degradation that causes removal of the phytol side chain by the chlorophyllase (CLH) and loss of the central Mg^{2+} ion due to the activity of the Mg^{-} dechelatase (MCS), second, the oxidative cleavage of the macrocyclic tetrapyrrole by a pheophorbidea oxygenase (PAO). The biochemistry of Chl degradation to linear tetrapyrroles is well understood in higher plants [6]. However, there are only a few studies that have applied an online technique, combining liquid chromatography with UV-DA-Detector and atmospheric pressure chemical ionization (APCI) tandem mass spectrometry (MS^n)

that allows collecting structural data for many components within a single run [7].

In both regurgitate and frass of feeding herbivorous insects it has been shown that most of the ingested Chl have been partly degraded to derivatives lacking the phytol side chain, the Mg^{2+} ion, and the carbonyl group at the isocyclic ring. An ecological role of ingested Chls in the interactions between insects, their food plant, and other insects has been described recently [8].

Our previous work has shown that the overall early Chl degradation processes in the selected insects are similar, but clearly differ on the quantitative level of the early metabolites for Phe a/b and Pph a/b. Despite that, the results indicate that the early Chl breakdown pathway is comparable to higher plants [9]. We now report the detection, isolation and characterization of late steps Chl catabolites, one new primary fluorescent Chl catabolite (pFCC) together with one known pFCC from senescent leaves of lima bean (*Phaseolus lunatus)* which grew in the greenhouse. Moreover, we tracked the late stages of Chl degradation products in the regurgitate and also in both freshly deposited and old frass of the larvae of Cotton leafworm *Spodoptera littoralis* (Lepidoptera) larvae, which were fed on fresh lima bean leaves.

2. EXPERIMENTAL

2.1. Materials

Commercially available HPLC grade solvents were used for extraction and chromatographic separation. Potassium dihydrogen phosphate puriss. p.a. and potassium phosphate dibasic-anhydrous p.a. were from Fluka (Buchs, Switzerland) and ammonium acetate 7.5 M soln were purchased from Sigma-Aldrich. SepPak-C18, Chromabond C18, and HR-X cartridges were purchased from Waters Associates and Macherey-Nagel, respectively. The pH values were measured with a WTW Sentix 21 electrode connected to a WTW pH535 digital pH meter.

2.1.1. Cultivation of Plants and Insects

Lima bean (*Phaseolus lunatus* 'Ferry Morse' var. Jackson Wonder Bush) was grown in soil. Individual plants were grown in plastic pots in a growth chamber at 23°C (160 μEm^{-2} s^{-1} during a 14 h photoperiod; relative humidity 60%) for the whole time. Larvae of *S. littoralis*, were reared on an artificial diet in a plastic box (25°C±1°C; 14:10 Light:Darkness) until their third instar [10]. Next, the third instars larvae of *S. littoralis* were fed (two or three larvae on one leaf) on green lima bean leaves (2 weeks old) for 2 days before collecting regurgitate and frass.

2.1.2. Preparation of Senescent Plant Samples

The cultivated plants were kept in the climate chamber until their leaves became yellow (160 μEm^{-2} s^{-1} during a 14 h photoperiod; relative humidity 60%). Such senescent plants were pulverized in liquid N_2 and the material stored at -80°C for further extraction.

2.1.3. Samples from Insect Digestive Tracts

The digestive products from insects were collected 48 h after placing the caterpillars onto their fresh food plants. The regurgitate (0.1 mL) or frass (0.1 g) was covered with acetone (2 mL) and stirred for 1 h in the dark at 4°C. After centrifugation at 14000 × g for 10 min at 4°C the solvent was removed by a stream of argon. The residue redissolved in 0.3 mL methanol was filtered through membrane filters (PVDF, Millipore, 0.22 μm). Samples were diluted with methanol (1:10 frass. 1:5 regurgitate) and injected (10 μL) for LC-MS analysis.

2.2. Methods

Crude extracts were fractionated by Sephadex LH20 with a water-MeOH gradient mixture. Concentrating and desalting were done with the SepPak C18 (Waters) and on the HR-X, with 150 mg silica material for 4 mL (Chromabond) cartridges. Chl catabolites were filtered and analyzed by LC-MS with online DAD monitoring at 320 nm and 450 nm. Analysis of early events Chl catabolites was described in our previous work [9, 24].

2.2.1. HPLC Methods

Separation of late events Chl catabolites was achieved on an Agilent HP1100 HPLC system equipped with a Kinetex Gemini C18 5 μm, 250 mm x 2 mm i.d. column (Phenomenex). Catabolites were eluted by a programmed gradient and varying flow rate starting at 0.30 mL/min and 20% solvent B (acetonitrile) and 80% solvent A (water) for 3 min, then started to 80% B for 47 min with an increased flow rate of 0.40 mL/min and ending at 100% B with a flow at 0.45 mL/min, maintained for 10 min and followed from 0.45 mL/min to 0.3 mL/min within 2 min, which was achieved within 8 min. Chl

catabolites were purified by reversed-phase semi-preparative HPLC using 50 mM ammonium acetate buffer (solvent A) and methanol (solvent B) by using a Kinetex C182.6 µm, 150 mm x 4.6 mm i.d. column (Phenomenex) with the same gradient as used in analytic HPLC.

