55. Biosynthesis of the Spermine Alkaloid Aphelandrine

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Aphelandrine (**1**) is shown to be biosynthesized in the root cells of *Aphelandra tetragona* (VAHL) NEES from labelled putrescine (**4**), spermidine (**5**), and cinnamic acid (**3**). Whether spermine (**6**) and the (*p*-hydroxycinnamoyl)spermidine **8** are precursors of **1** is uncertain, since the latter is hydrolysed to a large extent before incorporation, and the former is metabolized to **4** and **5**. Methionine (**7**) is the source of the 3-aminopropyl unit of **5** and **6**.

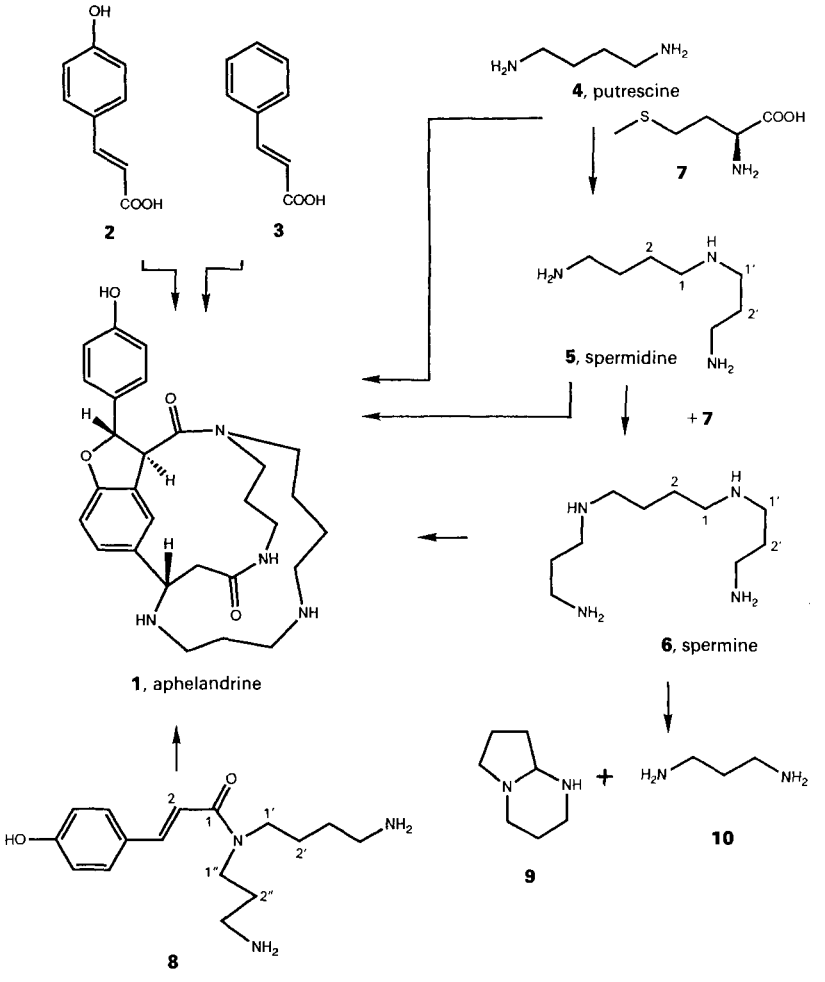
Introduction. – The roots of several *Aphelandra* species have been found to contain aphelandrine (**1**) [1] as the major alkaloid (0.2% of dry weight). Structure elucidation by chemical degradation [2] revealed that **1** belongs to the quite rare class of macrocyclic spermine alkaloids [3]. The absolute configuration of the three chiral centers of **1** was determined by CD spectra in comparison to the diastereoisomeric orantine [4], and more recently by X-ray analysis of the perchlorate of **1** [5]. Nothing is known about the biosynthetic origin and the metabolism of spermine alkaloids like **1**.

The structure of **1** is suggesting a biosynthetic pathway connecting building blocks like *p*-hydroxycinnamic acid (**2**), spermine (**6**), spermidine (**5**), and putrescine (**4**; *Scheme 1*). It certainly is of primary importance to establish optimal feeding methods in order to determine, whether **1** is synthesized *'de novo'* from these simple precursors. Moreover, information is needed on the site of biosynthetic activity, and it would be of great advantage to identify 'later-stage' intermediates, like **8**, which would provide insight into the reaction sequence of coupling these building blocks to the chiral macrocycle.

