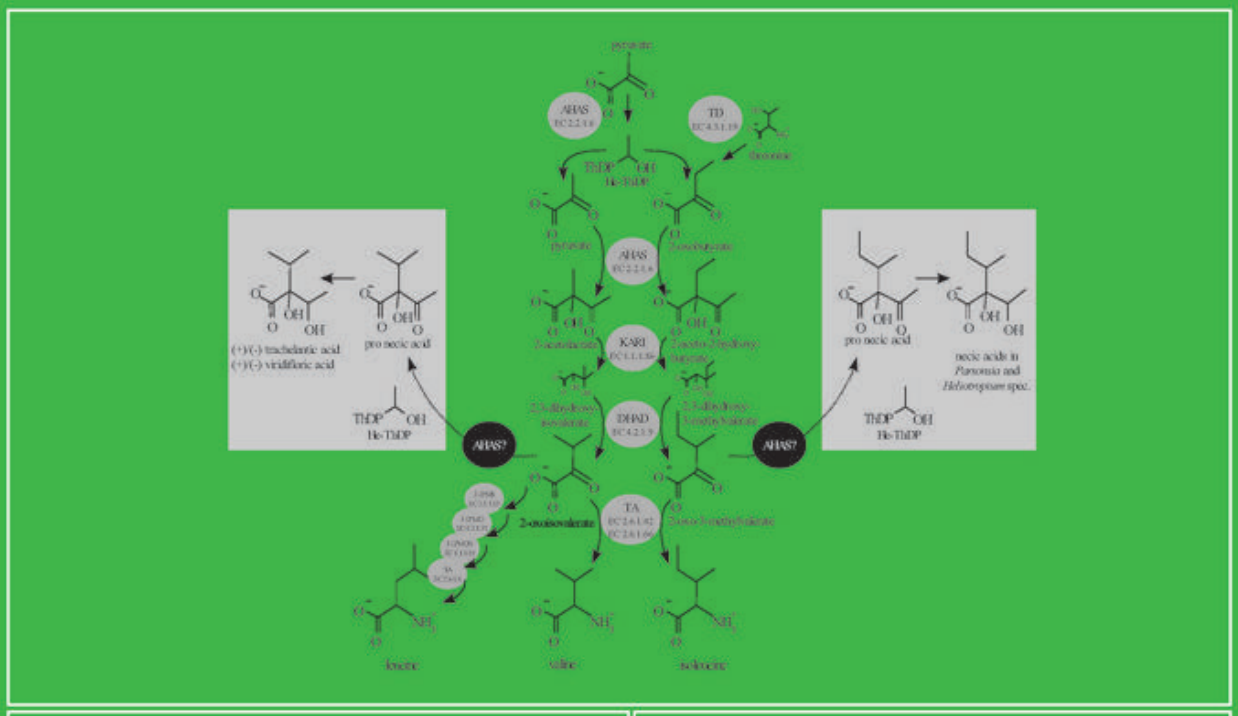
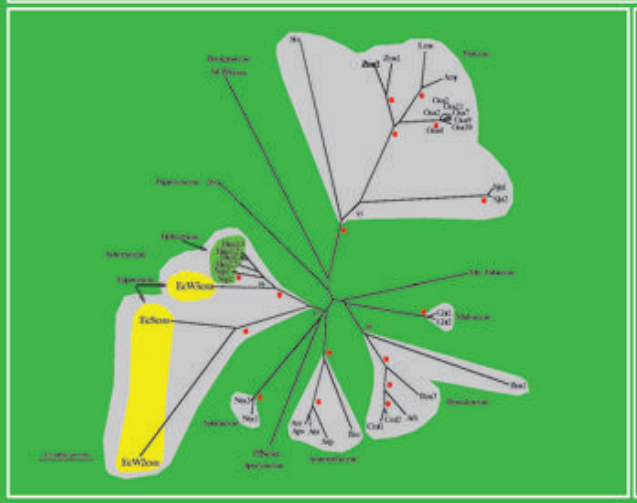
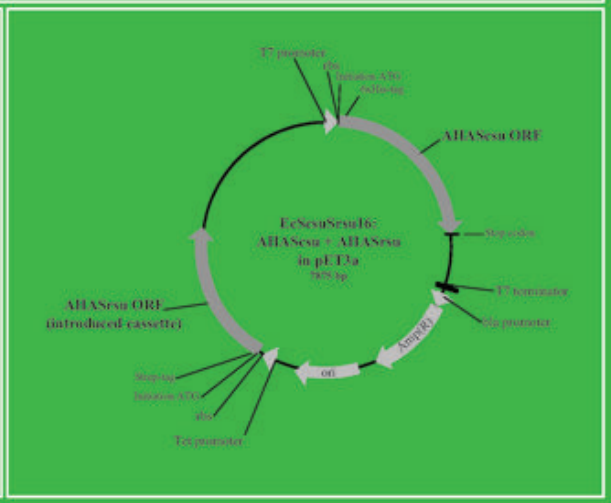
Dorothee Langel

Biosynthesis of the Unique Necic Acid Moiety in Lycopsamine Type Pyrrolizidine Alkaloids – a Molecular Approach –







Cuvillier Verlag Göttingen

Biosynthesis of the Unique Necic Acid Moiety in Lycopsamine Type Pyrrolizidine Alkaloids - a Molecular Approach -

Von der Fakultät für Lebenswissenschaften
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
zur Erlangung des Grades einer
Doktorin der Naturwissenschaften
(Dr. rer. nat.)
genehmigte

D i s s e r t a t i o n

von Dorothee Langel
aus Fulda

Bibliografische Information der Deutschen Nationalbibliothek
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über http://dnb.ddb.de abrufbar.
1. Aufl. - Göttingen : Cuvillier, 2008
Zugl.: (TU) Braunschweig, Univ., Diss., 2008

978-3-86727-646-7

1. Referent:	Professor Dr. Dietrich Ober
2. Referent:	Professor Dr. Thomas Hartmann
eingereicht am:	28.04.2008
mündliche Prüfung (Disputation) am:	20.06.2008

Druckjahr 2008

Nonnenstieg 8, 37075 Göttingen
Telefon: 0551-54724-0
Telefax: 0551-54724-21
www.cuvillier.de

1. Auflage, 2008
Gedruckt auf säurefreiem Papier

978-3-86727-646-7

Contents

Danksagung

An dieser Stelle danke ich allen sehr herzlich, die zur Entstehung der vorliegenden Arbeit beigetragen haben:

Prof. Dr. Dietrich Ober, für die Begeisterung weckende Einführung in Thema und Methodik, die ständige Bereitschaft zu Diskussion und Rat, die Übertragung von Verantwortlichkeit für "unsere" Diplomanden und das stete Vertrauen in meine Person;

Prof. Dr. Thomas Hartmann, für das in mich gesetzte Vertrauen, eine Lösung der Problemstellung zu erzielen, die stete Bereitschaft, über wirklich alle möglichen Lösungen nachzudenken, auch wenn sie der ursprünglichen Theorie diametral entgegen liefen und nicht zu letzt für die Übernahme des Co-Referats;

Dr. Till Beuerle, für die "Entwicklungshilfe" beim spezifischen Assay und die ständige Bereitschaft zur ultra schnellen GC-MS Vermessung der Proben;

Prof. Dr. Ronald G. Duggleby, University of Brisbane, Australia, for providing valuable information on recombinant AHAS and for supplying us with the pET GM construct harbouring the *E. coli* AHASII that was essentially helpful as a positive control for the enzyme assay;

Dr. Barbar J. Mazur and Dr. Robert A. LaRossa, Dupont, USA, for kindly providing us with the AHAS-deficient *E. coli* strain MF2000;

Prof. Dr. Jens Christoffers und Dipl.-Chemiker Michael Rössle, Carl von Ossietzky Universität Oldenburg (Niedersachsen) vormals Universität Stuttgart, für die Einladung zum Erlernen der Synthese der benötigten Referenzsubstanzen;

Anita Backenköhler, für die einleuchtende Einarbeitung, die ständige Bereitschaft, den Teufel im Detail zu finden und für die darüber hinaus entstandene außergewöhnliche Freundschaft;

allen Mitarbeitern des Instituts für Pharmazeutische Biologe der TU Braunschweig für das angenehme Arbeitsklima und die gute Zusammenarbeit; insbesondere Frau Dr. Hoda Mohagheghi, für die guten, erkenntnisreichen Stunden im Büro, bei Exkursionen und im Labor; Frau Dipl.-Apothekerin Dagmar Enß, für ihre unbeirrbare Zähigkeit und Ausdauer während ihrer Diplomarbeit und ihre Stützkraft bei noch so unerfreulichen Ergebnisquoten; Herrn Dipl.-Apotheker Uwe Genzel, für die tatkräftige Unterstützung bei den Expressionsversuchen im Rahmen seiner Diplomarbeit sowie Frau Apothekerin Jessica Steinert, für die Hilfe bei den katalytischen Untereinheiten.

allen Mitarbeitern der Arbeitsgruppe Ober am Botanischen Institut der Christian-Albrechts Universität zu Kiel für das herzliche Willkommen, der tatkräftigen Mithilfe bei Aufbau und Einrichtung von Labor und Arbeitsplätzen und für das außerordentlich gute Arbeitsklima.

1 Introduction

Plants produce a vast and diverse assortment of chemical compounds. These compounds can be classified as products or intermediates from primary and secondary metabolism. Primary metabolism is necessary for growth and development, is universal, uniform, and hardly changed during evolution. Examples of plant primary metabolites are simple carbonic acids and sugars, amino and nucleic acids (Croteau et al. 2000).

On the other hand, secondary metabolism is not necessary for growth and development but is essential for continued existence of a species within its environment (Hartmann 1985). Plant secondary metabolites serve as chemical signals that enable the sessile plant to respond to environmental cues. Secondary compounds can function in the defense against herbivores, pathogens, and competitors while others provide protection from sun radiation, aid in pollen and seed dispersal or can submit informations acting as pheromone like signals and phytohormones (Hartmann 1991, Harborne 1993).

Plant secondary compounds are derived from central primary metabolites and, based on their biosynthetic origins, they can be divided into three major groups: the terpenoids, the phenylpropanoids including phenolic compounds, and the alkaloids. Terpenoids originate from carbohydrate metabolism (carbonic acids and sugars) and functionally include toxins and feeding deterrents against herbivores like cardenolides or antibacterial, fungicide and viricidal mono- and sesquiterpenes in essential oils. In lower dosages, essential oil components are important to attract pollinators and seed dispersers. Those are visually attracted by many flavonoid pigments that belong to the phenolic compound group, e.g., anthocyanins can be responsible for red and blue, while chalcones count for yellow flower pigments. Besides this visual function, phenolic compounds like tannins, lignans, and flavonoids serve as defense against herbivores and pathogens. Moreover, lignins strengthen cell walls mechanically, and naphthaquinones like juglone have allelopathic activity and may adversely influence the growth of neighbouring plants.

The fascinating, chemical diverse group of alkaloids is synthesized from compounds of the amino and nucleic acid metabolism. These bitter-tasting, nitrogenous alkaloids protect plants from a variety of herbivorous animals, and many possess dramatic physiological effects on vertebrates including humans.

Most alkaloids are easily resorbed and many of them interfere with essential parts of the nervous system, acting on pre- and/or post-synaptic receptors, inactivating neurotransmitters, inhibiting the post-synaptic signal transduction pathways, or hampering a proper function of ion channels. Depending on the targeted nerve, this can result in paralysis of selected muscle tissues like skeleton muscles and heart muscles, or it can result in the paralysis of pain-conductive nerves providing an analgesic effect, or it can result in the paralysis of the central nervous system causing psychological effects. Moreover, some alkaloids interfere with the assembly or disassembly of essential structures within the cytoskeleton, hindering cell division and awarding some cytostatic effects while other

alkaloids are able to alkylate DNA probably causing cancer (Hänsel and Sticher 2004).

The first identified alkaloid was morphine from the latex of opium poppy (*Papaver somniferum*). Until today, morphine is used in medicine as an analgesic and cough suppressant. However, excessive use of this drug can lead to strong addiction and, if overdosed, it provokes respiratory paralysis and death.

More than 12,000 alkaloids have been isolated since the discovery of morphine by the German pharmacist Fiedrich Sertürner in 1806 and the questions, how and why alkaloids are made by plants, have fascinated generations of researchers within the fields of biology, chemistry, and pharmacy.

1.1 Pyrrolizidine Alkaloids - Typical Compounds of Plant Secondary Metabolism

Pyrrolizidine alkaloids (PAs) are characterized as typical compounds of plant secondary metabolism (Croteau et al. 2000). PAs do fulfill the four essential demands on secondary compounds as proposed by Hänsel and Sticher (2004).