2.2.2. Mass Spectrometry

TIC and LC/MS^n analysis was carried out using a ThermoFinnigan LCQ with atmospheric pressure chemical ionization (APCI), in the full scan mode (vaporizer temperature: 450ºC; capillary temperature: 145ºC; discharge current: 5 eV; capillary voltage: 10 V; the tube lens offset: 20 V). HR-MS data were acquired on an LTQ-Orbitrap XL mass spectrometer connected to the Ultimate 3000 series RSLC (Dionex, Sunnyvale, CA, USA) system. UHPLC was accomplished on an Acclaim C18 Column (150x2.1 mm, 2.2 µm; Dionex) equipped with a C18 3.5 µm guard column (2.1x10 mm, Waters, Dublin, Ireland). As a solvent system (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid (eluent additive for LC-MS, Sigma Aldrich, Steinheim, Germany) were used. The flow rate was set to 500 µL min^{-1}. One µL of the sample was injected into the gradient system. Starting conditions were set to 99% A and 0.5% B. Gradient was linearly increased within 8 min to 10% B, followed by 7 min from 10% to 80% B which was held for 6 min, before decreasing to the initial conditions, which were kept for 5 min to re-equilibrate the column. Ions were generated using an Atmospheric pressure chemical ionization (APCI) source that was operated at 400°C, at a heated capillary temperature of 220°C and a corona discharge of 4.5 mA. High- resolution full scan mass spectra were acquired applying 30,000 m/Δm resolving power in a mass range of *m/z* 150 – 1000 using the Orbitrap mass analyzer. MS/MS data acquisition was performed using CID and HCD with various collision energies (5, 15, 20, 25). Data were interpreted using XCALIBUR software (Thermo Fisher Scientific, MA, USA).

2.2.3. Spectroscopy

UV/Vis spectra: Jasco UV 550; λ_{max} [nm] (log ε/rel. ε). Fluorescence spectra: F750. CD spectra: Jasco J810; λ_{max} and λ_{min} [nm], Δε). 1H- and 13C-NMR, LC-SPE-NMR: Bruker Avance DRX 500MHz spectrometer; Bruker Avance AV-500 MHz II+ (δ (CD_3OH)).

