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Tissue distribution and biosynthesis of 1,2-saturated pyrrolizidine alkaloids in *Phalaenopsis* hybrids (Orchidaceae)

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Abstract

Phalaenopsis hybrids contain two 1,2-saturated pyrrolizidine monoesters, T-phalaenopsine (necine base trachelanthamidine) and its stereoisomer Is-phalaenopsine (necine base isoretronecanol). T-Phalaenopsine is the major alkaloid accounting for more than 90% of total alkaloid. About equal amounts of alkaloid were genuinely present as free base and its *N*-oxide. The structures were confirmed by GC–MS. The quantitative distribution of phalaenopsine in various organs and tissues of vegetative rosette plants and flowering plants revealed alkaloid in all tissues. The highest concentrations were found in young and developing tissues (e.g., root tips and young leaves), peripheral tissues (e.g., of flower stalks) and reproductive organs (flower buds and flowers). Within flowers, parts that usually attract insect visitors (e.g., labellum with colorful crests as well as column and pollinia) show the highest alkaloid levels. Tracer feeding experiments with ^{14}C-labeled putrecine revealed that in rosette plants the aerial roots were the sites of phalaenopsine biosynthesis. However active biosynthesis was only observed in roots still attached to the plant but not in excised roots. There is a slow but substantial translocation of newly synthesized alkaloid from the roots to other plant organs. A long-term tracer experiment revealed that phalaenopsine shows neither turnover nor degradation. The results are discussed in the context of a polyphyletic molecular origin of the biosynthetic pathways of pyrrolizidine alkaloids in various scattered angiosperm taxa. The ecological role of the so called non-toxic 1,2-saturated pyrrolizidine alkaloids is discussed in comparison to the pro-toxic 1,2-unsaturated pyrrolizidine alkaloids. Evidence from the plant-insect interphase is presented indicating a substantial role of the 1,2-saturated alkaloids in plant and insect defense.

Keywords: Phalaenopsis hybrids; Orchidaceae; 1,2-Saturated pyrrolizidine alkaloid; Pyrrolizidine alkaloid biosynthesis; Alkaloid tissue distribution; Phalaenopsine; Molecular evolution

1. Introduction

Pyrrolizidine alkaloids represent an excellent system to study not only phytochemical and biochemical aspects of plant secondary metabolism (Hartmann and Witte, 1995; Hartmann and Ober, 2000) but also its molecular evolution (Hartmann and Ober, 2000; Ober et al., 2003; Reimann et al., 2004). Using these alkaloids as a model system we are trying to understand in more detail, how a biogenetic pathway was recruited during evolution and integrated into the plant metabolism.

Pyrrolizidine alkaloids are esters of a necine base with one or more necic acids. They are produced constitutively by the plant most probably as defense against herbivores. Pyrrolizidine alkaloids are strong feeding deterrents for most herbivores and are part of many fascinating interactions between the producing plant and adapted herbivores (Hartmann, 1999; Hartmann and Ober, 2000). The occurrence of more than 400 structures is restricted to certain unrelated angiosperm taxa. About 95% of the species containing pyrrolizidine alkaloids belong to only five families, viz. the Asteraceae (tribes Senecioneae and Eupatorieae), the Boraginaceae, the Apocynaceae, the genus *Crotalaria* within the Fabaceae and in certain genera of the Orchida-

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ceae. Further occurrences are reported from single species of other families like the Celastraceae, Santalaceae, Sapotaceae, Ranunculaceae and Convolvulaceae (Hartmann and Witte, 1995; Jenett-Siems et al., 2005). In plants the pyrrolizidine alkaloids are mostly found in the form of their polar, salt-like *N*-oxides (Hartmann and Ober, 2000).

The occurrence of pyrrolizidine alkaloids in the Orchidaceae have been first described in the early 1960s and the 1970s by Brandänge and coworkers (Luning et al., 1966; Brandänge and Luning, 1969, 1970; Brandänge et al., 1970, 1971, 1972; Brandänge and Granelli, 1973). To date, in the Orchidaceae pyrrolizidine alkaloids have been found in eight genera, viz. *Liparis*, *Malaxis*, *Cysis*, *Phalaenopsis*, *Vanda*, *Vandopsis* (Hartmann and Witte, 1995), *Pleurothallis* (Borba et al., 2001), and *Cremastra* (Ikeda et al., 2005) belonging to five tribes of the subfamily Epidendroideae (Cameron, 2004). More than ten unique orchid alkaloids are known, representing the phalaenopsine type of pyrrolizidine alkaloids (Hartmann and Witte, 1995). Alkaloids of the phalaenopsine type are monoesters of 1,2-saturated simple necines bases like trachelanthamidine and its stereoisomers (Fig. 1). The typical necic acids are mostly aralkyl acids (Fig. 1).

