# Indole Alkaloids from *Ochrosia elliptica* Plant Cell Suspension Cultures

Karl-Heinz Pawelka and Joachim Stöckigt

Institut für Pharmazeutische Biologie, Ludwig-Maximilians-Universität, Karlstraße 29,
D-8000 München 2, Bundesrepublik Deutschland

From cell suspension cultures of *Ochrosia elliptica* Labill. eight different monoterpenoid indole alkaloids were isolated and comprised three alkaloid classes *viz. Corynanthé/Heteroyohimbine* (tetrahydroalstonine, cathenamine, pleiocarpamine and two methoxylated heteroyohimbines), *Strychnos* (norfluorocurarine), *Apparicine* (apparicine and epchrosine). All these compounds except the Strychnos alkaloid are biogenetically closely related to the known alkaloids occurring in *Ochrosia* plants, although such alkaloids have not previously been detected in *O. elliptica*. Epchrosine has not before been described as a natural product.

## Introduction

*Ochrosia elliptica* Labill. [syn. *Bleekeria elliptica* (Labill.) Koidz., *Excavatia elliptica* (Labill.) Mgf.] belongs within the monoterpenoid indole alkaloid bearing subfamily Plumerioideae of the tribe Rauwolfieae. To date 16 different species of the genus *Ochrosia* have been described and their alkaloid patterns have been examined in detail [1]. From these species more than 45 different indole alkaloids have been isolated and identified, including pyridocarbazole alkaloids (*e.g.* ellipticine, 9-methoxyellipticine) showing cytostatic activities [2]. Recently the alkaloid production by *O. elliptica* callus and suspension cultures has been investigated and in the resultant reports [3, 4] the formation of five alkaloids of two classes were described. Three of the compounds were the carbazoles ellipticine, 9-methoxyellipticine and elliptinine. During our phytochemical screening of the alkaloid composition in cultured plant cells we also identified the alkaloids of *O. elliptica* suspensions but found a quite different pattern. In this paper we report on the detection of eight *Ochrosia* alkaloids, which can be divided into three alkaloid classes.

## Material and Methods

### Cell cultures

Callus cultures were initiated from leaves of *O. elliptica* and grown on agar plates on DAX-medium [5] which was modified as previously reported [6]. The

cultures were transferred to new media every 4 weeks. DAX-medium was used to obtain cell suspensions, which were transferred into fresh medium at weekly intervals. Larger amounts of tissue (kg range) were grown in 1 l Erlenmeyer flasks with 300 ml nutrient medium. After a growth period of 21 days at 25 °C (600 lux, 100 rpm shaking) an average of 280 g fresh cells (13.2 g dry weight) were harvested from 1 l medium. Cells were frozen with liquid nitrogen and stored at −25 °C.

### Isolation of alkaloids

Alkaloids were isolated by a procedure described earlier [7]. 1.5 kg tissue was extracted at 5 °C with 2 l ethyl acetate with stirring for 2 days. After a second extraction (ethyl acetate/5% methanol) the organic layers were pooled and concentrated to dryness. The residue was processed as described earlier [7] and the resulting crude alkaloid mixture was chromatographically purified.

### Chromatography

Column chromatography was carried out on 200 g Silica gel 60 (particle size < 0.063 mm, Merck), column 3.7 × 70 cm. The crude alkaloid mixture was dissolved in 10 ml chloroform and the solution chromatographically fractionated using the mobile phase (a) chloroform/methanol/ammonia (90:10:0.2) at a flow rate of 0.9 ml/min. 90 fractions (each 13.5 ml) were collected and analysed for alkaloidal products by thin layer chromatography (TLC) and ceric ammonium sulphate (CAS) detection reagent.

TLC was performed on Polygram Sil G/UV$_{254}$ plates (0.25 mm, Macherey-Nagel) using two sys-

tems, the above mentioned solvent system (a), and solvent system (b) petroleum ether (40 °C−60 °C)/acetone/diethylamine (7:2:1).

For high pressure liquid chromatography (HPLC) a Spectra Physics instrument and a Hibar Lichro Cart RP 18 column (0.4 × 25 cm) were employed. The following conditions were used for alkaloid separation: solvent system acetonitrile/10 mM ammonium carbonate, linear gradient 25:75 to 99:1 in 40 min, flow rate 1 ml/min.

*Spectroscopic methods*

UV spectra were obtained using a Perkin Elmer Spectrophotometer 551 S with methanol-Uvasol as solvent. All mass spectra (MS) were recorded on a quadrupole instrument (Finnigan MAT 44 S) at 70 eV in EI-mode. $^1$H-NMR analyses were measured with a Bruker 200 MHz instrument and optical rotation values were determined using a Perkin Elmer Polarimeter 241 and chloroform as solvent.

**Results and Discussion**

The crude alkaloid mixture from 1.5 kg *Ochrosia* cell suspensions yielded four alkaloid containing main fractions (**F I**−**F IV**) by column chromatography and the combining of fractions No 10−13 (**F I**), No 10−17 (**F II**), No 17−24 (**F III**), No 24−35 (**F IV**) in order of increasing polarity.