1. Differential distribution among limited taxonomic groups within the plant kingdom

PAs were discovered in more than 6,000 plant species (Chou and Fu 2006) distributed among certain unrelated Angiosperm taxa. More than 95% of the PA-producing species investigated so far belong to just five families (marked with a red disc in Figure 1.1) namely the Asteraceae (1), the Boraginaceae (2), the Apocynaceae (3), the Orchidaceae (4), and the Fabaceae (5). Further isolated occurrences were reported from single species of other families (marked with a pink disc in Figure 1.1) like the Convolvulaceae (6), the Santalaceae (7), the Sapotaceae (8), the Ranunculaceae (9), the Celastraceae (10) (reviewed by Hartmann and Witte, 1995), and recently from the Lamiaceae (11) discovered by Nawaz et al. (2000).

The scattered occurrence of PAs among the Angiosperms has been particularly puzzling and provoked the question whether the PA biosynthetic pathway was invented just once and lost several times during evolution or whether it evolved several times independently in distinct, separate lineages. To date, this question was answered in favour of an independent evolution. Homospermidine synthase (HSS, EC 2.5.1.44), catalyzing the first step in PA biosynthesis, was found to be of polyphyletic origin within the Angiosperms (Ober and Hartmann 1999b). Up to now, the HSS has been shown to be recruited at least four times independently, once early in the evolution of the Boraginaceae, once within the monocots, and twice within the Asteraceae separating the tribes of Senecioneae and Eupatorieae (Reimann et al. 2004).

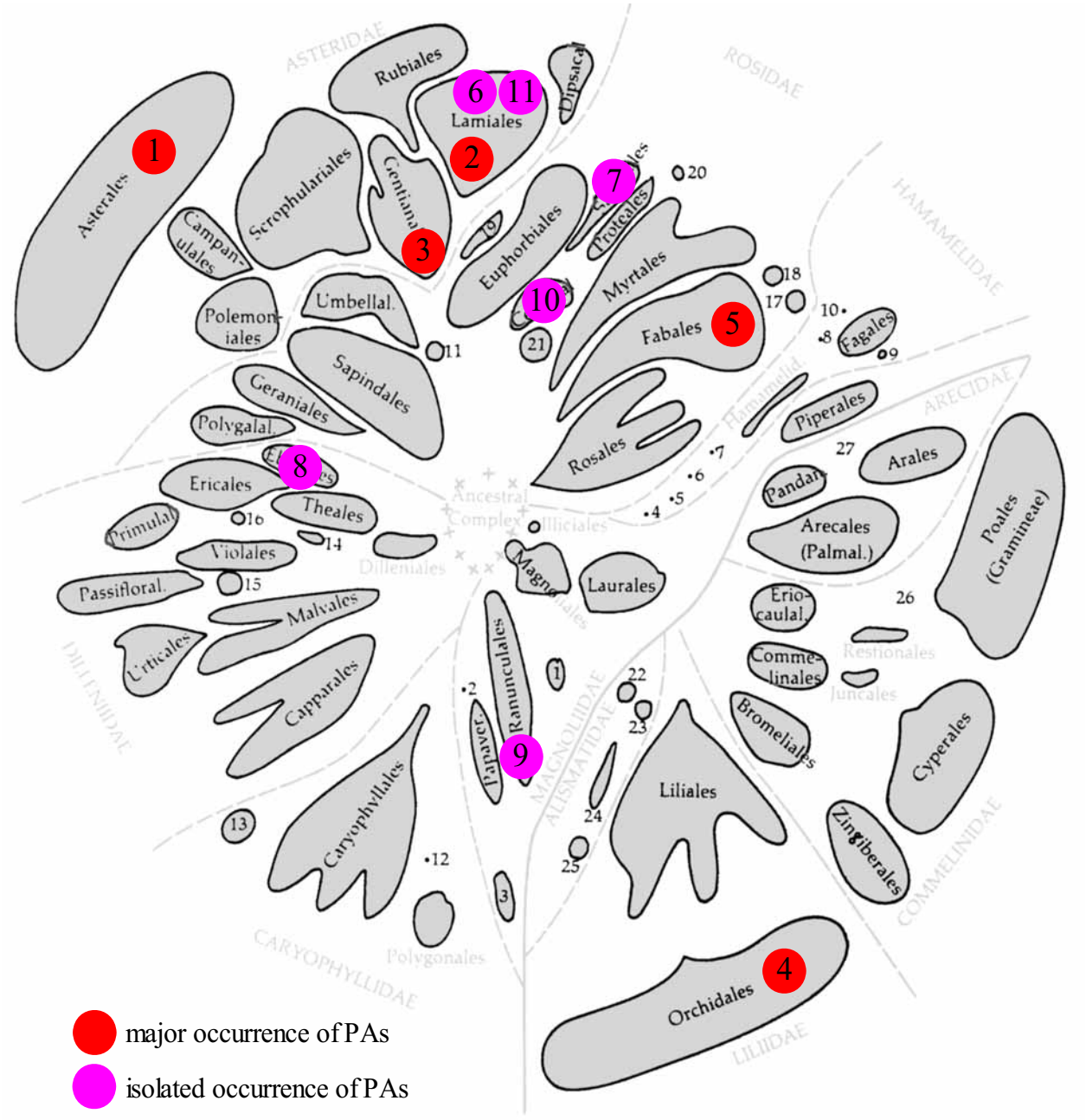


Figure 1.1: Major and isolated occurrences of pyrrolizidine alkaloids within the angiosperms, modified from Ober and Hartmann 1999b and Stebbins 1974: Asteraceae (1), Boraginaceae (2), Apocynaceae (3), Orchidaceae (4), Fabaceae (5), Convolvulaceae (6), Santalaceae (7), Sapotaceae (8), Ranunculaceae (9), Celastraceae (10), and Lamiaceae (11).

2. Great structural diversity and complexity

More than 660 different chemical structures of PAs are known so far (Jiang et al. 2006). Basically, plant PAs are composed of a "necine base" moiety that is esterified with one or more "necic acids" (Panel B of Figure 1.2, Crout 1966).

In 1995, the main PA-types (see Figure 1.2) comprising senecionine, triangularine, monocrotaline, lycopsamine, and phalaenopsine type PAs were described by Hartmann and Witte (1995) based on Culvenor's chemosystematic suggestions (Culvenor 1978). The growing number of discovered PA-structures as well as the increased understanding of the mechanisms involved in biosynthesis, biological activities and functions of PAs led to a new modified classification introduced by Hartmann (2006) which emphasizes the biosynthetic relationship within the necic acids:

I. Macrocyclic or open-chain diester types with eleven or more members including their related monoesters comprising senecionine (**S**), triangularine (**T**), and monocrotaline (**M**) types esterified with isoleucine derived necic acids (Panel I in Figure 1.2).

