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DISTRIBUTION, BIOSYNTHESIS AND TURNOVER OF PYRROLIZIDINE ALKALOIDS IN *CYNOGLOSSUM OFFICINALE*

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Key Word Index—*Cynoglossum officinale*; Boraginaceae: Hound's tongue; pyrrolizidine alkaloids; distribution; biosynthesis; turnover.

Abstract—The facultative biennial *Cynoglossum officinale* contains the *N*-oxides of the following pyrrolizidine alkaloids (PAs): trachelanthamine, viridiflorine, 7-angeloylheliotridine, rinderine, echinatine, 3′-acetylechinatine and heliosupine. The inflorescences contain the highest levels of PAs. At the vegetative rosette stage, the youngest leaves have higher PA levels than the older leaves. Both isolated roots and isolated shoots of rosette plants were able to produce PAs from ^{14}C-labelled putrescine. PA production patterns differed between roots and shoots. In intact plants, there was no significant turnover of PAs within 32 days. Within the shoot, PAs were reallocated from the ageing leaves into the newly formed, youngest leaves. Shoot to root transport of PAs was observed, which indicated that PAs are transported via the phloem.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are studied because of their toxic effects on livestock [1] and their role in insect–plant interactions [2]. PAs are ester alkaloids consisting of a necine base moiety, esterified with one or two necic acids [3]. Tracer studies have shown that the necine base is biosynthesized from two putrescine molecules via homospermidine [2, 4].

Relative to other members of the Boraginaceae, *Cynoglossum officinale* contains high levels of PAs [5]. It is a facultative biennial herb, which is widely distributed in the temperate zones of Western Europe, Asia and Canada [6]. In this species, PAs serve as deterrents against a range of generalist herbivores [7]. It is not known whether the specialist herbivores on *C. officinale*, such as the monophagous weevil *Mogulones cruciger*, store PAs for their own defence.

Although several studies report on total PA level [5, 8] or on identification of purified PAs of *C. officinale* [9, 10], information on the relative abundance of the different PAs is still lacking. Moreover, it is unclear whether the biosynthesis of PAs in *C. officinale* is restricted to the shoots, as in other Boraginaceae, such as *Heliotropium* spp. [11, 12]. In several studies on the biosynthesis of necine bases [13] and necine acids [14–16] in *C. officinale*, plants were fed with tracer and extracted as a whole. It is thus impossible to discriminate between root and shoot biosynthesis.

In this paper, we have studied the occurrence, distribution and site of biosynthesis of PAs in *C. officinale*. Moreover, our experiments provide information about turnover and transport of PAs. This physiological knowledge is essential when studying ecological aspects of PAs in the plant.

RESULTS AND DISCUSSION

Occurrence and distribution of PAs

We identified seven major PAs in *C. officinale* (Fig. 1 and Table 1). All PAs were present predominantly as *N*-oxides. It is now generally accepted that plants synthesize, translocate and store PAs in the form of the polar *N*-oxides [2]. The 1,2-unsaturated PAs were all esters of the necine base heliotridine, while both trachelanthamine (**1**) and viridiflorine (**2**) represent esters of the saturated base (−)-trachelanthamidine. Except for 7-angeloylheliotridine (rivularine in ref. [2]) (**7**), which was classified as a triangularine-type PA, all PAs found in *C. officinale* belong to the lycopsamine group [2]. It must be realized, however, that there may exist pronounced differences in the PA composition and total PA levels between populations from different geographical areas [8, 17]. Man'ko [10] showed that plants from Kazachstan contained only one PA, while plants from Leningrad (Russia) and Kharkov (Ukraine) contained four different PAs.

It is generally accepted that the biosynthesis of PAs in plants proceeds from saturated necines to unsaturated necines [4]. In *Eupatorium cannabium*, this conversion