The genus *Phalaenopsis* with about 70 species is native in the humid forests from South Asia to North Australia. *Phalaenopsis* species grow as epiphytes mainly on trees without parasitizing them. *Phalaenopsis* species are monopodial plants as they grow as a single stem without pseudobulbs. The inflorescence emerges from the leaf rosette. Aerial roots allow the plants to attach to their support and ensure water and nutrient supply. *Phalaenopsis* species are popular as ornamental plants and are used for many decades for breeding, resulting in several thousands of intra and interspecific hybrids. Since Brandänge's pioneering work no other studies dealt with the orchid alkaloids. The occurrence of pyrrolizidine alkaloids in monocots deserves closer attention since we showed that the first specific enzyme of pyrrolizidine alkaloid biosynthesis, i.e. homospermidine synthase (EC 2.5.1.45), was recruited at least four times independently during angiosperm evolution. The independent origin of the pathways leading to pyrrolizidine alkaloids in these lineages is likely and would account for the scattered occurrence of pyrrolizidine alkaloids in isolated, unrelated families of the angiosperms (Reimann et al., 2004). As a basis for more detailed molecular studies the present investigation was carried out. Using *Phalaenopsis* hybrids as experimental plant we established the alkaloid profiles and distribution pattern within the various plant organs and performed tracer feeding studies to outline the biosynthetic pathway and identify its tissue localization. The results are discussed in an evolutionary context, particularly addressing the ecological role of the so-called non-toxic 1,2-saturated pyrrolizidine alkaloids.

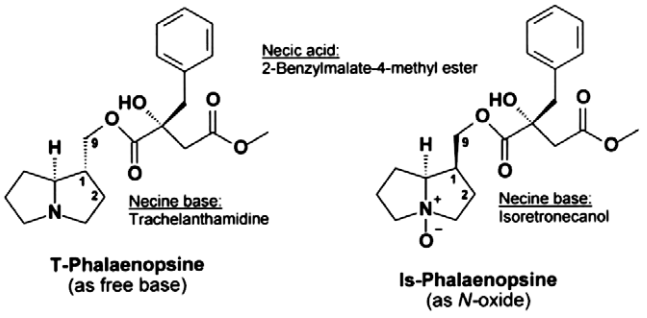


Fig. 1. Structures of the 1,2-saturated pyrrolizidine alkaloids found in *Phalaenopsis* hybrids. Both alkaloids are found as mixture of the free base and its *N*-oxide.

2. Results

2.1. Alkaloid profiles

Alkaloid extracts of *Phalaenopsis hybrids* revealed the presence of two pyrrolizidine alkaloids which could be identified by GC–MS as T-phalaenopsine and Is-phalaenopsine (Brandänge et al., 1972; Hartmann et al., 2005a,b) (Fig. 1). These two alkaloids are characterized by a unique necic acid, 2-benzylmalate-4-methyl ester, esterified to the epimeric necine bases trachelanthamidine and isoretronecanol, respectively. In all studied *Phalaenopsis* hybrid plants T-phalaenopsine was found to be the dominating alkaloid, generally accounting for more than 90% of total alkaloids. The same ratio was found in different organs and tissues of individual plants analyzed. Since a separation of the two isomeric phalaenopsines was only achieved by GC but not HPLC or TLC, the term 'phalaenopsine' used in the following text refers to the mixture of both isomers.

In the various plant tissues phalaenopsine was always present as a mixture of the free base and its *N*-oxide (Fig. 1). The proportion of *N*-oxide accounted for 40–60% of total alkaloid. Since the *N*-oxides are easily reduced in the presence of weak reducing agents (Hartmann and Toppel, 1987), a control experiment was performed in which the standard extraction procedure was carried out in the presence of added ^{14}C-labeled phalaenopsine *N*-oxide. Analysis of the final extract by TLC confirmed that no artificial reduction had occurred during the extraction procedure. Furthermore, no phalaenopsine could be detected by GC if its *N*-oxide was injected. Direct HPLC analysis of crude plant extracts confirmed the presence of almost equal

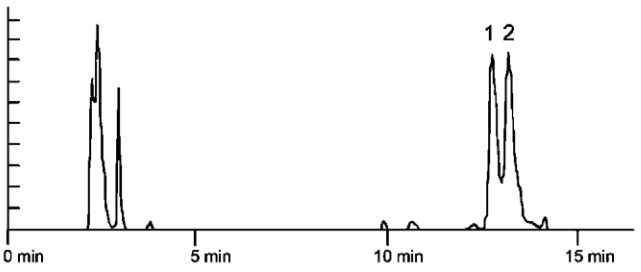


Fig. 2. HPLC separation of phalaenopsine (1) and its *N*-oxide (2) in crude methanolic extracts of *Phalaenopsis* roots. Separation was achieved using the gradient system; detection by UV at 280 nm.

amounts of the alkaloid free base and its *N*-oxide as illustrated in Fig. 2.

2.2. Tissue-specific alkaloid distribution

The quantitative distribution of phalaenopsine between various organs and tissues as well as its tissue concentrations were analyzed for a four-month-old *Phalaenopsis* plant in the rosette stage (Table 1A), a flowering plant (Table 1B) and an inflorescence with flower buds (Table 1C). In addition various organs and tissues of a single flower were analyzed (Table 1D). The major results are summarized as follows. In the rosette plant (Table 1A) about equal portions of phalaenopsine are found in roots (34%), leaves (34%), and the shoot apex (32%), respectively. The highest phalaenopsine concentration, i.e. 6-fold higher than the average concentration of the whole plant, was found in the shoot apex. About 75% of total leaf-alkaloid was found in the youngest leaf compared to only 6% in the oldest one. The phalaenopsine content and concentration in root tips is twice as high as the value measured for the basal part of the roots. Thus, in the rosette plant, the young still developing and growing organs or tissues, i.e. apical root parts, young leaves, shoot meristems show the highest alkaloid concentrations.