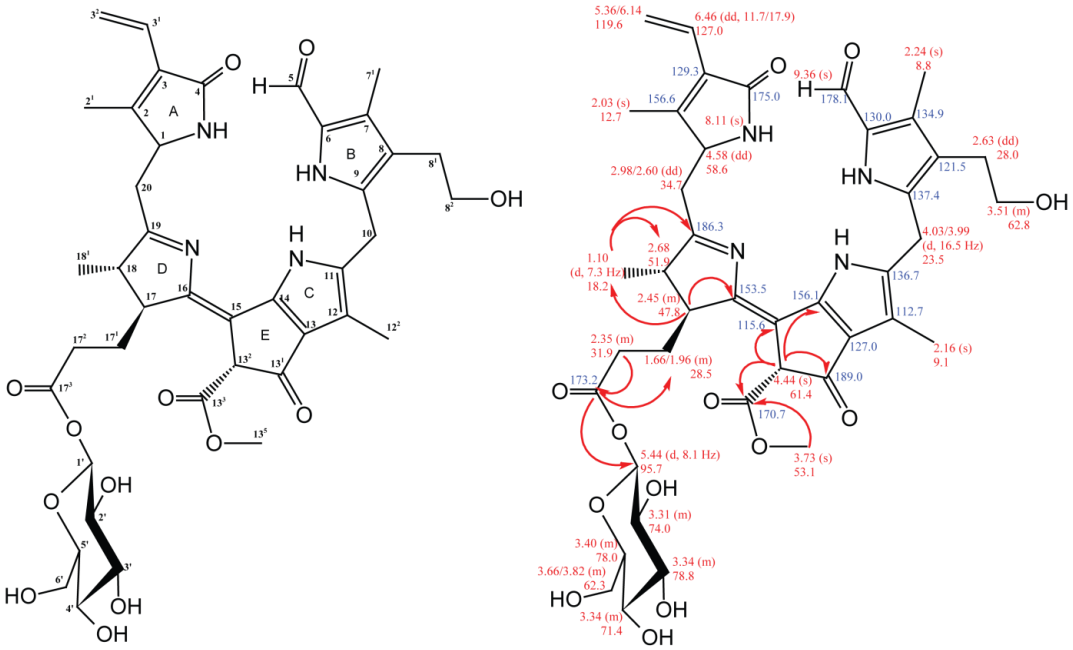
2.3. Isolation of Late Chl Catabolites

2.3.1. Isolation of Chl Catabolites from The Senescent Leaves

70 g (frozen weight) of yellow lima bean (*Phaseolus lunatus*) leaves were worked up. The leaves were frozen in liquid nitrogen and ground. 90 mL of MeOH were added to each sample, the mixture was filtrated with suction over a Buechner funnel. The filter cake was suspended in 90 mL MeOH and extracted, as before. This extraction was repeated four times (totally 450 mL). The juice was concentrated on a rotary evaporator to 50 mL. The residual mixture was diluted with 50 mL of water and 100 mL of potassium phosphate buffer 20 mM pH 7 (KPi). After centrifugation (6000 rpm, 2x10 min) another 100 mL of water was added and the preparation was applied to a 5 g SepPak Vac C18 cartridge, and desalted by washing with water [11, 12]. Further purification by semi-preparative HPLC gave crude samples of Pl-FCC-**1** from both batches, which were desalted and concentrated over SepPak cartridges (as described above) and purified further by the second round of semi-preparative HPLC. The solvents were evaporated *in vacuo* and a sample of 4.2 mg of Pl-FCC-**1** was obtained, which was used for recording UV, Fluorescence, and APCI mass spectra, as well as NMR spectra.

2.3.2. Isolation of Chl Catabolites from The Excretions of S. Littoralis Larvae

Freshly collected regurgitate (3 mL) was diluted with 27 mL ultra-pure water. The extraction was repeated four times and the combined mixture was filtered with suction over a Celite. The filter cake was suspended again in 50 mL of MeOH and filtered, as before. 6.9 water containing fresh and 9.4 g old-frass collected from the larvae reared on green lima bean were used for extraction. The extractions of old frass with 70 mL and fresh frass with 40 mL of MeOH-KPi mixture 5:2 (v/v) were stirred in the cold room (4°C) under dim light for 4 h. The juice was concentrated on a rotary evaporator to 25 mL and evaporated under 25°C. The residual mixture was diluted with 50 mL of water and 100 mL of KPi and partitioned with n-Hexane (20) mL to remove unpolar Chlorins. After centrifugation (6000 rpm, 2x10 min) another 100 mL of water was added, the preparation was applied to SPE described above. The solvents were evaporated *in vacuo* and a crude extract was analyzed by HPLC-UV-DAD-MS application.



Scheme 1. The structure of *Pl*-FCC-**1** isolated from the senescent leaves of lima bean (*Phaseolus lunatus*)

3. RESULTS

3.1. Chlorophyll Catabolites in Senescent Lima Bean Leaves

In order to investigate the whole degradation pathway of Chl in the gut of insects, we used the comparative analysis of linear tetrapyrroles isolated from the senescent food plants and insect gut fluid and frass.

First, we analyzed the senescent leaves of lima bean and characterized two major primary fluorescent Chl catabolites (FCC). One of them was isolated for the first time from this species (*Pl*-FCC-**5**) and the other one was identified as a novel FCC structure (Pl-FCC-**1**) containing a glucose unit in Scheme 1.

The structure was elucidated by NMR spectra using the data on the constitution of Pl-FCC-**1** and assigned H atoms with heteronuclear correlations (^{1}H,^{13}C HMBC). The spectra were measured at 600 MHz in CD_3OH. For the corresponding data with ^{1}H,^{13}C NMR in Table 1.

The yellow senescent lima bean leaves extract was compared with excretions of *S. littoralis*, chromatographic profiles were detected by LC-UV/DAD-MS on the basis of their characteristic fluorescence and absorbance [6, 13]. We observed no specific characteristic in comparison of excretions with the senescent leaf extracts in Figure 1. We, then further identified the major FCCs of the senescent leaves and characterized their identical absorbance and MS-fragments by using spectral methods that might could be useful as references for further indications.

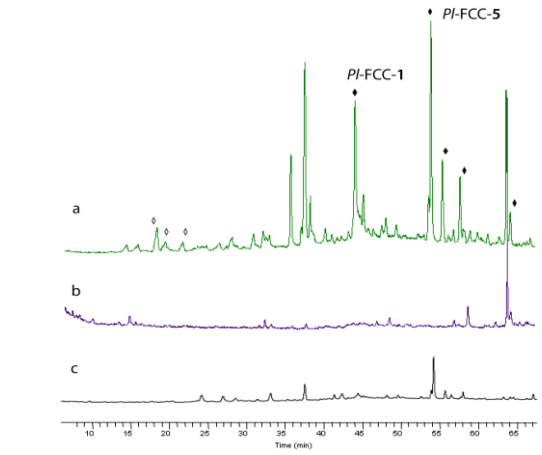


Figure 1. The TIC chromatogram of lima bean, larval egestion extracts. UV-DAD detection at λ=320 nm. a) senescent leaves of lima bean b) regurgitate of S. littoralis c) old frass of S. littoralis. ◊- unidentified non-fluorescent Chl catabolites (NCC) types, ♦- unidentified FCC types.