II. Lycopsamine (**L**) type PAs occurring as open-chain mono- and diesters, and macrocyclic triesters esterified with a unique C7 necic acid (Panel II in Figure 1.2).

III. Special type PAs characteristic to selected genera enclosing ipanguline (**I**) and phalaenopsine (**P**) types, occurring as mono- and open-chain di- and triesters with aryl, aralkyl, and rarely alkyl necic acids (Panel III in Figure 1.2).

The distribution of PA types within the Angiosperm families is shown in Table 1.1. Remarkably, the alkaloid pattern within the Asteraceae differs significantly between its two PA-producing tribes, the Senecioneae and the Eupatorieae. While the vast majority of PA types within the Eupatorieae belongs to the lycopsamine (L) type, PAs within the Senecioneae are exclusively found in class I with an accumulation of senecionine (S) types. These distinct alkaloid patterns are reflected in the finding that the homospermidine synthase (HSS) catalyzing the first step in PA biosynthesis was recruited independently within the Eupatorieae and the Senecioneae. Moreover, the inability of Senecioneae species to produce lycopsamine type PAs leads to the hypothesis that the HSS might not be the only enzyme specialized in PA biosynthesis. The formation of lycopsamine type PAs requires a second specialized enzyme that is important for the formation of the unique C7 necic acids characterizing the lycopsamine type PAs. Thus, this second specialized enzyme might not be present in the Senecioneae, the Orchidaceae, the Convolvulaceae (*Ipomoea*), Ranunculaceae, Celastraceae, and Lamiaceae but within the Eupatorieae as well as in the Boraginaceae, the Apocynaceae, the Santalaceae, the Sapotaceae, the Convolvulaceae (*Merremia*), and in the Fabaceae (*Laburnum anagyroides*).

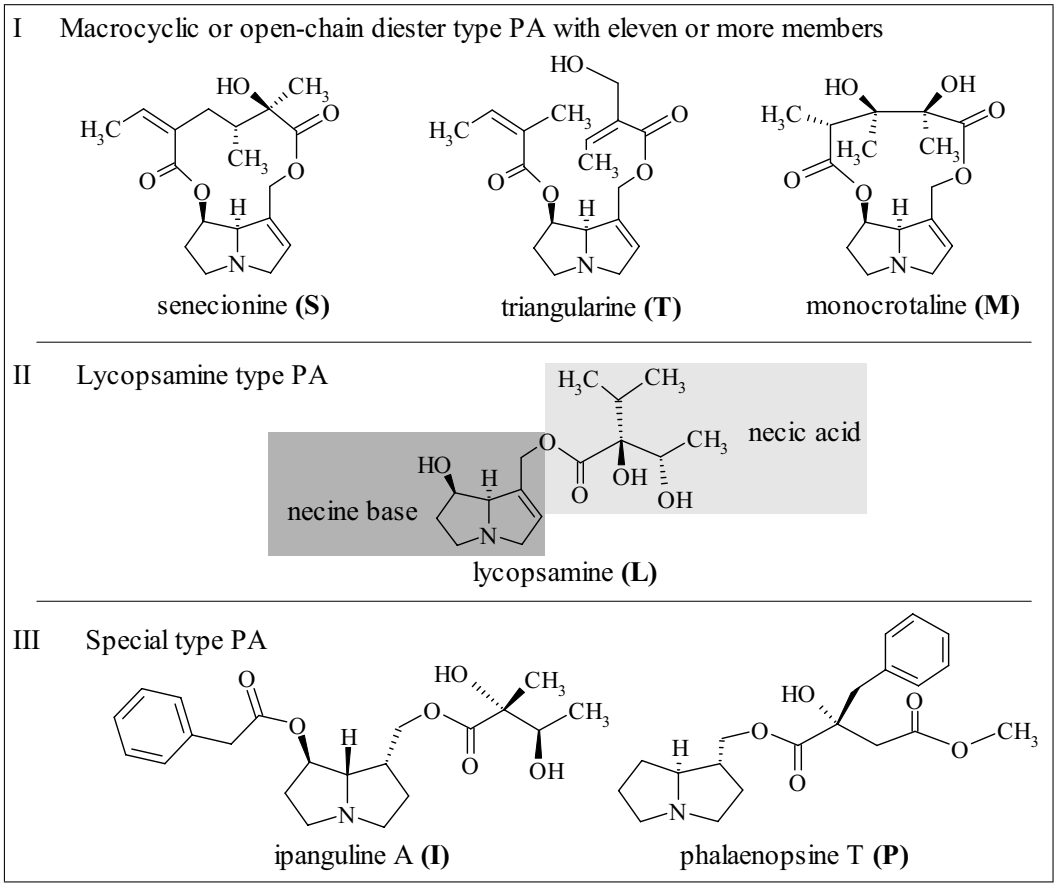


Figure 1.2: The three classes of pyrrolizidine alkaloids (PAs): **I.** Macrocyclic or open-chain diesters comprising the senecionine (S), triangularine (T), and monocrotaline (M) type PAs; **II.** Lycopsamine (L) type PAs; and **III.** Special type PAs enclosing the ipanguline (I) and phalaenopsine (P) type PAs.

family of PA occurrence	prevalent genera (Hartmann and Witte 1995)	distribution of PA types [%]						
		I			II	III		others
		S	**T**	**M**	**L**	**P**	**I**	
Asteraceae, tribe Senecioneae	*Senecio*	82	16	1	-	-	-	1
Asteraceae, tribe Eupatorieae	*Eupatorium*, *Ageratum*	8	5	-	87	-	-	
Boraginaceae	virtually all genera[1]	< 1	7	1	90	1	-	
Apocynaceae	*Parsonsia*	4	-	-	77	4	-	15
Orchidaceae	subfamily of Epidendroideae[2]	-	-	-	-	95	-	5
Fabaceae	*Crotalaria*	39	2	45	1	-	-	13
Convolvulaceae	*Ipomoea*, / *Merremia*[3]	-	-	-	- / 100	-	100 / -	
Santalaceae	*Thesium*, *Amphorogyne*[4]	-	-	-	14	86	-	
Sapotaceae	*Planchonella*, *Minusops*	-	-	-	13	75	-	12
Ranunculaceae	*Caltha*, *Trollius*[5]	100	-	-	-	-	-	
Celastraceae	*Bhesa*	-	100	-	-	-	-	
Lamiaceae	*Ajuga*[6]	100	-	-	-	-	-	

[1]Röder (1995), [2]Frölich et al. (2006), [3]Mann et al. (1996),
[4]Thu Huong et al. (1998), [5]Liddell and Stermitz (1994), [6]Nawaz et al. (2000)

Table 1.1: The distribution of PA types within the Angiosperm families.

