Biotransformation of Cadaverine and Potential Biogenetic Intermediates of Lupanine Biosynthesis by Plant Cell Suspension Cultures

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Abstract

Alkaloid extracts obtained from cell suspension cultures of Lupinus polyphyllus, Conium maculatum and Symphytum officinale were separated by glass capillary gas chromatography and identified by GC/MS. Cadaverine is incorporated into the quinolizidine alkaloids by L. polyphyllus cell cultures only; identified transformation products are sparteine, 17-oxosparteine, lupanine and 4 other alkaloids which probably are α-isosparteine, α-isolupanine, 12,13-dehydrosparteine and 12,13-dehydro-17-oxosparteine. Sparteine displays a biotransformation pattern in all cell cultures which is similar to the pattern after cadaverine feeding. 17-Oxosparteine is converted into lupanine indicating its intermediacy in lupanine biosynthesis. A possible pathway of lupanine biosynthesis and alkaloid transformation is discussed.

Introduction

Cell suspension cultures of Lupinus polyphyllus accumulate quinolizidine alkaloids to a limited extent only [1]. This does not mean, however, the absence of the respective biosynthetic capacity. We found that precursors were readily incorporated into the alkaloids and subsequently, we could isolate an enzyme system from these cultures which is capable of the synthesis of the tetracyclic alkaloids [2, 3]. Therefore these cultures provide a means to study the biochemistry of lupin alkaloid biosynthesis. The low alkaloid production offers the advantage that background alkaloids do not interfere and this allows the omission of labelled precursors or intermediates, resulting in an easier experimental system.

Lupin alkaloids derive from lysine via its intermediate cadaverine. Three cadaverine units are combined into the tetra-
cyclic quinolizidine skeleton; intermediates of this pathway have not been detected in the in vivo experiments so far [4, 5]. Our enzymatic experiments indicate that 17-oxosparteine, an alkaloid almost unknown for Lupinus species, is the key intermediate in lupanine biosynthesis [2, 3, 6].

In this study we report on qualitative aspects of the biotransformation of cadaverine, sparteine and 17-oxosparteine by cell suspension cultures of Lupinus polyphyllus, to gain information on their potential enzymatic activities and alkaloid metabolism. Cell cultures of Conium maculatum, and Symphytum officinale which do not produce quinolizidine alkaloids were included in these studies to distinguish between specific and non-specific metabolic steps.

Material and Methods

Lupinus polyphyllus, Conium maculatum, and Symphytum officinale cell suspension cultures were kept according to [1].

Alkaloid extraction and isolation was performed using standard methods described in [1].

Alkaloid extracts were separated on CpSil 5 glass capillary columns (0.25 mm i.d. × 25 m) employing a Perkin Elmer F 22 gas chromatograph. Alkaloids were identified by GC/mass spectrometry (AEI MS 30 DATA system DS 50) [1].

About 15 g cells (fresh weight) derived from stationary cultures were inoculated into 50 ml autoclaved medium in 300 ml Erlenmeyer flasks. Filter sterilized precursors (Millipore filter 0.45 μm) were added directly after subculturing. Suspension cultures were kept on rotary shakers (120 r.p.m.) at 25° C, 70 % rH and continuous illumination. After 20 days of culture the cells were filtered through cheese cloth and then subjected to alkaloid extraction.

Cadaverine was purchased from Fluka, sparteine from Roth; 17-oxosparteine was prepared from sparteine according to [7].

Results and Discussion

Identification of the Metabolites

Alkaloid extracts of cell suspension cultures previously fed on cadaverine, sparteine or 17-oxosparteine for 20 days were separated by glass capillary gas chromatography (Fig. 1 a–d). More than 10 nitrogen containing compounds could be detected in the range where quinolizidine alkaloids are found in this chroma-