The LC-UV/DAD-MS studies of samples from senescent leaves and *S. littoralis* excretions provided evidence for the major Chl catabolite of FCCs, whose spectral characteristics provide information on fluorescent open-chain linear tetrapyrroles [6]. The

UV/Vis spectrum of *Pl*-FCC-**1** shows absorbance maximum at 316 and 358 nm.

The long wave absorption maximum at 363 nm and the photoluminescence at 442 mn are due to a conjugated π system extending over the C and D rings of the structure Scheme 1 and Figure 2 [13].

These FCCs were used as references for further MS fragmentation. Both structures were holding a number of epimers in the LC-MS analysis. The crude extract of senescent leaves contained 5 FCCs and 3 non-fluorescent Chl catabolites (NCCs). We identified two of the major peaks, which were further used for comparison with polar extracts of frass and regurgitate of *S. littoralis* on Figure 1.

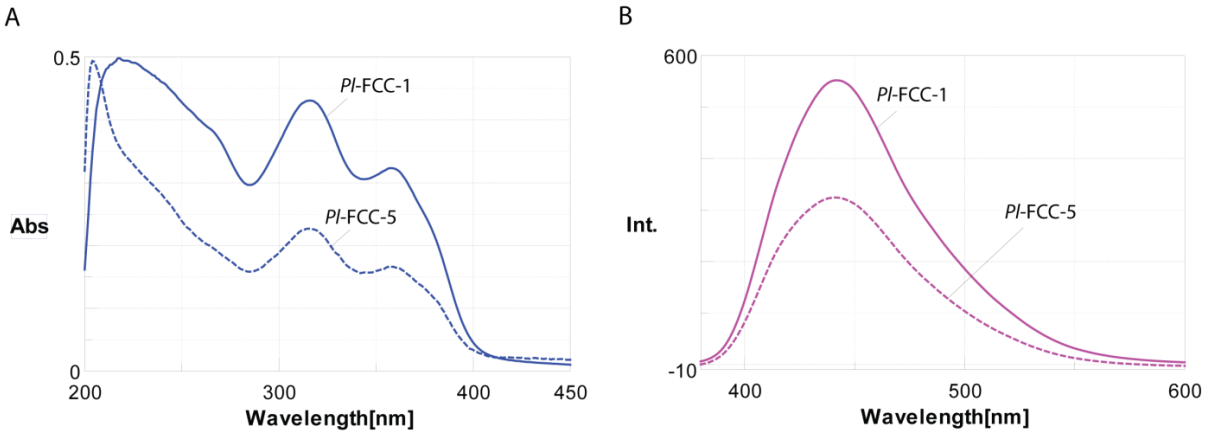


Figure 2. The two major FCCs in senescent lima bean leaves, *Pl*-FCC-**1** and *Pl*-FCC-**5**, are *hm*FCCs and have similar UV/Vis spectra (A). The fluorescence (excitation at 350 nm) spectra (B) of the glucosyl-carrying *Pl*-FCC-**1** is similar with *Pl*-FCC-**5** (dashed line); spectra of FCCs in MeOH

