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Biogenesis of infractine alkaloids in *Cortinarius infractus:* Importance of 5-Hydroxytriptophane pathway in biogenesis of alkaloids in mushrooms

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ABSTRACT

Cortinarius infractus (Pers.: Fr.) Fr. (brown spored mushrooms in Agaricales, Basidiomycota) are characterized by an overall olivaceous color on the basidiocarp, globose to subglobose spores, and a more or less bitter taste. The bitter taste may be caused by the presence of the bitter indole alkaloid infractopicrine. *C. infractus* is classified into the subgenus *Phlegmacium* according to the traditional infrageneric systematic of *Cortinarius*. Secondary metabolites were analyzed using high performance liquid chromatography (HPLC) equipped with a diode array detector (DAD) and mass spectrometry (MS) with pneumatically assisted electrospray ionization EI source, and gas chromatography-MS with a supersonic beams (GC-MS with SMB). HPLC-MS recorded the presence of β-carboline-1-propionic acid, 6-hydroxy-β-carboline-1-propionic acid, 5-hydroxytryptophan, and infractopicrine in *C. infractus*. The fraction that tentatively included 5-hydroxytryptophan was collected and analyzed by GC-MS with SMB to verify the hypothesis that *C. infractus* contains β-carboline-1-propionic acid-like alkaloids. The recorded spectral data were consistent with previously published information [1-3]. The presence of 6-hydroxy-β-carboline-1-propionic acid and 5-hydroxytryptophan in *C. infractus* was recorded by direct measurement. The biogenesis of 5-hydroxytryptophan and 6-hydroxy-β-carboline-1-propionic acid from tryptophan in *C. infractus* is proposed and is explained in schemas. The biogenesis of alkaloids and toxins via the 5-hydroxytryptophan pathway in plants may have been underestimated because of the general belief that 5-hydroxytryptophan is a rare amino acid that is seldom found in living systems and that exists mainly as an intermediate transmitter in the nervous system.

KEYWORDS: *C. infractus*, 5-hydroxytryptophan, 6-hydroxy-β-carboline-1-propionic acid

1. INTRODUCTION

A preliminary chemotaxonomic study of *Cortinarius infractus* and *C. subtortus* in 2006 [1] revealed some similarities and differences between *C. infractus* and *C. subtortus*. The absence of infractine and the discovery of β-carboline-1-propionic acid in *C. infractus* have been reported [1-3], but both substances are absent in *C. subtortus* [1]. In 1984, Steglich *et al*. proposed that infractine is present in *C. infractus* [4], but it has not been found in either species [1-3].

β-carboline-1-propionic acid-like alkaloids, which belong to the indole alkaloid group, may be typical for *C. infractus* and may have value as chemotaxonomic markers in the genus *Cortinarius.* Because of their potential value and because of

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the apparent inconsistency in [4] mentioned above, we investigated whether *C. infractus* contains β-carboline-1-propionic acid-like alkaloids. β-Carboline-1-propionic acid is found in *C. infractus*, and the absence of infractine has been explained as an artifact [2] caused by careless preparation of biological materials [4]. Steglich *et al*. [4] also described the presence of hydroxy-infractine (derivative from 6-hydroxy-β-carboline-1-propionic acid), but this was also explained as an artifact [2].

The presence of 6-hydroxy-β-carboline-1-propionic acid in *C. infractus* was not shown directly in the study by Brondz *et al*. [2], however, the presence of its artifacts, the ethyl- and methyl-derivatives of 6-hydroxy-β-carboline-1-propionic acid, indicated the presence of this substance. Evidence in the form of mass spectra of the derivatives of 6-hydroxy-β-carboline-1-propionic acid were presented, but the presence of 6-hydroxy-β-carboline-1-propionic acid has not been confirmed analytically.