<table>
<thead>
<tr>
<th>GC-Peak</th>
<th>M⁺</th>
<th>Most abundant ions</th>
<th>Reference</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>234</td>
<td>137 98 193 234 110</td>
<td>[18], [19]</td>
<td>α-Isosparteine</td>
</tr>
<tr>
<td>2</td>
<td>234</td>
<td>137 98 193 234 110</td>
<td>[1], [18], [19]</td>
<td>Sparteine</td>
</tr>
<tr>
<td>3</td>
<td>232</td>
<td>134 97 232 149 175</td>
<td>–</td>
<td>12,13-Dehydrosparteine</td>
</tr>
<tr>
<td>5</td>
<td>246</td>
<td>97 98 246 136 148</td>
<td>–</td>
<td>12,13-Dehydro-17-oxosparteine</td>
</tr>
<tr>
<td>6</td>
<td>248</td>
<td>136 149 248 97 110</td>
<td>[19]</td>
<td>α-Isolanpine</td>
</tr>
<tr>
<td>7</td>
<td>248</td>
<td>136 149 248 97 110</td>
<td>[1], [9], [19]</td>
<td>Lupanine</td>
</tr>
</tbody>
</table>

* Arranged in order of decreasing intensity, first one being the base peak
tographic system [1]. Seven of these compounds could be identified by GC/MS. The characteristic fragment ions of these compounds and the literature source in which reference spectra have been recorded are listed in Table 1.

GC-peaks 2 and 7 were found to be identical with sparteine and lupanine [1]. Both alkaloids are accompanied by compounds (GC-peaks 1 and 6) with identical fragmentation patterns. Mass chromatograms of the respective molecular ions (m/z 234, Fig. 2 and m/z 248, Fig. 3) indicate that these compounds are isomers of sparteine and lupanine; the isomers are supposed to be α-isosparteine and α-isolupanine, compounds known to occur in Leguminosae [8].

GC-peak 4 results in a mass spectrum (Fig. 4a) identical with that of 17-oxosparteine [3]. Note the elimination of CO (M⁺-28) which is characteristic for an oxosparteine carrying the oxygen in 12- or 17-position [9].

Two unknown unsaturated alkaloids with molecular ions of m/z 232 and m/z 246 could be identified (Fig. 2 and 3; Fig. 4b, c). Compound 3 has similar retention times as sparteine and could easily be identified as a dehydrosparteine. The mass spectrum of 12-hydroxyisparteine (epiretamine) [10] offers similar charac-

Figure 1. Separation of alkaloid extracts from cell suspension cultures by glass capillary gas chromatography. For identity of the numbered peaks see Table 1. GC-conditions: 0.25 mm × 25 m CpSyl 5 columns 190–270°C, 4°C C/min, 16 min isothermal; PND (phosphorous nitrogen detector).

a. control: *L. polyphyllus* culture without precursor
b. *L. polyphyllus* culture fed on 10 mM cadaverine
c. *L. polyphyllus* culture fed on 5 mM sparteine
d. *C. maculatum* culture fed on 3 mM 17-oxosparteine.
ristic fragment ions as compound 3 such as m/z 232, 175, 134, 122. Since the hydroxyl group is lost by water elimination, the subsequent fragmentation is based on 12,13-dehydrosparteine. The presence of hydroxysparteine in the alkaloid extracts can be excluded, since M⁺250 does not turn up in the GC/MS runs; furthermore it would display longer retention times due to the polar hy-
Figure 4. Mass spectra of 17-oxosparteine (a), 12,13-dehydro-17-oxosparteine (b) and 12,13-dehydrosparteine obtained by GC/MS from alkaloid extracts of cell cultures after precursor feeding.

droxyl group. Due to these similarities we would tentatively describe compound 3 as 12,13-dehydrosparteine.

GC-peak 5 must represent either a dehydro-oxosparteine or a dehydro-lupanine. Fragments m/z 110 and 84 are characteristic for quinolizidine alkaloids with unsubstituted A and/or D rings; in the mass spectrum of GC-peak 5 both fragments are present, but m/z 108 and m/z 82 additionally. This implicates that the unsaturation should be localized in either ring A or D. Fragment m/z 136 of 17-oxosparteine can be related to the unsubstituted ring A and B; assuming the same fragmentation pattern, the double bond should be localized in ring D therefore.
The mass spectrum of compound 5 shows more similarities with that of 17-oxosparteine (Fig. 4a) than those of lupanine (Table I) or the only 5,6-dehydrolupanine so far described [11]. Therefore we would tentatively call compound 5 a dehydro-17-oxosparteine. In dehydrosparteine the unsaturation was supposed to be located in the 12,13-position; assuming the same dehydrogenation process, we would call compound 5 a 12,13-dehydro-17-oxosparteine. These assignments of unsaturation need further clarification.