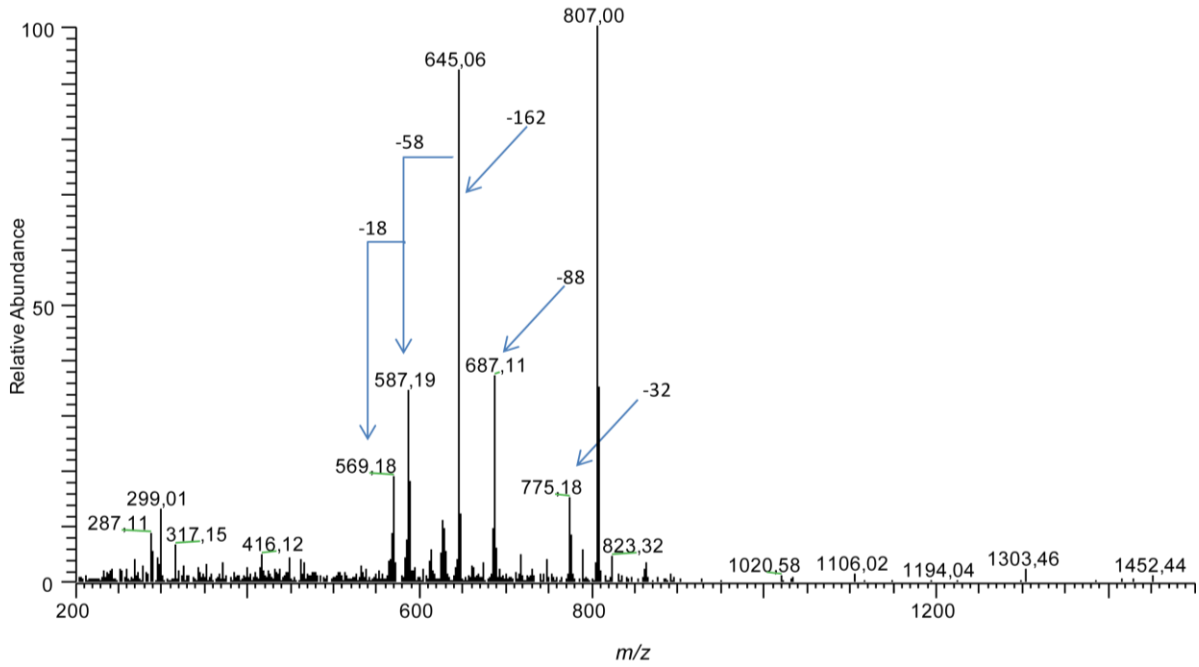


Figure 3. The LC-APCI-MS of *Pl*-FCC-**1** eluting at the 44.72 min

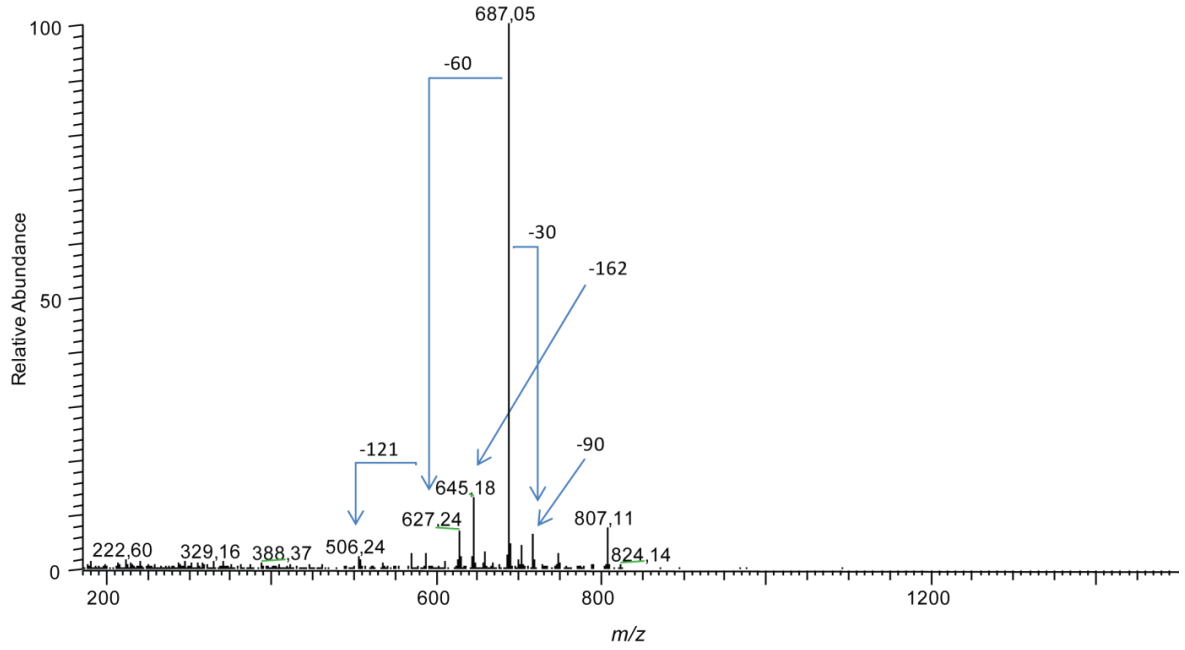


Figure 4. The Tandem spectra of *Pl*-FCC-**1** at *m/z* 807

Regarding retention time (t_R) and the mass spectral data, the old frass contained products similar to the two FCCs shown above. In contrast, in the regurgitate none of the characteristics feature was detected. This may indicate that light- and oxygen-dependent degradation did not occur in the gut [14]. APCI-MS^n spectra of *Pl*-FCC-**1** indicated pseudo-molecular ions $[M+H]^+$ at *m/z* 807 (base peak), consistent with a molecular formula $C_{41}H_{50}N_4O_{13}$. Loss of a glucopyranosyl moiety $[M-162]^+$ was observed at *m/z* 645 suggesting a tetrapyrrole-sugar moiety. A fragment at *m/z* 687 indicated loss of an A ring Figure 3 and 4.

The ^{1}H NMR spectra (600 MHz) of *Pl*-FCC-**1** and *Pl*-FCC-**5**, dissolved in CD_3OH, showed each a set of characteristic signals of the tetrapyrrole moiety, among them signals at low field account for one formyl and one vinyl group, three singlets and one doublet of four methyl groups at high field, as well as a sharp singlet of the methyl ester group at 3.73 ppm in Figure 5.