The sequence of biogenesis of 6-hydroxy-β-carboline-1-propionic acid is also unclear, specifically whether β-carboline-1-propionic acid is generated first and oxidization at position 6 occurs next or vice versa; and whether the oxidized substance is transformed to 6-hydroxy-β-carboline-1-propionic acid later. The tentative hypothesis is that β-carboline-1-propionic acid and 6-hydroxy-β-carboline-1-propionic acid are end products from two separate pathways. Biogenesis of both substances starts from tryptophan. As shown in Scheme 1 in Fig. 1,

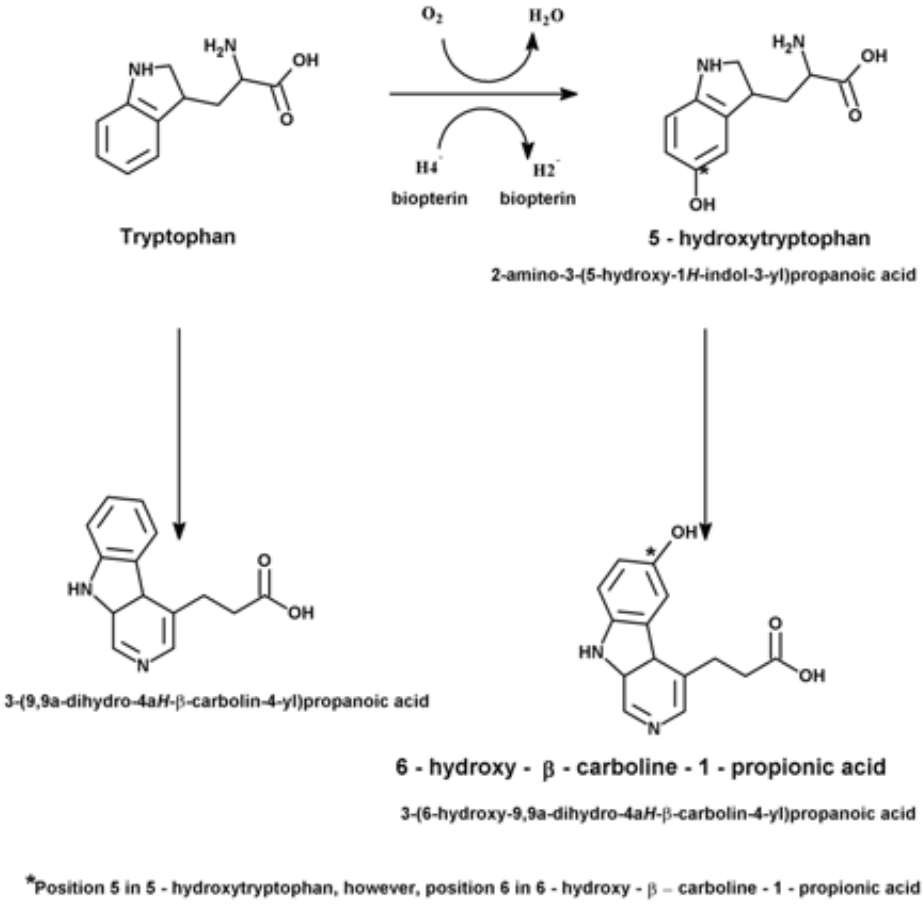

Fig. 1. Oxidation of tryptophan to 5-hydroxytryptophan. β-carboline-1-propionic acid originates from tryptophan. In the case of 6-hydroxy-β-carboline-1-propionic acid, the tryptophan is first oxidized at position 5.

β-carboline-1-propionic acid originates directly from tryptophan, and in the case of 6-hydroxy-β-carboline-1-propionic acid, the tryptophan is first oxidized in position 5. The process of oxidation of tryptophan in position 5 has been shown in brain tissues [5], and this process leads to the biogenesis of the precursors of nerve signal transmitters. Transformation of tryptophan to serotonin in the mammalian brain involves transformation via 5-hydroxytryptophan as an intermediate [6]. Close examination showed that position 5 in the aromatic ring of 5-hydroxytryptophan is equivalent to position 6 in the aromatic ring of 6-hydroxy-β-carboline-1-propionic acid (Scheme 1 in Fig. 1).

