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Zheming Ying, Xixiang Ying, Guanlin Yang, Didier Stien. The first tripyrrolic chlorophyll catabolites isolated from *Crataegus pinnatifida* Bge. var. major brown leaves. *Phytochemistry Letters*, 2020, 35, pp.197-199. 10.1016/j.phytol.2019.12.006 . hal-02413667

**HAL Id: hal-02413667**

**<https://hal.science/hal-02413667>**

Submitted on 16 Dec 2019

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**The first tripyrrolic chlorophyll catabolites isolated from  
*Crataegus pinnatifida* Bge. var. *major* brown leaves**

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## **ABSTRACT**

Two diastereoisomers, namely, *Crataegus pinnatifida* tripyrrolic Chl catabolites or *Cp*-TCC-1 (**1**) and *Cp*-TCC-2 (**2**), were isolated from the leaves of *Crataegus pinnatifida* Bge. var. *major*. Compounds **1** and **2** were identified using spectroscopic methods including HR-ESI-TOF-MS, 1D-NMR, 2D-NMR. A probable biosynthetic pathway to *Cp*-TCC-1 (**1**) and *Cp*-TCC-2 (**2**) is expounded.

## **KEYWORDS**

*Crataegus pinnatifida* Bge. var. *major*; Brown leaves; Tripyrrolic chlorophyll catabolites; Chlorophyll a biodegradation

## 1. Introduction

Hawthorn leaf is the dried leaf of *Crataegus pinnatifida* Bge. var. *major* or *Crataegus pinnatifida* Bge. belonging to the Rosaceae family and is a well-known traditional Chinese medicine used for the treatment of qi-stagnancy and blood stasis, chest distress, palpitation, loss of memory, dizziness and tinnitus (Chinese Pharmacopoeia Commission, 2005). It is used in clinics for the treatment of chronic cardiac insufficiency, congestive heart failure (Pittler et al., 2003) and arrhythmia (Veveris et al., 2004), decreasing blood pressure (Walker et al., 2002), antioxidation (Bahorun et al., 1994), among others.

The study of the chemical constituents of hawthorn began in 1921. Before the 1950s, the main study concentrated on the nutritional ingredients including vitamins, carbohydrates, fats, proteins, inorganic ingredients, tannins, flavonoids and triterpenoids (Liu, 1985). Now, a large diversity of secondary metabolites have been isolated from hawthorn leaves. In the present study, brown hawthorn leaves were examined to investigate on possible metabolite composition differences depending of the age and desiccation conditions of the leaves used in clinical applications. Two diastereoisomeric chlorophyll catabolites have been isolated. This work has also led to the discovery of a new chlorophyll a biodegradation pathway.

## 2. Results and discussion

Compounds **1** and **2** were obtained as a  $\approx 1.2:1$  mixture of stereoisomers (Figure 1). The compounds had a molecular formula of  $C_{24}H_{27}N_3O_6$ , as deduced from the HR-ESI<sup>+</sup>-MS pseudomolecular ion at  $m/z$  454.1962  $[M + H]^+$  (calcd. for  $C_{24}H_{28}N_3O_6^+$ , 454.1973), corresponding to a compound with 13 degrees of unsaturation.

A partial structure of the furo[2,3-b]pyrrol-5-one subunit was built from the sequence of COSY correlations from protons H-22 to H-28 (Figure 2, Table 1). The HMBC correlations of methyl protons H-28 with C-24, C-25 and C-26, along with the correlation of N<sub>27</sub>-H with C-26, demonstrated the existence of an amide group in C-26. The N<sub>27</sub>-H correlated to all carbons of the  $\gamma$ -lactam, which could then be completed with quaternary carbon C-20 at  $\delta$  98.8 in **1** and 97.9 in **2**. The distinctive chemical shift of this carbon indicated that it should be linked to an oxygen atom. This was demonstrated by the observation of a HMBC correlation between proton H-22b at  $\delta$  3.88 (**1**)/3.80 (**2**) and C-20 in both isomers. Carbon C-19 turned out to connect C-20 to a pyrrole moiety, as demonstrated by the correlations H-19/C-9, C-10, C-20, C-24 and H-18/C-9, C-10, C-11.