3. Characteristic supply with PAs depending on the developmental stage

PA biosynthesis in the Asteraceae is strictly coordinated to root growth (Hartmann et al. 1988, Sander and Hartmann 1989) and is terminated when flowers open (Anke 2004). PA backbone structures like senecionine *N*-oxide are formed constitutively and under high constraint. Their biosynthesis is not inducible by wounding nor by microbial attack (van Dam and Vrieling 1994, Tinney et al. 1998). Additionally, PA backbones are diversified via specific one- or two-step reactions such as hydroxylation, epoxidation, O-acetylation, or otonecine formation which proceed in a molecule position-specific and stereoselective manner (Hartmann and Dierich 1998). Any genetic variability affecting the PA transforming enzymes modifies the PA bouquet without affecting the overall quantity. Such a highly plastic system may indicate a powerful strategy in constitutive plant defense against differential herbivory (Hartmann 1999, Hagen 2003).

4. Biosynthesis in specialized tissues, accumulation in unique, defined patterns

PAs are synthesized in specific tissues (see Table 1.2) and are accumulated in cell vacuoles of various tissues (Ehmke et al. 1987) with up to 80% of total plant PA content. Such accumulation tissues are important for species or individual survival, e.g., buds, seeds or roots (Hartmann and Zimmer 1986). Translocation from tissues of biosynthesis to those of accumulation is enabled by PAs existing as hydrophilic *N*-oxides (Figure 1.3) which allows transport via phloems (Hartmann et al. 1989, Witte et al. 1990). A specific PA *N*-oxide carrier, responsible for selective uptake into the vacuoles, has been characterized (Ehmke et al. 1988). Phloem loading and unloading is still under investigation and predicted to be carrier-mediated since species, which do not produce PAs, are unable to translocate PAs via the phloem (Hartmann et al. 1989). Stored PAs are stably retained and show no metabolic turnover except for PA-protected seedlings from *Crotalaria scassellatii* using a PA-specific *N*-oxygenase to start the mobilization of the bound nitrogen utilized for growth and development (Chang 1997).

1.1.1 Toxicity of Pyrrolizidine Alkaloids and Role in Plant Protection

In mammals, PAs are hepatotoxic and carcinogenic (Mattocks 1972, Culvenor et al. 1976, Wiedenfeld and Röder 1984, Steenkamp et al. 2001). The toxic compound is formed after ingestion via bioactivation by microsomal liver cytochrome P450 monooxygenases producing unstable pyrrolic intermediates (Figure 1.3). These highly reactive intermediates are only formed when the alkaloid substrates display the following essential features (Winter and Segall 1989): C1-C2 double bond (boxed area A in the pro-toxic PA structure in Figure 1.3), esterification of the allylic hydroxyl group at C9 (boxed area B), and free or esterified hydroxyl group at C7 (boxed area C). The necic acids are cleaved off and the remaining nucleophilic necine base derived compound is able to alkylate DNA (Fu et al. 2004). The attacked liver develops necrosis, fibrosis, and eventually neoplastic growth. The loss of liver function means an overflow of brain toxic substances like ammonia and

families	organs of PA synthesis	tissues of PA synthesis[1]	organs of highest[2] PA accumulation
Asteraceae, tribe Senecioneae	roots	specific groups of endodermis + adjacent cortex cells opposite the phloem[3]	infl.[4] (90%)
Asteraceae, tribe Eupatorieae	roots	cortex of annually sprouted roots[5]	infl.[6] (90%)
Boraginaceae	Sof roots[7]	endodermis[8]	?
	Cof shoots[9], roots[8]	endodermis + pericycle[8]	?
	Hin shoots[7], leaves[8] + buds[8]	epidermis of shoots + leaves[8]	infl. (71%)[7]
Orchidaceae	aerial root tips	basic meristem[10]	buds[11] (52%)
	buds	epidermis[10]	
Fabaceae	roots and/or leaves and/or shoot tips[12]	nd	seeds[13]
Convolvulaceae	shoots[14], roots[15]	nd	young leaves + shoot tips (60%)[14]

[1]deduced from the localization of the first pathway specific enzyme, the homospermidine synthase (HSS), [2]PAs were found in all parts of the plants
[3]Moll et al. (2002), [4]Hartmann and Zimmer (1986), [5]Anke et al. (2004), [6]Biller et al. (1994), [7]Frölich et al. (2007), [8]Niemüller (2007), [9]Van Dam et al. 1995, [10]Anke (2004), [11]Frölich et al. (2006), [12]Nurhayati and Ober (2005), [13]Toppel et al.(1988), [14]Jenett-Siems et al. (1998), [15]Jenett-Siems et al. (2005)

Table 1.2: Plant organs and tissue of PA biosynthesis vary significantly among the PA producing families or even genera while plant organs of PA accumulation are preferentiably reproductive tissues. Abbreviations: infl. = inflorescence (includes buds, flowers + fruits), Sof = *Symphytum officinale*, Cof = *Cynoglossum officinale*, Hin = *Heliotropium indicum*, nd = not determined, ? = unfortunately, most phytochemical reports do not contain any quantitive information on PA levels or even specifications of the analyzed plant organs.

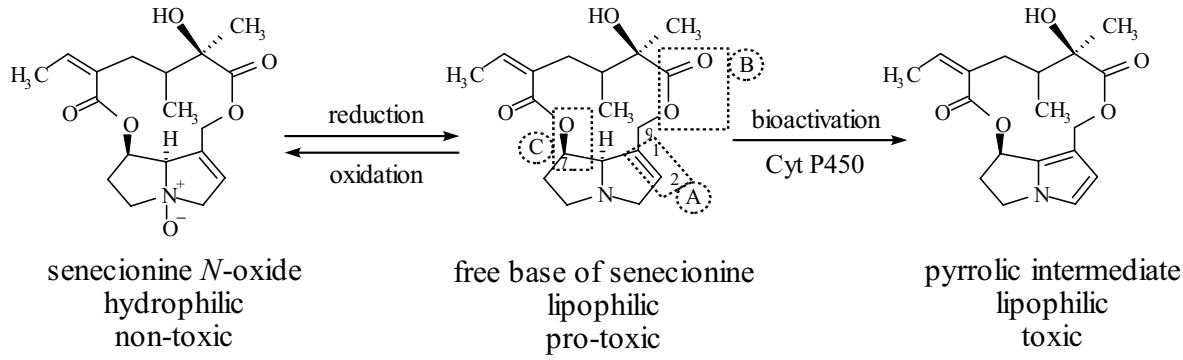


Figure 1.3: In plants, pyrrolizidine alkaloids exist in two molecular forms: the hydrophilic, non-toxic *N*-oxide and the lipophilic, pro-toxic free base. During bioactivation in vertebrates, the toxic pyrrolic intermediate is generated by liver microsomal monooxygenases. This toxic form is only produced when the pro-toxic form displays the following essential features: C1-C2 double bond (boxed area A), esterification of the allylic hydroxyl group at C9 (boxed area B), and free or esterified hydroxyl group at C7 (boxed area C).