The same biogenic oxidative reaction might lead to the production of RNA toxins such as α-amanitine [6], 3-(2-dimethylaminoethyl)-1H-indol-5-ol (bufotenine), 3-[2-(trimethyl azaniumyl) ethyl]-1*H*-indol-5-olate (bufotenidine), or dehydro-bufotenine (Scheme 2 in Fig. 2) or nerve signal transmitters. This hypothesis would be supported by verification of the presence of the end product 6-hydroxy-β-carboline-1-propionic acid and the start product 5-hydroxy tryptophan in the biosynthetic process of infractine-like alkaloids. Because infractine may not exist naturally and its presence may be an artifact [2], we use the expression “carboline-like alkaloids” instead of infractine and infractine-like alkaloids in this paper.

Carboline-like alkaloids are members of the indole alkaloid family. The indole alkaloids possess a broad array of physiological activities in fish, insects, and mammals including humans. The indole alkaloids include toxins, narcotics, analgesics, bitter-flavored substances, and substances with unpleasant odors. Because such substances in plants can have defensive functions against herbivores, these substances are called by us “defensives”. Indole alkaloids are important in medicine and farming, and they serve as good taxonomic markers.

2. MATERIALS AND METHODS

2.1. Biological material and preparation of samples for analysis

Basidiocarps of *C. infractus* were collected in the vicinity of Oslo, Southeast Norway at about 10 m asl in a broadleaf deciduous forest dominated by lime and hazel on Ordovician calcareous clay schist. Voucher specimens will be deposited in the Mycological Herbarium at The Natural History Museum, University of Oslo, Norway.

The basidiocarps were dried in air at 40°C.

2.2. Preparation of extracts for high - performance liquid chromatography - mass spectrometry (HPLC-MS) analysis

One hundred mg of dried tissue was obtained from basidiocarps, pulverized, and extracted in 1 mL of deionized water of ultra-pure quality under vigorous shaking for 1 h and then placed in an ultrasonic bath for 10 min. The solid material was removed from the extracts, and the extracts were analyzed on the same day.

2.3. Preparation of fractions for GC-MS with supersonic molecular beams (SMB) analysis

The fractions were monitored by HPLC-MS, and those that indicated the presence of a substance with MW 220 gram-mol were collected. The mobile phase was evaporated and subjected to gas chromatography GC–MS with SMB analysis.

2.4. HPLC-MS

The chromatographic system comprised an HP 1100 instrument (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode array detector (190-900 nm range) and ChemStation Rev. A. 10. 02. software. UV detection was performed at 254 nm with continuous scanning from 190 to 400 nm. The samples were analyzed with a Discovery ZR-PS 3 μm column measuring 150 mm × 4.6 mm i.d. (Supelco, Bellefonte, PA, USA). The composition of the mobile phase was acetonitrile p.a. quality (Merck, Darmstadt, Germany), 0.1 N ammonium hydroxide (Merck) in deionized water, and 0.5 mM phosphate buffer, pH 2.5 (Merck) (60:20:20, v:v:v). The fractions were eluted isocratically with a flow rate of 0.75 mL x min^{-1}. The injection volume was 20 μL. The HPLC system was coupled online to a Quattro MS/MS triple-quadruple mass spectrometer (Micromass, Altrincham, UK) equipped with a pneumatically assisted electrospray ionization source. Data acquisition and processing were performed using

