Prelandrine, the Key-Step Intermediate in the Biosynthesis of the Macrocyclic Spermine Alkaloid Aphelandrine

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Dedicated to Professor *Elmar Vilsmaier* on the occasion of his 60th birthday

By means of the high sensitive on-line-coupled high-performance liquid chromatography and atmospheric-pressure chemical-ionization mass spectrometry (HPLC/APCI-MS and HPLC/APCI-MS/MS) techniques, the new macrocyclic spermine alkaloid prelandrine (**5**) was detected in the roots of *Aphelandra squarrosa* (Acanthaceae), and its structure was elucidated as 4′-hydroxyprotoverbine (=8-(4-hydroxyphenyl)-1,5,9,13-tetraazacycloheptadecan-6-one). It was further demonstrated that protoverbine (**6**) is enzymatically hydroxylated to prelandrine (**5**) in a reaction catalyzed by microsomes from the roots of *A. squarrosa*. The chemical synthesis of (−)-(*S*)-prelandrine is also described. The possible key role of prelandrine (**5**) as an intermediate in the biosynthesis of aphelandrine (**1**) is discussed.

Introduction. – The macrocyclic spermine alkaloid aphelandrine (**1**; *Scheme*) is a member of a rather rare class of natural products. Aphelandrine (**1**) has been found as the major alkaloid in the roots of several *Aphelandra* species [1–3]. Although the structure of aphelandrine (**1**) displays *p*-coumaric acid and spermine (or spermidine and putrescine) as building blocks, little is known about the biosynthesis of this type of alkaloids. The feeding experiments with ^{3}H- and ^{14}C-labelled putrescine, spermidine, and coumaric acid confirmed the biogenetic structural units of aphelandrine; however, the question of whether mono- or dicoumaroylspermine is the precursor remained unanswered [4–6]. For the further steps, there are two hypotheses: the first involves cyclization of *N*(1),*N*(5)-di(*p*-coumaroyl)spermine (**3**) leading to the species **2**, which subsequently undergoes phenol coupling to give aphelandrine (**1**; *Scheme*) [7]. The second possibility is transformation of *N*(1)-cinnamoylspermine (**4a**) or *N*(1)(*p*-coumaroyl)spermine (**4b**) to the macrocycle **6** or **5**, respectively, followed by acylation to give **2**, which again undergoes phenol coupling to give aphelandrine (**1**; *Scheme*). When *N*(1)-cinnamoylspermine (**4a**) is cyclized by *Michael* addition, a hydroxylation step leading from **6** to **5** would be expected.

So far, there exists no evidence to support one of these possibilities, presumably because of the concentration limits of the conventional analytical methods. Application of high-sensitive on-line-coupled HPLC/APCI-MS (atmospheric-pressure chemical-ionization mass spectrometry) or ESI-MS (electrospray-ionization mass spectrometry) techniques allows the detection of substances, in some cases, at picomolar concentrations. Moreover, HPLC co-eluted substances can be easily differentiated by their molecular weights. The MS/MS spectra of selected quasi-molecular ions ($[M+H]^+$) very often provide substantial structural information without isolation.