results in a destroyed central nervous system, in an inability to breathe and finally in death. The potential health hazard for humans caused by unsaturated PAs was the reason for the Federal Health Department of Germany (Kommission E 1992) to recommend a limited use of leaves and buds from coltsfoot (*Tussilago farfara*) in bronchial tea (maximum use is 6 weeks per year with a daily uptake of 1 μg), in the use of roots from comfrey (*Symphytum officinale*) as extracts in ointments treating distortions and contusions (with external use on intact skin, daily dose is 100 μg of PAs), and of butterbur extracts (*Petasitis hybridus*) used for the treatment of migraine (petasitenine daily allowance is 0.1 μg).

The described effects on mammals work very slow and do not prevent the individual plant from being eaten. Hence, reported livestock intoxications with PA-producing plants (Cheeke 1989) might have been interpreted rather as a man-made accident with locked up cattle forced to feed on run-down pastures. Grazing animals, however, usually avoid PA plants, apparently because of their deterrent taste (Boppré 1986).

Considering herbivorous insects, PAs are hypothesized to play a protective role in plant defense as well. Most plant secondary compounds are thought to be co-evolved in the cretaceous period (130 million years before present) during the appearance and rapid diversification of both Angiosperms and insects which appeared to Darwin (Darwin and Seward 1903) as an "abominable mystery". While the Angiosperm-specific flower attracted the facultative herbivorous insect, essential for pollination, it was necessary for the plant to ensure intact tissues important for species survival and reproduction (Fraenkel 1959). Hence, protective PAs are cytotoxic for adult insects and mutagenic for larvae and pupae (Frei et al. 1992). Within one generation, the herbivore attack is completely stopped which is an optimal result for the plant.

The most convincing evidence favoring an anti-feeding role of plant PAs can be observed from specialized herbivorous insects. A number of these insects from diverse taxa have evolved adaptations not only to overcome the defensive barrier of PA-protected plants, but to sequester and utilize PAs for their own defense against predators. Plant-derived PA-sequestering species are found among butterflies and moths (Lepidoptera), leaf beetles (Coleoptera), aphids (Homoptera), and the African grasshopper *Zonocerus* (Orthoptera). These plant PA sequestering insect species advertise their unpalatability to potential insect predators by conspicuous warning coloration (Hartmann 1999) with the effect that predators with previous contact to the taste of PAs avoid preying on aposematic coloured insects (Hare and Eisner 1993).

The giant tropical orb-weaving spider *Nephila clavipes* (Araneae, Tetragnathinae), for instance, liberates PA sequestering adults of lepidopteran Ithomiinae, Danainae, and Arctiidae unharmed from its web (Belt 1888, Vasconcellos-Neto and Lewinsohn 1984, Eisner 1982). When PA-free, dead and wing-less workers of honey bee *Apis mellifera* (Apidae) were coated topically with methanolic solutions of various PAs and tossed onto the web of a female spider, the potential prey was released from the web depending on the type and amount of encoated PAs (Silva and Trigo 2002).

Besides of their function as antifeeding compounds, PAs play a role in mating behavior. In the arctiid species *Utetheisa ornatrix,* the content of PAs inside a male evolved as a criterion for attractiveness (Boppré 1986, Boppré 1990). PAs are reconstructed by males to the pheromone hydroxydanaidal and advertised to females that now can estimate the total systemic PA-load of males (Dussourd et al. 1991). During copulation PAs are transmitted from male to female. The eggs receive both the male and the female load and are protected in the best way their parents were able to do (Eisner et al. 2002).

1.1.2 Pyrrolizidine Alkaloid Biosynthesis

Pyrrolizidine alkaloid (PAs) biosynthesis was shown to root in the amino acid biosynthesis. Proven and proposed parts of the principle metabolic pathway of the lycopsamine type PAs are shown in Figure 1.4.

1.1.2.1 Necine Base Biosynthesis

At first sight, necine bases reveal the identity of PAs due to their characteristic bicyclic ring system containing a nitrogen at position 4 within the 1-hydroxymethyl pyrrolizidine (see Figure 1.3).

Tracer studies with ^{14}C-labelled putative precursors (Hartmann and Toppel 1987, Sander and Hartmann 1989) revealed a pathway that starts from arginine which is decarboxylated to agmatine and then transformed to *N*-carbamoyl putrescine (CAP, Figure 1.4). The amido group is removed and putrescine is released. It can be converted to spermidine and reverted to putrescine again. With the help of homospermidine synthase (HSS), homospermidine is produced by transferring the aminobutyl moiety from spermidine to putrescine, releasing diaminopropane (Böttcher et al. 1993, Ober and Hartmann