When fraction **F I** was analysed by TLC, 4 alkaloids were separated (Oe 1−4) in solvent system b. The less polar alkaloid ($R_f = 0.9$) exhibited a yellow-brownish CAS-reaction, an UV spectrum with maxima at 272, 280 and 292 nm and the same mass fragmentation pattern as for the known heteroyohimbine alkaloid tetrahydroalstonine. The identity of Oe 1 as tetrahydroalstonine was further substantiated by co-TLC and HPLC, the latter technique nicely separating seven of the eight possible stereoisomers of tetrahydroalstonine ($R_t = 26.72$ min) when an acetonitrile/ammonium carbonate gradient analysis is performed. Two further alkaloids (Oe 2, Oe 3) were observed in fraction **F I**, which exhibited a CAS-reaction very similar to that of Oe 1 but showed a slightly higher polarity (Oe 2 $R_f = 0.8$, Oe 3 $R_f = 0.7$). The UV data of both compounds were identical and strongly related to those of tetrahydroalstonine. These data suggested a heteroyohimbine structure for Oe 2 and Oe 3, which

was clearly confirmed by analysis of the MS fragmentation patterns, which were almost identical for both alkaloids (M$^+$ 382 *m/z*). The total fragmentation pattern was that of ajmalicine isomers except that all ions were shifted by 30 m/z to higher masses. Since this was particularly striking for fragment ions arising from the β-carboline part of the molecule (156 → 186 *m/z*, 169 → 199 *m/z*, 184 → 214 *m/z*) it is obvious that both alkaloids are substituted at the A ring by a methoxy group. Unfortunately the isolation of only trace amounts of Oe 2 and Oe 3 prohibited any NMR measurements. Therefore the point of OCH$_3$-attachment to position 9, 10, 11 or 12 remains uncertain. However, the isolation of exclusively 10 and/or 11 substituted Corynanthé-alkaloids from *Ochrosia* plants strongly favours those positions to be methoxylated in Oe 2 and Oe 3. Moreover, the fact that $2^3$ stereoisomers theoretically exist for example for tetrahydroalstonine, complicates the final structure elucidation. Assuming for biogenetic reasons a fixed stereochemistry at C-15, a monosubstitution of the aromatic system would finally lead to 32 possible different structures. A more detailed analysis of the MS spectra of both alkaloids however reduces this number to 16 possibilities, because the absence of the ion at 255 *m/z* in conjunction with a significant signal at 253 *m/z* strongly points to a (S)-configuration at C-20 (D/E-*cis*) as found in tetrahydroalstonine [8]. In this context it is noteworthy, that structurally related alkaloids from *Ochrosia* species possess in most cases this D/E-*cis* system. Significantly only two species, *O. nakaiana* and *O. poweri*, have been found to synthesize the D/E-*trans* alkaloids serpentine and 10-methoxy-serpentine resp. [9−11]. Regarding the compounds discussed here their structures seem to agree with the known *Ochrosia* alkaloids, although most of their relatives are bis-substituted in the intact plant. Such dimethoxylated products were not detectable in our cultured cells whereas 10,11-dimethoxytetrahydroalstonine (iso-reserpiline) and its 3-*iso* derivative (reserpiline) have been detected in several cell strains of *O. elliptica* cell suspensions [4].
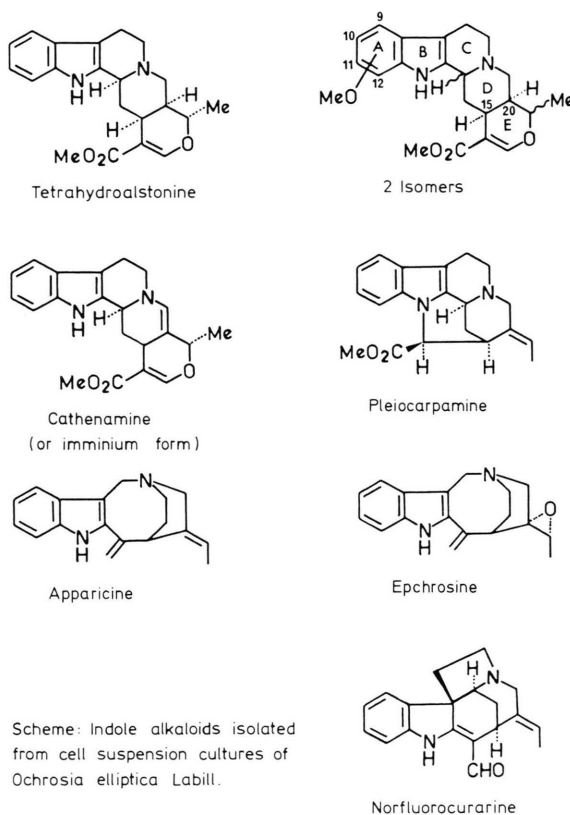
Purification of alkaloidal products of fraction **F II** in solvent system (b) indicated two major alkaloids Oe 4 ($R_f = 0.5$) and Oe 5 ($R_f = 0.7$). Oe 4 also exhibited a yellow CAS-reaction, a typical indole chromophore and MS data which were in agreement with those published for cathenamine [12, 13]. In addition, co-TLC and the reduction of Oe 4 with