Metabolism of Cadaverine and Potential Intermediates in L. polyphyllus Cell Suspension Cultures

Cadaverine and potential intermediates of quinolizidine biosynthesis such as sparteine and 17-oxosparteine were added in concentrations of 3 to 10 mM. In relation to untreated controls, growth of cadaverine fed cultures was reduced by only 23%, that of sparteine cultures by 7%. It is noteworthy that the cells could grow with these high concentrations of exogenous alkaloids. One explanation may be that only small amounts of the precursors had been taken up. After three weeks of growth only 0.6% of cadaverine and 0.1–0.25% of exogenously applied alkaloids were taken up as can be judged from the intracellular alkaloid content. No corrections were made for cadaverine or alkaloid metabolism into non-alkaloidal compounds. However, the great majority of the added precursors was still left in the medium.

Cultures fed upon cadaverine or alkaloids displayed distinct alkaloid patterns whereas untreated controls were free of detectable alkaloids (Fig. 1; Table II).

Table II

Metabolism of cadaverine, sparteine and 17-oxosparteine by cell suspension cultures of Lupinus polyphyllus, Conium maculatum, and Symphytum officinale.

Total alkaloid content of the cells (minus precursor) was set 100%. To calculate the transformation rate of the precursor, the concentration of the transformation products was compared with that of the intracellular precursor. Assignment of the GC-peaks (number 1–7) as in Table I. tr = traces

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Substrate</th>
<th>Transformation rate %</th>
<th>Alkaloids produced (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1   2   3</td>
<td>4   5   6    7</td>
</tr>
<tr>
<td><em>L. polyphyllus</em></td>
<td>Cadaverine</td>
<td>4   51  17*</td>
<td>0.8  0.1</td>
</tr>
<tr>
<td><em>C. maculatum</em></td>
<td>Cadaverine</td>
<td>–   –   –</td>
<td>–   –    –</td>
</tr>
<tr>
<td><em>S. officinale</em></td>
<td>Cadaverine</td>
<td>–   –   –</td>
<td>–   –    –</td>
</tr>
<tr>
<td><em>L. polyphyllus</em></td>
<td>Sparteine</td>
<td>4.9 tr</td>
<td>58   26   3</td>
</tr>
<tr>
<td><em>C. maculatum</em></td>
<td>Sparteine</td>
<td>0.8 –</td>
<td>33   50   10</td>
</tr>
<tr>
<td><em>S. officinale</em></td>
<td>Sparteine</td>
<td>0.8 tr</td>
<td>50   36   –</td>
</tr>
<tr>
<td><em>L. polyphyllus</em></td>
<td>17-Oxosparteine</td>
<td>3.0 –</td>
<td>–     –   –</td>
</tr>
<tr>
<td><em>C. maculatum</em></td>
<td>17-Oxosparteine</td>
<td>2.0 –</td>
<td>–     –   –</td>
</tr>
</tbody>
</table>

* GC-peak 3 (Fig. 1b) represents 2 compounds; one is a 12,13-dehydrosparteine, the other an unidentified compound with a molecular ion at m/z 208.
** Mainly due to GC-peak 8 (Fig. 1b), which corresponds to an unknown product with M* at m/z 153.
Upon feeding of cadaverine at least 6 alkaloids can be identified in *L. polyphyllus* cell cultures. These include lupanine, the main alkaloid of differentiated plants and cell suspension cultures [1]. Sparteine which can be detected in cell suspension cultures and plants in traces only, figures as the main alkaloid in these experiments. The other alkaloids have not been detected in *Lupinus polyphyllus* plants or cell suspension cultures thus far.