The analysis of a plant in bloom (Table 1B) revealed that the five flowers contained more than 50% of total plant phalaenopsine, the stalk and leaves about 20% and total roots a bit less than 30%. The highest alkaloid concentrations (i.e., 1.2 mg/g) were found in the flowers; they were about two to six times higher than those found in the vegetative organs. However, the total alkaloid concentration measured in the flowering plant was more than twice the concentration found in the rosette plant (Table 1A and B). By far the highest phalaenopsine levels are found in inflorescences with young flower buds (Table 1C). The alkaloid concentration of the whole inflorescence was about 10-fold higher than in rosette plants and 4–5-fold higher than the mean value of a plant in bloom. Young flower buds (6.3 mg alkaloid per g) contain about 2–3-fold higher alkaloid concentrations than old buds (i.e., 2.8 mg per g). Within the inflorescence stalk the alkaloid concentration decreased from the top (2 mg/g) to less than 0.3 in the basal part. The peripheral stem tissue showed a 6-fold higher alkaloid concentrations than the central tissue.

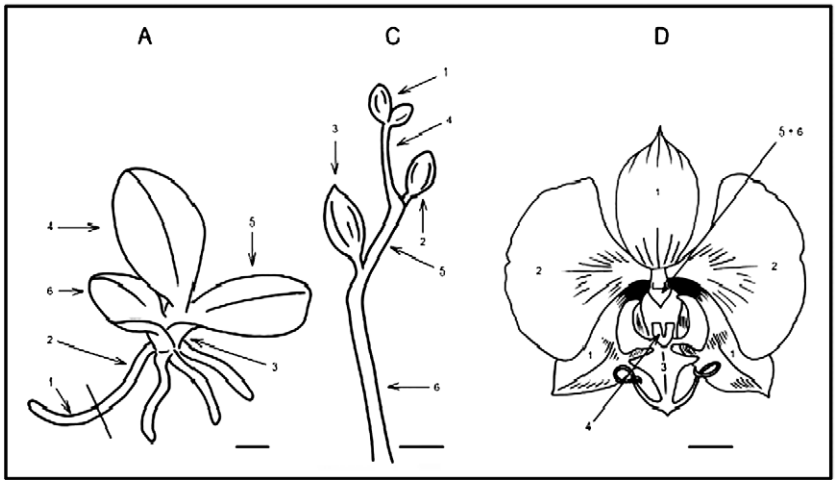
To get a picture of phalaenopsine distribution within flowers, a single flower was dissected and analyzed as shown in Table 1D. Alkaloid was present in all flower organs. Almost equal alkaloid concentrations were measured in sepals and petals, including the labellum. The whole perianth contained 58% of total alkaloid at tissue concentrations of 0.5–0.7 mg/g, whereas the remaining 42% were concentrated in the crests on the upper surface of the labellum and in the reproductive organs, i.e. the column and anther cup with the pollinia. These tissues reach 2–3-fold higher phalaenopsine concentration than the average of the whole flower.

A dramatic decrease in the phalaenopsine content was observed in withering flowers (Table 1D). Whether this loss is due to alkaloid export or degradation remains ambiguous.

2.3. Biogenetic tracer feeding experiments

To identify the site of phalaenopsine synthesis in *Phalaenopsis* plants, detached organs were incubated with ^{14}C-labeled putrescine as it was described earlier for *Senecio vulgaris* (Hartmann et al., 1989) and *Cynoglossum officinale* (Van Dam et al., 1995). Although the applied tracer was completely absorbed by detached roots, leaves, flowers, shoot apices and inflorescences within five days, no labeled phalaenopsine could be isolated from the tissues. The same negative results were obtained with [^{14}C]arginine as an alternative alkaloid precursor. In *Senecio* roots the diamine oxidase inhibitor, β-hydroxyethylhydrazine (HEH), causes an accumulation of homospermidine, the first pathway-specific intermediate of pyrrolizidine alkaloid biosynthesis, by preventing its incorporation into the alkaloids (Böttcher et al., 1993). In none of the orchid tissues did tracer feeding in the presence of 2 mM HEH cause accumulation of labeled homospermidine. Substantial tracer incorporation was only obtained when the tracer was fed via the aerial roots of undisturbed intact plants (Table 2). In this experiment the tracer was completely absorbed within two days and after another five days 38% of applied radioactivity could be recovered from the roots; 26% of the recovered radioactivity accounted for phalaenopsine. This corresponds to an incorporation rate of ca. 10%. Labeled putrescine was no longer detectable in the alkaloid extracts. The labeled phalaenopsine was present in the state of the free base; phalaenopsine *N*-oxide could not be detected. The identity of the labeled alkaloid was confirmed by radio TLC and radio HPLC in comparison to reference alkaloid. Since small amounts of labeled phalaenopsine (less than 3% of the recovered radioactivity) were also detected in young leaves, the ability of leaves to synthesize alkaloids could not be excluded. In a comparative experiment the potential biosynthetic capacity of roots and shoots were compared. The results are summarized in Table 3. Plant A received the tracer via an aerial root, plant B via a leaf. In both plants the tracer was completely absorbed within four days. Small amounts of tracer were only detectable in those organs, to which the tracer was applied and in which most of the recovered radioactivity was detected. This suggests a slow transport of the labeled compounds within the plant. In plant A, as expected, newly biosynthesized phalaenopsine was found in root extracts, whereas in plant B no alkaloid could be detected in the leaves. However the roots of plant B contained small but substantial amounts of labeled alkaloids. This indicates that the leaf is not capable of synthesizing phalaenopsine, but the tracer taken up by the leaf must have been translocated to the roots where it was incorporated into phalaenopsine. As traces of labeled phalaenopsine were also detected in the basal part