borohydride leading to tetrahydroalstonine and 19-epi-ajmalicine, supported the structure determination of this compound. This alkaloid is now reported for the first time as occurring in *Ochrosia*. One has however to realize that apart from the high instability of this alkaloid, cathenamine could also be formed from dehydrogeissoschizine during the work-up procedure employed and an artificial formation of the enamine (or its immonium form) therefore cannot be excluded. Nevertheless, the presence of this alkaloid, which is a biosynthetic intermediate in the heteroyohimbine pathway, fits structurally and biogenetically well with the above mentioned alkaloids. The second compound of **F II,** Oe 5, gave a violet reaction with CAS, and its MS fragmentation did not coincide with any of our MS-Library collection of data on 1800 indole alkaloids and was therefore expected to be a novel alkaloid. The UV spectrum of this compound was superimposible with that of apparicine and the overall fragmentation pattern pointed to an oxygenated derivative of it ($M^+$ 280 $m/z$). NMR analyses clearly revealed the new product to be 19($R$), 20($R$)-epoxyapparicine, now named epchrosine. Details of its structure determination will be published elsewhere [14].

TLC of the alkaloid fraction **F III** (solvent system b) lead to the separation of Oe 6 ($R_f = 0.3$) and Oe 7 ($R_f = 0.2$). The less polar alkaloid Oe 6 showed a purple reaction with CAS and a characteristic indole-UV with maximum absorption at 230 and 285 nm. A MS-Library search suggested Oe 6 to be pleiocarpamine. Its overall mass fragmentation and the prominent signals at 322 $m/z$ ($M^+$), 263 $m/z$ ($M^+-CO_2CH_3$) and 180 $m/z$ (quinolium ion) were in agreement with published data for pleiocarpamine [15]. This alkaloid has already been detected in cell suspension cultures of *Catharanthus roseus* (L.) G. Don [16] and we also isolated it from cultivated *Stemmadenia tomentosa* Greenman var. palmeri (Rose) Woods cells. Within the genus *Ochrosia* the presence of pleiocarpamine has not been demonstrated before. However, its close biogenetic relation to open E-ring *Corynanthé* alkaloids, which are widely distributed in *Ochrosia* species, sufficiently explains its formation in this culture system. The alkaloid Oe 7 revealed a blue colour with CAS. The UV data (242, 290 sh, 299, 360 nm), a reversible bathochromic shift ($\lambda_{max}$ 360 → 367 nm) in the presence of base, the MS data and the highly negative optical rotation ($-1210°$) were in full accord with the

structure of norfluorocurarine. Although this alkaloid has not been isolated from *Ochrosia* plants and obviously is the first example of a *Strychnos* alkaloid of this genus, it is well known as a constituent of other *Apocynaceae* cell cultures, such as *S. tomentosa* Greenman var. palmeri (Rose) Woods and *Rhazya stricta* Decaisne [18]. From the most polar fraction (**F IV**) an indole base (Oe 8) was isolated exhibiting the same chromatographic and spectroscopic features as described for apparicine [19]. Determination of its optical rotation indicated that the alkaloid belongs to the ($-$)-enantiomeric series of apparicines. The formation of this alkaloid in the *Ochrosia* culture as well as that of its epoxy-derivative *epchrosine,* is not surprising because the apparicine-type has already been demonstrated as 10-hydroxy- and 10-methoxyapparicine in *O. oppositifolia* (Lam.) K. Schum. leaves [20].

Summarising, we have isolated eight different indole alkaloids of the *Heteroyohimbine/Corynanthé, Strychnos* and *Apparicine* types from cell suspension cultures of *O. elliptica*. At least six of these alkaloids



Tetrahydroalstonine



2 Isomers



Cathenamine
(or imminium form)



Pleiocarpamine



Apparicine



Epchrosine

Scheme: Indole alkaloids isolated from cell suspension cultures of Ochrosia elliptica Labill.



Norfluorocurarine

have not been previously isolated from this particular species. Apart from the *Strychnos* alkaloid norfluorocurarine, all the compounds can be related biogenetically to the known structures of indole bases of plants of the genus *Ochrosia*. In contrast to the published work on *O. elliptica* cell suspensions, in which the formation of known *Ochrosia* alkaloids is reported [4], the alkaloid pattern discussed here is completely different (scheme). It is therefore obvious that no prediction of the formation of secondary compounds in such cell systems can be made.

Even if the knowledge of indole alkaloid composition in cell cultures is still very limited, it would appear from the data of our work that most of the alkaloids originate from earlier sequences of the main biosynthetic pathways than in the intact plant. In this respect cell cultures studies are an attractive complement to the studies of the whole plant. Moreover, during our phytochemical characterization of *Apocynaceous* cell suspensions we have found no culture so far that was unable to synthesize monoterpenoid indole alkaloids.

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