Results and Discussion. – After some unsuccessful attempts to feed precursors by roots to intact *Aphelandra tetragona* (VAHL) NEES, the 'cotton-wick' method proved to yield up to 0.13% incorporation of [1,4-$^{14}C_2$,3′,4-$^{3}H_2$]spermidine ([1,4-$^{14}C_2$,3′,4-$^{3}H_2$]-**5**) into aphelandrine (**1**, see *Table 1, Entry 2*). [3-$^{14}C_1$]Cinnamic acid ([3-$^{14}C_1$]-**3**; *Entry 1*), [1,4-$^{14}C_2$,2,3-$^{3}H_2$]putrescine ([1,4-$^{14}C_2$,2,3-$^{3}H_2$]-**4**; *Entry 4*), and [U-^{14}C]methionine ([U-^{14}C]-**7**, *Entry 6*), were incorporated into **1** in rates of *ca.* 0.01%. The radioactive aphelandrine could be isolated exclusively from the roots, indicating good transport of the precursors in the stem and, moreover, suggesting that the roots of *A. tetragona* contain the only biosynthetic active cells concerning the formation of **1**. This result is supported by the already established fact, that aphelandrine is found in a ratio of *ca.* 30:1 in the roots and in the green parts of *A. tetragona*, respectively [6].

In most cases, doubly labelled precursors were applied, since no suitable degradation methods of **1** are available in order to localize the label, and it was reasoned that the

Scheme 1. *Schematic Representation of the Results of the Incorporation Experiments*



retention of $^{3}H/^{14}C$ would be sufficient to prove a specific incorporation. As expected, doubly labelled putrescine [1,4-$^{14}C_2$,2,3-$^{3}H_2$]-**4** was incorporated into **1** with retention of $^{3}H/^{14}C$ (*Table 1, Entry 4*). However, depending on feeding conditions, $\leqslant 47\%$ of the ^{3}H-label was lost, when the spermidine [1,4-$^{14}C_2$,3′,4-$^{3}H_2$]-**5** was converted to aphelandrine (**1**). At first sight, this result seems quite puzzling, but subsequent experiments of the metabolism of the polyamines in *A. tetragona* provide a reasonable interpretation, *vide infra*.

To investigate the metabolism of **1** and its possible biosynthetic precursors **4**–**6**, the green parts and the roots of *A. tetragona* were separately and quantitatively analyzed *i*) for metabolites of **1**, after feeding [^{3}H,^{14}C]-**1**, originating from earlier biosynthetic experiments, and *ii*) for the polyamines **4**–**6**, after application of [1,4-$^{14}C_2$,3′,4-$^{3}H_2$]-**5**, [2,3-$^{3}H_2$]-**4**, [U-^{14}C]-**7**, and a mixture of [3′,4-$^{3}H_2$]-**5** and [1,4-$^{14}C_2$]-**6**. It could be clearly

Table 1. *Incorporation of Labelled Precursors into Aphelandrine* (**1**) *in* Aphelandra tetragona

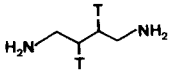
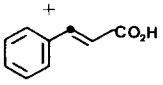
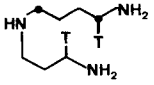
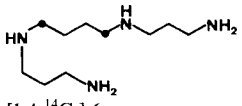
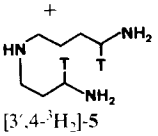
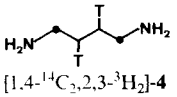
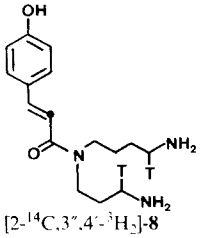
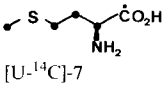
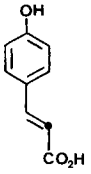
Entry	Precursor	Time of exposure [days]	$^3H/^{14}C$ Ratio of		Incorporation rate [%]	
			precursor	**1**	^3H	^{14}C
1	 [2,3-$^3H_2$]-**4** +  [3-^{14}C]-**3**	16	1.0	10.22	0.102	0.01
2	 [1,4-$^{14}C_2$,3′,4-$^3H_2$]-**5**	22	2.0	1.06	0.07	0.13
		3	3.53	2.2	0.004	0.006
		21	3.53	2.37	0.026	0.034
3	 [1,4-$^{14}C_2$]-**6** +  [3′,4-$^3H_2$]-**5**	22	2.4	3.40	0.126	0.093
4	 [1,4-$^{14}C_2$,2,3-$^3H_2$]-**4**	14	1.0	1.096	0.0107	0.0098
5	 [2-^{14}C,3″,4′-$^3H_2$]-**8**	13	5.97	46.75	0.0087	0.0011
6	 [U-^{14}C]-**7**	14	–	–	–	0.012
7	 [2-^{14}C]-**2**	7	–	–	–	0.0008
		14	–	–	–	0.0015