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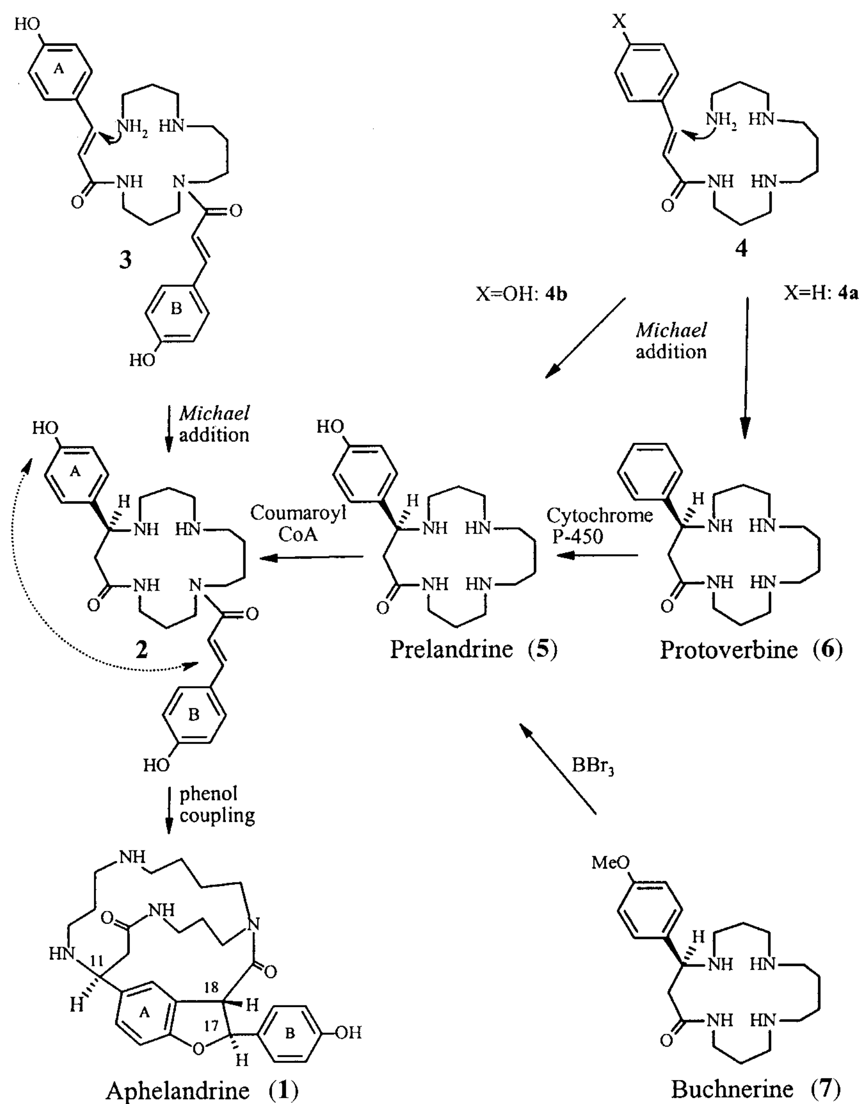
[1]) Part of the thesis of *L.N.*, Universität Zürich, 2000.

Scheme



A central step in the biosynthesis of aphelandrine (**1**) is the cyclization *via Michael* addition of the disubstituted intermediate **3** or the monosubstituted **4** to give species **2** and **5**, respectively (*Scheme*). Studies related to these two possibilities are the subject of the present paper. Enzymatic studies and the analysis of the plant root extracts from *A. squarrosa* by means of on-line-coupled HPLC/APCI-MS technique favor the second of the above-mentioned pathways for the biosynthesis of aphelandrine (**1**), *i.e.*, the conversion of **4** to **5**.

Results and Discussion. – So far, aphelandrine (**1**) has been found exclusively in the roots of different *Aphelandra* species. The cell cultures from *A. tetragona* and *A. sinclairiana* have been shown to be devoid of any alkaloids [8]. Aphelandrine (**1**) is easily detectable in the roots of *A. squarrosa* already 4 weeks after planting by shoot propagation. In *A. tetragona*, its content increases until 17 weeks of planting, afterwards it decreases [9]. On the basis of these results, 12-weeks-old roots of *A. squarrosa* were selected for the biochemical studies.

The biosynthesis of aphelandrine (**1**) presumably proceeds according to one of the pathways shown in the *Scheme*. The first hypothesis, *i.e.*, cyclization of the dicoumaroyl spermine **3** to the species **2**, followed by phenol coupling [7], is based on the established large distribution of mono-, di-, and polycinnamoyl-substituted polyamines as natural secondary metabolites in the plant kindgom (*cf.* [10] and the refs. cit. therein) and by the involvement of *N*(1),*N*(10)-di(*p*-coumaroyl)spermidine in the biosynthesis of the spermidine alkaloid lunarine [11]. Moreover, a large group of similar compounds, including coumaroyl spermidines, has been isolated from the anthers of different *Aphelandra* species [12]. Unfortunately, all attempts to detect the above mentioned dicoumaroylspermine **3** or any of its homologs in the root extracts of *A. squarrosa* were unsuccessful. Thus, its role as precursor remains uncertain.

According to the second possibility, *i.e.*, cyclization of the monocinnamoylspermine **4a** or monocoumaroylspermine **4b** to the species **6** and **5**, respectively, followed by acylation (coumaroyl CoA, corresponding acyl transferase) to **2**, and phenol coupling, an additional hydroxylation step in the case of **6** is necessary.