Table 1
Total content, concentration and relative abundance (alkaloid distribution) of phalaenopsine in various organs and tissues of *Phalaenopsis* hybrids



Plant organ	Fresh weight (g)	Alkaloid content (mg)	Alkaloid concentration (mg/g FW)	Alkaloid distribution (%)
(A) Rosette plant (4-months)				
Root tips (1)	0.57	0.11	0.20	22
Root basal parts (2)	0.53	0.06	0.12	12
Shoot apex (3)	0.14	0.16	1.15	32
Young leaf (4)	0.84	0.13	0.15	26
Middle leaf (5)	0.46	0.03	0.07	6
Old leaf (6)	0.22	0.01	0.03	2
Whole plant	2.76	0.50	0.18	100
(B) Flowering plant				
5 Flowers	28.9	33.8	1.2	52
Stalk	9.0	5.3	0.6	8
3 Leaves	54.9	8.5	0.2	13
Roots	60.5	18.8	0.3	28
Whole plant	153.3	66.4	0.4	100
(C) Inflorescence with buds				
Young flower buds (1)	0.16	1.01	6.31	20.7
Middle flower bud (2)	0.24	0.91	3.80	18.6
Old flower bud (3)	0.51	1.43	2.80	29.3
Stalk tip (4)	0.32	0.65	2.03	13.3
Stalk middle part (5)	0.50	0.64	1.28	13.1
Stalk basal part (6)				
Total	1.03	0.24	0.23	4.9
Peripheral tissues	0.41	0.20	0.49	
Central tissues	0.62	0.04	0.06	
Whole inflorescence	2.76	4.88	1.77	100
(D) Single flower				
3 Sepals (1)	1.00	0.69	0.69	24.6
2 Petals (2)	1.13	0.63	0.56	22.5
Labellum (3)	0.55	0.27	0.49	9.6
Crests (4)	0.19	0.31	1.63	11.1
Column (5)	0.39	0.89	2.28	31.8
Pollinia (6)	0.006	0.01	2.00	0.4
Whole flower	3.27	2.58	0.86	100
Flower slightly withered	2.41	0.15	0.07	
Flower completely withered	2.03	tr	tr	

(A) Rosette plant (vegetative state); the term 'shoot apex' refers to the apical shoot part engulfed by the leave sheaths; numbers in parenthesis refer to illustration A above the table. (B) Flowering plant. (C) Abscised inflorescence with flower buds; numbers in parenthesis refer to illustration C. (D) Single flower, numbers in parenthesis refer to the illustration D. Scale bars in illustrations: 1 cm.
tr, traces.

Table 2
Incorporation of [^{14}C]putrescine (6 μCi) into phalaenopsine by a young *Phalaenopsis* plant

Plant organ analyzed	Recovery of radioactivity applied (%)	^{14}C-labeled phalaenopsine	
		% of recovered radioactivity	Total incorporation (% of radioactivity applied)
Roots and shoot apex	38.2	25.6	9.8
2 Young leaves	0.7	2.7	tr
3 Old leaves	0.1	n.d.	n.d.

The tracer was applied via aerial root (2 days). Analysis after five days further incubation in water.
tr, traces; n.d., not detectable.

of the tracer root of plant A, this tissue was tested separately for its capacity to synthesize phalaenopsine. The apical part of an aerial root was removed. The remaining basal part of the root was fed with tracer for five days as well as the detached root tip. Another undamaged aerial root of the plant was fed as control (Table 3, plant C). As observed earlier the detached root tip was in contrast to the control unable to synthesize alkaloids. In comparison to the control root the decapitated root showed a 7-fold lower incorporation of tracer into phalaenopsine, indicating a reduced but significant ability of the basal root part to synthesize alkaloid.