Table 2. *Polyamine Metabolism in* Aphelandra tetragona

Entry	Precursor				Polyamines isolated from the stem					
	time of exposure [days]	total activity $\times 10^8$ [dpm]		$^3H/^{14}C$	total activity $\times 10^5$ [dpm]			$^3H/^{14}C$		
					4	**5**	**6**	**4**	**5**	**6**
1	16	^3H	5.55	1.0	^3H	21.9	3.13 2.13	–	–	–
[2,3-$^3H_2$]-**4** + [3-^{14}C]-**3**		^{14}C	5.55		^{14}C	–	– –			
2	22	^3H	2.22	2.0	^3H	5.22	1.45 0.28	1.24	1.21	1.49
		^{14}C	1.11		^{14}C	4.22	1.20 0.19			
[1,4-$^{14}C_2$,3′,4-$^3H_2$]-**5**	3	^3H	4.73	3.53	^3H	23.96	1.56 0.37	2.29	2.88	2.47
		^{14}C	1.34		^{14}C	10.43	0.54 0.15			
	21	^3H	4.73	3.53	^3H	4.59	0.67 0.31	2.40	2.69	2.71
		^{14}C	1.34		^{14}C	1.91	0.25 0.12			
3	22	^3H	2.664	2.40	^3H	15.90	8.92 11.00	2.91	3.32	4.09
[1,4-$^{14}C_2$]-**6** + [3′,4-$^3H_2$]-**5**		^{14}C	1.11		^{14}C	5.48	2.68 2.69			
4	14	^{14}C	1.24		^{14}C	0.36	0.06 0.1			
[U-^{14}C]-**7**										
5	13	^3H	10.17	5.97	^3H	3.50	4.90 2.52	–	–	–
[2-^{14}C,3″,4′-$^3H_2$]-**8**		^{14}C	1.70		^{14}C	–	– –			

shown that **1** is not metabolized in the green parts of *A. tetragona*; moreover, **1** seems not to be transported in its free form from the stem to the roots.

In contrast, the polyamines are metabolized in the stem of *A. tetragona*. After application of [1,4-$^{14}C_2$,3′,4-$^3H_2$]spermidine ([1,4-$^{14}C_2$,3′,4-$^3H_2$]-**5**), doubly labelled spermine (**6**), spermidine (**5**), and putrescine (**4**) could be isolated after dilution with non-radioactive reference compounds and counted as their pure 4-bromobenzamides (see *Exper. Part*). The remarkable change of the $^3H/^{14}C$ ratio (see *Table 2*) indicates the loss of the ^3H-labelled 3-aminopropyl group from **5** to give putrescine **4** (32–38% ^3H-loss), which is subsequently alkylated by methionine (**7**) to give spermidine (**5**) and spermine (**6**). The alkylation of **4** to produce **5** and **6** is known from other organisms [7] and also established in the stem of *A. tetragona* by feeding [U-^{14}C]methionine ([U-^{14}C]-**7**; see *Table 2*).

The change of $^3H/^{14}C$ in the polyamine metabolism is not exactly reproducible even when the plants were harvested within the same time after feeding [1,4-$^{14}C_2$,3′,4-$^3H_2$]-**5**. Consequently, no conclusions can be drawn from these experiments with regard to the relative rates of interconversion **4**→**5**→**6**. However, is its firmly established that the 3-aminopropyl unit is easily added and removed biosynthetically from the polyamines. It is also obvious from the competitive feeding experiment with spermine/spermidine that spermine (**6**) is a less efficient precursor for aphelandrine (**1**) compared to putrescine (**4**) and spermidine (**5**). From the change of the $^3H/^{14}C$, it seems that **6** is prone to oxidative cyclization to give **9** and **10** (*Scheme 1*) [8] rather than being incorporated into **1** or metabolized to **4** and **5**.

