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Short communication

# Identification of illudins in *Omphalotus nidiformis* and *Omphalotus olivascens* var. *indigo* by column liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry

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**Abstract**

Reversed-phase liquid chromatography was used to separate toxins in mushrooms of the genus *Omphalotus*. Crude ethyl acetate extracts of cultures were injected directly onto a 150×2 mm I.D. column packed with 3 μm octadecylsilica and eluted with a gradient of acetonitrile in 0.1% aqueous acetic acid at a flow-rate of 200 μl/min. Monitoring of the column effluate by atmospheric pressure ionization tandem mass spectrometry allowed the identification of the toxins. The fungal toxins illudin M and illudin S were detected and identified for the first time in cultures of the Australian *Omphalotus nidiformis* and the North American *Omphalotus olivascens* var. *indigo* (Boletales, Basidiomycetes) and confirmed the valuable taxonomic character of illudins for the genus *Omphalotus*. 

*Keywords:* *Omphalotus*; Fungi; Basidomycetes; Tandem mass spectrometry; Illudins; Toxins

## 1. Introduction

Illudins are low molecular mass natural products isolated from certain species of the genus *Omphalotus* Fayod and the closely related *Lampteromyces japonicus* (Kawamura) Sing. [1]. They are the main toxins of these fungi and are considered as one of the most important features for classifying a mushroom as a member of the family *Omphalotaceae* [2]. Until recently, the Australian *Omphalotus nidiformis* (Berk.) O.K. Miller was classified in the genus *Pleurotus* (Fr.) Quél. Miller transferred it to *Omphalotus* [3] because of its well-known toxicity [4] and because of morphological characteristics. Nevertheless, the toxic substances of *Omphalotus nidiformis* were unknown until now. If this fungus is placed right in the genus mentioned above it is very likely that its toxic substances are illudins. Moreover, the presence of illudins has not been investigated so far in the recently described *Omphalotus olivascens* var. *indigo* Moreno, Esteve-Raventós, Pöder and Ayala [5]. The occurrence of these toxins in the two taxa would increase the taxonomic value of this character.

Column liquid chromatography (LC) is often used to separate natural compounds in complex mixtures and its combination with mass spectrometry (MS) or tandem mass spectrometry (MS–MS) allows the direct analysis of crude biological extracts. Among

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various types of interfaces used in LC–MS, the electrospray ionization (ESI) [6,7] and heat-assisted pneumatic nebulization [8] interfaces are most frequently used. During atmospheric pressure chemical ionization (APCI), ions are formed in the gas phase by electron capture or through proton- and charge transfer reactions between analyte molecules and reactand ions, which have been formed in the ion-source from eluent constituents by corona discharge [9,10]. LC–APCI–MS has been successfully applied to the analysis of a variety of synthetic and biological compounds which include microbial metabolites and plant metabolites [11]. Draisici et al. identified a polyether–lactone toxin in algae by LC–MS [12]. Tanaka et al. studied the reactions of illudin S isolated from *Lampteromyces japonicus* with cysteine derivatives and characterized the addition products by NMR and LC–ESI–MS–MS [13]. The LC–MS analysis of illudins in mushrooms of the genus *Omphalotus* has, however, not been described in the literature so far. In this paper, we report on the use of LC–APCI–MS for the separation of illudin M and illudin S in extracts of the mushrooms *Omphalotus nidiformis* and *Omphalotus olivascens* var. *indigo*.

## 2. Experimental

### 2.1. Chemicals

The malt extract liquid medium contained 30 g malt extract (Merck, Darmstadt, Germany), 3 g pepton from soymeal papain-digested (Merck) in 1 l bidistilled water. Ethyl acetate (p.a.), acetonitrile (gradient grade), anhydrous sodium sulfate (p.a.), and acetic acid (p.a.) were from Merck. High purity water was supplied by a NANOpure unit (Barnstead, Newton, MA, USA). Illudin S and M standards were kindly provided by Trevor C. McMorris, San Diego, USA.

### 2.2. Cultures and extraction of the toxins

The different strains listed in Table 1 were cultivated for 22 days in malt extract liquid medium at 20°C. The cultures in 100 ml medium were shaken at 80 rpm in 500 ml Erlenmeyer flasks. For each strain, 300 ml culture fluid were extracted three times with 300 ml ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulfate, filtered, evaporated to dryness (vacuum, 50°C), and redissolved in 1 ml acetonitrile.