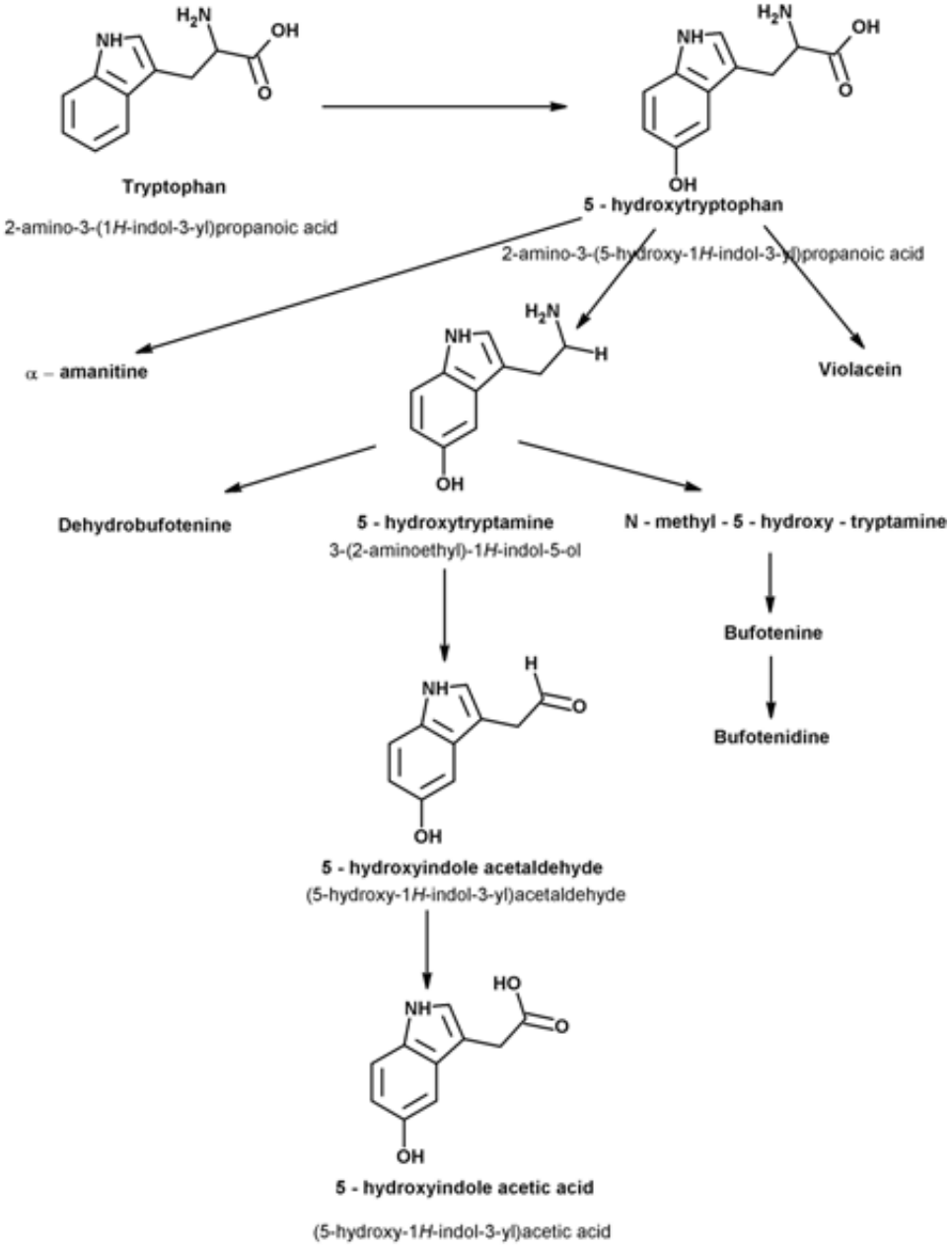

Fig. 2. Biogenic oxidative reaction leads to production of RNA toxins such as α-amanitine, 3-(2-dimethylaminoethyl)-1*H*-indol-5-ol (bufotenine), 3-[2-(trimethyl azaniumyl)ethyl]-1*H*-indol-5-olate (bufotenidine), or dehydrobufotenine and other.

a MassLynx 4.0 SP4 data system (Waters). The effluent entered the mass spectrometer through an electrospray capillary set at 3.0 kV at a source block temperature of 90°C and a desolvation gas temperature of 150°C. Nitrogen was used as both the drying gas and the nebulizing gas at flow rates of about 610 and 80 L h^{-1}, respectively. The source temperature and desolvation temperature were 120°C and 250°C, respectively. The ion source parameters were optimized with respect to the positive molecular ions, and the cone voltage was set to 60 V. The mass spectra between *m/z* 80 and *m/z* 1000 were obtained at a scan speed of 200 *m/z* unit scan/s with a mass resolution

corresponding to 1 unit at half peak height. The instrument was calibrated earlier with sodium iodide. The chromatogram is shown in Fig. 3.