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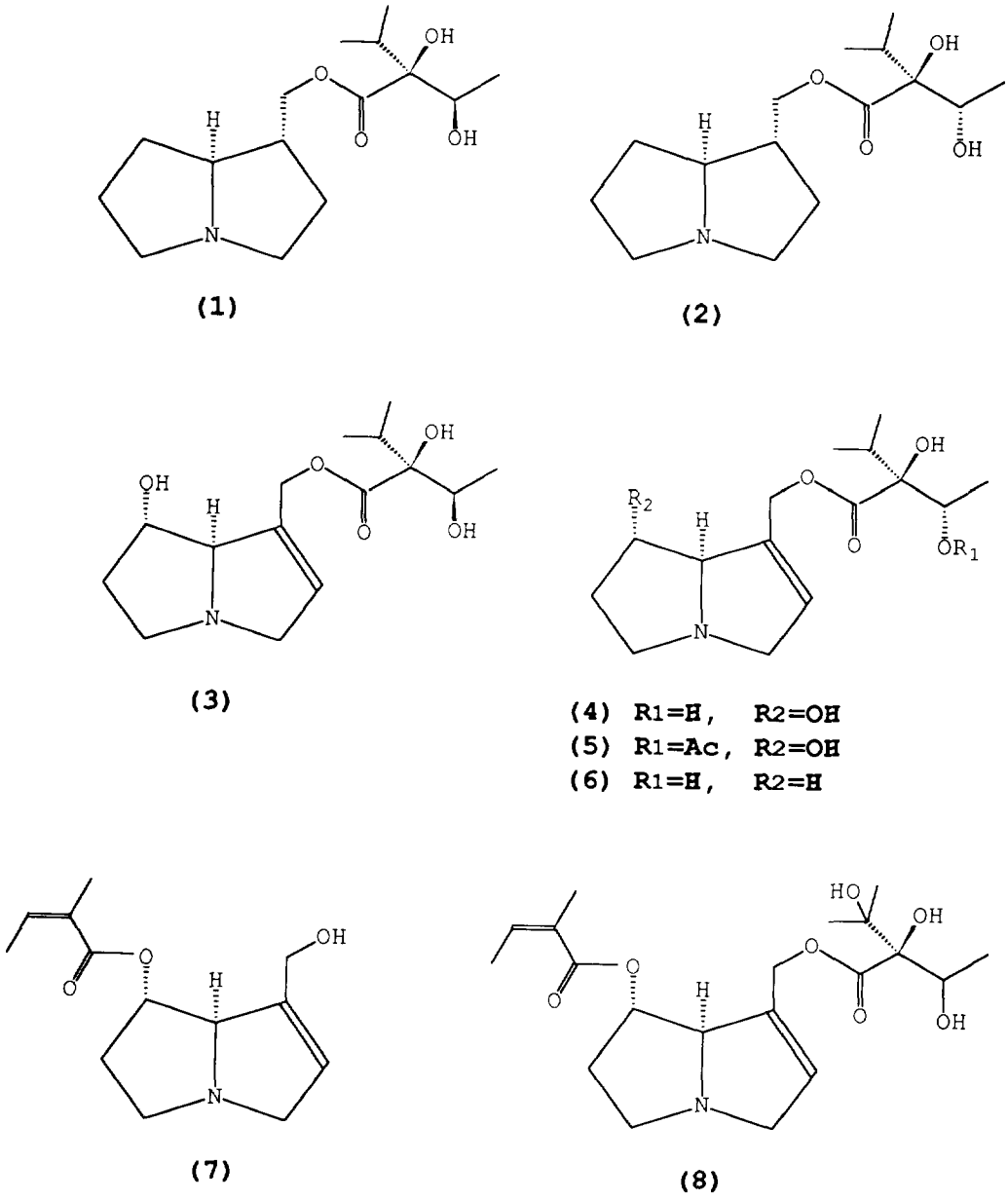

Fig. 1. Structures of pyrrolizidine alkaloids in *C. officinale*, trachelanthamine (**1**), viridiflorine (**2**), rinderine (**3**), echinatine (**4**), 3'-acetylechinatine (**5**), amabiline (**6**), 7-angeloylheliotridine (**7**) and heliosupine (**8**).

takes place at the level of the O-9 esters [2]. In *C. officinale*, viridiflorine (**2**) and echinatine (**4**) are both esterified at the O-9 position with (−)-viridifloric acid, viridiflorine (**2**) could thus be the precursor for (**4**). The same applies to trachelanthamine (**1**) and rinderine (**3**), both containing (+)-trachelanthic acid at the O-9 position (Fig. 1).

In the leaves of rosettes, **2** and **4** are indeed relatively more abundant than the second few PAs (Table 1). In contrast, the roots contain relatively more **1** and **3** than the leaves. This indicates that the stereochemistry of the acids is preserved during conversion from saturated to unsaturated O-9 esters. More evidence for this hypothesis may be obtained by tracer-feeding studies. Heliosupine (**8**) and 7-angeloylheliotridine (**7**) are also closely related [15], (**7**) probably being the precursor for (**8**) . However, there was no relationship between the distribution patterns of these two PAs. While the relative abundance of **7** was low in all organs, **8** was predominant in the old leaves (Table 1).