**Table 1.**

<sup>1</sup>H-NMR (600 MHz) and <sup>13</sup>C-NMR (125 MHz) data for compounds **1** and **2** in DMSO-*d*<sub>6</sub>

Position	<i>Cp</i> -TCC-1 ( <b>1</b> )		<i>Cp</i> -TCC-2 ( <b>2</b> )	
	$\delta_C$ , type	$\delta_H$ mult. ( <i>J</i> in Hz)	$\delta_C$ , type	$\delta_H$ mult. ( <i>J</i> in Hz)
1	NH	9.57 s		9.95 s
2	170.4, C		171.2, C	
3	128.2, C		129.7, C	
4	142.5, C		143.3, C	
5	127.7, C		126.6, C	
6	108.9, C		107.5, C	
7	146.4, C		148.7, C	
8	NH	11.67 s		14.30 s
9	137.1, C		135.8, C	
10	112.4, C		112.6, C	
11	131.6, C		128.0, C	
12	191.1, C		191.8, C	
13	47.4, CH <sub>2</sub>	a: 3.41 d (20.6) b: 3.45 d (20.6)	44.7, CH <sub>2</sub>	3.63 s
14	8.2, CH <sub>3</sub>	1.80 s	8.0, CH <sub>3</sub>	1.82 s
15	24.2, CH <sub>2</sub>	2.87 m	21.5, CH <sub>2</sub>	2.78 brt (8.0)
16	38.8, CH <sub>2</sub>	2.09 m	33.4, CH <sub>2</sub>	2.40 brt (8.0)
17	174.7, C		173.5, C	
18	9.2, CH <sub>3</sub>	2.10 s	9.2, CH <sub>3</sub>	2.09 s
19	33.4, CH <sub>2</sub>	a: 2.95 d (14.3) b: 3.17 d (14.3)	35.3, CH <sub>2</sub>	a: 3.00 d (14.6) b: 3.04 d (14.6)
20	98.8, C		97.9, C	
22	65.4, CH <sub>2</sub>	a: 3.65 m b: 3.88 brt (8.0)	65.9, CH <sub>2</sub>	a: 3.67 m b: 3.80 brt (8.0)
23	32.7, CH <sub>2</sub>	a: 1.68 brdd (7.5, 4.7) b: 1.98 m	32.7, CH <sub>2</sub>	a: 1.65 brdd (7.1, 4.5) b: 1.53 m
24	47.7, CH	2.27 brdd (8.2, 5.0)	48.4, CH	2.31 brdd (8.1, 5.0)

25	42.4, CH	2.08 qd (7.4, 4.5)	42.2, CH	2.18 qd (7.4, 4.1)
26	177.8, C		177.7, C	
27	NH	8.48 s		8.42 s
28	16.3, CH <sub>3</sub>	0.60 d (7.4)	16.8, CH <sub>3</sub>	0.97 d (7.4)

On the other side of the molecule, an ethylene subunit C-15–C-16 was found based on the COSY experiment. In HMBC, H-16 correlated with a carboxylic acid in C-17 at  $\delta$  174.4 (1)/173.5 (2). The correlations N<sub>1</sub>-H/C-3, C-4, C-5, H-14/C-2, C-3, C-4 and H-15/C-4, C-5, C-16 defined a pyrrol-2-one moiety in which C-5 at  $\delta$  127.7 (1)/126.6 (2) is an olefinic carbon. HMBC correlation of protons H-13 with C-5 made it possible to place these protons in the allylic position, with C-13 linked to C-6. The very large <sup>2</sup>*J* coupling between H-13 protons observed in compound **1** is typical of a constrained 5-membered ring. H-13 also correlated to C-6, C-7 and a conjugate carbonyl group in C-12 at  $\delta$  191.1 (1)/191.8 (2). Furthermore, H-13 correlated to C-11 in compound **1**; this correlation confirmed the junction between the cyclopentenone ring and the indole moiety.