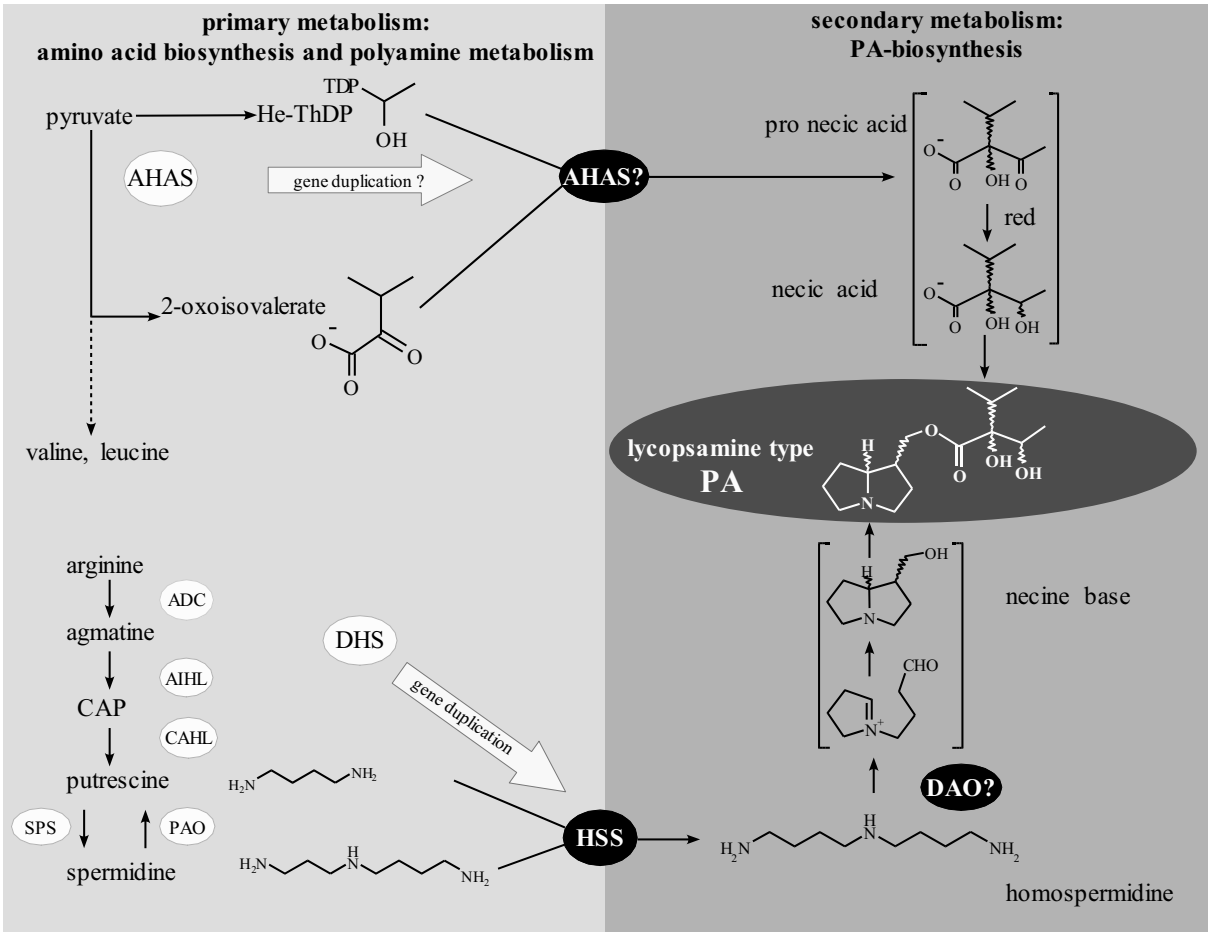


Figure 1.4: PA-biosynthesis is deduced from enzymes and substrates from primary metabolism, i.e., the amino acid biosynthesis and polyamine metabolism according to Hartmann and Ober 2000. Postulated parts of the pathway are tagged by a question mark, postulated intermediates are enclosed in square brackets (ADC = arginine decarboxylase, AHAS = acetohydroxyacid synthase, AIHL = agmatine iminohydrolase, CAHL = *N*-carbamoylputrescine amidohydrolase, CAP = *N*-carbamoylputrescine, DAO = diamine oxidase, He-ThDP = hydroxyethyl thiamine diphosphate, HSS = homosperimidine synthase, PA = pyrrolizidine alkaloid, PAO = a putrescine producing polyamine oxidase, SPS = spermidine synthase).

1999b, Hartmann and Ober 2000).

The next step in the biosynthesis of the necine base has not yet been clarified at the enzyme level. In the presence of β-hydroxyethylhydrazine (HEH), which is a diamine oxidase inhibitor, *Senecio* root cultures were shown to accumulate homospermidine (Böttcher et al. 1993). More recently, the same result were confirmed with various organs from *Heliotropium indicum*. Inhibiting trials combined with ^{14}C-putrescine tracer technique revealed that HEH completely interrupted PA biosynthesis directly after the homospermidine synthesis, homospermidine accumulated and appeared to function exclusively as unique necine base precursor (Frölich et al. 2007). Hence, the postulated intermediate dialdehyde might be formed by a diamine oxidase like enzyme (DAO? in Figure 1.4). The postulated dialdehyde might undergo cyclization resulting in the 1-hydroxymethylpyrrolizidine main body of necine bases via an intermediate iminium ion.

HEH inhibitor studies had provided evidence that the HSS is the first pathway specific enzyme in the formation of the necine base and, moreover, in the PA biosynthesis. Thus, the HSS is active at the interface between primary (amino acid) and secondary (PA) metabolism (Figure 1.4).

The HSS of *Senecio vernalis* was cloned, sequenced, and overexpressed in *E. coli* (Ober and Hartmann 1999b). High sequence similarities were shown between plant HSS and deoxyhypusine synthase (DHS) which catalyzes the first step in the activation of the eukaryotic initiation factor 5A (eIF5A, Chen and Liu 1997). DHS transfers an aminobutyl moiety from spermidine to the ϵ-amino group of a specific lysine residue in eIF5A. HSS catalyses exactly the same reaction but uses putrescine instead of eIF5A as an acceptor for the aminobutyl moiety (Ober et al. 2003b). While DHS is capable of accepting both substrates, eIF5A and putrescine, HSS only utilizes putrescine as an acceptor molecule for the transfer of an aminobutyl group. Hence, the HSS can be considered to be a modified DHS that lost its ability to bind one of the two original substrates with a still very well conserved catalytic activity. Noteworthy, the DHS main function is seen in the post-translational activation of eIF5A that was shown to be essential for cell growth and proliferation in *Saccharomyces cerevisiae* (Park et al. 1998) which is clearly part of the primary metabolism. The synthesis of homospermidine is considered a side reaction of the DHS which might explain the occurrence of homospermidine at least in traces even in non-PA-producing plants (Ober et al. 2003a). Hence, the detection of the PA precursor homospermidine in a plant is no indicator that this plant species is at the border of evolving the PA biosynthesis.