A more sophisticated extraction method, which allowed extraction of more polar alkaloids, and analysis by the highly sensitive on-line-coupled HPLC/APCI-MS technique paved the way leading to the decision concerning the possible pathways. Analysis of the alkaloid extract from young roots of *A. squarrosa* (400 g of 12-week-old roots) revealed an additional peak with the quasi molecular ion $[M+H]^+$ at *m/z* 349, besides the signals corresponding to known alkaloids. This *m/z* value corresponds to the intermediate (−)-(*S*)-4′-hydroxyprotoverbine (**5**) proposed in the second of the above-mentioned pathways (*Scheme*). To establish the structure of **5**, (−)-(*S*)-4′-hydroxyprotoverbine was synthesized from synthetic (−)-(*S*)-buchnerine (**7**) [13] through *O*-demethylation with Br_3B (*Scheme*). The data obtained from HPLC-UV (DAD)/APCI-MS and MS/MS measurements demonstrated that the synthetic and the natural products are identical (*Table 1*). The MS/MS experiments showed identical fragmentation patterns for the synthetic and the natural compounds (*Fig. 1*). Since (−)-(*S*)-4′-hydroxyprotoverbine (**5**) is a natural product, it was named prelandrine (**5**; *Scheme*). No trace of compounds **4** or **6** could be detected. The scarcity of material prevented us from establishing the configuration of the naturally occurring prelandrine (**5**). The presence of **5** in the root extracts of *A. squarrosa* investigated indicates its possible key role in the biogenesis of aphelandrine (**1**) and favors the second pathway.

The similarly constituted alkaloids protoverbine (**6**; 4′-unsubstituted analogue of prelandrine (**5**)) and buchnerine (**7**; *O*-methylated analogue of prelandrine (**5**)) were isolated earlier from *Verbascum pseudonobile* (Scrophulariaceae) [14] and *Clerodendrum buchneri* (Verbenaceae) [15]. They were isolated together with their corresponding *N*(1)-cinnamoylated derivatives, which are analogous to the expected ultimate precursor **2** of aphelandrine (**1**). This observation indicates that this metabolic

