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# Systematic identification of alkaloids in *Macleaya microcarpa* fruits by liquid chromatography tandem mass spectrometry combined with the isoquinoline alkaloids biosynthetic pathway

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ABSTRACT

Alkaloids in *Macleaya microcarpa* were characterized systematically by combining liquid chromatography tandem mass spectrometry (LC–MS/MS) with the biosynthetic pathway of isoquinoline alkaloids. The mass spectral fragmentation behaviors of 16 references belonging to eight types of alkaloids that exist in the biosynthetic pathway of isoquinoline were investigated in detail. The benzyltetrahydroisoquinoline and aporphine alkaloids were distinguished by characteristic losses of the $NHR_1R_2$ ($R_1$ and $R_2$ represent the substituent groups of the nitrogen atom) radical and the fragment ions below *m/z* 200. Tetrahydroprotoberberine, *N*-methyltetrahydroberberine and protopine alkaloids were differentiated by the retro-Diels–Alder (RDA) reaction, α-cleavage and the $[M–H_2O]^+$ and $[M–CH_4]^+$ ions. Discrimination of protoberberine, benzophenanthridine and dihydrobenzophenanthridine-type alkaloids can be realized through the characteristic [fragment ion-2H]$^+$, $[M–H_2O]^+$, $[M–CH_4]^+$, $[M+H–CH_3CH_2CH_2OH]^+$ and $[M+H–CH_3COCH_3]^+$ ions. Forty-one alkaloids, including one benzyltetrahydroisoquinoline, one aporphine, nine protopines, seven protoberberines, one tetrahydroprotoberberine, three *N*-methyltetrahydroprotoberberines, five benzophenanthridines and fourteen dihydrobenzophenanthridines, were separated and identified simultaneously. Thirty-three of these were reported for the first time in *M. microcarpa*. The benzyltetrahydroisoquinoline, aporphine, tetrahydroprotoberberine and *N*-methyltetrahydroprotoberberine-type alkaloids have not been reported previously in *M. microcarpa*. This method can be applied to the analysis of herbal medicines that possess the biosynthetic pathway of isoquinoline alkaloids.



## 1. Introduction

*Macleaya microcarpa* (Maxim) Fedde belongs to the genus *Macleaya* of the Papaveraceae family. This perennial herb is a traditional folk medicine and is distributed widely in south and northwest China [1]. Isoquinoline alkaloids, including protopine, allocrytopine, sanguinarine, chelerythrine, dihydrosanguinarine, dihydrochelerythrine, macarpine, chelirubine and chelilutine are its major chemical components [1–3]. In addition to these well-known components, a series of compounds in the isoquinoline alkaloids biosynthetic pathway is still unknown and requires further researched. Several biological activities have originated from these alkaloids, including anti-inflammatory, anti-parasitic, anti-tumor, anti-leukemic and anti-microbial activities, against fish pathogenic bacteria and *Dactylogyrus intermedius* [4–8]. Moreover, this medical plant has been used to promote animal growth as a popular natural feed additive (*e.g.* Sangrovit®) in Europe and Asia since 2002. With increasingly attention on food safety, the drug residue has become one of the main issues that could affect human health. Therefore, a study by mass spectrometry of the behavior of alkaloids in *M. microcarpa* provideds a perfect foundation for research into the drug residue and metabolism.

In the Papaveraceae plant family, most of isoquinoline alkaloids are biosynthesized from the benzyltetrahydroisoquinoline alkaloids, including aporphine, tetrahydroprotoberberine, *N*-methyltetrahydroprotoberberine, protopine, protoberberine, benzophenanthridine and dihydrobenzophenanthridine [9,10] (Fig. 1). However, benzyltetrahydroisoquinoline,

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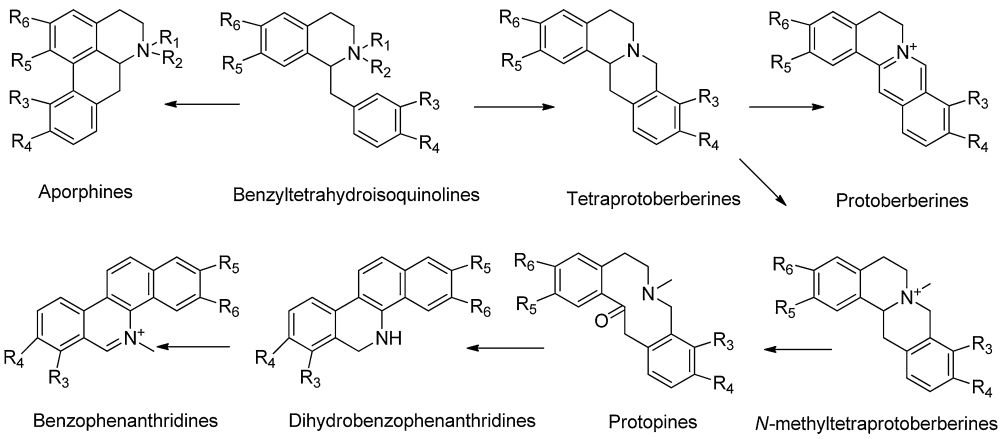


**Figure 1.** Biosynthetic pathways to various isoquinoline alkaloid.

aporphine tetrahydroprotoberberine and *N*-methyltetrahydroprotoberberine-type alkaloids have not been reported previously in *M. microcarpa*. It is difficult to separate and identify compounds by traditional phytochemical methods because these alkaloids are microscale constituents. Liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) is a sensitive and useful tool for identifying the micro, or even trace components.

