Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 1026–1037
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Tissue distribution, core biosynthesis and diversification of pyrrolizidine alkaloids of the lycopsamine type in three Boraginaceae species

Cordula Frölich [a], Dietrich Ober [b], Thomas Hartmann [a,*]

[a] *Institut für Pharmazeutische Biologie, Technische Universität Braunschweig, Mendelssohnstrasse 1, D-38106 Braunschweig, Germany*
[b] *Botanisches Institut, Universität Kiel, Olshausenstrasse 40, D-24098 Kiel, Germany*

Received 12 November 2006; received in revised form 4 January 2007
Available online 22 February 2007

Abstract

Three species of the Boraginaceae were studied: greenhouse-grown plants of *Heliotropium indicum* and *Agrobacterium rhizogenes* transformed roots cultures (hairy roots) of *Cynoglossum officinale* and *Symphytum officinale*. The species-specific pyrrolizidine alkaloid (PA) profiles of the three systems were established by GC–MS. All PAs are genuinely present as *N*-oxides. In *H. indicum* the tissue-specific PA distribution revealed the presence of PAs in all tissues with the highest levels in the inflorescences which in a flowering plant may account for more than 70% of total plant alkaloid. The sites of PA biosynthesis vary among species. In *H. indicum* PAs are synthesized in the shoot but not roots whereas they are only made in shoots for *C. officinale* and in roots of *S. officinale*. Classical tracer studies with radioactively labelled precursor amines (e.g., putrescine, spermidine and homospermidine) and various necine bases (trachelanthamidine, supinidine, retronecine, heliotridine) and potential ester alkaloid intermediates (e.g., trachelanthamine, supinine) were performed to evaluate the biosynthetic sequences. It was relevant to perform these comparative studies since the key enzyme of the core pathway, homospermidine synthase, evolved independently in the Boraginaceae and, for instance, in the Asteraceae [Reimann, A., Nurhayati, N., Backenkohler, A., Ober, D., 2004. Repeated evolution of the pyrrolizidine alkaloid-mediated defense system in separate angiosperm lineages. Plant Cell 16, 2772–2784.]. These studies showed that the core pathway for the formation of trachelanthamidine from putrescine and spermidine via homospermidine is common to the pathway in *Senecio* ssp. (Asteraceae). In both pathways homospermidine is further processed by a β-hydroxyethylhydrazine sensitive diamine oxidase. Further steps of PA biosynthesis starting with trachelanthamidine as common precursor occur in two successive stages. Firstly, the necine bases are structurally modified and either before or after this modification are converted into their O^9-esters by esterification with one of the stereoisomers of 2,3-dihydroxy-2-isopropylbutyric acid, the unique necic acid of PAs of the lycopsamine type. Secondly, the necine O^9-esters may be further diversified by O^7- and/or $O^{3'}$-acylation.

Keywords: *Heliotropium indicum*, Boraginaceae; *Cynoglossum officinale*; *Symphytum officinale*; Hairy root cultures; Pyrrolizidine alkaloid biosynthesis; Alkaloid tissue distribution; Structural diversification

1. Introduction

Within the diverse class of pyrrolizidine alkaloids (PAs) the two major types are represented by macrocyclic diesters either with a 12-membered ring (senecionine type) or an 11-membered ring (monocrotaline type) and monoesters or open-chain diesters containing the unique branched aliphatic C_7 necic acid, 2,3-dihydroxy-2-isopropylbutyric acid. Macrocyclic PAs are found in the Asteraceae (tribe Senecioneae) and the Fabaceae (e.g., genus *Crotalaria*); PAs of the lycopsamine type are known from the families Asteraceae (tribe Eupatorieae) and many genera of the

* Corresponding author. Tel.: +49 531 391 5694; fax: +49 531 391 8104.
E-mail address: t.hartmann@tu-bs.de (T. Hartmann).

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.01.002

Boraginaceae and Apocynaceae (Hartmann and Witte, 1995; Hartmann and Ober, 2000).

Recently it was shown that the PA-specific biosynthetic pathways evolved polyphyletically within the angiosperms. The first pathway-specific enzyme of PA biosynthesis, homospermidine synthase (HSS, EC 2.5.1.45), was recruited at least four times independently: once within the Boraginaceae and within the Orchidaceae, respectively, and even twice within the Asteraceae, i.e. independently within the Senecioneae and the Eupatorieae (Reimann et al., 2004). Homospermidine synthase which till now has been only detected in PA-producing plant species, catalyzes the formation of the polyamine homospermidine, the unique precursor of the necine base moiety of all PAs representing 1-hydroxmethylpyrrolizidine derivatives. The enzyme has been isolated, purified, cloned and characterized from root cultures of *Senecio vulgaris* (Böttcher et al., 1993, 1994; Ober et al., 2000) and *S. vernalis* (Ober and Hartmann, 1999; Ober et al., 2003b). In *Senecio* senecionine *N*-oxide, the backbone structure of macrocyclic PAs of the senecionine type, is exclusively synthesized in the roots and translocated to shoots through the phloem (Hartmann et al., 1989). In the shoots senecionine *N*-oxide is biochemically modified to yield the species-specific PA patterns (Hartmann and Dierich, 1998; Pelser et al., 2005). In *Senecio* PAs preferentially accumulate in the inflorescences (Hartmann and Zimmer, 1986) and are stored within cellular vacuoles (Ehmke et al., 1988). The site of PA biosynthesis in roots is restricted to small groups of endodermal and adjacent cortex cells located opposite the phloem as demonstrated by immunolocalization of homospermidine synthase (Moll et al., 2002).

Although the biochemistry and physiology of PAs in *Senecio* is quite well understood only little information is available about PAs in other plant taxa. The polyphyletic origin of homospermidine synthase within the angiosperms provokes intriguing questions regarding evolutionary and mechanistic aspects about the origin of the biosynthetic pathways in PA containing species of distant angiosperm families. In this study we compare the core pathway of PA biosynthesis in three species of the Boraginaceae and the species-specific strategies of structural diversification. By means of classical tracer techniques the alkaloid distribution between major plant organs, identification of the sites of PA biosynthesis and the sequence of structure diversification in *Heliotropium indicum* (major object) are studied. For comparison PA producing hairy root-cultures of *Cynoglossum officinale* and *Symphytum officinale* are included.

2. Results and discussion

2.1. Tissue-specific alkaloid levels in H. indicum

H. indicum is a phytochemically well studied species, containing PA profiles that greatly vary between populations (Hartmann and Witte, 1995). The alkaloid profile of the *H. indicum* population used in our experiments includes six PAs, i.e. indicine, and its 3′-acetylesters as major alkaloids. These are accompanied by minor quantities of lycopsamine and the three indicine aralkyl esters, 3′-benzoyl-, 3′-cinnamoyl- and 3′-dihydrocinnamoyl-indicine that are previously not known from *H. indicum*. PAs are detectable in all plant organs. Indicine is the dominating PA in the inflorescences (>75% of total PAs) whereas the vegetative plant tissues contain about equal proportions of indicine and its 3′-acetylester. The minor alkaloids generally account for less than 5–10% of total PAs. The inflorescences with all developmental flower stages possess 71% of total plant PAs (Table 1), whereas the vegetative shoot including leaves and all stem parts display only 10%; roots account for the remaining 19%. Consequently inflorescences, especially the flowers and flower buds hold the highest PA tissue concentrations, reaching up to 1.5% of dry weight. This PA tissue distribution roughly corroborates the results of earlier studies (Catalfamo et al., 1982). Preferential accumulation of PAs in the reproductive tissues is also known from species of the Asteraceae, e.g. *Senecio vulgaris* and *S. vernalis* (Hartmann and Zimmer, 1986) and Orchidaceae, e.g. *Phalaenopsis* hybrids (Frölich et al., 2006).

2.2. Biogenetic tracer feeding experiments with H. indicum

Since the PA distribution all over the plant does not indicate a preferential site of synthesis, detached plant organs were incubated with ^{14}C-labelled putrescine as described earlier for *Senecio vulgaris* (Hartmann et al., 1989). Abscised inflorescences, leaves and stems were allowed to take up the tracer via the transpiration stream.