To get more information on the transport, turnover and *N*-oxidation of phalaenopsine a biogenetic long-term experiment was performed. ^{14}C-labeled putrescine was applied to aerial roots of two rosette plants. In both plants the tracer was completely absorbed within 24 h. The two plants were dissected and analyzed after two and four weeks, respectively. No tracer was detected in any of the alkaloid extracts. The results are summarized in Table 4. Three major points need to be stressed: (1) There is a slow but substantial translocation of the newly synthesized alkaloid from the 'tracer-root' to the other roots, the shoot apex and young leaves, whereas older leaves did not receive newly synthesized alkaloids. (2) Phalaenopsine (free base) is accompanied by a small proportion of its *N*-oxide. However, in the various tissues, with one exception, the fractions of *N*-oxide are between 8% and 14% of total alkaloid and thus considerably lower than the 40–60% normally found in *Phalaenopsis* (see Fig. 2). It might be possible that *N*-oxidation is a slow continuous process affecting total plant phalaenopsine and is not yet recognizable within the population of newly synthesized labeled molecules even after four weeks. (3) The total incorporation of phalaenopsine is almost 3% after 2 weeks. This value does not change after another two weeks indicating that there is no substantial degradation or turnover of the alkaloids during this time interval.

The incorporation of various ^{14}C-labeled potential alkaloid precursors into phalaenopsine was tested to establish the polyamine-dependent alkaloid biosynthesis in *Phalaenopsis* (Table 5). Besides putrescine homospermidine and less pronounced spermidine are alkaloid precursors, whereas the amino acids arginine and ornithine, the direct precursors of putrecine and spermidine, are not incorporated at all. Labeled trachelanthamidine and isoretronecanol are incorporated into phalaenopsine but with low efficiency, i.e. 5%, respectively, 1% total incorporation. The higher rate with trachelanthamidine in comparison to isoretronecanol may reflect the dominating role of T-phalaenopsine in the mixture of the two stereoisomeric orchid alkaloids.

Table 3
Biosynthesis of phalaenopsine by *Phalaenopsis* rosette plants

Plant organ analyzed	Recovery of radioactivity applied (%)	% of recovered radioactivity		Total incorporation into alkaloid
		Putrescine	Phalaenopsine	
Plant A				
Tracer root (apical)	27.1	5.8	28.7	7.8
Tracer root (basal)	1.3	tr	1.8	0.02
Remaining roots	0.2	n.d.	n.d.	
Shoot apex	0.2	n.d.	n.d.	
Leaves	0.5	n.d.	n.d.	
Plant B				
Tracer leaf	23.5	7.7	n.d.	
Remaining leaf	n.d.	n.d.	n.d.	
Shoot apex	3.0	tr	tr	
Roots	2.6	n.d.	5.6	0.2
Plant C				
Undamaged root	29.3	6.1	28.2	
Decapitated root	29.7	7.0	3.9	
Detached root tip	18.9	10.0	n.d.	

Plant A received [^{14}C] putrescine (3 μCi) via an aerial root; plant B received the same amount via a leaf. In plant C one root received the tracer in the same manner as plant A (undamaged root, control), a second root was decapitated and both, the detached root tip and the basal root part still attached to the plant, were fed with tracer. Analysis was carried out after a feeding time of four days.
tr, traces; n.d., not detectable.

Table 4
Biosynthesis of phalaenopsine and its *N*-oxide by *Phalaenopsis* rosette plants

Plant organ analyzed	Analysis after 2 weeks				Analysis after 4 weeks			
	Total ^{14}C-activity recovered (%)	% of recovered radioactivity		Total incorporation into alkaloid (%)	Total ^{14}C-activity recovered (%)	% of recovered radioactivity		Total incorporation into alkaloid (%)
		Phalaenopsine	Phalaenopsine *N*-oxide			Phalaenopsine	Phalaenopsine *N*-oxide	
Tracer root (apical)	10.5	20.7	2.6	2.5	7.7	23.6	2.4	2.0
Tracer root (basal)	1.8	2.3	6.7	0.2	2.5	3.1	tr	0.1
Remaining roots	2.1	2.9	n.d.	0.1	3.3	3.9	n.d.	0.1
Shoot apex	0.5	10.0	n.d.	0.1	0.7	40.8	n.d.	0.3
Young leaves	0.8	3.8	n.d.	tr	0.8	40.5	4.8	0.4
Middle old leaves	0.5	tr	n.d.		0.4	n.d.	n.d.	
Old leaves	0.3	n.d.	n.d.		0.2	n.d.	n.d.	
Whole plant	16.5	14.3	2.4	2.9	15.6	16.9	1.4	2.9

Plants received [^{14}C]putrescine (150 μmol; 6 μCi per plant) via an aerial root. Plants were harvested and analyzed after 2, respectively, 4 weeks as indicated. The [^{14}C]putrescine has been completely absorbed by the roots after 24 h; it was no longer detectable in the tissue extracts after 2 weeks.
tr, traces; n.d., not detectable.