2.5. GS-MS with SMB

The GC-MS with SMB method has been described in detail [7, 8]. The compounds were separated with a VF-5HT column with 0.25 mm i.d. with 0.1 μm film thickness and 4 m length (Varian, Inc., Middleburg, The Netherlands). The reduction of column length was performed in the laboratory. The helium flow rate was 8 mL/min. The 1 μL sample of *C. infractus* (water extract, fractions collected during HPLC with MS monitoring) at a concentration of ~100 ppm was injected with a split ratio of 10:1 using the Varian 1079 injector at 250°C. The GC oven temperature was programmed to increase from 120°C to 300°C at 30°C/min [9]. Ion source degradation was prevented with a contact-free fly-through ESI ion source [10]. The separation and detection of analytes are shown as a chromatogram in Fig. 4, and the mass spectrum is shown in Fig. 5.

3. RESULTS AND DISCUSSION

In chemotaxonomy, fingerprint chromatography is often used as the first approach [1, 3]. This technique can provide important information or important hints for further studies. A preliminary chemotaxonomic study in 2006 [1] revealed differences in the fingerprint analysis of *C. infractus* and *C. subtortus*. β-carboline-1-propionic acid and infractopicrine were found in *C. infractus*, but both substances were absent in *C. subtortus*. Infractine was absent in both species. To perform further chemotaxonomic studies of *Cortinarius*, it is important first to verify whether infractine is found in the basidiocarps of these mushrooms. Previous work on this question [2, 3] showed that the presence of infractine and 6-hydroxyinfractine reported in the study by Steglich *et al*. [4] is an artifact caused by the sample preparation method. Bross *et al*. [11] attempted to correct this problem, although they did not report any experimental data or descriptions of analytical procedures, but they refer only to the analytical procedures and data reported by Blaskó *et al*. [12]. However, the study by Blaskó *et al*. [12] did not isolate β-carboline-1-propionic acid or infractine, but presented only a hypothesis, as follows. “Examination of the isolation processes reported for β-carboline-1-propionate (1a) (1) and methyl β-carboline-1-propionate (1b) (2) allows us to suppose that β-carboline-1-propionic acid (1c) is the true natural product in both cases, the ester derivatives are only artifacts of the isolation procedure depending on the alcohol used during the manipulation” [12]. Blaskó *et al*. [12] presented no practical analytical evidence of the existence of β-carboline-1-propionic acid or the artificial nature of esters in *C. infractus* to support the hypothesis. This publication [12] is about the synthesis of other similar substances and does not provide background information for the taxonomic evaluation, discussion, or explanation of the biogenesis of 6-hydroxy-β-carboline-1-propionic acid.