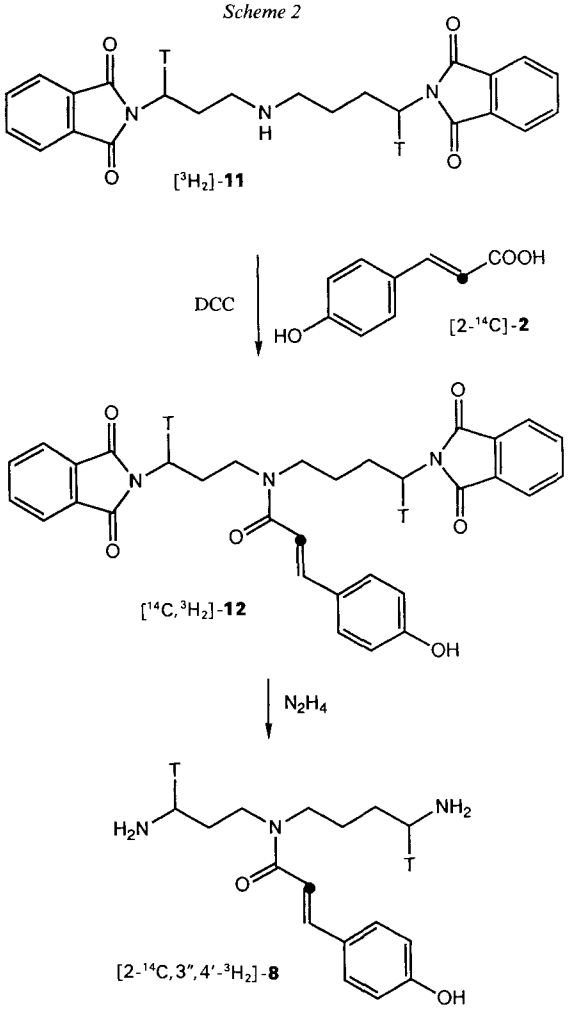
It follows that at present there is no unequivocal proof, that spermine (**6**) is a biosynthetic precursor of aphelandrine (**1**). It seems even possible, that the second 3-aminopropyl group, necessary for the construction of the spermine macrocycle is only added after condensation of spermidine (**5**) with *p*-hydroxycinnamic acid (**2**). In order to test this hypothesis hydroxy[2-$^{14}C_1$]cinnamic acid ([2-$^{14}C_1$]-**2**) was prepared following the procedure of *Humora* and *Quick* [9] and condensed with the doubly protected ^3H-labelled spermidine [$^3H_2$]-**11** to furnish the amide [^{14}C,$^3H_2$]-**12**. Subsequent hydrazinolysis of the protecting groups afforded the desired doubly labelled amide [2-$^{14}C_1$,3″,4′-$^3H_2$]-**8** [10] (*Scheme 2*). After feeding of the latter spermidine derivative to *A. tetragona*, the polyamines **4**–**6** were isolated and found to be only ^3H-labelled, whereas aphelandrine (**1**) was doubly labelled, however, displaying an eigth-fold increase of $^3H/^{14}C$ compared to the precursor.

Since the incorporation of the ^3H- and ^{14}C-label of **8** into **1** are of the same order as for *p*-hydroxy[2-$^{14}C_1$]cinnamic acid ([2-$^{14}C_1$]-**2**) and [3′,4-$^3H_2$]spermidine ([3′,4-$^3H_2$]-**5**), when these compounds were administered to *A. tetragona* in separate experiments, it is quite intriguing to assume efficient hydrolysis of the amide bond of **8** in the stem of the plant much before the compound can reach its destination in the roots of *A. tetragona*.

The incorporation rates of *p*-hydroxycinnamic acid (**2**; 0.001%) and cinnamic acid (**3**; 0.01%) are quite low and differ from each other by a factor of ten. Possible explanations are as follows: the lower incorporation of **2** in comparison to **3** may be due to the fact that **3** is hydroxylated after the formation of an intermediate amide conjugate. On the other hand, the transport of the hydroxylated compound **2** may be less efficient.