Table 1
Contents and concentrations of total PAs in the different organs of a single representative four-months-old specimen of *Heliotropium indicum*

Plant organ analyzed	Total PAs		
	Content (mg)	Concentration (mg/g dry wt)	Rel. abundance (%)
Inflorescence			
Flower buds	7.1	11.3	71
Open flowers	47.1	15.2	
Withered flowers	13.0	5.2	
Fruits	7.3	3.5	
Stems			
Upper part	2.5	1.4	3
Lower part	0.6	0.1	
Leaves			
Young	1.4	2.1	7
Medium	1.6	1.0	
Old	4.3	0.4	
Roots			
Young	8.8	4.9	19
Old	11.7	4.7	
Whole plant	105.4	3.2	100

Roots absorbed the tracer from the incubation medium. Preliminary experiment revealed that all shoot organs but not roots were able to incorporate ^{14}C-labelled putrescine into PAs. Indicine *N*-oxide accompanied by a small proportion of its 3′-acetyl derivative was the only detectable alkaloid. Since alkaloid biosynthesis was most efficient in young leaves all subsequent experiments were performed with young leaves. The result of a time course tracer experiment is summarized in Table 2. Within two days the applied tracer has been metabolized and its incorporation into indicine *N*-oxide completed. During the following days total radioactivity in the alkaloid fraction remained stable indicating the absence of PA degradation or turnover. The only metabolic activity is a slow but steady conversion of indicine into its 3′-acetyl ester, first detectable at day 2 and reaching a portion of 12% at day 4. 'Metabolite X' represents a labelled transiently occurring intermediate of still unknown structure (see discussion in Section 2.5).

In addition to putrescine biosynthetically related amines such as agmatine (intermediate between arginine and putrescine), spermidine (precursor of homospermidine) and homospermidine itself were found to be efficiently incorporated into indicine *N*-oxide if applied as radioactively labelled tracer. The role of homospermidine as the first pathway-specific precursor in the biosynthesis of PAs in Asteraceae has previously been demonstrated in experiments with the diamine oxidase inhibitor β-hydroxyethylhydrazine (HEH) (Böttcher et al., 1993). Addition of HEH completely blocks PA biosynthesis and causes accumulation of homospermidine, indicating participation of a HEH-sensitive diamine oxidase. Therefore, similar HEH inhibitor experiments were performed with *H. indicum*. A comparison of the genuine levels of the three amines involved in PA biosynthesis in trials with and without addition of HEH revealed informative results (Table 3). Whereas the tissue-specific levels of putrescine and spermidine increased only slightly in the presence of HEH the homospermidine levels were affected drastically in all organs that actively synthesize PAs. Roots that have the highest level of genuine homospermidine (i.e., 34 nmol/g in comparison to 11 nmol/g in young leaves) are only slightly affected by HEH. In an additional experiment the effect of HEH on the incorporation of labelled putrescine into polyamines and indicine *N*-oxide was studied (Table 4). In all tissues that are capable of PA synthesis the formation of indicine *N*-oxide was totally inhibited by HEH, concomitantly substantial amounts of labelled amines, not detectable in the absence of HEH, were recorded. In contrast roots, that

Table 2
Time course of the incorporation of [^{14}C]putrescine (0.5 nmol; 0.5 μCi) into indicine *N*-oxide (indicine *N*ox) of young leaves of *H. indicum*

Days	Recovery of radioactivity applied (%)[a]	% of recovered radioactivity[b]			Total incorporation into PA (%)[a]
		Putrescine	'Metabolite X'	Indicine *N*ox	
0.25	25.7	–	32.9	14.7	2.7
0.5	31.6	14.3	22.4	13.8	3.4
1	29.5	5.3	15.8	32.4	8.6
2	25.6	tr	tr	73.6[c]	18.2
3	22.7	nd	nd	78.2[c]	17.0
4	23.3	nd	nd	38.3[c]	18.7

The abscised leaves received the tracer in 50 μl via the cut end.
After tracer absorption (ca. 24 h) the incubation was continued in tap water until harvest.
–, not analyzed; tr, traces; nd, not detectable.
[a] Total radioactivity applied = 100%.
[b] Total radioactivity in MeOH extract = 100%.
[c] Containing an increasing proportion of 3′-acetylindicine.

Table 3
Effect of of the diamine oxidase inhibitor, β-hydroxyethylhydrazine (HEH), on the genuine concentrations of putrescine (put), spermidine (spd) and homospermidine (hspd) amines in different organs of *H. indicum*

Plant organ	Amine concentrations (nmol/g fr wt)					
	−HEH			+HEH		
	put	spd	hspd	put	spd	hspd
Leaf (young)	95	117	11	184	172	252
Leaf (old)	22	80	tr	32	120	14
Stem (upper part)	45	50	2	95	63	132
Inflorescence	455	245	4	917	342	101
Young roots	46	46	34	54	53	46

Analysis was performed two days after application of 2 mM HEH.
tr, traces.

Table 4
Incorporation of [^{14}C]putrescine into amines and indicine *N*-oxide in the absence (−HEH) and presence (+HEH) of the diamine oxidase inhibitor, β-hydroxyethylhydrazine (HEH)

^{14}C-labeled products	% of total recovered radioactivity[a]							
	Leaf		Stem		Inflorescence		Root	
	−HEH	+HEH	−HEH	+HEH	−HEH	+HEH	− HEH	+ HEH
Putrescine	nd	5.4	nd	nd	nd	34.5	29.1	36.3
Spermidine	nd	10.1	nd	8.8	nd	11.3	14.4	20.5
Homospermidine	nd	69.2	nd	83.2	nd	31.4	12.1	22.6
Indicine *N*-oxide	76.7	nd	77.3	nd	56.4	nd	nd	nd

The various organs of *H. indicum* were incubated with 50 nmol (1 μCi) [^{14}C]putrescine for 5 days; HEH (2 mM) was added 2 h prior to addition of the tracer and maintained during the runtime of the experiment.
nd, not detectable.
[a] Radioactivity in MeOH extracts = 100%.

do not synthesize PAs, show already in absence of HEH substantial levels of labelled amines which are only slightly increased in the presence of HEH. The inhibition by HEH is reversible and does not affect the viability of the treated tissues (Fig. 1). After replacement of HEH by water young leaves synthesize labelled indicine *N*-oxide at the expense of labelled homospermidine that accumulated in the presence of HEH. Within six days the level of labelled homospermidine decreased from ca. 60% to less than 20% while indicine increased simultaneously from zero to about 50% of total radioactivity. The sum of homospermidine and indicine *N*-oxide remains stable indicating no competing degradation of homospermidine.

The results of the inhibitor experiments indicate that PA biosynthesis in *H. indicum* behaves like that in *S. vulgaris* (Böttcher et al., 1993). In both species HEH completely arrests PA biosynthesis and causes the accumulation of homospermidine, indicating that the biosynthetic pathway must be interrupted directly after homospermidine. In both systems the only fate of homospermidine appears to be its function as unique alkaloid precursor. A distinctive feature of *H. indicum* is its ability to synthesize homospermidine but not PAs in the roots. Molecular studies are needed to prove whether homospermidine synthase activity is expressed in roots or whether it originates as by-product of deoxyhypusine synthase (Ober et al., 2003a).

2.3. Sequence of indicine N-oxide biosynthesis in H. indicum

Trachelanthamidine has been demonstrated as first core-intermediate in the biosynthesis of retronecine monoesters (Kunec and Robins, 1986). To get a more detailed insight into the sequence of indicine *N*-oxide biosynthesis in *H. indicum* tracer feeding experiments with trachelanthamidine and other potential necine base intermediates and monoesters were performed. The results are illustrated in Fig. 2. The structures on the left comprise the ^{3}H- and ^{14}C-labelled alkaloids that were applied as free bases to young leaves of *H. indicum*. After four days the leaves were extracted and analyzed. The square boxes show the produced ester alkaloids. The figures in parenthesis indicate the percentage of radioactivity in the extracts associated with the respective alkaloid fraction. The figures in the circles show the total tracer incorporation thus indicating biosynthetic efficiency. The major results are summarized as

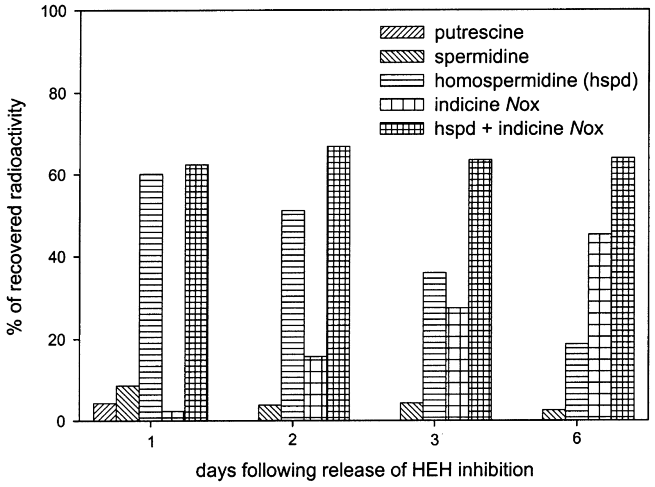

Fig. 1. The diamine oxidase inhibitor β-hydroxyethylhydrazine (HEH) total blocks the formation of radioactively labeled PAs from labeled putrescine in *H. indicum* leaves and causes accumulation of homospermidine. After release of the HEH inhibition labeled indicine *N*-oxide is produced at the expense of labeled homospermidine.