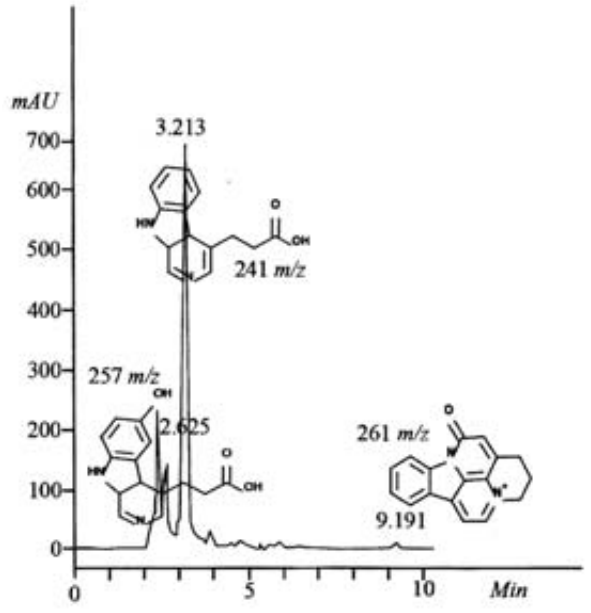

Fig. 3. HPLC chromatogram of water extracts from *C. infractus.*

If our hypothesis is correct that 6-hydroxy-β-carboline-1-propionic acid present in species of *Cortinarius* is derived from tryptophan via 5-hydroxytryptophan, then 5-hydroxytryptophan should be detectable in *C. infractus*.

The presence of 5-hydroxytryptophan in species of *Cortinarius* may lead to alkaloids with hydroxyl moieties in positions 4, 5, and 6 in the ring. The position of the hydroxyl will depend in

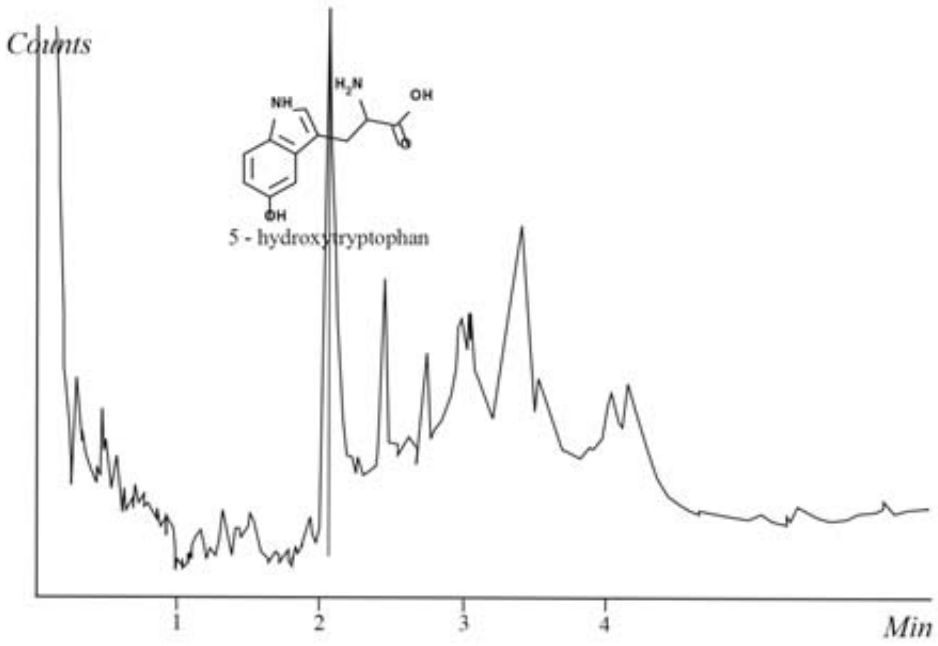

Fig. 4. GC-MS with SMB chromatogram of the HPLC-MS fraction with *m/z* 221.