Since it is not possible to explain the changes of $^3H/^{14}C$ ratio during the transformation of certain precursors into aphelandrine (**1**), the original assumption that feeding doubly labelled precursors would be sufficient to prove a specific incorporation is not

Scheme 2



valid in some cases. Consequently, chemical degradations for **1** must be developed. Stable isotope techniques should be applied, if incorporation rates could be enhanced using tissue cultures. This technique would be also of advantage concerning a possible fluctuation of the biosynthetic activity in a years circle of *A. tetragona* [11].

We thank the members of our analytical laboratories (microanalysis, MS, NMR, and isotope lab) for their service and the *Swiss National Science Foundation* for financial support. We are very thankful to *Gärtnerei Weber AG*, CH–5416 Kirchdorf, for cultivating *Aphelandra* plants.

Experimental Part

Plants and Radiochemicals. Aphelandra tetragona (VAHL) NEES, 3–15-weeks old, were commerically available. [1,4-$^{14}C_2$]Spermine ([1,4-$^{14}C_2$]-**6**) and [3-$^{14}C_1$]cinnamic acid ([3-$^{14}C_1$]-**3**) were obtained from *Amersham Radiochemi-*

cals, England. [3′,4-$^3H_2$]Spermidine ([3′,4-$^3H_2$]-**5**), [1,4,-$^{14}C_2$]spermine ([1,4-$^{14}C_2$]-**6**), [2,3-$^3H_2$]putrescine ([2,3-$^3H_2$]-**4**, [1,4-$^{14}C_2$]putrescine ([1,4-$^{14}C_2$]-**4**), [U-^{14}C]methionine ([U-^{14}C]-7), and [2-$^{14}C_1$]malonic acid were purchased from *New England Nuclear*, U.S.A. The radioactivity of individual samples was measured by liquid-scintillation counting using a *Packard Tri-Carb* scintillation analyzer, model *2000 CA*. IR (cm^{-1}; $CHCl_3$): *Perkin Elmer 298*. ^{1}H-NMR: *Varian XL-200* (200 MHz); δ in ppm (TMS, internal standard) and J in Hz. ^{13}C-NMR: *Varian XL-200* (50.4 MHz).

Feeding Experiments. A H_2O soln. of the labelled compounds was administered near the base of the stem using the 'cotton-wick' method. In general, the precursors were taken up by the plant within 24 h after 2–22 days (see *Tables 1* and *2*), the plants were harvested, and the roots, stem, and leaves lyophilized separately.

Extraction and Purification of **1** *and the Polyamines* **4**, **5**, *and* **6**. After adding unlabelled aphelandrine (**1**), the lyophilized parts of *A. tetragona* were exhaustively extracted with 5% AcOH/MeOH. The extract was evaporated at 40°/0.01 Torr, toluene was added, evaporated again, and the residue finally dissolved in 0.25M HCl. After extraction with hexane and Et_2O, the aq. layer was adjusted to pH 9.5 and extracted with $CHCl_3$. To isolate **1**, the $CHCl_3$ extract was dried (Na_2SO_4), evaporated, and the residue chromatographed twice on prep. TLC ($CHCl_3$/MeOH/NH_4OH 7:7:1 and 78:19:3) yielding **1** which was crystallized from MeOH/H_2O to constant specific activity. To isolate **4**–**6**, the basic aq. layer was concentrated to 2 batches of 100 and 50 ml, and aliquots diluted with 0.1 mmol of each polyamine **4**, **5**, and **6**. Subsequently, 4-bromobenzoyl chloride/2M NaOH was added to each probe and the resulting 4-bromobenzamides were isolated by prep. TLC ($CHCl_3$/MeOH 9:1 and 19:1) and crystallized from MeOH/H_2O to constant specific activity. Incorporation rates are given in % of the total activity administered by the 'cotton-wick' method.