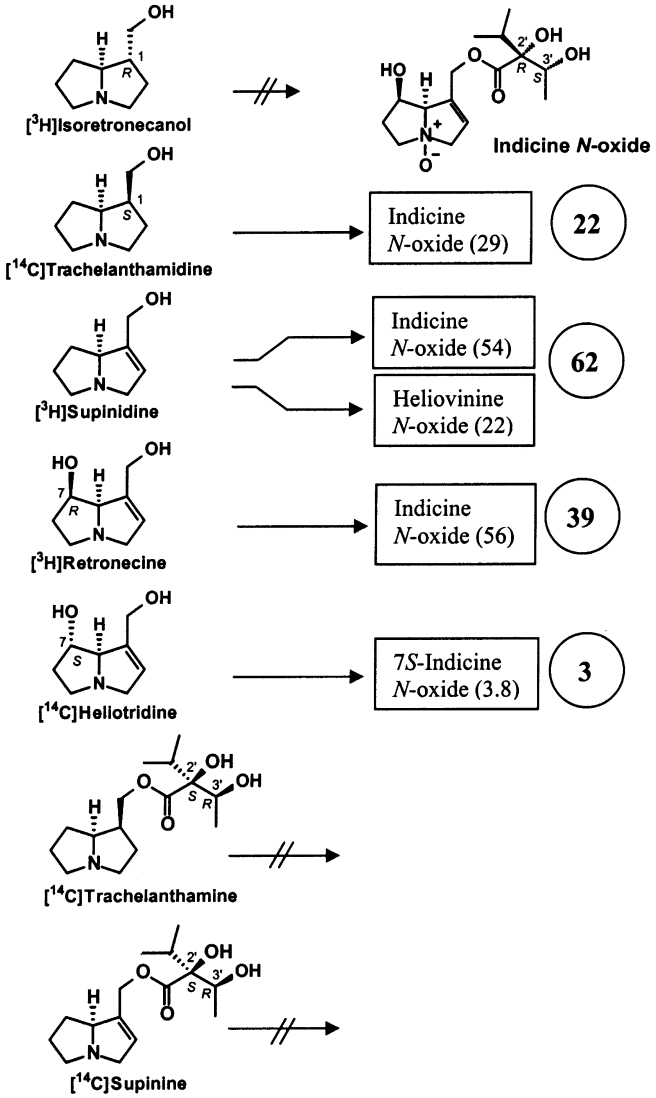

Fig. 2. Conversion of various radioactively labeled necine bases and potential ester alkaloid intermediates into indicine *N*-oxide or corresponding monoesters by young leaves of *H. indicum*. The structures illustrated on the left are applied as labeled tracers (administered as free bases). After 4 days the leaves were harvested and analyzed. The square boxes show the synthesized ester alkaloids. Figures in parenthesis give the percentage of recovered radioactivity associated with the respective compounds (radioactivity in MeOH extract = 100%). Figures in circles show the total tracer incorporation into PA *N*-oxides (radioactivity applied = 100%) indicating the efficiency of biosynthesis. Heliovinine, 7-deoxyindicine.

follows: (1) all synthesized alkaloids are present as *N*-oxides; (2) trachelanthamidine is converted into indicine *N*-oxide without detectable intermediates, whereas supinidine yields in addition to indicine *N*-oxide the respective supinidine ester, i.e. heliovinine *N*-oxide; (3) retronecine is more efficiently esterified than its 7*S*-epimer heliotridine; (4) the ester alkaloids trachelanthamine and supinine are not converted into the respective retronecine esters.

The results suggest that the indicine biosynthesis in *H. indicum* leaves proceeds in a channelled manner without detectable accumulation of free intermediates. This corroborates the genuine PA profile that only shows retronecine esters. Obviously 1,2-dehydrogenation and 7-hydroxylation of the necine bases precedes O^9-esterification which itself appears not to be specific for retronecine since both supinidine and heliotridine are esterified although the respective esters, i.e. heliovinine *N*-oxide and 7*S*-indicine *N*-oxide do not show up in the genuine PA pattern. The results indicate the following biosynthetic sequence: trachelanthamidine–supinidine–retronecine–O^9-esters.

2.4. PA biosynthesis in hairy root cultures of Cynoglossum officinale

Roots cultures of *C. officinale* transformed with *Agrobacterium rhizogenes* were included for comparative reasons. *C. officinale* synthesizes PAs like *H. indicum* in the shoots (van Dam et al., 1995) but also, as shown here, in the roots. In contrast to *H. indicum* the PA profile of *C. officinale* does not contain retronecine esters but instead heliotridine, supinidine and trachelanthamidine esters. The PA profile of a one-week-old *C. officinale* root culture contains rinderine *N*-oxide (72%) and its 3′-acetylester (25%) as major alkaloids accompanied by traces of trachelanthine *N*-oxide (<2%) and supinine *N*-oxide (<2%). Total PA concentration account for 0.19 mg per g fresh wt.

A long-term tracer experiment (3 h–18 days) with ^{14}C-labelled putrecine was performed with the *C. officinale* root culture, following the same strategy as described for *H. indicum*. The results are illustrated in Fig. 3. Five labelled PA *N*-oxides were detected, comprising the four alkaloids found in the genuine PA profile of hairy roots and in addition a small but substantial proportion of intermedine *N*-oxide that was not genuinely detected in root cultures. 'Metabolite X' was detectable transiently until day four. The appearance of the five alkaloids during the course of the experiment shows distinct characteristics: (1) The concentration of rinderine *N*-oxide, the major alkaloid, increased until day 4 and then remains almost stable; (2) the minor alkaloids trachelanthamine *N*-oxide and supinine *N*-oxide increased in concentration until day 2, the point of time when the tracer putrescine was almost

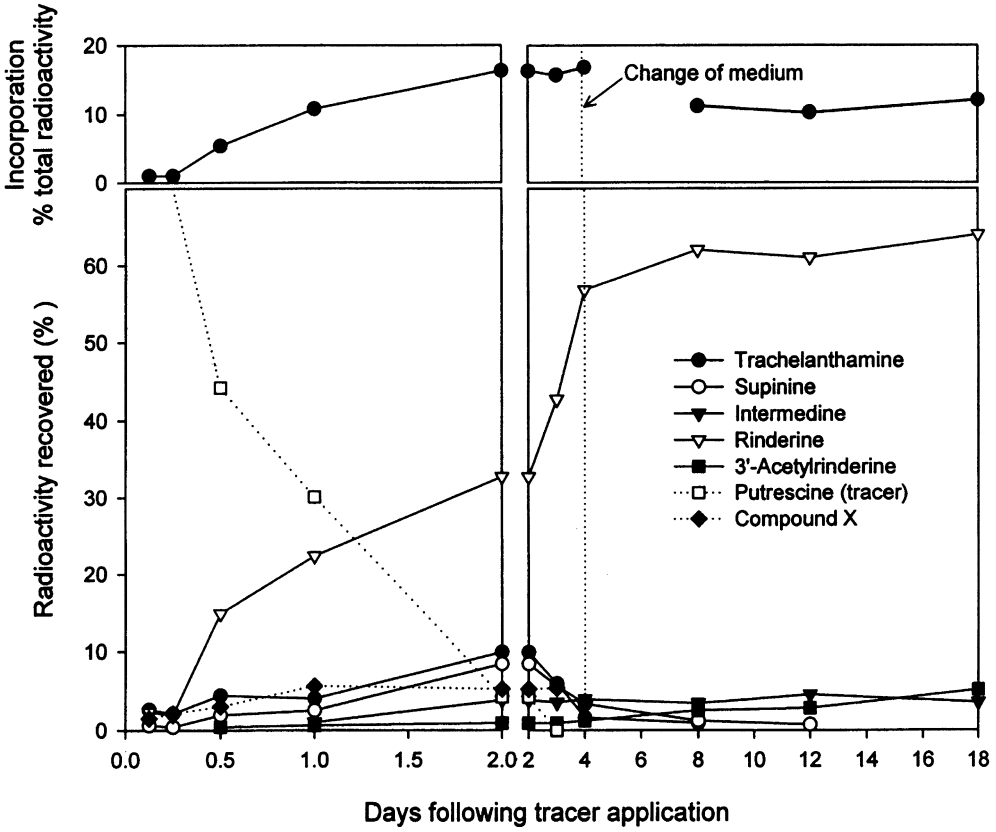

Fig. 3. Incorporation of [^{14}C]putrescine into pyrrolizidine alkaloids by hairy root cultures of *C. officinale*. Left panel: short-term (0–2 days) kinetics; right panel long-term (2–18 days) kinetics. Radioactivity is expressed as percent recovered from the MeOH extracts. The top panels show the total incorporation into PAs, i.e. percent of radioactive applied. After 4 days roots had been transferred into fresh growth medium; indicated by the dotted line.

completely consumed, then they sharply decreased in concentration and were no longer detectable after 8 and 12 days, respectively; (3) 3′-acetylrinderine *N*-oxide increased slowly but continuously over the whole incubation period, and (4) intermedine *N*-oxide increased until day 2 and then remained almost stable. A comparison of the total incorporation of radioactivity into the alkaloid *N*-oxide fraction (Fig. 3, top graphs) shows no decrease after day 2 indicating the absence of alkaloid degradation or turnover. The transient occurrence of trachelanthamine and supinine indicate their role as intermediates in the formation of rinderine which is slowly but continuously further converted into its 3′-acetyl derivative. Intermedine *N*-oxide appears to be synthesized independently of rinderine *N*-oxide.