### 2.3. High-performance liquid chromatography and atmospheric pressure chemical ionization mass spectrometry

The HPLC system consisted of a low-pressure gradient micro pump (model Rheos 4000, Flux Instruments, Karlskoga, Sweden) controlled by a personal computer, a vacuum degasser (Knauer, Berlin, Germany), a column oven (model S4110, Sykam, Gilching, Germany), a microinjector (model 8125, Rheodyne, Cotati, CA, USA) with a 20 μl sample loop, a variable wavelength detector (model Linear UV-VIS 200, Linear Instruments, Fremont, CA, USA) with a 3 mm/1.2 μl detector cell, and a PC-based data system (GynkoSoft, Ver. 5.22, Gynkotek, Germering, Germany). The column temperature was held at 30°C and the UV-absorbance of the eluate was monitored at 320 nm with a detector rise time of 0.1 s. LC separations were performed with a gradient of 10–90% acetonitrile in 0.1% aqueous

Table 1
List of strains used in this study

| Species | Location | Substrate | Date | Strain |
|---|---|---|---|---|
| *Omphalotus olivascens* var. *indigo* | Baja California, Mexico | *Quercus agrifolia* | 1995-01-14 | IND1[a] |
| *Omphalotus olivascens* var. *indigo* | Baja California, Mexico | *Quercus agrifolia* | 1995-02-01 | IND2[a] |
| *Omphalotus nidiformis* | Western Australia | Burned wood | – | VT19468[b] |
| *Omphalotus nidiformis* | Western Australia | *Acacia* | – | VT1949[b] |
| *Omphalotus nidiformis* | Western Australia | n.a. | – | VT1490[b] |

[a] IND, Institute of Microbiology, University of Innsbruck, Austria.
[b] VT, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA.

acetic acid in 10 min at a flow-rate of 200 μl/min. The Nucleosil ODS (3 μm, 100 Å) column-packing material was obtained from Macherey and Nagel (Düren, Germany) and packed into a 150×2 mm i.d. stainless-steel column (Grom, Herrenberg, Germany) with the help of an air-driven high-pressure packing pump (Knauer, Berlin, Germany).

APCI–MS–MS was performed on a Finnigan MAT TSQ 7000 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with the atmospheric pressure chemical ionization ion source. For LC–MS analysis with heat-assisted pneumatic nebulization, a corona current of 5 μA at 4 kV and a sheath gas pressure of 410 kPa were employed. The temperatures of the heated nebulizer and the heated capillary were set at 300 and 200°C, respectively. Mass spectra were recorded by scanning the third quadrupole Q3 from $m/z$ 150–300 at a rate of 150 $m/z$ $s^{-1}$. Tandem mass spectra of ions preselected by Q1 were obtained with a collision energy of 20 eV and an argon collision gas pressure of 0.26 Pa by scanning Q3 from $m/z$ 100–250 at a rate of 150 $m/z$ $s^{-1}$. Total ion chromatograms and mass spectra were recorded on a DEC-Alpha 3000 workstation with ICIS software Ver. 7.01 (Finnigan).

## 3. Results and discussion

Illudin S and illudin M are sesquiterpenes whose structures were determined after isolation from *Omphalotus illudens* (Schw.) Bresinsky and Besl, by McMorris and Anchel in 1963 [14,15]. The chromatographic retention times of illudin S and illudin M were obtained by injection of reference solutions in acetonitrile. Separation was achieved using a 3 μm Nucleosil ODS stationary phase and a 10 min gradient of 10–90% acetonitrile in 0.1% aqueous acetic acid. Because of its higher polarity (one additional hydroxyl group), illudin S eluted first at 3.7 min and illudin M at 8.1 min. Scanning the quadrupole analyzer at 150–300 amu in 1 s in the positive ion mode allowed the detection of protonated molecules, solvent adducts and fragment ions of illudin S and illudin M. The spectra of both compounds showed protonated molecules of relatively low abundance at $m/z$ 265 and 249, respectively (Table 2). The base peak in the spectrum of illudin S at $m/z$ 217 is a result of elimination of water and formaldehyde. The ion of highest mass ($m/z$ 288) is an adduct with acetonitrile after loss of water. Elimination of formaldehyde indicates the presence of a hydroxymethyl group and is observed in the spectrum of illudin S only. Illudin M yields an acetonitrile adduct of the intact molecular ion at $m/z$ 290. The base peak at $m/z$ 231 is attributed to the loss of water.