Liquid chromatography/mass spectrometry (LC–MS) has been widely used for structural elucidation and determination in herbal medicine and prescription [11,12]. Several compounds were preferentially chosen as the authentic references for the investigation of fragmentation pathways, and then the structures of chemical constituents were determined by the fragmentation behaviors. Two obvious shortages existed in this routine method. Firstly, systematically characterization of compounds was difficult for the reason of the unknown types of components and the limited of authentic references. Secondly, the related-structure compounds were uneasy determined for the similar fragmentation behaviors [13]. However, in this study, LC–MS combined with the biosynthetic pathway of isoquinoline alkaloids not only can determination all types of alkaloids existed in the biosynthetic pathway, but also can distinguish the similar compounds well though the investigation of MS/MS fragmentation pathways of alkaloids existed in the biosynthetic pathway.

High-performance liquid chromatography/quadrupole-time-of flight mass spectrometry (HPLC–Q-TOF-MS) combined with a screening procedure is a powerful means for speculating on and identifying compound structure. In this method, the alkaloid structure is divided into three parts, namely, the skeleton, the substitute groups and the linkage sites between the skeleton and substitute groups. The skeleton is determined from the ultraviolet (UV) spectra and tandem mass spectrometry (MS/MS) fragmentation patterns. The substitute groups are determined through a screening procedure and from the MS/MS fragmentation pathways. The linkage sites are deduced from the MS/MS fragmentation behavior and from the biosynthetic pathway of isoquinoline alkaloids [11]. This method has been used as a powerful and rapid tool for elucidating and identifying the alkaloid structure in *M. microcarpa*.

## 2. Experimental

### 2.1. Materials and reagents

HPLC-grade acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany) and ROE (Newark, DE, USA), respectively. Deionized water was purified using a Milli-Q system (Bedford, MA, USA). The chemicals were used for HPLC–Q-TOF-MS and HPLC–Q-TOF-MS/MS analysis. The reference substances, *S*-reticuline, demethylcoclaurine, magnoflorine, corydaline, tetrahydropalmatine, phellodendrine, berberine and jatrorrhizine were purchased from National Institutes for Food and Drug Control (Beijing, China). The other reference alkaloids, including protopine, allocryprotopine, sanguinarine, chelerythrine, dihydrosanguinarine, dihydrochelerythrine, oxysanguinarine and 6-acetonylsanguinarine, were separated and identified in our laboratory. Their purity was found to be more than 98% by HPLC/MS and $^1$H-nuclear magnetic resonance analyses.

### 2.2. HPLC conditions

Chromatography was performed using an Aglient 1290 HPLC system (Aglient Technologies, Palo Alto, CA, USA) consisting of a auto-sampler, a rapid resolution binary pump, vacuums degasser, thermostatted column compartment and a tunable UV detector. The separation was carried out on an Agilent ZORBAX SB-C18 column (150 mm × 4.6 mm, 5 μm, Agilent Technologies). The elution system was 0.2% formic acid aqueous solution (A) and acetonitrile (B). The linear gradient elution was as follow: 0–40 min, 10–34% (B); 40–50 min, 34–57% (B); 50–65 min, 57–95% (B). The sample injection volume was 5 μl. The column temperature was maintained at 30 °C and the flow rate was set at 1.0 ml/min.

### 2.3. Q-TOF-MS conditions

Mass spectrometric experiments were performed using a 6530 Q-TOF-MS accurate-mass spectrometer (Agilent Technologies) in the positive electrospray ionization ($ESI^+$) mode, and the TOF data were collected between *m*/*z* 100 and 1700 in centroid mode. The conditions of the Q-TOF-MS were optimized as follows: gas temperature: 300 °C; drying gas: 8 L/min; nebulizer pressure: 35 psi; sheath gas temperature: 350 °C; sheath gas flow rate: 11 L/min; capillary voltage: 3500 V; fragmentor voltage: 175 V; skimmer voltage: 65 V; OCT1 RF Vpp: 750 V. The TOF mass spectrometer was calibrated routinely in the $ESI^+$ mode before sample analysis using reference masses at *m*/*z* 121.0508, 922.0097 to obtain high-accuracy mass measurements. The targeted MS/MS experiments were operated using variable collision energy (18–45 eV), which was optimized for each individual compound.

### 2.4. Plant material and sample preparation

Twenty plants with *M. microcarpa* fruits were collected from Hunan Agriculture University (Hunan, China) in August, 2013, and