These conclusions are further substantiated in a feeding experiment with radioactively labelled potential alkaloid intermediates (Fig. 4). The experiment was performed and evaluated in the same ways as described for *H. indicum* (Section 2.3). The major results can be summarized as follows: (1) Again, all synthesized ester alkaloids are exclusively present as *N*-oxides. (2) Trachelanthamidine and

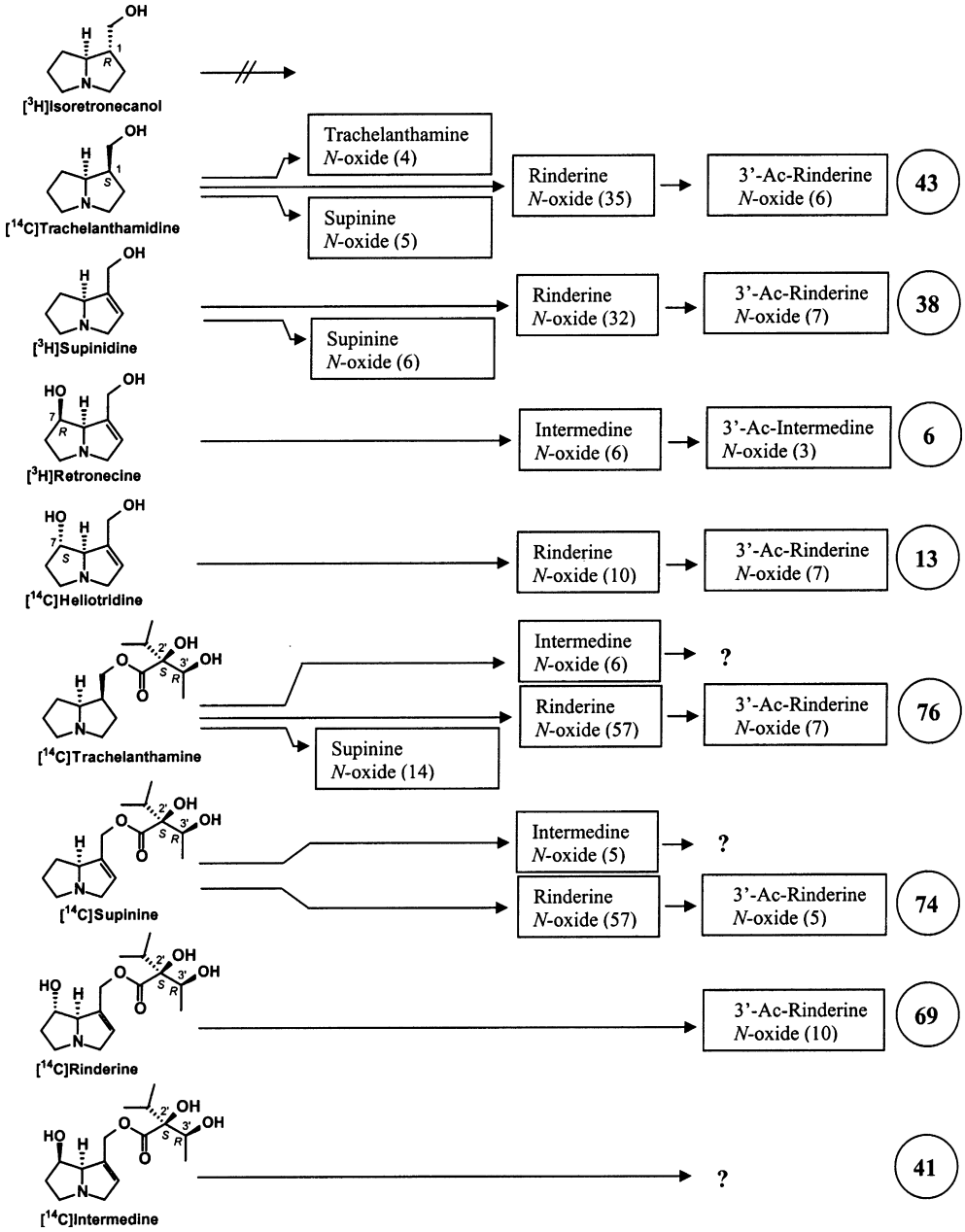

Fig. 4. Conversion of various radioactively labeled necine bases and potential ester alkaloid intermediates into ester alkaloid *N*-oxides by hairy root cultures of *C. officinale*. The structures illustrated on the left are applied as labeled tracers (administered as free bases). After 4 days (necine bases) and 7 days (O^9-esters) the roots were harvested and analyzed. The square boxes show the synthesized, respectively, further processed ester alkaloids. Figures in parenthesis give the percentage of recovered radioactivity associated with the respective compounds (radioactivity in MeOH extract = 100%). Figures in circles show the total tracer incorporation into PA *N*-oxides (radioactivity applied = 100%) indicating the efficiency of biosynthesis.

supinidine are efficiently converted into rinderine *N*-oxide; however low but substantial portions of trachelanthamine *N*-oxide, respectively, supinine *N*-oxide could be detected as biosynthetic intermediates. (3) Retronecine and heliotridine are poorly incorporated into the *N*-oxides of intermedine and rinderine, respectively. (4) Both trachelanthamine and supinine are efficient precursors of rinderine *N*-oxide. The latter is always accompanied by a small portion of intermedine *N*-oxide indicating that the 7-hydroxylation of the necine base generates both epimers. (5) Rinderine *N*-oxide is always accompanied by a small proportion of its 3′-acetyl derivative whereas the 3′-acetylation of intermedine *N*-oxide remains ambiguous; 3′-acetyl intermedine could be detected in traces in a few but not all trials.

The results indicate that in *C. officinale* root cultures O^9-esterification of the necine base with (+)-trachelanthic acid occurs most efficiently at the stage of trachelanthamidine and thus prior to necine 1,2-dehydrogenation and 7-hydroxylation. O^7-Hydroxylation generates preferentially the 7*S*-configurated necine moiety, i.e. heliotridine but not with absolute stereoselectivity. Thus, the heliotridine ester, rinderine, is accompanied by a small fraction of the respective retronecine ester, intermedine. The results confirm the biosynthetic sequence: trachelanthamidine–trachelanthamine–supinine–rinderine (intermedine)–3′-acetylrinderine.