LC–UV–APCI–MS of an extract of *Omphalotus olivascens* var. *indigo* revealed a large peak at 8.2 min in the UV trace corresponding to illudin M which demonstrates the high sensitivity and selectivity of UV detection at 320 nm for illudin M (Fig. 1a). A number of unresolved peaks was seen in the total ion chromatogram (Fig. 1b). Extraction of a selected ion chromatogram at $m/z$ 231, the base peak in the spectrum of illudin M (Fig. 1c), showed one large peak eluting at 8.2 min and some small peaks spread over the whole chromatogram. The APCI mass spectrum obtained upon addition of nine scans under the peak at 8.2 min clearly identifies this peak as illudin M (Fig. 2a). Moreover, analysis of the mass spectra under the smaller peaks made the presence of illudin M in these peaks very unlikely. Illudin S was tracked in *Omphalotus olivascens* var. *indigo* from a selected ion trace at $m/z$ 288 (Fig. 1d) and its identity was confirmed by the extracted mass spectrum depicted in Fig. 2b. The splitting of the illudin S peak is due to injection of the sample as solution in acetonitrile and could be alleviated by using a weaker solvent for dissolution of the sample. However, since the splitting does not interfere with the identification of illudin S and, on the other hand, acetonitrile as solvent excluded any loss of sample components due to precipitation, no attempt was

Table 2
Interpretation of the APCI mass spectra of illudin S and illudin M

| Illudin S | | Illudin M | |
|---|---|---|---|
| $m/z$ | Interpretation | $m/z$ | Interpretation |
| 288 | $(M-H_2O+ACN+H)^+$ | 290 | $(M+ACN+H)^+$ |
| 265 | $(M+H)^+$ | 272 | $(M-H_2O+ACN+H)^+$ |
| 247 | $(M-H_2O+H)^+$ | 249 | $(M+H)^+$ |
| 229 | $(M-2H_2O+H)^+$ | 231 | $(M-H_2O+H)^+$ |
| 217 | $(M-H_2O-CH_2O+H)^+$ | 213 | $(M-2H_2O+ACN+H)^+$ |
| 199 | $(M-2H_2O-CH_2O+H)^+$ | | |

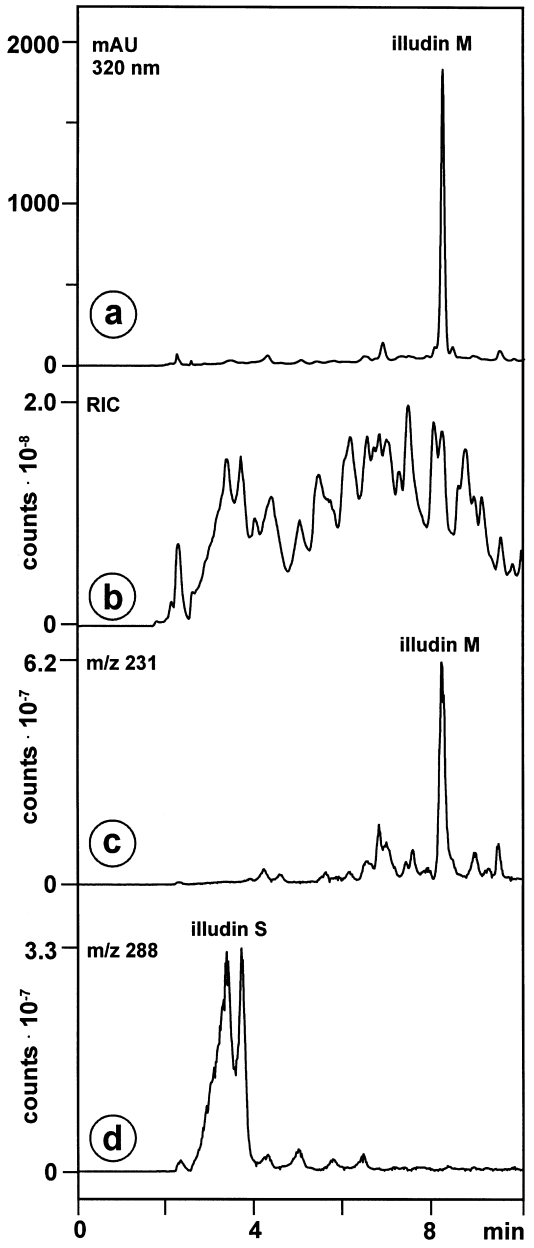


Fig. 1. LC–UV–APCI–MS analysis of an ethyl acetate extract of *Omphalotus olivascens* var. *indigo*. (a) UV trace; (b) total ion chromatogram; (c) trace of *m*/*z* 231; (d) trace of *m*/*z* 288. Scan, 150–300 amu in 1 s; sample, 2.5 μl ethyl acetate extract of *Omphalotus olivascens* var. *indigo*.