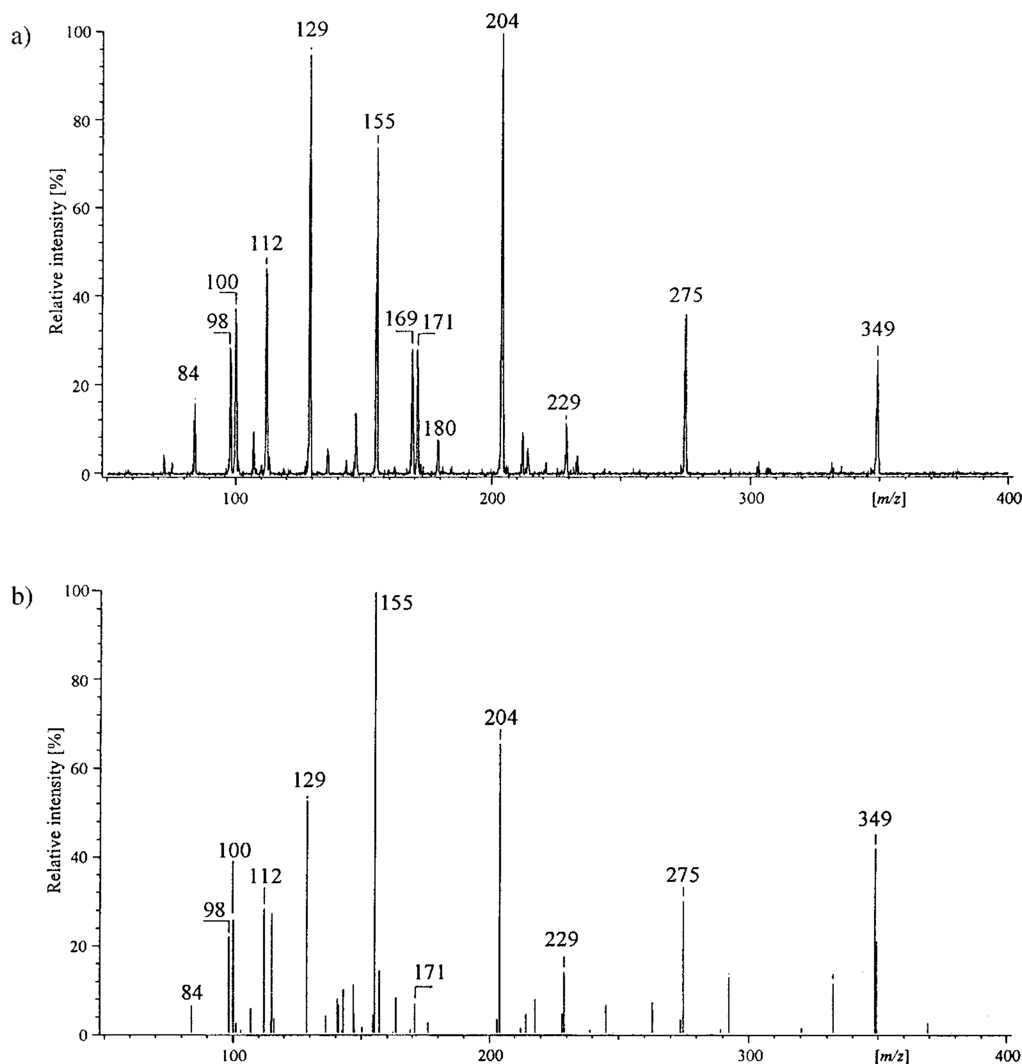


Fig. 1. *MS/MS Results of synthetically prepared prelandrine* (**5**) *as a standard* (*a*) *and prelandrine* (**5**) *detected in the root extract of* A. squarrosa (*b*). The synthetically prepared prelandrine (**5**) was used as a finger-print.

reaction sequence that occur in different plant families is widely spread in nature.

If *N*(1)-cinnamoylspermine (**4a**) is the substrate for the macrocyclization by *Michael* addition, then a hydroxylation step from **6** to **5** is to be expected. In the plant tissues, hydroxylations and several oxidative biochemical transformations are catalyzed by membrane-bound cytochrome P-450 mono-oxygenases. To test this assumption, microsomes were isolated from young roots of *A. squarrosa.* The incubation of the microsomes with the synthetically obtained (−)-(*S*)-protoverbine (**6**) in the presence of NADPH led to the hydroxylated derivative prelandrine (**5**). This was shown by the on-

Table 1. *Summary of the HPLC-UV (DAD)/MS Results*

	t_R [min]	UV_{max} [nm]	$[M+H]^+$
Prelandrine (**5**) as standard	1.20	280	349
Prelandrine (**5**) prepared enzymatically	1.30	280	349
Prelandrine (**5**) in the root extract	1.22	280	349

line-coupled HPLC/UV(DAD)/APCI-MS technique, with the synthetically obtained prelandrine (**6**) as a reference (*Table 1*).

To answer the question of whether this hydroxylation is enzymatically catalyzed by a microsome-bound cytochrome-P-450, several key criteria were tested. The significant hydroxylation activity was found in the microsomal fraction compared with the crude cell-free extracts. No activity was found with the concentrated soluble fraction. Heat inactivation of microsomes suppressed, or the presence of H_2O_2 did not result in product formation (data not shown).

Hydroxylation of protoverbine requires as cofactor NADPH, which could not be replaced by NADH. When the reaction mixture was purged with N_2 gas to displace O_2, no hydroxylation activity was detected. An absolute requirement for O_2 was additionally demonstrated by removal of O_2 from the assay through addition of the glucose/glucose oxidase/catalase system [16]. In this case, the hydroxylation activity was completely suppressed. No reduction in the enzyme activity was observed when the preincubation was performed with heat-inactivated glucose oxidase. Thus, hydroxylation activity is strictly dependent on molecular O_2 (*Table 2*).

Table 2. *Relative Enzyme Activities*

Assay conditions	Protoverbine hydroxylase activity[a])
Air bubbled (standard assay)	+++
N_2 bubbled	0
+ glucose + glucose oxidase + catalase	0
+ glucose + heat-denatured glucose oxidase + catalase	+++
NADPH + NADH	+++
NADH	+
CO/O_2 9 : 1, dark	0
CO/O_2 9 : 1, white light	++

[a]) The signs +, ++, and +++ indicate increasing activities.