Inflorescences had the highest total PA concentration (Table 1). High levels of PAs in the flowers have been observed in several other plant species, such as *Heliotropium* spp. [12, 18], *Chromolaena odorata* [19] and

Table 1. Total pyrrolizidine alkaloid (PA) concentration and relative abundance (in %) of individual PAs in rosette plants and flowering plants of *C. officinale*

			Rosette plants			Flowering plants	
	$[M]^+$ (*m/z*)	RI on DB-1	Roots (%)	Old leaves (%)	Young leaves (%)	Stem (%)	Inflorescences (%)
Saturated PAs							
1 Trachelanthamine	285	1970	23	4	1	14	18
2 Viridiflorine	285	1986	12	15	29	2	4
Unsaturated PAs							
7 7-Angeloylheliotridine	237	1820	3	6	3	4	3
3 Rinderine	299	2152	22	4	2	10	19
4 Echinatine	299	2172	27	24	39	35	47
5 3′-Acetylechinatine	341	2220	3	3	1	22	6
8 Heliosupine	397	2557	10	44	25	13	3
Total PA ($mg\,g^{-1}$ fr. wt)			0.60–1.80	0.58–0.94	1.41–4.30	1.30–1.55	2.93–6.91

*The relative abundance of the PAs in rosette plants was determined on a combined extract of two rosette plants grown in the greenhouse in Braunschweig, while the data of the vegetative parts were assessed on a combined extract of three plants from the experimental garden in Leiden. Since the data for roots and leaves of flowering plants were comparable to those of the rosettes, they are omitted from the table. The range of total PA concentration is based on observations in field plants and plants in growth rooms from 1990 to 1994.

Senecio spp. [20]. In monocarpic species, such as *C. officinale*, flowering is a single event. Loss of flowers by herbivory cannot be compensated fully, which makes the inflorescence highly valuable to the plant [21]. The plant can decrease this risk of fitness-loss by protecting its flowers with high PA levels. Within rosette plants, the youngest leaves had the highest PA levels (Table 1, see also ref. [22]), which may reflect the plant's effort to protect its future photosynthetic output against herbivory [23].

Biosynthesis in isolated organs

Although the uptake of ^{14}C-labelled putrescine in both isolated roots and shoots was more than 90%, the recovery in the methanolic extract was very low (Table 2). For example, in *Senecio vulgaris* plants, about 21–41% of the tracer was recovered [24]. It is, however, generally found that incorporations into plants of the Boraginaceae are an order of magnitude lower than those obtained with *Senecio* spp. [4]. Measurements of the residual plant material revealed that over 60% of the label could not be recovered and probably had disappeared as $^{14}CO_2$ after breakdown of putrescine via 4-aminobutyric acid [25]. However, the greater part of the extracted label was incorporated into PAs (Table 2). The rest of the label was found in putrescine and polyamines.

Both isolated roots and shoots produced PAs from ^{14}C-labelled putrescine. In other Boraginaceae, such as *Heliotropium* spp., PA biosynthesis takes place exclusively in the shoots [11, 12]. In the Asteraceae, in contrast, the roots are the exclusive site of biosynthesis [2, 24]. To our knowledge, *C. officinale* is the first plant species known to be able to synthesize PAs in both shoots and roots. Moreover, roots have a different PA production pattern than shoots. First, isolated roots were unable to produce 7-angeloylheliotridine (**7**) and heliosupine (**8**) (Table 2). Presumably, the enzymes of this branch of the

Table 2. Relative abundance of labelled pyrrolizidine alkaloids (PAs) in isolated roots and shoots (rosettes) of *C. officinale*, after feeding experiments with ^{14}C-labelled putrescine. Identification of PAs by GC-MS

	Roots		Shoots	
Incubation time (hr)	27	72	27	81
Number of replicates (*n*)	2	2	2	2
Label in MeOH (%)	3	2	13	8
Label in PAs (%)	78	85	63	72
Alkaloid (%)				
1 Trachelanthamine and **2** Viridiflorine*	32	29	46	43
7 7-Angeolylheliotridine	—	—	16	5
3 Rinderine	44	44	—	—
4 Echinatine	15	14	26	35
5 3′-Acetylechinatine	8	13	5	9
8 Heliosupine†	—	—	7	8
6 Amabiline	—	—	—	tr

*On TLC, trachelanthamine and viridiflorine were not separated. GC-MS analysis of spot extracts: in roots, trachelanthamine–viridiflorine = 2:1; in shoots, viridiflorine only.