2.5. PA biosynthesis in hairy root cultures of Symphytum officinale

A root culture of *S. officinale* was included as a third example because of its complex PA profile which shows a mixture of retronecine O^9-monoesters and O^7,O^9-diesters. In roots of a one-week-old culture monoesters like intermedine (7%) together with traces of lycopsamine (<1%) are only minor alkaloids. They are accompanied by a rich array of O^7,O^9-diesters such as 7-acetylintermedine (>56%), 7-senecioylintemedine (echiupinine) and 7-tigloylintermedine (myoscorpine) (19%) and their respective 3′-acetyl derivatives (9%) as well as 7-propionylintermedine (4%). Five more PAs are present in trace amounts (<1%), among them 7-tigloyl- and 7-angeloyllycopsamine. All

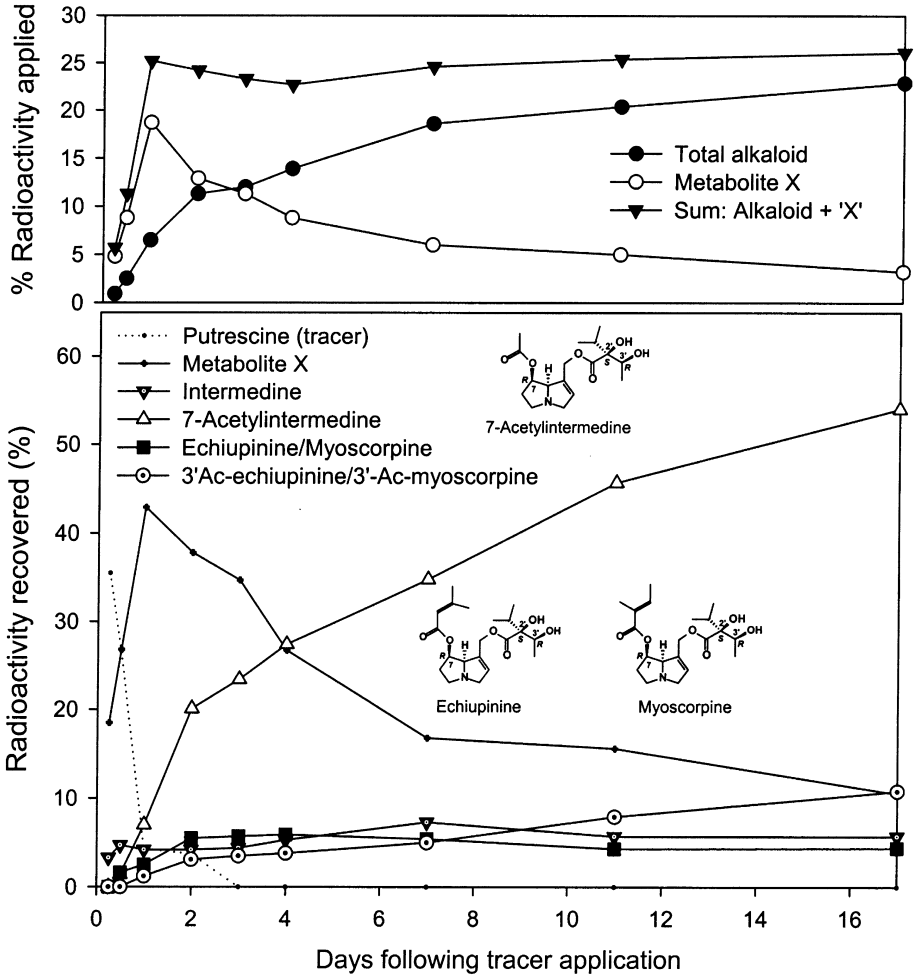

Fig. 5. Incorporation of [^{14}C]putrescine into pyrrolizidine alkaloids by hairy root cultures of *S. officinale*. Lower panel: kinetics of the various labeled metabolites determined. Radioactivity is expressed as percent recovered from the MeOH extracts. The top panel shows total tracer incorporation (radioactivity applied = 100%) into 'metabolite X', total PA *N*-oxides and sum of both. After 4 days roots had been transferred to fresh growth medium.

alkaloids are exclusively present as *N*-oxides. Total PA concentration of a one-week-old root cultures reaches 0.4 mg per g fresh wt.

A long-term (17 days) tracer feeding experiment with ^{14}C-labelled putrescine was performed in the same manner as in the preceding trials. Despite the complexity of the genuine PA profiles in roots the result provides a quite concise picture (Fig. 5): Intermedine *N*-oxide and echiupinine/mypscopine *N*-oxide are present as minor PAs which after two days reach levels of 5–7% that remain constant over the duration of the experiment. In contrast, the major alkaloid 7-acetylintermedine *N*-oxide increases steadily and reaches after 17 days a level of >50%. The 3′-acetyl derivatives of echiupinine/mypscopine *N*-oxide show a similar kinetic and slowly but continuously increase to a level of about 10% in 17-days-old root cultures. 'Metabolite X' which displays the same chromatographic behaviour as the respective labelled intermediate detected in feeding experiments with *H. indicum* (Table 2) and *C. officinale* is very prominent in *S. officinale* roots. It is rapidly produced at the expense of putrescine. Within the first 24 h the level of labelled putrescine decreased to less than 5% while compound X increased to about 42% of total radioactivity. However, in contrast to *H. indicum* and *C. officinale* where compound X was detectable transiently only during the first two to four days, it decreased only slowly in *S. officinale* root cultures. After 17 days about 10% were still detectable. A comparison of total incorporation of radioactivity into alkaloids and 'metabolite X' (Fig. 5, top panel) confirms the increase of labelled alkaloid *N*-oxide at the expense of label from compound X confirming its role as biosynthetic intermediate. 'Metabolite X' is also formed in tracer feeding experiments with spermidine and homospermidine but not trachelanthamidine. Consequently it must be an intermediate between homospermidine and the first necine base. Its isolation and structural characterization is currently under investigation.

Tracer studies with potential alkaloid intermediates were performed to elucidate the conversion of the core alkaloids into the peripheric products of structural diversification. The same experimental strategy was applied as for the other two species. The results are illustrated in Fig. 6.

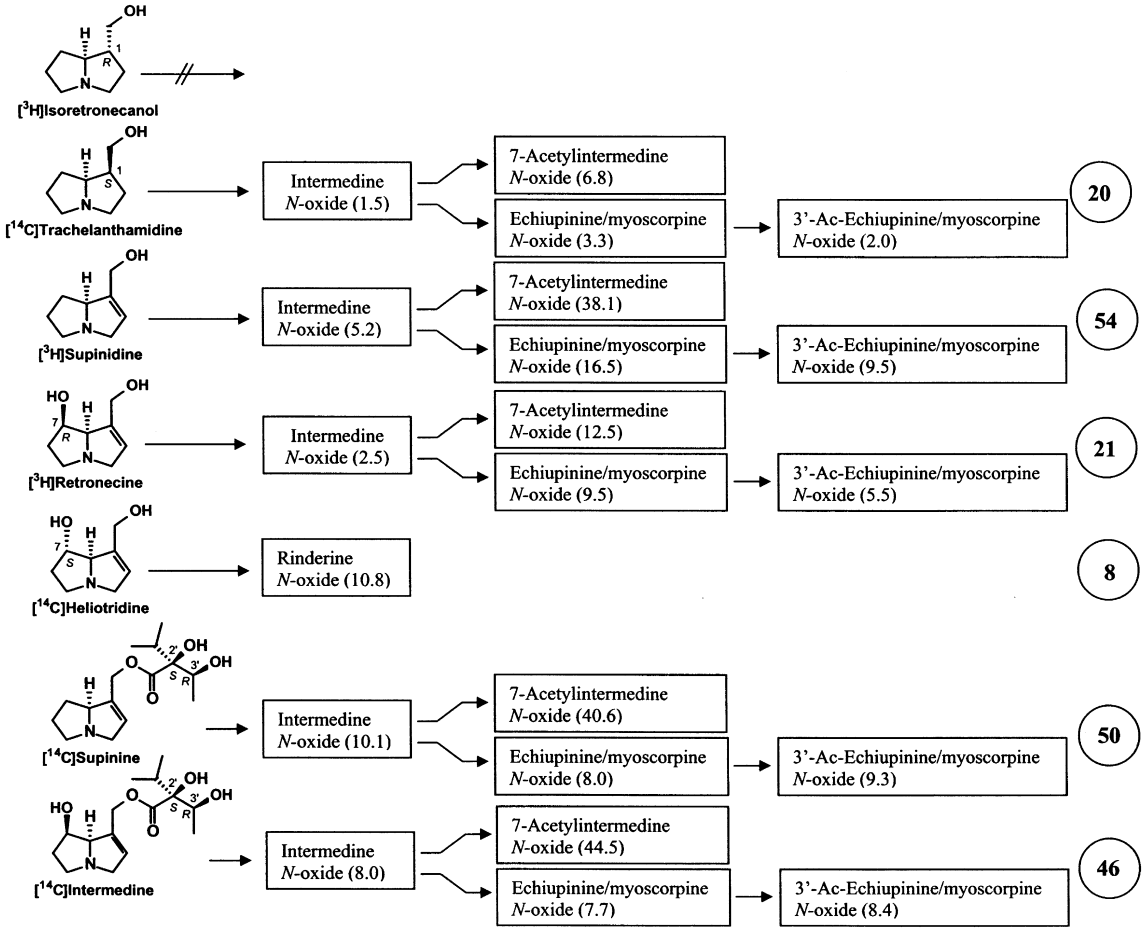

Fig. 6. Conversion of various radioactively labeled necine bases and potential ester alkaloid intermediates into ester alkaloid *N*-oxides by hairy root cultures of *S. officinale*. The structures illustrated on the left are applied as labeled tracers (administered as free bases). After 4 days the roots were harvested and analyzed. The square boxes show the synthesized, respectively, further processed ester alkaloids. Figures in parenthesis show the percentage of recovered radioactivity associated with the respective compounds (radioactivity in MeOH extract = 100%). Figures in circles show the total tracer incorporation into all PA *N*-oxides (radioactivity applied = 100%) indicating the efficiency of biosynthesis.