Based on the ROESY experiment, it was possible to demonstrate that these two compounds were configurational stereoisomers along the C-5–C-6 double bond and to ascertain that the relative configuration of the furo[2,3-*b*]pyrrol-5-one moiety was identical in both compounds (Figure 3). The double bond was unambiguously defined as (*E*) for compound **1** and (*Z*) for compound **2** based on the observed ROE correlation between H-13 and N<sub>1</sub>-H in **1** and between H-13 and H-15/H-16 in **2**. In the furo[2,3-*b*]pyrrol-5-one subunit, the ROE correlations of the central proton H-24 with H-18, H-19 and H-28 demonstrated the *cis* ring junction and the relative configuration at C-25. The latter was further confirmed upon observation of the ROE correlation H-22a/H-25, demonstrating that these protons are on the concave side of the bicycle.

Interestingly, the biological origin of compounds **1** and **2** can be traced back from phyllobilins, and, eventually, chlorophyll a (Chl a). In plants, Chl a will degrade into pheophorbide a; oxidative cleavage of the pheophorbide a ring will generate primary fluorescent Chl catabolites (*p*FCC) (Hörtensteiner, 2013). Further degradation steps will generate a series of nonfluorescent Chl catabolites (NCCs) generally viewed as the final breakdown products of chlorophyll a. However, structural similarities between known NCCs such as *Brassica napus*-NCC-3 (Mühlecker and Kräutler, 1996), also found in *Arabidopsis thaliana* (Pružinská et al., 2005), and compounds **1** and **2** suggest that **1** and **2** are tripyrrolic Chl a catabolites deriving from NCCs. The A-ring of *Bn*-NCC-3 can be removed through oxidative cleavage with a pheophorbide a oxygenase (PAO)-like enzyme to provide the tripyrrolic intermediate **3** (Scheme 1). A double Baeyer-Villiger oxidative cleavage and a subsequent – presumably spontaneous – decarboxylation would then lead to both configurational diastereoisomers of intermediate **4**. Reduction of the B-ring can produce the enelactam **5** that undergoes spontaneous diastereoselective cyclization to provide compounds **1** and **2** (Gossauer et al., 1989). Since these compounds were isolated from the leaves of *Crataegus pinnatifida* Bge var. *major* and are the first Chl catabolites of their class, we propose to name them following the nomenclature of Chl catabolites, *i.e.*, *Crataegus pinnatifida* tripyrrolic Chl catabolites, or *Cp*-TCC-1 (**1**) and *Cp*-TCC-2 (**2**).

### 3. Conclusion

In summary, two new compounds, *Cp*-TCC-1 (**1**) and *Cp*-TCC-2 (**2**) were isolated from the brown leaves of *Crataegus pinnatifida* Bge var. *major*. The structures of these compounds were established using comprehensive spectroscopic methods. This is the first report of post-NCCs Chl a catabolites.

## 4. Experimental

### 4.1. General experimental procedures

The NMR spectra were recorded using an AVANCE 600 MHz instrument (Bruker Corporation, Switzerland). The compounds were dissolved in DMSO-*d*<sub>6</sub>. Molecular mass was recorded by using a 6520 quadrupole-time-of-flight mass spectrometer (Agilent, Palo Alto, CA). In the separation process, column chromatography (CC) included silica gel (100-220 and 200-300 mesh, Qingdao Marine Chemical Co., Qingdao, China), a polyamide resin (80-100 mesh, Taizhou Luqiao Sijia Biochemical Plastic Factory, Zhejiang, China) and ODS (20-40 μm, GE Healthcare, Marlborough, MA). TLC was performed on silica gel GF254 (Qingdao Marine Chemical Co., Qingdao, China). A final purification step was conducted with a Shimadzu Nexera X2 UHPLC LC-30A system (Shimadzu, Kyoto, Japan) equipped with a Kromasil C18 (4.6 × 150 mm, 5 μm) column (Dalian, China).

### 4.2. Plant material

The dried brown leaves of *Crataegus pinnatifida* Bge. var. *major* were collected in Sujiatun (Shenyang, China) in October 2015, and identified by Prof. Xixiang Ying. Voucher specimens (No. 20151012) were deposited in our laboratory at the Liaoning University of Traditional Chinese Medicine.