N-*(4-Amino[4-$^3H_1$]butyl)*-N-*(3-amino[3-$^3H_1$]propyl)-3-(4-hydroxyphenyl)[2-^{14}C]prop-2-enamide* ([2-$^{14}C_1$, 3″,4′-$^3H_2$]-**8**). According to [10], N-{*4-{3-(phthalimido)[3-$^3H_1$]propylamino}[1-$^3H_1$]butyl*}*phthalimide* ([$^3H_2$]-**11**) was prepared in 80% yield from 1 mCi (16.34 mCi/mmol) of [3′,4-$^3H_2$]spermidine ([3′,4-$^3H_2$]-**5**). Subsequent addition of 0.13 mCi (4.76 mCi/mmol) of 4-hydroxy[2-$^{14}C_1$]cinnamic acid ([2-$^{14}C_1$]-**2**) to a soln. of [$^3H_2$]-**11** in THF in the presence of dicyclohexylcarbodiimid (DCC) gave in 73% yield N-{*4-{3-(4-hydroxyphenyl)*-N-{*3-(phthalimido)[3-$^3H_1$]propyl*}*[2-^{14}C]prop-2-enamido*}*[1-$^3H_1$]butyl*}*phthalimide* ([^{14}C, $^3H_2$]-**12**) as a (*E*/*Z*)-mixture. Deprotection by hydrazine hydrat in EtOH [10] yielded [2-$^{14}C_1$,3″,4′-$^3H_2$]-**8** which was isolated and purified as its dioxalate; $^3H/^{14}C = 5.97$. Compounds [$^3H_2$]-**11**, [$^{14}C_1$,$^3H_2$]-**12**, and [2-$^{14}C_1$,3″,4′-$^3H_2$]-**8** were identified by comparison with authentic samples originating from a synthesis with non-labelled material IR (KBr; measured as dioxalate): 3400 (br.), 3000 (br.), 1640, 1605, 1585, 1515, 1465, 1380, 1260, 1225, 1165, 1155, 980, 830. ^{1}H-NMR (CD_3OD; free base): 7.63–6.68 (*m*, 6 H); 4.86 (OH); 3.71–3.50 (*m*, 4 H); 3.35–3.29 (4 NH); 3.03–2.93 (*m*, 4 H); 2.07–1.69 (*m*, 6 H). ^{13}C-NMR ((D_6)DMSO; free base): 170.86, 170.61, 168.28, 167.95, 156.42, 156.37 (6*s*); 144.06 (*d*); 132.45 (*s*); 131.19, 130.50, 118.14, 117.06, 116.42, 113.39, 113.36 (7*d*); 48.33, 46.41, 46.11, 43.80, 38.62, 38.54, 38.44, 35.46, 31.43, 29.78, 29.74, 29.62, 29.59, 29.52, 29.49, 28.45, 28.18, 27.55, 26.75, 26.56, 25.68, 25.41 (22*t*). CI-MS: 292 ($[M + 1]^+$).

REFERENCES

[1] H. Bosshardt, A. Guggisberg, S. Johne, M. Hesse, *Pharm. Acta Helv.* **1978,** *53,* 355.

[2] P. Dätwyler, H. Bosshardt, H.O. Bernhard, M. Hesse, *Helv. Chim. Acta* **1978,** *61,* 2646.

[3] A. Guggisberg, M. Hesse, in 'The Alkaloids', Ed. A. Brossi, Academic Press Inc., New York, 1983, Vol. 22, p. 85–188.

[4] P. Dätwyler, H. Bosshardt, S. Johne, M. Hesse, *Helv. Chim. Acta* **1979,** *62,* 2712.

[5] A. Guggisberg, R. Prewo, M. Hesse, *Helv. Chim. Acta* **1986,** *69,* 1012.

[6] G. Papazoglou, Ph. D. Thesis, Universität Zürich, 1990.

[7] T.A. Smith, *Biochem. Biophys. Res. Commun.* **1970,** *41,* 1452; E. Leete, *Phytochemistry* **1985,** *24,* 957; J. Negrel, *ibid.* **1989,** *28,* 477; T.A. Smith, in 'Encyclopedia of Plant Physiology', Eds. E.A. Bell and B.V. Charlwood, Springer Verlag, Berlin, 1980, Vol. 8, p. 8.

[8] T.A. Smith, *Phytochemistry* **1972,** *11,* 899; S.J. Croker, R.S.T. Loeffler, T.A. Smith, R.B. Sessions, *Tetrahedron Lett.* **1983,** *24,* 1559.

[9] M. Humora, J. Quick, *J. Org. Chem.* **1979,** *44,* 1166.

[10] G. Sosnovsky, J. Lukszo, *Z. Naturforsch., B* **1986,** *41,* 122.

[11] A. Lorenzi-Riatsch, M. Hesse, to be published; W. Fiedler, A. Lorenzi-Riatsch, M. Hesse, *Planta Med.* **1990,** *56,* 493.