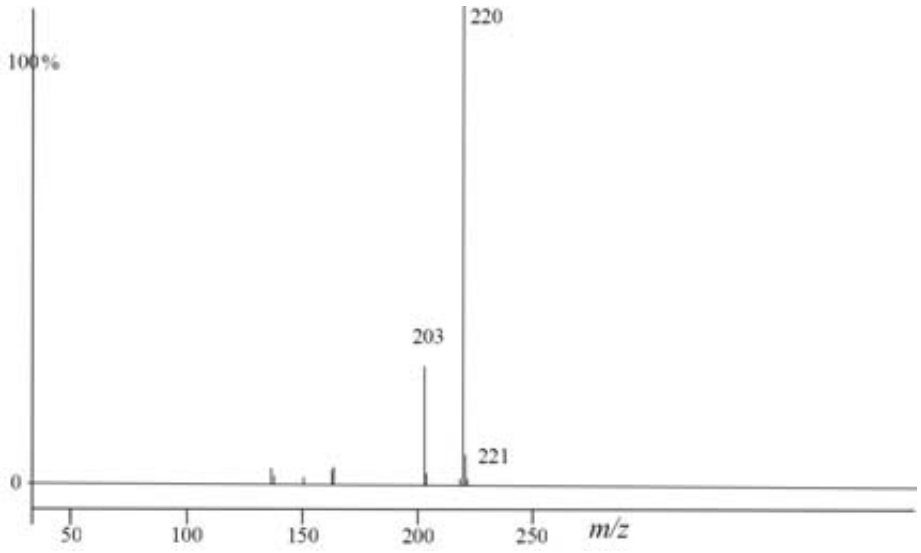

Fig. 5. Mass spectrum from GC-MS with SMB (fragmentation of the peak containing substance with *m/z* 221 by HPLC-MS fractionation).

part of the accepted chemical nomenclature (see the explanation in the scheme 1 in Fig. 1). Usually, tryptophan hydroxylase in the mammalian brain catalyzes the formation of 5-hydroxytryptophan, the first step in the synthesis of the neuro-transmitter 5-hydroxytryptamine [13].

During the HPLC-MS analysis, specific ion monitoring was performed in four independent channels for *m/z* 221, 241, 257, and 261. Based on the fragmentation patterns and comparison with previous results [3], it was possible to identify the compounds eluting as peaks in the extract of

C. infractus, and suggest that infractopicrine is the compound in the peak eluted at 9.191 min in Fig. 3. The fragmentation pattern of the compound suggests that it has a fused aromatic structure. The $[M]^+$ *m/z* 261 of the base peak of the spectrum correlates well with that of infractopicrine, a quaternary ammonium internal salt with a MW of 261 gram-mol [3].

In the chromatogram of *C. infractus*, the presence of *m/z* 257 was detected at 2.625 min; this could be explained by the presence of 6-hydroxy-β-carboline-1-propionic acid as an $[M+H]^+$ ion [2, 3].

The substance with a peak at 3.213 min exhibited poor fragmentation at a cone voltage of 60 V, and this result is difficult to interpret. However, the $[M+H]^+$ base peak at *m/z* 241 in the spectrum is clearly visible. The gram-mol of this compound should be 240 [2, 3].

In the biosynthetic pathway, tryptophan should be the common precursor to β-carboline-1-propionic acid and 6-hydroxy-β-carboline-1-propionic acid. Because the peak was not resolved from other substances and it was thus difficult to obtain a decisive answer about the nature of the substance at *m/z* 221 (the substance of 220 gram-mol) based on the HPLC-MS results, it was necessary to confirm the nature of the molecule. The combined peak was collected as a fraction. After vaporization, the crystalline residue contained a large amount of phosphate from the buffer, which prevented its successful analysis by HPLC-MS, and conventional GC-MS. GC-MS with SMB was used. GC-MS with SMB revealed the presence of the major substance $[M]^+$ of 220 gram-mol, as shown in the chromatogram in Fig. 4; the MS spectrum is shown in Fig. 5. The fragmentation of 5-hydroxytryptophan agrees with the rules of fragmentation for aromatic acids, as described in [2, 14, 15], and the instrumentation used. The molecular $[M]^+$ ion peak of the aromatic acid is visible. The prominent peak is formed by the loss of an OH (M - 17).

The L-form of 5-hydroxytryptophan is used as antidepressant and an antiepileptic. In several species of *Cortinarius*, the substances derived from hydroxytryptophan have been described [16] for example in *C. brunneus*. These substances are represented broadly in plants and mushrooms as toxins such as α- and β-amanitine and amatoxin. 7-hydroxy-β-carboline-1-propionic acid was found in *Eurycoma harmandiana* [17]; 6-hydroxy indole-3-carboaldehyde was isolated from the edible mushroom *Agrocybe cylindracea* [18].

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