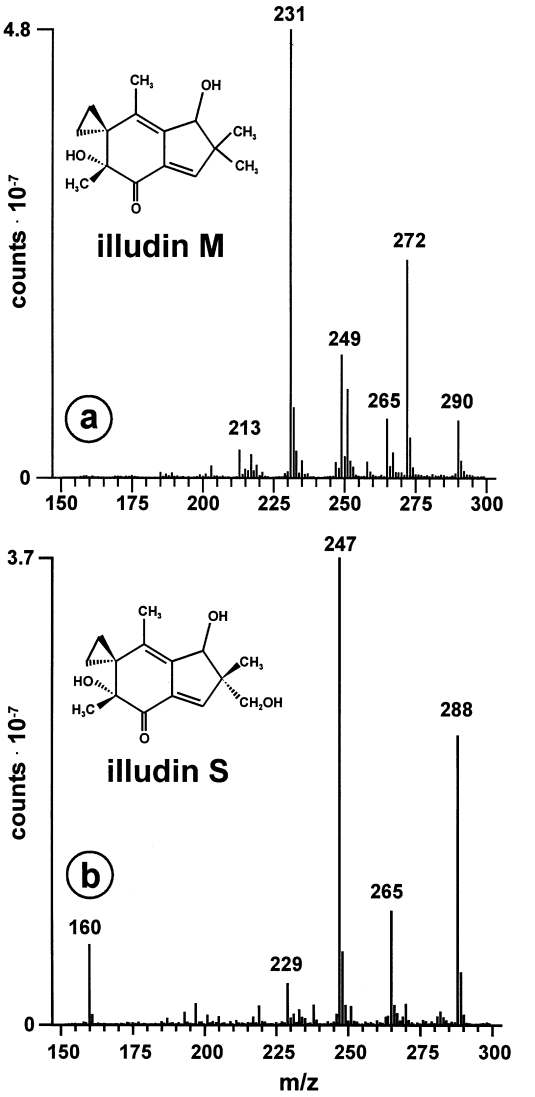


Fig. 2. Structures and APCI mass spectra of (a) illudin M, monoisotopic mass=248.14 g/mol, and (b) illudin S, monoisotopic mass=264.14 g/mol, detected in an ethyl acetate extract of *Omphalotus olivascens* var. *indigo*. For peak identification see Table 2.

made to dissolve the sample in a weaker solvent. Moreover, analysis of the mass spectra under the peak profile indicated that the split peak is chemically homogenous and composed mainly of illudin S and a coeluting minor impurity of *m*/*z* 160.

The identity of illudin M was additionally corroborated by tandem mass spectrometry with collisionally induced dissociation. Fig. 3a depicts the tandem mass spectrum of illudin M obtained by direct infusion of the standard solution. Daughter