3. Discussion

3.1. Tissue distribution of phalaenopsine suggests ecological relevance

T-phalaenopsine accompanied by a small portion of its stereoisomer Is-phalaenopsine were the only pyrrolizidine alkaloids detected in *Phalaenopsis* hybrids. Phalaenopsine was found in all plant organs and tissues. The allover tissue distribution indicates always highest concentrations in young and developing tissues (i.e., root tips, shoot apex and young leaves), peripheral tissues (e.g., of the flower stalk) and reproductive organs. The highest alkaloid concentrations with 0.63% (fresh weight basis) were found in the youngest flower buds. This is more than 7-fold of the concentrations found in fully extended flowers (0.09%). The apparent decrease in the alkaloid concentration during flower development is not due to alkaloid loss or export, it is a 'dilution' by increase of fresh weight. In fact, during flower development a 'middle old bud' (Table 1C) increases its fresh weight almost 14-fold but its alkaloid content only 3-fold (see Table 1C and D). Within the open flower highest alkaloid concentrations were found in the column, the pollinia and the crests of the labellum. The latter exhibits a conspicuous bait for insect visitors. These are precisely the tissues that are visited by desired and undesired orchid pollinators and that are most important for the reproductive success of the plant. In general, the quantitative alkaloid distribution within an orchid plant resembles the distribution reported for pro-toxic 1,2-unsaturated pyrrolizidine alkaloids in *S. vulgaris* (Asteraceae), i.e. highest concentrations in flowers and peripheral stem tissues (Hartmann and Zimmer, 1986; Hartmann et al., 1989). It is well in accordance with the "optimal defense theory" that explains the allocation of defense metabolites preferentially in those tissues that have a high probability of herbivore attack and in those of higher value for reproductive fitness, e.g., meristems, other young organs, flower parts near ovaries and pollen (McKey, 1974, 1979).

3.2. Are 1,2-saturated pyrrolizidine alkaloids defense compounds? Chemoecological evidence

An assumed role of phalaenopsine in plant defense against herbivores raises the question of its biological activity. Pyrrolizidine alkaloids are feared for their toxicity. Pyrrolizidine alkaloid-containing plants are probably the most common poisonous plants affecting livestock, wildlife, and humans (Mattocks, 1986; Stegelmeier et al., 1999). The molecular basis of pyrrolizidine alkaloid toxicity is well understood. There are at least two essential structural features for toxicity: (1) a 1,2-double bond and (2) associated with it an allylic hydroxyl group at C-9 that needs to be esterified. A second OH-group at C-7, free or esterified, intensifies the toxicity. Alkaloids of this structure are not toxic per se but they require metabolic activation to exert toxicity. In vertebrates this bioactivation is mediated

Table 5
Incorporation of potential ^{14}C-labeled precursors into phalaenopsine

Tracer applied	Recovery of radioactivity applied (%)	Percent of radioactivity recovered		Total incorporation into alkaloid (%)[a]
		Tracer	Phalaenopsine	
L-[U–^{14}C]Arginine	13.2	30.2	n.d.	
L-[U–^{14}C]Ornithine	14.3	6.2	n.d.	
[^{14}C]Putrescine	25.6	tr	40.4	10.3
[^{14}C]Spermidine	23.0	16.4	1.8	0.3
[^{14}C]Spermine	27.4	18.8	n.d.	
[^{14}C]Homospermidine	55.9	13.6	13.4	7.5
[^{14}C]Trachelanthamidine	26.7	68.6	18.8	5.0
[^{3}H]Isoretronecanol	25.3	63.6	4.1	1.0

The tracer was applied to the root-tip of an aerial root of an intact plant. After 4 days the tracer root was analyzed.
tr, traces; n.d., not detectable.
[a] Radioactivity applied, 100%.

by liver cytochrome P-450 enzymes that convert the pro-toxic alkaloids into pyrrolic intermediates which rapidly react with biological nucleophiles, i.e. proteins and nucleic acids, causing severe cell toxicity and even liver cancer (Fu et al., 2004). Since insects possess a similar cytochrome P-450 system like vertebrates (Brattsten, 1992) pyrrolizidine alkaloids are also cytotoxic and genotoxic for insects (Frei et al., 1992; Narberhaus et al., 2005).

The 1,2-saturated pyrrolizidine alkaloids like phalaenopsine lack the 1,2-double bond and thus an allylic OH-group and therefore are regarded as non-toxic, at least as far as P-450-dependent bioactivation is concerned. Generally they are neglected when toxicity of pyrrolizidine alkaloids is discussed. Many species of the poisonous genera *Senecio, Eupatorium* (Asteraceae) and *Heliotropium* (Boraginaceae) contain mixtures of pro-toxic 1,2-unsaturated and non-toxic 1,2-saturated pyrrolizidine alkaloids (Hartmann and Witte, 1995). Among them there are even single species like *Heliotropium floridum* (Reina et al., 1997) that contain only 1,2-saturated alkaloids. From two plant families only 1,2-saturated pyrrolizidine alkaloids are known. These are the Orchidaceae discussed here, and the Convolvulaceae, where a few species belonging to the subgenus Quamoclit of the large genus *Ipomoea* contain the ipangulines, i.e. monoesters and diesters of 1,2-saturated platynecine (Jenett-Siems et al., 1998, 2005).