1.1.2.2 Necic Acid Biosynthesis

In contrast to the biosynthesis of the necine base, the knowledge concerning the biosynthesis of the necic acids is rather fragmentary and has not yet been clarified at the enzyme level. Tracer studies revealed that all aliphatic necic acids are derived from branched-chain amino acids (Hartmann and Witte 1995). Best examined are class I necic acids (Figure 1.2 on page 13) from senecionine, monocrotaline, and triangularine type PAs. Crout and co-workers showed that isoleucine is incorporated once into monocrotalic acid

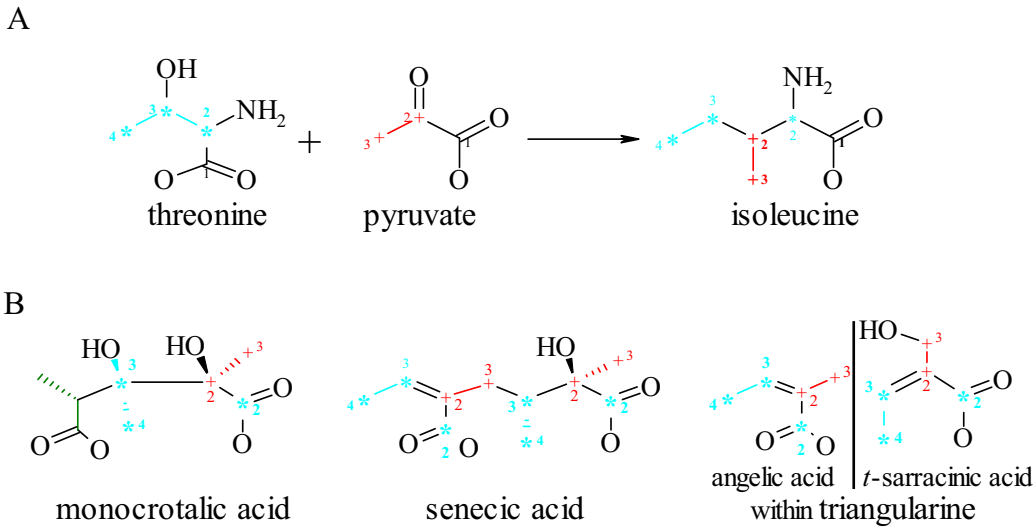


Figure 1.5: Isoleucine is incorporated into necic acids of group I PAs: carbon atoms highlighted in cyan (*) originate from threonine, while carbon atoms highlighted in red (+) originate from pyruvate.

(Robins et al. 1974) as well as into triangularine type necic acids (angelic acid: Crout 1967), where in macrocyclic senecionine type necic acids (seneciphyllic acid: Crout et al. 1966, 1970, senecic and isatinecic acid: Crout et al. 1972, Bale et al. 1978, Cahill et al. 1980) isoleucine is incorporated twice (Figure 1.5).

Moreover, leucine was shown to be introduced to senecioic acid (O'Donovan and Long 1975) while threonine/isoleucine labeled one half and valine/leucine labeled the other half of the trichodesmic acid from senecionine type PAs (Devlin and Robins 1984).

Studies by Crout (1966) on the biosynthesis of lycopsamine type necic acids performed on hound's tongue (*Cynoglossum officinale*, Boraginaceae) indicated that valine is incorporated, but as valine only provides 5 carbon atoms, Crout suggested that the missing two-carbon-atom unit for a complete C7 necic acid is inserted at the α-carbon atom of either valine or the corresponding product of transamination, 2-oxoisovalerate, via an "active acetaldehyde" in an acyloin condensation. Tracer studies with ^{13}C-labelled glucose (Weber et al. 1999) provided further indications that the "active acetaldehyde" introduced into lycopsamine type necic acids is the same used in branched-chain amino acid biosynthesis (Figure 1.6): the hydroxyethyl thiamine diphosphate (He-ThDP).

The enzyme transferring the He-ThDP in primary metabolism is an acetohydroxyacid synthase (AHAS, EC 2.2.1.6). In catalyzing the first step of the biosynthesis of valine, leucine, and isoleucine (Umbarger and Brown 1958), the AHAS is able to recognize two different substrates: pyruvate and 2-oxobutyrate (Umbarger and Brown 1958, Radhakrishanan and Snell 1960). Supposing an enlarged substrate specificity, the enzyme may accept 2-oxoisovalerate to form an intermediate in necic acid biosynthesis (Figure 1.6).

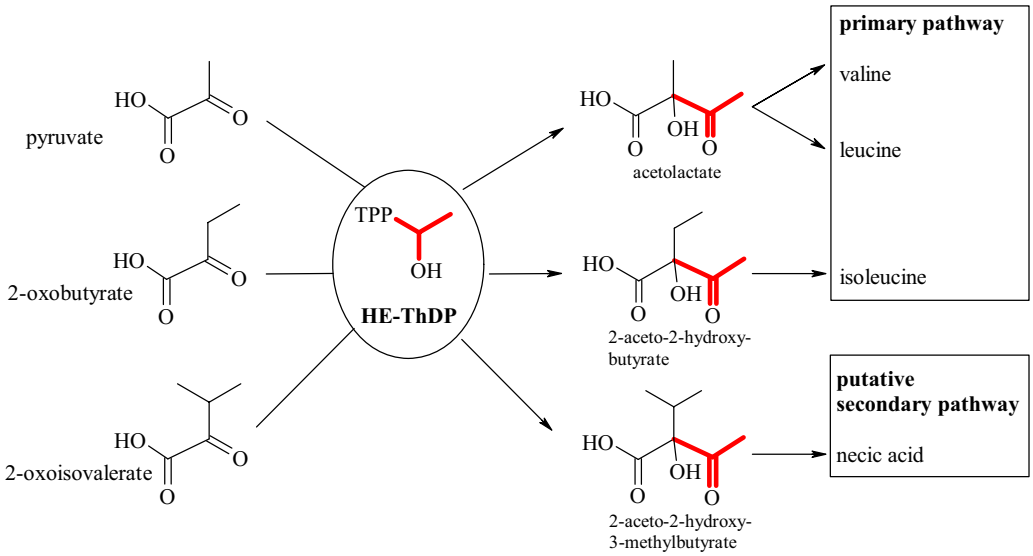


Figure 1.6: Postulated broadening of the substrate specificity of a postulated AHAS-like enzyme catalyzing the first step in necic acid biosynthesis within PA-producing plants. As shown by Crout 1966, a two-carbon-atom unit (highlighted in red) is inserted at the α-carbon atom of 2-oxoisovalerate via the "active acetaldehyde" He-ThDP (hydroxyethyl thiamine diphosphate). Tracer studies with ^{13}C-labbelled glucose, performed by Weber et al. (1999), confirmed that valine (precursor: acetolactate) shows the same labbelling patterns as the lycopsamine type necic acids (precursor: 2-aceto-2-hydroxy-3-metylbutyrate).