### 4.3. Extraction and Isolation

The dried leaves of *Crataegus pinnatifida* Bge. var. *major* (40 kg) were extracted twice with water for 1 h per run and then concentrated. The aqueous extract was later absorbed onto a polyamide resin (80–100 mesh, Taizhou Luqiao Sijia Biochemical Plastic Factory, Zhejiang, China) column eluted with water and hot water, respectively. The hot water fraction (150 g)



was evaporated and applied to a silica-gel (200–300 mesh, approximately 2.5 kg) column (8 × 120 cm), eluted with ethyl acetate:butanone:formic acid:water (6:2:1:0.2) to obtain 30 fractions (500 ml for each). Fractions 20-25 were gathered, evaporated and separated using a Sephadex LH-20 250 g column (φ5 × 80 cm) eluted with methanol to obtain 5 fractions (200 mL each). The fraction 4 was applied again to a Sephadex LH-20 150 g column (φ2 × 150 cm) using methanol as eluent. Final separation was performed using an UHPLC system equipped with an autosampler and eluted with the mobile phase consisting of acetonitrile-water (49:51, v/v), yielding 5 mg of a mixture of compounds **1** and **2**.

#### 4.4. Physical and spectroscopic data of new compounds

Compounds **1** and **2**. <sup>1</sup>H and <sup>13</sup>C NMR in DMSO, see Table 1, and Table S1 in supplementary material; HR-ESI-MS *m/z* 454.1962 [M + H]<sup>+</sup> (calcd. for C<sub>24</sub>H<sub>28</sub>N<sub>3</sub>O<sub>6</sub><sup>+</sup>, 454.1973).

#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:

#### Acknowledgments

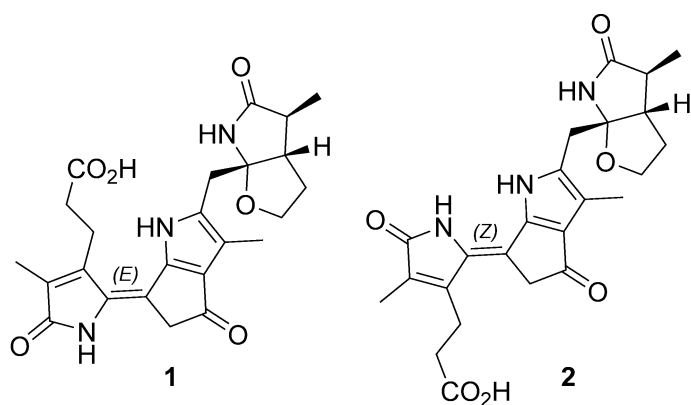
Funding: This work was supported by the National Natural Science Foundation of China [grant number 81573546].

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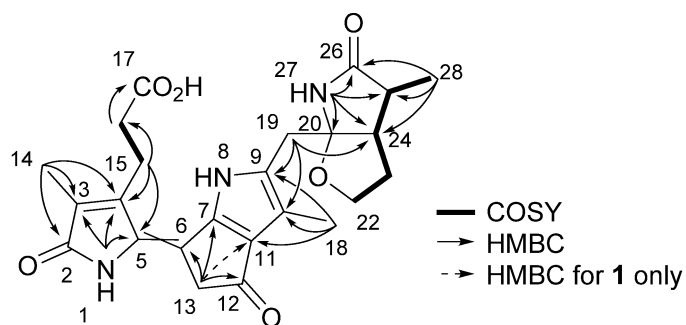
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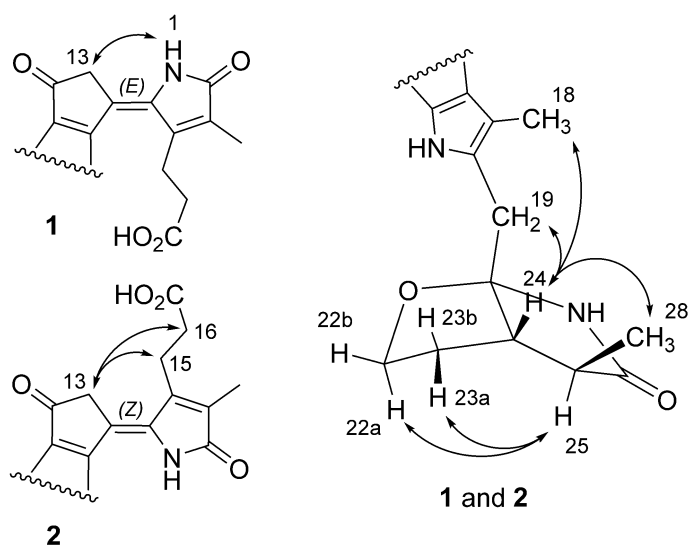
## Graphics