The biological activity of 1,2-saturated pyrrolizidine alkaloids is largely unknown. We are aware only two reports: insect antifeedant activity has been demonstrated for some trachelanthamidine esters (Reina et al., 1997) and most interestingly cremastrine, an isoretronecanol ester with isocaproic acid, isolated from the orchid *Cremastra appendiculata* inhibits the binding of anticholinergic drugs to the muscarinic M3 receptor (Ikeda et al., 2005). The tissue-specific alkaloid distribution as outlined above for *Phalaenopsis,* represents a strategy one would expect for a plant chemical defense system that evolved under the pressure of herbivory. Further strong but indirect evidence suggesting a defense role of 1,2-saturated pyrrolizidine alkaloids comes from the fascinating field of chemical ecology of plant-insect interactions. A number of specialized insects, e.g. leaf beetles, butterflies, moths, developed highly specific adaptations that allow them to sequester pro-toxic pyrrolizidine alkaloids from their host-plants and utilize them for their own benefit particularly as defense against their predators (Hartmann, 1999, 2004; Hartmann and Ober, 2000; Eisner et al., 2002). Among tiger moths (Arctiidae) there are species that as larvae not only sequester pro-toxic 1,2-unsaturated pyrrolizidines from their food plants but also 1,2-saturated alkaloids. The highly polyphagous larvae of the arctiids *Estigmene acrea* and *Grammia geneura* locate pyrrolizidine alkaloid-containing plants incidentally but immediately recognize them by taste receptors in their mouthparts that are phagostimulatory (Bernays et al., 2002a,b). These receptors specifically recognize all kinds of pro-toxic pyrrolizidine alkaloids but interestingly also non-toxic 1,2-saturated alkaloids (Hartmann et al., 2005b). Both kinds of pyrrolizidine alkaloids are sequestered by larvae and are transmitted to the adult stages during metamorphosis. However pyrrolizidine alkaloids that do not fulfill certain structural requirements cannot be directly transmitted to the pupal stage, before transmission; they need to be converted into transmittable 'insect alkaloids' (i.e., creatonotines and callimorphines) by trans-esterification. Surprisingly, not only the 1,2-unsaturated pyrrolizidines are recovered in this way but also 1,2-saturated structures, including phalaenopsine (Hartmann et al., 2005a,b). This specific biochemical behavior suggests strongly that not only the 1,2-unsaturated alkaloids are involved in the well documented defense of herbivorous insects against their predators and parasitoids (Eisner et al., 2002) but that also the largely neglected class of 1,2-saturated alkaloids must be of ecological importance.

3.3. Aspects of phalaenopsine biosynthesis

When discussing phalaenopsine biosynthesis it should be recalled that pyrrolizidine alkaloid biosynthesis in angiosperms is of polyphyletic origin. Evidence has been presented that the gene encoding homospermidine synthase, the first pathway-specific enzyme of necine base

formation, has been recruited several times independently from gene duplicates of ubiquitous deoxyhypusine synthase (EC 2.5.1.46) in dicots and at least once within monocots (Ober and Hartmann, 1999; Ober et al., 2003; Reimann et al., 2004; Ober, 2005). Putrescine, spermidine and homospermidine are precursors of phalaenopsine indicating that the basic route of necine biosynthesis should be the same as in *Senecio* that has been studied in detail (for review see Hartmann and Ober, 2000). However, there are some differences. In contrast to *S. vulgaris* roots (Hartmann et al., 1988) spermidine is a much less efficient precursor in *Phalaenopsis* and the polyamine precursors arginine and ornithine are not incorporated at all. This may reflect differences of polyamine metabolism at the interphase between primary and secondary metabolism that need to be studied in more detail. In the rosette plant the root is the only site of alkaloid biosynthesis and active synthesis requires the intact root-shoot connection and comes to rest if the root is detached. This behavior shows some parallels to alkaloid biosynthesis in *Senecio* and *Eupatorium* roots that only synthesize alkaloids when they are actively growing (Hartmann et al., 1988; Sander and Hartmann, 1989; Anke et al., 2004). Since in both plants the alkaloids synthesized in roots and are translocated into the shoot and do not underlie any turnover it is important to adjust the rate of alkaloid synthesis to plant growth. In *Phalaenopsis* alkaloid biosynthesis requires apparently an endogenous signal transmitted from the root base to the apex.

4. Experimental

4.1. Plant material

Young up to 4-month-old rosette plants of *Phalaenopsis* hybrids were obtained from local orchid breeding stations (Hennis Orchideen, Hildesheim and Wichmann Orchideen, Celle, Germany). The plants were kept in a greenhouse till use. Flowering *Phalaenopsis* hybrids were obtained from the same breeders or as ornamental plants from the local market.

4.2. Alkaloid extraction and purification for GC and HPLC analysis (Witte et al., 1993)

Fresh plant material was 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). The alkaloid 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 pyrrolizidine alkaloids, i.e. free base plus *N*-oxide.