Sparteine transformation leads to lupanine and α-isolupanine, but in a higher proportion to 12,13-dehydrosparteine and 17-oxosparteine. In comparison to sparteine only a smaller amount of 17-oxosparteine was taken up by the cells. This might explain the lack of other transformation products than lupanine.

*Comparison with Conium maculatum and Symphytum officinale Cell Cultures*

Both species don’t contain quinolizidine alkaloids and are not able to produce them upon feeding of cadaverine (Table II).

*Conium maculatum* cultures possess, however, the ability to transform 17-oxosparteine and sparteine into a similar alkaloid pattern as do lupin cultures. The quantitative patterns are significantly different (Table II); the amount of 17-oxosparteine released is higher and that of the lupanine produced lower in *Conium* cultures as compared to lupin cultures.

*Symphytum officinale* cultures convert sparteine to dehydrosparteine and 17-oxosparteine predominantly, and only in traces into lupanine.

The transformation of the sparteine skeleton by non-quinolizidinal plants indicates a wider distribution or unspecificity of the respective enzyme systems. The efficiency of lupanine production seems to be enhanced in the lupin cultures, however, possibly due to a selection of the respective enzymes.

*Metabolic Pathway of Alkaloid Transformation*

In scheme 1 the possible sequence of quinolizidine transformation in cell cultures is sketched. It has to be recalled that these feeding experiments may deviate significantly from in vivo conditions. If exogenous compounds are not or not completely transported into the intracellular compartment, where the endogenous synthesis takes place, unrelated enzymes may catalyze unspecific reactions. This situation may readily occur if we exceed the normal in vivo concentration of a precursor in feeding experiments.

The occurrence of a dehydrosparteine and a dehydrooxosparteine might imply their intermediary role in 17-oxosparteine or lupanine biosynthesis. These transformations however occurred in lupin alkaloid producing and non producing cell cultures; furthermore Corynebacteria and horseraddish peroxidase were also reported to convert sparteine into dehydrosparteine [12, 13]. From these data it can be assumed that the dehydroderivatives of sparteine and 17-oxosparteine are unspecific conversion products and not of biogenetical importance.

The occurrence of sparteine and lupanine isomers raises the question whether these are artifacts or products of specific biosynthetic enzymes.

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1 The concentrations of endogenously produced alkaloids in cell suspension cultures are about 1–5 mg/100 g dry weight; thus not detectable in the GC assays employed here.
Experiments with \(^4\)CO\(_2\) could exclude sparteine as an intermediate of lupanine biosynthesis in *L. polyphyllus* and other lupin species [17]. This is in agreement with our studies on isolated chloroplasts which convert cadaverine directly into lupanine [6]. Additionally cell suspension cultures of *Baptisia australis* and *Sarothamnus scoparius* produce lupanine as the main alkaloid, although \(\alpha\)-pyridone alkaloids or sparteine figure as major alkaloids in these species [15, 16]. Therefore lupanine synthesis seems to be the main pathway of quinolizidine alkaloids. This is contradictory to the concept of Nowacki and Waller [4, 5] who proposed sparteine as a necessary intermediate of lupanine biosynthesis. They reached this conclusion because application of exogenous sparteine to some legumes led to its incorporation into lupanine [14]. From our biotransformation experiments with both quinolizidinal and nonquinolizidinal plants we can conclude that the sparteine-lupanine transformation is quite non specific and may not necessarily reflect the in vivo biosynthetic pathway.

From experiments with isolated lupin chloroplasts which convert cadaverine and 17-oxosparteine into lupanine it can be concluded that 17-oxosparteine is the key intermediate in lupanine biosynthesis [6]. This pathway seems to be highly channelled in vivo since membrane dissociation leads to the occurrence of 17-oxosparteine and sparteine instead of lupanine. In the cell suspension cultures employed, chloroplast formation is incomplete. Feeding of cadaverine to these cultures in unphysiological concentrations may lead to the situation that their capacity to transform 17-oxosparteine into lupanine is insufficient. This may result in an overflow of the 17-oxosparteine pool, leading to the release of sparteine observed.