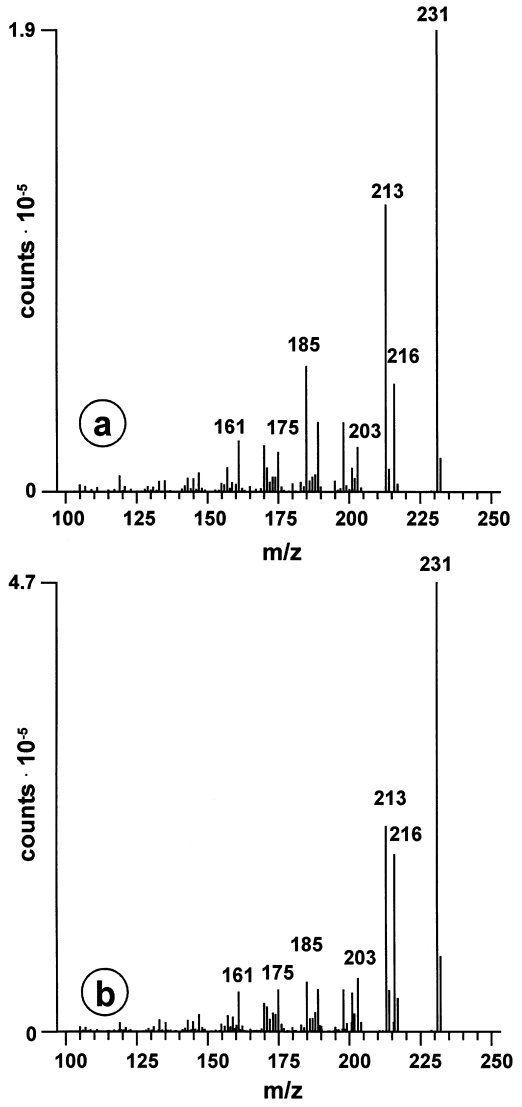


Fig. 3. Tandem mass spectrum of (a) directly infused illudin M standard, and (b) illudin M in an ethyl acetate extract of *Omphalotus olivascens* var. *indigo*. Daughter ions of *m*/*z* 231; collision energy, 20 eV; collision gas pressure, 0.26 Pa; scan, 100–250 amu in 1 s; sample: (a) standard solution of illudin M; (b) 20 μl ethyl acetate extract of *Omphalotus olivascens* var. *indigo*.

ions of *m*/*z* 231 were generated at a collision energy of 20 eV and a collision gas pressure of 0.26 Pa. In a second MS–MS experiment under chromatographic conditions, daughter ions from the peak at 8.2 min were generated under otherwise identical conditions. The extracted tandem mass spectrum is depicted in Fig. 3b and its high similarity with the spectrum in Fig. 3a clearly ascertains its identity as illudin M.

The presence of illudin M in an extract from *Omphalotus nidiformis* was not as obvious in the UV trace (Fig. 4a) as in *Omphalotus olivascens* var. *indigo* (Fig. 1a). The total ion chromatogram was, however, less complicated than that obtained with *Omphalotus olivascens* var. *indigo* and had six dominant peaks (Fig. 4b). Of these, the one at 8.6 min indicated the presence of illudin M which was confirmed by the extracted APCI mass spectrum. Retention time of illudin M most probably shifted because of adsorption of strongly retained compounds during more than 10 runs of raw extracts and the original retention time could be restored after washing with acetonitrile for ca. 40 min. The split peak eluting at 3.2 and 3.4 min corresponds to illudin S. Analysis of the mass spectra under the peaks revealed signals at *m*/*z* 288, 265, 247 and 229 which are characteristic of illudin S.

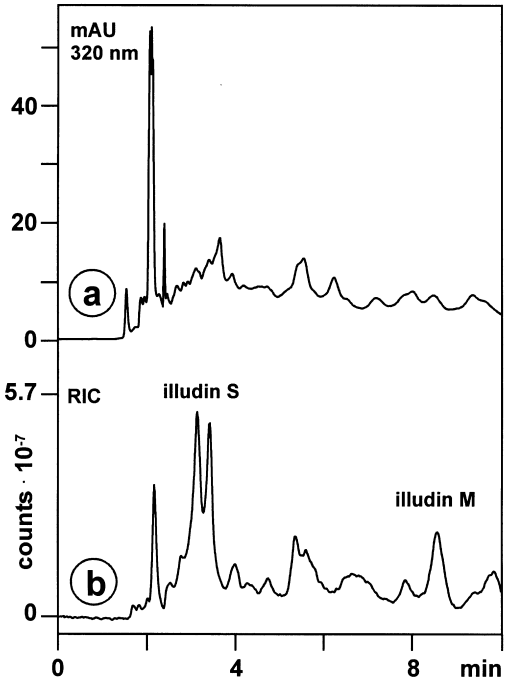


Fig. 4. HPLC–UV–APCI–MS analysis of an ethyl acetate extract of *Omphalotus nidiformis*. (a) UV trace; (b) total ion chromatogram. Scan, 150–300 amu in 1 s; sample, 2.5 μl ethyl acetate extract of *Omphalotus nidiformis*.

## 4. Conclusions

It has been shown that reversed-phase column liquid chromatography combined with APCI mass spectrometry is a valuable tool for identification of toxins in fungi of the genus *Omphalotus.* The high selectivity of APCI mass spectrometry and tandem APCI mass spectrometry allowed the confident identification of the toxins even in crude extracts of complex composition. Illudin M and illudin S were detected both in *Omphalotus nidiformis* and *Omphalotus olivascens* var. *indigo* which indicates that the presence of illudins is highly characteristic for the genera *Omphalotus* and *Lampteromyces.* Moreover, illudins have not been found so far in any other group of basidiomyces. Therefore, the detection of these toxins in *Omphalotus nidiformis* which was just recently transferred to this genus because of its morphological characteristics is confirmed chemically by the detection of illudins.

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