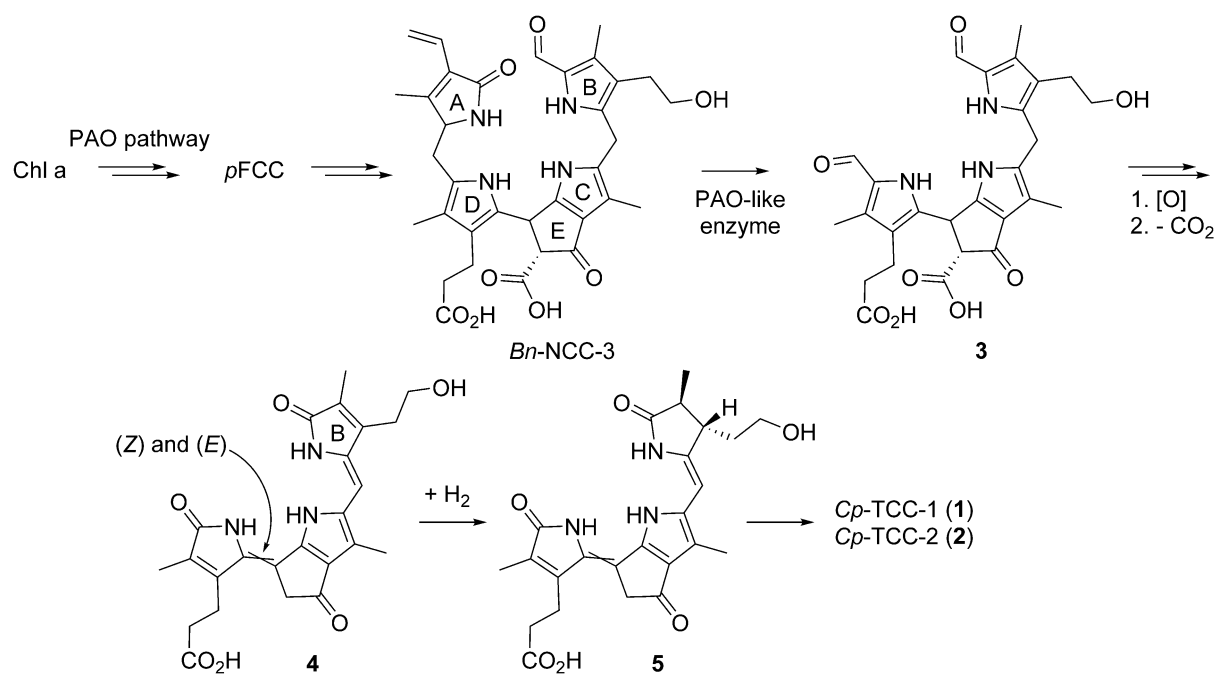
**Figure 1.** Tripyrrolic chlorophyll catabolites from leaves of *Crataeguspinnatifida* Bge. var. *major*.



**Figure 2.** COSY and key HMBC correlations for both compounds 1 and 2.



**Figure 3.** Key ROE correlations for both configurational diastereoisomers 1 and 2.



**Scheme 1.** Probable biosynthetic pathway to *Cp*-TCC-1 (**1**) and *Cp*-TCC-2 (**2**).