A further specific reaction to substantiate cytochrome P-450 involvement is the inhibition of the enzyme by a CO/O_2 9 : 1 mixture [17]. The enzyme activity was strongly inhibited by CO/O_2 9 : 1 in the dark, and this inhibition could be partly reversed by illumination with white light (*Table 2*).

The presence of cytochrome P-450 in the microsomal preparation was shown by the CO difference spectrum of sodium-dithionate-reduced microsomes [18]. The spectrum exhibited the typical absorption maximum at 450 nm (*Fig. 2*).

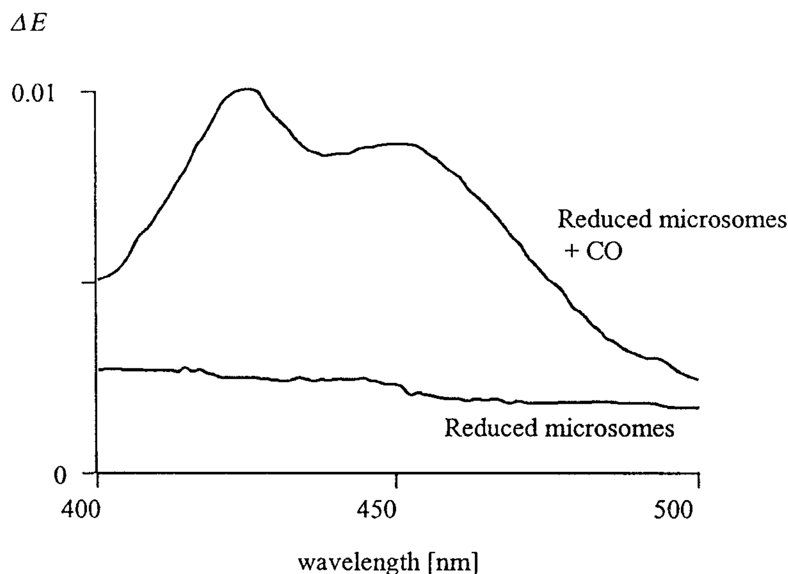


Fig. 2. *CO Difference spectrum of sodium-dithionite-reduced cytochrome P-450 from the roots of* Aphelandra squarrosa

Thus, it was shown that protoverbine (**6**) is hydroxylated to prelandrine (**5**) in a reaction catalyzed by a membrane-bound enzyme, which requires NADPH and O_2. The results indicate that this enzyme, protoverbine hydroxylase, is a cytochrome P-450 enzyme. Although protoverbine (**6**) is a substrate for cytochrome P-450, no trace of it could be detected in the extracts from the roots of *A. squarrosa* studied. Therefore, the question of whether prelandrine (**5**) is derived directly from coumaroylspermine **4b** by cyclization, or from protoverbine (**6**) by hydroxylation remains unanswered. The presence of a cytochrome P-450 protoverbine hydroxylase in the roots favors the second pathway, but it cannot completely be ruled out that an unspecific hydroxylase can catalyze this reaction. Further studies concerning substrate specificity are necessary to answer this question.

Because of the (*S*)-configuration at C(11) in aphelandrine (**1**; *Scheme*), the (*S*)-isomers of synthetically obtained prelandrine (**5**) and protoverbine (**6**) were used as standards. The C(17)- and C(18)-centers of aphelandrine (**1**) are also (*S*)-configured.

The epimeric alkaloid orantine (11*S*,17*R*,18*R*) has been isolated from the same plant material together with aphelandrine (**1**). Obviously, the biogenesis of orantine follows the same pathways as proposed above.

In summary, the data reported here strongly suggest that the biosynthetic pathway leading to aphelandrine (**1**) follows the reaction sequences from monosubstituted spermine species **4** *via* prelandrine (**5**; *Scheme*). Prelandrine, as a key intermediate, was identified in the root extract, and it was also prepared enzymatically. Protoverbine

hydroxylase, an enzyme catalyzing the hydroxylation of protoverbine (**6**) to prelandrine (**5**), was detected in the microsomal fraction. We have sound evidence to propose that this reaction is mediated by a cytochrome P-450 enzyme. The compound **2** was not detected in the plant material examined. Nevertheless, the preliminary experiments with synthetically obtained compound **2** confirm its precursor role in the biogenesis of aphelandrine (**1**). Further experiments with deuterated **2** are in progress.