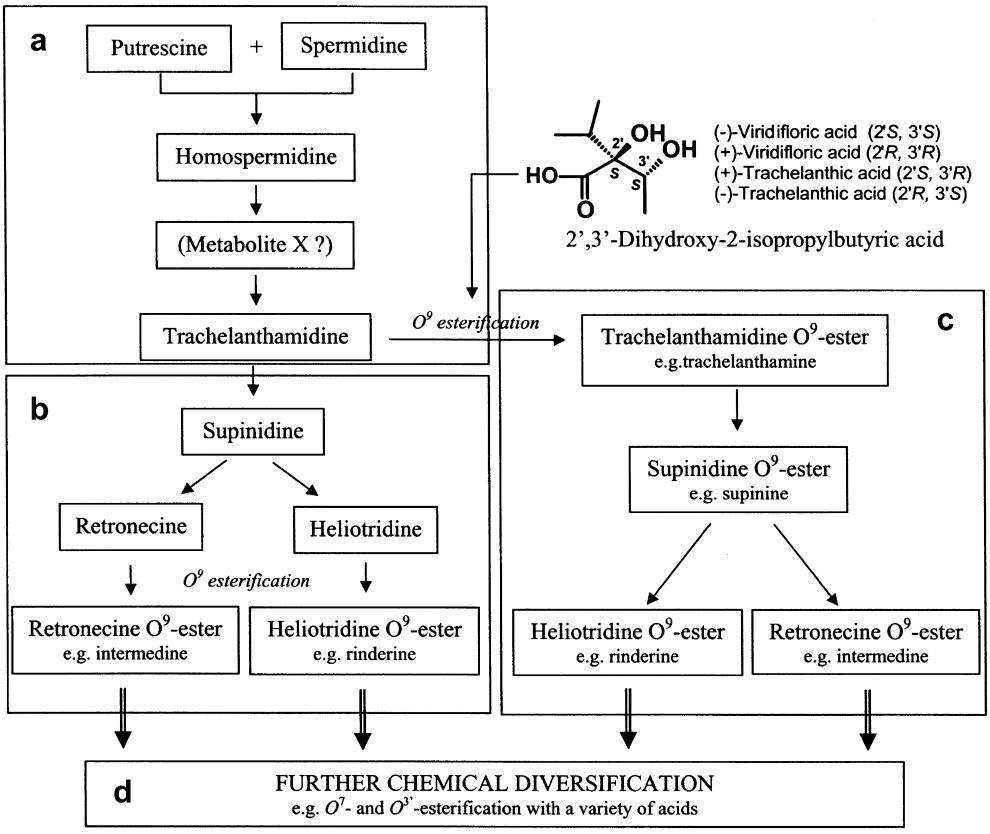

Fig. 7. The different stages of biosynthesis of pyrrolizidine alkaloids of the lycopsamine type in Boraginaceae. Stage 1 (a) Core biosynthesis yielding trachelanthamidine as first necine base. Stage 2 represented by two alternatives, (b) structural modification of trachelanthamidine followed by O^9-esterification. (c) O^9-Esterification of trachelanthamidine followed by structural modification of the necine base moiety. Stage 3 (d) Diversification of the various necine base O^9-esters by further O^7- and $O^{3'}$-esterification.

The most important findings are: (1) All synthesized ester alkaloids are exclusively present as *N*-oxides. (2) The necines trachelanthamidine, supinidine and retronecine are converted into intermedine *N*-oxide without detectable intermediates. (3) Intermedine *N*-oxide is efficiently esterified at C-7 yielding 7-acetyl intermedine *N*-oxide and echiupinine/myoscorpine *N*-oxides. (4) The latter alkaloids are further modified by 3′-acetylation; (5) Heliotridine (with 7*S* configuration) is converted to rinderine but less efficiently than retronecine to intermedine. Rinderine is not further esterified at C-7, indicating the stereoselective esterification of the 7*R* epimer. (6) The efficient conversion of supinine into intermedine *N*-oxide indicates that O^9-esterification is apparently an early step and may occur prior to 7-hydroxylation of the necine base moiety.

3. Conclusions

This study addresses three major aspects: (1) comparison of PA core biosynthesis in Asteraceae and Boraginaceae; (2) tissue-specificity of PA biosynthesis; (3) strategies of structural diversification of PAs of the lycopsamine type in three species of the Boraginaceae.

Homospermidine synthase (EC 2.5.1.45) the first pathway-specific enzyme in PA biosynthesis evolved independently in Asteraceae and Boraginaceae by gene duplication of deoxyhypusine synthase (EC 2.5.1.46) (Reimann et al., 2004). The characterization of the enzymatic steps between homospermidine and the first necine base, most likely trachelanthamidine still awaits elucidation. Involvement of a HEH-sensitive diamine oxidase appears likely in the pathways in both plant families. *Senecio vulgaris* (Asteraceae) (Böttcher et al., 1994) and the three species of the Boraginaceae studied here respond in an identical manner to HEH. In the presence of HEH PA biosynthesis is interrupted and homospermidine accumulated. After release of HEH inhibition PA biosynthesis continues at the expense of homospermidine. A unique feature of PA biosynthesis in Boraginaceae is the transient accumulation of the still unidentified 'metabolite X' between homospermidine and trachelanthamidine. Such an intermediate was not detected in the biosynthesis of senecionine *N*-oxide which proceeds in a channelled manner without detectable free intermediates (Hartmann and Toppel, 1987; Hartmann et al., 1989).

PA-containing species of the Asteraceae synthesize their alkaloids, as far as known, exclusively in the roots (Hartmann et al., 1989; Moll et al., 2002). In this respect, Boraginaceae are more variable. Depending on the species the site of biosynthesis may be the whole shoot (e.g., *H. indicum*), the roots (*S. officinale*) or both shoots (van Dam

et al., 1995) and roots in *C. officinale*. An interesting results is that *H. indicum* roots that do not synthesize PAs upon feeding of [^{14}C]putrescine accumulate labelled homospermidine. Probably the roots express homospermidine synthase but not the following enzymes of PA biosynthesis. Since we know, at least from studies with *Senecio* species, that PAs are spatially mobile and translocated from their sites of biosynthesis to the various sites of accumulation (Hartmann et al., 1989; Hartmann, 1996), there is no need for a restriction of PA biosynthesis to a particular plant organ. Although spatial mobility of PAs in Boraginaceae has not been studied in detail some mobility is indicated, it appears that roots of *H. indicum* (Table 1) must obtain their PAs by import from the shoot, and that *S. officinale* shoots import their PAs from the roots. Root-less shoot cultures of *S. officinale* are devoid of PAs (Hartmann, unpublished).

Despite the diverse PA profiles found in the genus *Senecio* most species synthesize senecionine *N*-oxide as macrocyclic backbone structure which subsequently is diversified to produce species-specific and even organ-specific PA patterns. This diversification is catalyzed by simple one-step or two-step reactions, mainly including position-specific hydroxylations, dehydrogenations, epoxidations, *O*-acetylations as well as retronecine-otonecine transformation of the necine-base moiety (Hartmann and Dierich, 1998; Pelser et al., 2005). In the Boraginaceae a different strategy for creating chemical diversity of PAs of the lycopsamine type can be suggested based on results obtained with the species compared in this study. Three stages of increasing diversification can be distinguished (Fig. 7). Stage 1 covers the core biosynthesis producing trachelanthamidine as first common necine base (Fig. 7a). Stage 2 covers the O^9-esterification with one of the stereoisomeric 2,3-dihydroxy-2-isopropylbutyric acids, the unique necic acid of lycopsamine type PAs. This esterification can occur after structural modification of the necine bases (Fig. 7b) as demonstrated for *H. indicum* (see Fig. 2) or before modification of the necine base moiety (Fig. 7c) as suggested for *C. officinale* roots (see Fig. 4). Finally in stage 3 (Fig. 7d) the various necine O^9-esters are further diversified by O^7-acylation as in *S. officinale* and/or $O^{3'}$-acylation with an array of organic acids as in *H. indicum* or simple acetylations as demonstrated for all three species.

4. Experimental

4.1. Plant material

Heliotropium indicum L. was grown in the greenhouse at about 24 °C and 70% humidity. Plants were propagated through shoot cuttings. Alkaloid analysis, i.e. evaluation of organ-specific PA profiles, was performed with three-months-old plants (height ca. 35 cm) that were in bloom and beginning to fruit. For tracer experiments cuttings were rooted in tap water and then maintained as hydroponics for another 2 weeks before use.