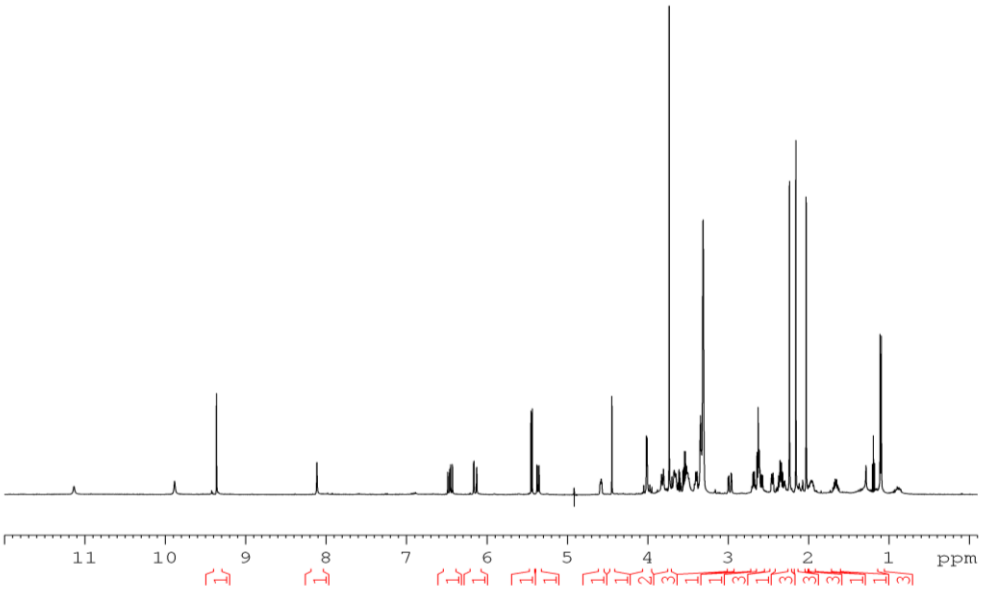


Figure 5. The proton NMR spectrum of the compound Pl-FCC-**1**

Further information on the constitution of *Pl*-FCC-**1** and *Pl*-FCC-**5** was gained from 2D-NMR spectroscopy, such as ^{1}H, ^{1}H COSY, ^{1}H, ^{13}C HSQC and HMBC spectra [15, 16]. A sharp singlet peak of the acidic proton at C(13^{2}) was detected at 4.44 ppm without any H/D exchange. The longest wavelength in UV/Vis spectrum is very characteristic for FCCs showing an intense band at 316 nm and with a shoulder at 358 nm Figure 2, which is also indicative of the presence of an *α*-formylpyrrole [4]. The UV/Vis and CD spectra of the less polar FCC, named *Pl*-FCC-**5**, were similar to those of the more polar analogues. HR-NSI-MS showed a molecular ion at *m/z*: 829.3268 $[M^{+}Na]^{+}$, appropriate for 807.3469 $[C_{41}H_{50}N_4O_{13}{}^{+}H]^{+}$ (calc. for $[C_{41}H_{50}N_4O_{13}{}^{+}H]^{+}$ 807.3447) which is consistent with the NMR data for the newly identified 3^{1},3^{2}-didehydro-8^{2}-hydroxy-13^{2}-(methoxycarbonyl)-17^{3}-(1'-ß-glucopyranosyl)-1,4,5, 10,17,18,20,22-octadhydro-4,5-seco-(22H)-phytoporphyrin.