4.3. Identification of phalaenopsines by GC–MS

The GC–MS data were obtained with a Hewlett Packard 5890A gas chromatograph equipped with a 2 m fused silica guard column (deactivated, ID 0.32 mm) and a 30 m × 0.32 mm analytical column (DB-1 J&W Scientific or ZB-1 Phenomenex). The capillary column was directly coupled to a triple quadrupole mass spectrometer (TSQ 700, Finnigan). The conditions applied were: Injector and transfer line were set at 280 °C; the temperature program used was: 100 °C (3 min)–300 °C at 6 °C min^{-1} for the separation of pyrrolizidine alkaloids and 70 °C (6 min)–300 °C at 10 °C min^{-1} for the separation of necic acid methyl esters, respectively. The injection volume was 1 μl. Depending on the concentration the split ratio was 1:20 or splitless, the carrier gas flow was 1.6 ml min^{-1} He, and the mass spectra were recorded at 70 eV.

The alkaloids were identified by their R_I, molecular ions and mass fragmentation patterns in comparison to reference data. In addition the purified alkaloids were hydrolyzed and the respective necine bases identified by GC–MS. Hydrolysis was performed in 8% NaOH at 60 °C for 90 min. GC–MS data of the phalaenopsines:

T-phalaenopsine: R_I (ZB-1), 2522; [M^+], 361; necine base, trachelanthamidine, R_I (DB-1), 1236; [M^+], 141.
Is-phalaenopsine: R_I (ZB-1), 2560; [M^+], 361; necine base, isoretronecanol, R_I (DB-1), 1257; [M^+], 141.

4.4. Alkaloid separation by GC and HPLC

Routine GC was performed using a capillary column (15 m × 0.25 mm fused-silica; DB-1, J&W Scientific) (Witte et al., 1993). All other GC conditions were the same as given for GC–MS. Detectors, FID and PND. Quantitative analyses were performed via the FID signals using heliotrine as internal standard.

The separation of phalaenopsine and phalaenopsine *N*-oxides was achieved by HPLC using the method according to Hartmann et al. (1988) that had to be adapted due to the low polarity of the phalaenopsines. For a gradient elution system two mobile phases were used. Mobile phase A: mixture of 150 ml acetonitrile and 850 ml H_3PO_4 (1.5% v/v). Mobile phase B: mixture of 500 ml acetonitrile and 500 ml H_3PO_4 (1.5% v/v). A linear gradient was formed starting with 100% eluent A leading in 30 min to 100% eluent B. For an isocratic system a mobile phase of 250 ml acetonitrile and 750 ml TFA in water (pH adjusted to 2.0) was applied. The flow-rate was 1.0 ml per min. The alkaloids were detected at 280 nm after a retention time for phalae-

nopsine and phalaenopsine *N*-oxide of 13.1 min and 13.4 min, respectively, with the gradient system, and 7.8 min and 8.5 min, respectively, with the isocratic system. Detection of ^{14}C-labeled phalaenopsine was achieved with a HPLC radioactivity monitor LB-506D (Berthold) equipped with a 2 ml flow cell and the splitt-mixer LB-5034. Rialuma (BAKER) was used as liquid scintillator.

4.5. Tracer-feeding experiments and product analysis

Most experiments were performed with [^{14}C]putrescine as tracer. Tracer was applied via aerial roots or leaves: the tip of an aerial root or a carved leaf tip of an intact plant was dipped into a reaction vial containing the tracer (3–6 μCi) solved in tap water. The absorption of the tracer-fluid was usually completed after 24 h. Afterwards the lost fluid volume was replaced by pure tap water till the end of the experiment. In experiments with β-hydroxyethylhydrazine the inhibitor was added 2 h prior to tracer application; the final inhibitor concentration was 2 mM.

For extraction, the plant organs 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 residue was dissolved in a defined volume of methanol and kept at −20 °C till analysis. Total radioactivity was determined by scintillation counting. 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 the solvent system CH_2Cl_2–MeOH–NH_3 (25%) (82:15:3). R_f values: phalaenopsine *N*-oxide, 0.56; phalaenopsine (free base), 0.65.

Other radiochemicals were obtained or prepared as follows: L-[U–^{14}C]arginine (320 mCi/mmol), L-[U–^{14}C] ornithine (282 mCi/mmol), [^{14}C]spermidine, and [^{14}C] spermine were purchase by Amersham Biosciences. [^{14}C]Homospermidine was prepared from [^{14}C]putrescine enzymatically using bacterial homospermidine synthase (EC 2.5.1.44) (Graser et al., 1998). [^{14}C]Trachelanthamidine was prepared by hydrolysis of [^{14}C]trachelanthamine obtained from *Eupatorium cannabinum* root cultures that had been fed with labeled putrescine (Hartmann, 1994) and [^{3}H]isoretronecanol prepared synthetically by Dr. Xia.

4.6. Preparation of phalaenopsine N-oxides

^{14}C-Labeled phalaenopsine was isolated, reduced and purified from a tracer experiment with [^{14}C]putrescine as described. The oxidation was performed according to Cymerman Craig and Purushothaman (1970). The purified [^{14}C]phalaenopsine was incubated in 1 ml 1 M *m*-chloroperbenzoic acid in chloroform for 30 min on an ice bath. The resulting *N*-oxide was purified via an alkaline aluminium oxide column using CH_1Cl_3–MeOH (3:1) as solvent. Radiochemical purity of the phalaenopsine *N*-oxide was confirmed by radio TLC and radio HPLC.

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