Hairy roots cultures of *C. officinale* L. and *S. officinale* L. were chosen because of rapid and reproducible growth and well expressed alkaloid profiles. Hairy root cultures of *C. officinale* L. were established by transformation with *Agrobacterium rhizogenes* (ATCC 15834). Hairy root cultures of *S. officinale* L. were established from plants that previously had been transformed with *Agrobacterium tumefaciens* (ATCC 23308) by transformation with *A. rhizogenes* (ATCC 15834). Standard protocols were used for transformation.

Hairy roots of *C. officinale* were grown in 300-ml conical flasks containing 80 ml modified MS medium (Murashige and Skoog, 1962) with 4% sucrose and reduction of NH_4NO_3 to 20% of its original amount, i.e. 330 mg l^{-1} instead of 1650 mg l^{-1}; the phytohormones were omitted. Hairy roots of *S. officinale* were grown in a modified B5 medium (Gamborg et al., 1968) with 2% sucrose and the phytohormones omitted.

4.2. Alkaloid analysis and identification

Fresh plant material (plant organs or in vitro cultured roots) were weighed and ground with liquid nitrogen and sea sand (Merck) in a mortar. Samples were extracted twice for 30 min each with 0.1 M H_2SO_4 and centrifuged. The supernatants were combined and half of the solution was made basic with 25% ammonia and applied to an Extrelut (Merck) column (1.4 ml solution/g Extrelut). PAs present as free bases were eluted with CH_2Cl_2 (6 ml/g Extrelut). The solvent was evaporated and the residue dissolved in 10–100 μl MeOH prior to GC, GC–MS or HPLC analysis. The remaining half of the acid supernatant was adjusted to 0.25 M H_2SO_4 and mixed with an excess of Zn dust and stirred for 3 h at room temperature for complete reduction of alkaloid *N*-oxides. Then the mixture was made basic and further processed as given above. This fraction contains total PAs, i.e. free bases plus *N*-oxides.

The pre-purified PA extracts were subjected to qualitative and quantitative GC and GC–MS analyses according to Witte et al. (1993) and as described recently (Frölich et al., 2006). Individual PAs were identified by their Kovats indices (R_I values), molecular ions and MS fragmentation patterns in comparison to reference compounds and our own comprehensive MS data base of PAs. GC-separation was obtained using a fused-silica column (WCOT, 30 m × 0.32 mm; DB-1, J&W Scientific CA).

GC–MS data of the PAs identified in the present study: (1) *H. indicum*: indicine R_I, 2125, [M$^+$], 299; lycopsamine R_I, 2147, [M$^+$], 299; 3′-acetylindicine R_I, 2195, [M$^+$], 341; 3′-benzoylindicine R_I, 2727, [M$^+$], 403; 3′-dihydrocinnamoylindicine R_I, 2910, [M$^+$], 431; 3′-*cis*-cinnamoylindicine R_I, 2925, [M$^+$], 429 (artifact, produced from the genuine trans-derivative during sample preparation); 3′-*trans*-cinnamoylindicine R_I, 2995, [M$^+$], 429. Analysis of the necine base following hydrolysis of the purified PA mixture confirmed retronecine (R_I, 1425, [M$^+$], 155) as sole necine base. (2) *C. officinale* root cultures: trachelanthamine R_I, 1970,

[M^+], 285; supinine R_I, 1978, [M^+], 283; rinderine R_I, 2152, [M^+], 299; 3′-acetylrinderine R_I, 2995, [M^+], 341. (3) *S. officinale* root cultures; figures in parenthesis following the name of the compounds indicate the relative abundance (total PAs = 100%): intermedine (7%) R_I, 2130, [M^+], 299; lycopsamine (<1%) R_I, 2145, [M^+], 299; 7-acetylintermedine (56%) R_I, 2216, [M^+], 341; 7-propionylintermedine (4%) R_I, 2300, [M^+], 355; echiupinine (7-senecioylintermedine)/myoscorpine (7-tigloylintermedine) (11 + 8%) R_I, 2480/2487, [M^+], 381 (sequence of elution from the column uncertain); 7-senecioyllycopsamine/7-tigloyllacopsamine (<1%) R_I, 2495/2503, [M^+], 381; 3′-acetylechiupinine/3′-acetylmyoscorpine (6 + 3%) R_I, 2557/2563, [M^+], 423.

4.3. Tracer experiments

H. indicum. Abscised plant organs of greenhouse-grown intact plants or hydroponics were put into a 2-ml-reaction-vial containing the respective tracer as indicated in 250 μl tap water. Most experiments were performed with young leaves. These were defined as follows: length, ca. 1 cm; fresh wt. ca. 20 mg; PA content: ca. 52 μg; PA concentration: ca. 3.2 mg/g fresh wt. The absorption of the tracer-fluid was usually completed within 24 h. Afterwards the lost fluid volume was replaced by pure tap water until termination of the experiment. Abscised roots of hydroponics were incubated in 5 ml water containing the tracer as indicated. In experiments with β-hydroxyethylhydrazine (HEH) the inhibitor (final conc. 2 mM) was always added 2 h prior to tracer application. After absorption of the tracer/inhibitor mixture the lost fluid was replaced by tap water containing 2 mM HEH.

Hairy root cultures of *C. officinale* and *S. officinale*: Roots (ca. 1 g fresh wt.) were collected aseptically from 7- to 10-days-old cultures and transferred into 5 ml medium containing the respective tracer. In long-term experiments (>4 days) roots were transferred aseptically into 100 ml fresh cultures medium without tracer. All incubations were performed on gyratory shakers (120 rpm) at 25 °C in the dark.

For extraction, the plant organs or cultured roots were washed with tap water, dabbed dry, weighed, and ground in a mortar with liquid nitrogen and sea sand (Merck) before they were extracted twice for 30 min each with methanol containing 1% HCl (25%) and centrifuged. The supernatant of the combined methanol extracts was evaporated. The resulting residue was dissolved in a defined volume of methanol. Total radioactivity was determined by scintillation counting and then aliquots were subjected to radio TLC or radio HPLC for separation and quantification of the labeled products according to Hartmann and Dierich (1998). TLC separation was achieved on silica gel 60 F_{254} with a combination of three solvent system. Solvent I: CH_2Cl_2–MeOH–NH_3 (25%) (82:15:3) ensured good separation of O^9-monoesters and O^9,O^7-diesters as free bases. With this solvent all necine bases showed lower R_f-values than the ester alkaloids and thus did not interfere. Solvent-II: CH_2Cl_2–MeOH–NH_3 (25%) (80:20:3) ensured good separation of O^9-monoesters and O^9,O^7-diesters as *N*-oxides. With this solvent all necine bases showed lower R_f-values than the ester alkaloids and thus did not interfere. Solvent III: EtOAc–*iso*-PrOH–NH_3 (25%) (45:35:20) provided good separation of the necine bases as free bases and *N*-oxides. The various feeding experiments with radioactively labeled precursor amines as well as necine bases and ester alkaloids were analyzed by TLC by combining the three solvent systems. In cases of doubt the labeled spot (labeled PA *N*-oxide) was scratched of the plate and analyzed in more detail as follows: (1) following hydrolysis the necine base was identified by TLC; (2) the *N*-oxide was reduced and the free base identified by TLC and in still doubtful circumstances by GC–MS. In the last case the extracts were co-chromatographed (TLC) with non labeled plant extracts or reference compounds and the dubious radioactive spots scraped off the plate and subjected to GC–MS. This method particularly helped to assign the various lycopsamine stereoisomers, i.e. indicine, rinderine, intermedine which are not separated by TLC.

4.4. HPLC and TLC analysis of polyamine and 'metabolite X'

Separation and quantification of polyamines was achieved through their benzoyl derivatives according to Flores and Galston (1982). An RP-18 column (Nucleosil, 25 cm, 4 mm i.d.; Macherey & Nagel) was applied. Elution: isocratically using the solvent system MeCN–H_3PO_4 (1.5%) (38:62); detection: (a) UV 230 nm, (b) radiolabeled samples UV 230 nm and in parallel radioactivity monitoring. The retention times (R_t, min) are: 'metabolite X', 3.3; putrescine, 6.5; spermidine, 11.5; homospermidine, 13.6.

TLC-separation of radioactively labeled polyamines was achieved on silica gel F_{254} with the solvent system Me_2O–MeOH–NH_3 (25%) (40:30:20). R_f values are: agmatine, 0.08; spermine, 0.15; 'metabolite X', 0.21; homospermidine, 0.22, spermidine, 0.29; putrescine, 0.60.