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Experimental Part

General. All solvents and reagents were of the highest purity commercially available. TLC: *Merck* precoated silica-gel *60 F_{254}* plates, 0.2 mm, detection by UV (254 or 366 nm), *Schlittler* [19], and *Dragendorff* reagents. $[\alpha]_D$: *Perkin-Elmer 241*. HPLC: *Waters 626 LC* System, *Waters 996* photodiode array detector and *Waters 600S* controller (*Waters Corp.*) with a *Millennium* Chromatography Manager Version 3.05 (*Waters*). MS, HPLC-MS, and HPLC-MS/MS: *Finnigan TSQ 700* triple-stage quadrupole instrument equipped with a *Finnigan* APCI or ESI ion source. ^{1}H-NMR: *Bruker AC-300 or ARX-300*. ^{13}C-NMR: *Bruker ARX-300*, chemical shifts (δ) in ppm, CD_3OD, Me_4Si as internal standards.

HPLC, HPLC-MS, and MS Conditions. Waters Symmetry® C_8 column (5 μm, 3.9 × 150 mm); flow rate 0.8 ml min^{-1}; mobile phase: *A:* 1% AcOH in H_2O, *B:* 1% AcOH in MeCN. The APCI operating conditions: positive mode; vaporizer temp.: 450°; corona voltage: 4.5 kV; heated capillary temp.: 220°; sheath gas: N_2 with an inlet pressure of 60 psi. ESI Operating conditions: positive-mode-heated capillary temp.: 200°; sheath gas: N_2 with an inlet pressure of 30 psi; flow; 3 μl min^{-1}. ESI- and APCI-MS/MS experiments: collision gas Ar with a relative pressure 2.0 to 3.2 m Torr; collision induced dissociation (CID) offset (coff): −27 to −32 eV.

Plant Material. Aphelandra squarrosa NEES (Acanthaceae) plants were cultivated by shoot propagation in the greenhouse of the Institute of Organic Chemistry, University of Zürich. For the experiments, 12-week-old plants were used.

(−)-8-(4-Hydroxyphenyl)-1,5,9,13-tetraazacycloheptadecan-6-one Trihydrochloride (**5**·3 HCl). A soln. of *(−)-(*S*)-buchnerine* (**7**; 10 mg, 0.028 mmol) in 3 ml of dry CH_2Cl_2 was cooled to −80° (dry ice/acetone bath). Then, 0.25 ml of a 1M soln. of Br_3B in CH_2Cl_2 (0.28 mmol) was added. The mixture was kept at −80° for 1 h, then allowed to warm. The reaction was quenched after 16 h by adding 3 ml of 90% aq. MeOH soln., and the mixture was evaporated to dryness *in vacuo* (50°). The excess Br_3B was removed by repetitive addition of MeOH sat. with gaseous HCl and evaporation *in vacuo* (50°): 11.4 mg (91%) of **5**·3 HCl. Colorless solid. TLC ($CHCl_3$/MeOH/25% aq. NH_3 soln. 4:3:1): R_f 0.16, $[\alpha]_D^{20} = -9.3$ ($c = 0.76$, MeOH). ^{1}H-NMR: 7.47 (d, $J = 8.6$, 2 H); 6.88 (d, $J = 8.6$, 2 H); 5.04 (d, 1 H); 2.95–3.75 (m, 13 H); 2.6–2.35 (m, 1 H); 2.25–1.75 (m, 8 H). ^{13}C-NMR: 174.9 (C=O); 160.2 (C−OH); 139.5, 130.8; 117.1; 58.17; 48.1; 46.7; 46.1; 44.1; 42.6; 37.9; 36.4; 28.5; 24.1; 23.0; 22.3. ESI-MS: 349 ($[M + H]^+$). ESI-MS/MS (−27 eV; rel. int. %): 349 (26), 275 (35), 229 (12), 204 (100), 179 (8), 169 (28), 155 (73), 129 (96), 112 (47), 100 (38), 98 (30), 84 (16).