†Heliosupine was identified by comparison of its R_f value with a reference sample.

Abbreviations: label in MeOH = % of the label methanolic extract, label in PAs = % of the extracted label incorporated into PAs (is set to 100% for relative abundance), – = not detectable, tr. = traces.

PA biosynthetic pathway are lacking in roots. Secondly, roots produced about three times more rinderine (**3**) than echinatine (**4**) (Table 2). Although trachelanthamine (**1**) and viridiflorine (**2**) could not be separated in our TLC system, GC-mass spectral analysis revealed that in the roots the combined spot contained twice as much **1** as **2**. Conversely, in the shoots the spot consisted exclusively of **2**, which is consistent with the observation that the shoots produce **4** and not **3** (Table 2). These results reinforce the assumption that trachelanthamine (**1**) is exclusively converted into rinderine (**3**), and viridiflorine (**2**) into echinatine (**4**). However, this conversion does not proceed until the precursor has disappeared, since both PAs occur simultaneously in the plant (Table 1).

In contrast to the roots, isolated shoots were able to synthesize both **7** and **8**. This again indicates that these two PAs are closely related. However, our results did not reveal whether **7** is a true precursor of **8**, rather than a breakdown product. Although the relative abundance of **7** decreased significantly between 27 and 72 hr after incubation, the percentage of **8** did not increase by the same order of magnitude. Additionally, at 72 hr after incubation traces of amabiline (**6**), known to be the intermediate between **2** and **4** [2, 4], could be detected in shoot extracts (Table 2).

It is essential for PA biosynthesis in the shoots that the structure of the rosette remains intact. Single leaves fed with putrescine were not able to produce PAs. PA production in the shoot is probably related to active metabolism in the central meristem of the rosette [26, 27]. Moreover, neither isolated inflorescences nor stem pieces could incorporate putrescine into PAs. Obviously, these parts receive their PAs from vegetative organs.

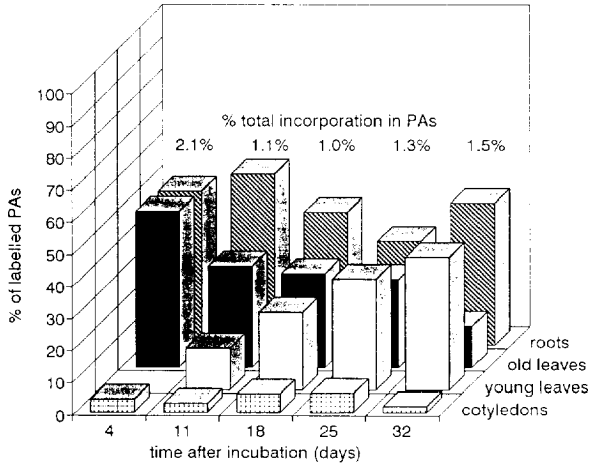

Fig. 2. Relative abundance of labelled pyrrolizidine alkaloids (PAs) after feeding rosette plants of *C. officinale* with ^{14}C-labelled putrescine. The percentage of label fed to the whole plant incorporated into PAs (total incorporation) is printed over the bars. This was set to 100% to calculate the relative abundance of labelled PA for every organ.

Biosynthesis and transport in intact plants

The recovery of radiolabel in intact plants was again very low (Fig. 2, numbers over bars). Four days after incubation there were still some labelled putrescine and polyamines present in the extract, but from 11 days after incubation all label extracted was found in PAs. There was no significant breakdown of labelled PAs; the percentage of labelled PAs extracted did not decrease with time after incubation (Fig. 2, numbers over bars). This means that from 11 days after incubation, changes in the distribution of PAs must result from transport within the plant.

The percentage of labelled PAs found in roots varied from 30 to 50%. The cotyledons, which were present on the hydroponically grown plants throughout the entire experiment, only received 5 to 10% of the labelled PAs. However, in both organs, the percentage of labelled PAs did not continuously increase or decrease with time. On the other hand, in old leaves, the percentage of labelled PAs gradually decreased with time, while it simultaneously increased in young leaves (Fig. 2). Obviously, the PAs are translocated from the ageing leaves into the newly formed, youngest three leaves, which were not even present at the time of tracer feeding. This means that the decrease of PA concentration with leaf age is not due to catabolism. PAs are generally found to be stable products which, except for transformation into species-specific derivatives, do not show any significant degradation or turnover [2, 28]. The removal of PAs from ageing leaves probably occurs simultaneously with the reallocation of nitrogen and other nutrients to the youngest leaves [29].