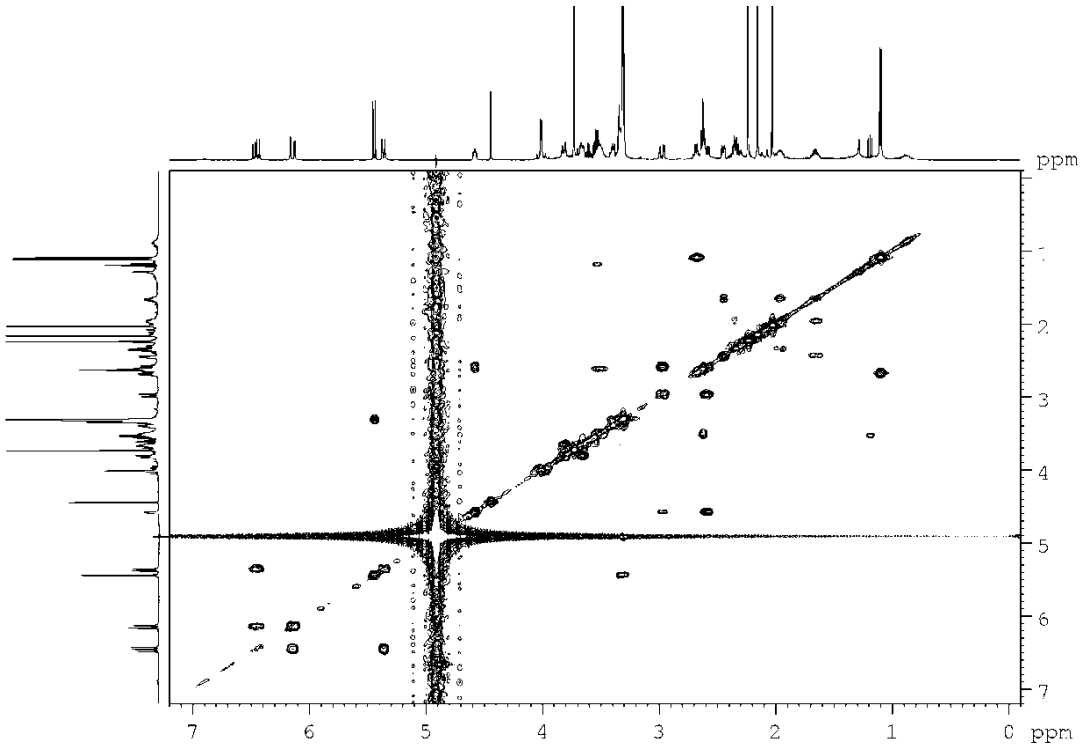


Figure 6. The ^{1}H-^{1}H COSY spectrum of *Pl*-FCC-**1**

Table 1. ^{1}H- and ^{13}C-NMR data of *Pl*-FCC-**1** and *Pl*-FCC-**5** in CD_3OH

***Pl*-FCC-1**						***Pl*-FCC-5**			
No.		**δ_C**	**δ_H**	***mult.***	**J_{HH} [Hz]**	**δ_C**	**δ_H**	***mult.***	**J_{HH} [Hz]**
1	CH	58.6	4.58	*dd*	4.0/7.8	58.1	4.59	*m*	-
2	C	156.6	-	-	-	156.0	-	-	-
2^{1}	CH_3	12.7	2.03	*s*	-	12.1	2.03	*s*	-
3	C	129.3	-	-	-	129.3	-	-	-
3^{1}	CH	127.0	6.46	*dd*	11.7/17.9	126.8	6.46	*dd*	11.7/17.8
3^{2a} **3^{2b}**	CH_2	119.6	5.36 6.14	*dd* *dd*	2.3/11.7 2.3/17.9	119.4	5.37 6.46	*dd* *dd*	2.0/11.7 2.0/17.8
4	C	175.0	-	-	-	175.3	-	-	-
5	CH	178.1	9.36	*s*	-	178.0	9.37	*s*	-
6	C	130.0	-	-	-	129.2	-	-	-
7	C	134.9	-	-	-	135.1	-	-	-
7^{1}	CH_3	8.8	2.24	*s*	-	8.6	2.24	*s*	-
8	C	121.5	-	-	-	121.0	-	-	-
8^{1}	CH_2	28.0	2.63	*dd*	6.7/7.5	27.7	2.63	*m*	-

***Pl*-FCC-1**						***Pl*-FCC-5**			
No.		**δ_C**	**δ_H**	***mult.***	**J_{HH} [Hz]**	**δ_C**	**δ_H**	***mult.***	**J_{HH} [Hz]**
8^2	CH_2	62.8	3.51	*m*	-	62.3	3.50	*m*	-
9	C	137.4	-	-	-	136.7	-	-	-
10a **10b**	CH_2	23.5	3.99 4.03	*d* *d*	16.5 16.5	75.2	4.01	*d* *d*	16.7 16.7
11	C	136.7	-	-	-	136.5	-	-	-
12	C	112.7	-	-	-	112.8	-	-	-
12^1	CH_3	9.1	2.16	*s*	-	9.0	2.16	*s*	-
13	C	127.0	-	-	-	126.9	-	-	-
13^1	C	189.0	-	-	-	n.d.	-	-	-
13^2	CH	61.4	4.44	*s*	-	52.1	3.85	*s*	-
13^3	C	170.7	-	-	-	170.6	-	-	-
13^5	C	53.1	3.73	*s*	-	52.8	3.73	*s*	-
14	C	156.1	-	-	-	n.d.	-	-	-
15	C	115.6	-	-	-	129.9	-	-	-
16	C	153.5	-	-	-	145.3	-	-	-
17	CH	47.8	2.45	*m*	-	47.6	2.41	*m*	-
17^{1a} **17^{1b}**	CH_2	28.5	1.66 1.96	*m* *m*	- -	28.7	1.65 1.93	*m* *m*	- -
17^{2a} **17^{2b}**	CH_2	31.9	2.35	*m*	-	31.3	2.27	*m*	-
17^3	C	173.2	-	-	-	174.9	-	-	-
17^5	-	-	-		-	51.9	3.60	*s*	-
18	CH	51.9	2.68	*ddd*	1.9/7.3/15.6	51.7	2.65	*m*	-
18^1	CH_3	18.2	1.10	*d*	7.3	17.6	1.10	*d*	7.5
19	C	186.3	-	-	-	186.5	-	-	-
20a **20b**	CH_2	34.7	2.60 2.98	*dd* *dd*	7.8/17.2 4.0/17.2	34.4	2.62 3.00	*m* *dd*	- 4.0/16.8
1'	CH	95.7	5.44	*d*	8.1	-	-	-	-
2'	CH	74.0	3.31	*m*		-	-	-	-
3'	CH	78.8	3.34	*m*		-	-	-	-
4'	CH	71.4	3.34	*m*		-	-	-	-
5'	CH	78.0	3.40	*m*		-	-	-	-
6'a **6'b**	CH_2	62.3	3.66 3.82	*m* *m*		-	-	-	-