Identification of Prelandrine (**5**) *in the Roots of* A. squarrosa. The 200 000*g* supernatant (of 4 microsomal fractions, each fraction was prepared from 400 g FW of 12-week-old roots; see below) was lyophilized. The residue was extracted 3 × with 1500 ml of MeOH/AcOH 98:2 in the dark at 4° (each for 12 h). The crude extracts were combined and concentrated *in vacuo* (30°). The residue was dissolved in H_2O, the pH was adjusted to 8.5 (K_2CO_3), and the soln. was extracted 6 × with $CHCl_3$/i-PrOH 4:1. The combined org. extracts were dried (Na_2SO_4) and evaporated *in vacuo*. The residue was dissolved in $CHCl_3$ and filtered. The filtrate was evaporated *in vacuo*, then dissolved in MeOH, and filtered through silica gel (2 g), first eluted with MeOH and then with $CHCl_3$/MeOH/25% aq. NH_3 soln. 4:3:1. The $CHCl_3$/MeOH/25% aq. NH_3 soln. eluate was evaporated and analyzed by HPLC and HPLC/APCI-MS/MS. HPLC: linear gradient *A*/*B* 97:3 to *A*/*B* 0:100 within 70 min. Detection: DAD 280 nm, APCI-MS and APCI-MS/MS.

Preparation of Microsomes. During enzyme isolation and preparation of the assays, all operations were carried out on ice or at 4°, the pestle, mortar, and all buffer solns. were pre-chilled. The microsomal membrane fraction was isolated from the roots of the plants. Washed roots (100 g FW) were cut into small pieces and shock-frozen with liquid N_2. Plant-tissue slices were homogenized in 400 ml of 0.1M K/Na phosphate buffer (pH 7.4),

containing 0.6M mannitol, 10 mM mercaptoethanol, 5 mM ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EDTA), and 1 mM (phenylmethyl)sulfonyl fluoride as inhibitor of serine proteases. The homogenate was filtrated through four layers of cheesecloth, and centrifuged for 20 min at 20000 × *g* to pellet mitochondria, plastids, and cellular debris. The 20000 × *g* supernatant was transferred to ultracentrifuge tubes, and microsomes were pelleted for 90 min at 200 000 × *g*. The microsomal pellet was resuspended twice in 0.1M K/Na phosphate buffer (pH 7.4), containing 1 mM EDTA and recentrifuged (200 000 × *g*, 30 min). Finally, the microsomal pellet was resuspended in 4 ml of 0.1M K/Na phosphate buffer (pH 7.4), containing 1 mM EDTA and 30% (*v*/*v*) glycerol and stored frozen at −80°.

Protein Content. Protein contents were determined according to the method of *Bradford* [20] with bovine serum albumin as the standard protein.

Microsomal Hydroxylation of (−)-(S)-Protoverbine (**6**). The incubation mixture contained resuspended microsomal pellet (1.54 to 2.25 mg of protein), 1 mM EDTA, 2.3 mM of (−)-(*S*)-protoverbine hydrochloride (**6**), 1 mM NADPH in a total volume of 1.5 ml. The tubes were incubated for 1 h at 25° with gentle shaking. The reaction was stopped by adding 100 µl of AcOH in 20 ml of MeOH, and the mixture was centrifuged for 10 min at 3000 × *g* to remove the precipitated protein. The MeOH/AcOH supernatant was evaporated to dryness *in vacuo* (30°). Dry samples were stored at −20° under Ar. The residues were taken up in 100 µl of MeOH and analyzed directly by HPLC/MS. Aliquots of 5–10 µl were injected onto an RP-C_8 column and eluted with a linear gradient *A*/*B* 97:3 to *A*/*B* 0:100 within 70 min. Detection: DAD 280, APCI-MS and APCI-MS/MS.

CO Difference Spectra. CO Difference spectra were obtained according to the procedure in [18] with sodium dithionite as reductant. For the investigations of the effects of O_2, the reaction mixtures without (−)-(*S*)-protoverbine (**6**) were placed into small glass vials, sealed with septum caps, kept on ice, and 200 ml of air, N_2, or a mixture of CO/O_2 9:1 [21] was gently bubbled through the soln. Then, the reaction was initiated by adding **6**. To examine the reversal of the CO inhibition, the tubes treated with CO/O_2 9:1 were illuminated with white light [17].

Enzymatic Oxygen-Scavenging System. To study the dependence of the aphelandrine synthase on molecular O_2, an enzyme assay lacking **6** was incubated for 30 min with 50 mM glucose, 5 U glucose oxidase, and 10 U catalase [16]. (−)-(*S*)-Protoverbine (**6**) was added to start the incubation and continued as described above.

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