4.5. Radiochemicals

Amines: [1,4-^{14}C]putrescine (113 mCi/mmol), spermidine {*N*-(3-aminopropyl)-[1,4-^{14}C]-1,4-diaminobutane} (108 mCi/mmol), spermine {*N*,*N*-bis-(3-aminopropyl)-[1,4-^{14}C]-1,4-diaminobutane} (115 mCi/mmol) were purchased from Amersham Biosciences. [U-^{14}C]Agmatine (330 mCi/mol) was enzymatically prepared from labeled arginine by means of arginine decarboxylase (Sigma). [^{14}C]Homospermidine was prepared enzymatically from labeled putrescine using bacterial homospermidine synthase (EC 2.5.2.44) (Graser et al., 1998).

Synthetic [9-^{3}H]isoretonecanol (48 Ci/mmol), [9-^{3}H]supinidine (48 Ci/mmol) and [9-^{3}H]retronecine (16 Ci/mmol) are from our stock collection. ^{14}C-labeled trachelanthamidine and heliotridine were prepared by hydrolysis of [^{14}C]trachelanthamine and [^{14}C]rinderine, respectively. Since the 1,2-saturated alkaloids proved rather unstable

hydrolysis was performed more gently, i.e. with conc. NH_4OH at room temp. for 72 h, whereas the 1,2-unsaturated necine bases could be obtained in good yield by more vigorous hydrolysis in 10% NaOH at 100 °C for 2 h. After hydrolysis the necine bases were extracted with EtOAc and purified by TLC.

^{14}C-labeled trachelanthamine, supinine and intermedine were prepared biosynthetically from [^{14}C]putrescine using root cultures of *Eupatorium clematideum* (Hartmann, 1994; Weber et al., 1999), ^{14}C-labeled rinderine by using hairy root cultures of *C. officinale*. All labeled PAs were purified as lipophilic free bases. The respective *N*-oxides were prepared using a modified procedure (Hartmann, 1994) originally described by Cymerman Craig and Purushothaman (1970). All prepared labeled PA free bases and *N*-oxides were radiochemically pure as tested by radio TLC and HPLC.

Acknowledgements

This work was supported by a grant of the Deutsche Forschungsgemeinschaft. We thank Claudine Theuring for excellent technical assistance particularly in the preparation of radiolabeled alkaloids and Ludger Witte for the GC–MS measurements.

References

Böttcher, F., Adolph, R.D., Hartmann, T., 1993. Homospermidine synthase, the first pathway-specific enzyme in pyrrolizidine alkaloid biosynthesis. Phytochemistry 32, 679–689.

Böttcher, F., Ober, D., Hartmann, T., 1994. Biosynthesis of pyrrolizidine alkaloids: putrescine and spermidine are essential substrates of enzymatic homospermidine formation. Can. J. Chem. 72, 80–85.

Catalfamo, J.L., Martin, W.B., Birecka, H., 1982. Accumulation of alkaloids and their necines in *Heliotropium curassavicum*, *H. spathulatum* and *H. indicum*. Phytochemistry 21, 2669–2675.

Cymerman Craig, J.C., Purushothaman, K.K., 1970. An improved preparation of tertiary amine *N*-oxides. J. Org. Chem. 35, 1721–1722.

Ehmke, A., Von Borstel, K., Hartmann, T., 1988. Alkaloid *N*-oxides as transport and vacuolar storage compounds of pyrrolizidine alkaloids in *Senecio vulgaris* L. Planta 176, 83–90.

Flores, H.E., Galston, A.W., 1982. Analysis of polyamines in higher plants by high performance liquid chromatography. Plant Physiol. 69, 701–706.

Frölich, C., Hartmann, T., Ober, D., 2006. Tissue distribution and biosynthesis of 1,2-saturated pyrrolizidine alkaloids in *Phalaenopsis* hybrids (Orchidaceae). Phytochemistry 67, 1493–1502.

Gamborg, O.L., Miller, R., Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151–158.

Graser, G., Witte, L., Robins, D.J., Hartmann, T., 1998. Incorporation of chirally deuterated putrescines into pyrrolizidine alkaloids: a reinvestigation. Phytochemistry 47, 1017–1024.

Hartmann, T., Zimmer, M., 1986. Organ-specific distribution and accumulation of pyrrolizidine alkaloids during the life history of two annual *Senecio* species. J. Plant Physiol. 122, 67–80.

Hartmann, T., Toppel, G., 1987. Senecionine *N*-oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of *Senecio vulgaris*. Phytochemistry 26, 1639–1644.

Hartmann, T., Ehmke, A., Eilert, U., von Borstel, K., Theuring, C., 1989. Sites of synthesis, translocation and accumulation of pyrrolizidine alkaloid *N*-oxides in *Senecio vulgaris* L. Planta 177, 98–107.

Hartmann, T., 1994. *Senecio* spp.: biochemistry of the formation of pyrrolizidine alkaloids in root cultures. In: Bajaj, Y.P.S. (Ed.), Medicinal and Aromatic Plants, vol. VI. Springer, Berlin, pp. 339–355.

Hartmann, T., Witte, L., 1995. Pyrrolizidine alkaloids: chemical, biological and chemoecological aspects. In: Pelletier, S.W. (Ed.), Alkaloids: Chemical and Biological Perspectives, vol. 9. Pergamon Press, Oxford, pp. 155–233.

Hartmann, T., 1996. Diversity and variability of plant secondary metabolism: A mechanistic view. Entomol. Exp. Appl. 80, 177–188.

Hartmann, T., Dierich, B., 1998. Chemical diversity and variation of pyrrolizidine alkaloids of the senecionine type: biological need or coincidence? Planta Berlin 206, 443–451.

Hartmann, T., Ober, D., 2000. Biosynthesis and metabolism of pyrrolizidine alkaloids on plants and specialized insect herbivores. Top. Curr. Chem. 209, 207–243.

Kunec, E.K., Robins, D.J., 1986. Evidence for different 1-hydroxymethylpyrrolizidines as intermediates in the biosynthesis of retronecine and rosmarinine. J. Chem. Soc. Chem. Commun., 250–252.

Moll, S., Anke, S., Kahmann, U., Hänsch, R., Hartmann, T., Ober, D., 2002. Cell specific expression of homospermidine synthase, the entry enzyme of the pyrrolizidine alkaloids in *Senecio vernalis* in comparison to its ancestor deoxyhypusine synthase. Plant Physiol. 130, 47–57.

Murashige, T., Skoog, F., 1962. Medium for growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Ober, D., Hartmann, T., 1999. Homospermidine synthase, the first pathway-specific enzyme of pyrrolizidine alkaloid biosynthesis, evolved from deoxyhypusine synthase. Proc. Natl. Acad. Sci. USA 96, 14777–14782.

Ober, D., Harms, R., Hartmann, T., 2000. Cloning and expression of homospermidine synthase from *Senecio vulgaris*: a revision. Phytochemistry 55, 305–309.

Ober, D., Gibas, L., Witte, L., Hartmann, T., 2003a. Evidence for general occurrence of homospermidine in plants and its supposed origin as by-product of deoxyhypusine synthase. Phytochemistry 62, 339–344.

Ober, D., Harms, R., Witte, L., Hartmann, T., 2003b. Molecular evolution by change of function: alkaloid-specific homospermidine synthase retained all properties of deoxyhypusine synthase except binding the eIF5A precursor protein. J. Biol. Chem. 278, 12805–12812.

Pelser, P.B., de Vos, H., Theuring, C., Beuerle, T., Vrieling, K., Hartmann, T., 2005. Frequent gain and loss of pyrrolizidine alkaloids in the evolution of *Senecio* section Jacobaea (Asteraceae). Phytochemistry 66, 1285–1295.

Reimann, A., Nurhayati, N., Backenkohler, A., Ober, D., 2004. Repeated evolution of the pyrrolizidine alkaloid-mediated defense system in separate angiosperm lineages. Plant Cell 16, 2772–2784.

van Dam, N.M., Witte, L., Theuring, C., Hartmann, T., 1995. Distribution, biosynthesis and turnover of pyrrolizidine alkaloids in *Cynoglossum officinale*. Phytochemistry 39, 287–292.

Weber, S., Eisenreich, W., Bacher, A., Hartmann, T., 1999. Pyrrolizidine alkaloids of the lycopsamine type: biosynthesis of trachelanthic acid. Phytochemistry 50, 1005–1014.

Witte, L., Rubiolo, P., Bicchi, C., Hartmann, T., 1993. Comparative analysis of pyrrolizidine alkaloids from natural sources by gas chromatography–mass spectrometry. Phytochemistry 32, 187–196.