3.2. Chlorophyll Catabolites in The Excretions of S. Littoralis Larvae

Abundant Chls and their pheo-derivatives were detected in the digested products of the cotton leafworm (*Spodoptera littoralis*), suggesting an incomplete degradation pathway of these pigments in the insect gut. Moreover, this phenomenon is also observed in many other Lepidopteran species, such as *Spodoptera eridania*, *Helicoverpa armigera*, *Heliothis virescens* and the tobacco hornworm (*Manduca sexta*) [9, 17]. In order to understand the whole degradation pathway of Chl in the gut of insects, I analyzed the different types of excretions for linear tetrapyrroles. As shown in Figure 1, the insect gut regurgitate has almost no polar compounds detectable by LC-UV/DAD-MS, in contrast, the frass material showing several peaks that resembled the spectral characteristics of linear tetrapyrroles found in senescent leaves, *Pl*-FCC-**1** *m/z* 807 ($[M+H]^+$) Figure 3 and *Pl*-FCC-**5** *m/z* 659 ($[M+H]^+$) Figure 4 and 5. Interestingly, only in the old frass (one-month-old) the compounds were detected. This result suggested that the oxygenolytic cleavage of tetrapyrroles can occur only with the presence of light and oxygen very likely in combination with yet uncharacterized enzymes which might be provided from microbes or plant enzymes that survived during their journey through the gut tubule.

4. DISCUSSION

The oxygenolytically opened linear tetrapyrroles were found to be identical in the frass of Egyptian Cotton Leafworm, *S. littoralis*, and in senescent food plants, *Phaseolus lunatus.* Ma and Dolphin hypothesized that Chl would be degraded in non-

basic (non-nucleophilic) and/or anaerobic conditions [18]. Since the dephytylated and demetallated catabolite Phe is still able to generate cell-toxic singlet oxygen by photo-activation [19], further degradation is accomplished by the Phe *a* oxygenase (P*a*O), which generates a red fluorescent Chl catabolite (RCC) by cleavage of the methene bridge between the A and B rings of the macrocyclic tetrapyrrole skeleton [20]. The latter are further processed primary to fluorescent Chl catabolites (FCCs) and then non-enzymatically to non-fluorescent Chl catabolites (NCCs).

The degradation of Chl in the digestive tract of *S. littoralis* was addressed by analysis of regurgitate and fecal excretions for the late Chl catabolites. In senescent plants, the Chls are degraded by two major steps namely early events and late events. We found inhibitory FCCs that feature the isomerization of FCCs to NCCs due to complex ester functions at the propionyl substituent also called typically 'hyper-modified' (*hm*) FCCs [21]. In insects, almost nothing is known about Chl degradation at the late stages. In our previous work, it was shown that certain Lepidopteran larvae possessed substantial amounts of the Chl catabolites Phe *a/b* and Pph *a/b* in their digestive products, but these compounds were not found in the fresh food plant leaves. Since the late Chl degradation products, such as FCCs and NCCs were neither found in the regurgitate nor in the fresh frass, insects appear to only degrade the Chl periphery. Insects obviously lack the factors in their gut that might promote further degradation due to be anoxic conditions. This occurs only after defecations due to the exposure to air and the microbial community of the frass, which is changed dramatically [14].

5. CONCLUSION

We demonstrate the inability of the insect gut to contribute to the late degradation processes of an ingested macrocyclic Chl because of lacking both oxygen and light. First, for the purpose of references, analyzed the open tetrapyrroles of the food plant lima bean leaves, which are generated during the senescence in plants where cell death promotes the macrocyclic Chlorin ring to oxidatively opened linear tetrapyrroles. Also analyzed the linear tetrapyrroles in the extracts of freshly collected egestion of the insect larvae of *S. littoralis*.

The two major FCCs were characterized for chemical structures and determined as a novel glycosylated FCC and one known structure. Interestingly, we found the same FCCs traces in old frass, which were characterized by LC-MS and UV spectra. This might be pushed on with the association of oxidative enzymes provided by plant origin and/or microbial production after exploiting to the air. Here we argue that Chl breakdown stops in insect gut due to the lack of oxygen and light. Oxidative degradation of Chl metabolites might, only, be further processed after defecation by bacteria, so that finally macrocyclic ring opening at the late stage may occur in pathways comparable to plants [9, 22].

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