The lack of turnover and the reallocation of PAs within *C. officinale* have significant implications for current theories on plant chemical defences. Alkaloids used to be considered as defences with high turnover rates [30, 31]. This general assumption seems invalid, since several studies revealed that in intact plants neither tobacco alkaloids (I. Baldwin, pers. comm.), indole alkaloids [32] or pyrrolizidine alkaloids [2] show large turnover rates. The physiological costs of maintaining a certain alkaloid concentration are thus likely to be overestimated in models of optimal defence allocation [33, 34].

The alkaloid pattern in *C. officinale* was already fixed at four days after incubation. After that there were no significant changes in pattern, and the data from all harvests were pooled (Table 3). As in other experiments, the roots typically contained **1** and **3** (Table 3). Unlike the isolated roots, the roots of the intact plants, which were all of the same full-sib family, contained relatively more **5** than **4**. This difference in relative abundance of **5** might be caused by genetic or physiological differences. The presence of labelled **8** in the roots clearly shows that there is PA transport from shoots to the roots (Table 3). Since shoot-root transport generally occurs via the phloem, this is an indication that the transport of PAs takes place via the phloem, as in *Senecio vulgaris* [24].

Surprisingly, we did not find labelled **3** or **1** to be transported from the roots into the leaves (Table 3),

Table 3. Relative abundance of labelled pyrrolizidine alkaloids (PAs) in rosette plants ($n = 5$) of *C. officinale*, 4 to 32 days after incubation with ^{14}C-labelled putrescine. Identification by GC-MS

Alkaloid	Roots (%)	Old leaves (%)	Young leaves (%)
1 Trachelanthamine and			
2 Viridiflorine*	28	25	37
7 7-Angeloylheliotridine	—	6	6
3 Rinderine	19	—	—
4 Echinatine	10	28	35
5 3′-Acetylechinatine	28	tr	tr
8 Heliosupine†	15	36	21
6 Amabiline	—	1	1

On TLC, trachelanthamine and viridiflorine were not separated. GC-MS analysis of spot extracts: in roots, mostly trachelanthamine with traces of viridiflorine; in shoots, viridiflorine only.

†Heliosupine was identified by comparison of its R_f value with a reference sample.

– = Not detectable, tr. traces.

although both PAs were present in leaf extracts (Table 1). However, this also indicates that the label, which was fed via the roots, has been transported as putrescine into the leaves, where it was transformed into the leaf-specific PAs. Heliosupine (**8**) was relatively more abundant in old leaves than in young leaves, indicating that the presence of **8** is related to ageing. Again, we found small quantities of the intermediate **6** in shoots.

Conclusions

This is the first study which relates the occurrence of PAs in *C. officinale* with their site of biosynthesis. The most important finding is that both roots and shoots are able to produce PAs and that these organs have different PA-production patterns. The differences in the biosynthetic pathways between roots and shoots are related exclusively to the selection of different necic acids, i.e. (–)-viridifloric acid and (+)-trachelanthic acid in roots, but only the former in shoots. Furthermore, only shoots seem to be able to produce heliosupine and its presumed side-product, **7**. The necine bases, i.e. trachelanthamidine and heliotridine, formed at the two sites of biosynthesis are identical.

EXPERIMENTAL

Plant material. For the pulse-labelling expts, seeds of *C. officinale* L. were collected in Leiden from selfed plants grown in a greenhouse. In each expt we used seeds of one plant to minimize genetic variation in PA content and other traits. The seeds were scarified and soaked in H_2O for two weeks at 20°/16 hr light, 10°/8 hr dark to enhance germination. Plants were reared on a hydroponic soln [35] in 1.2 l pots; the soln was renewed every week. Plants were grown in the period between the 15 February and 15 May 1993 in a greenhouse in Braunschweig, min. temp. 18°.

Extraction and detection of alkaloids. Rosette plants were collected in the greenhouse in 1992. Flowering plants were sampled in the experimental garden in Leiden in 1994. The fr. plant material was weighed, air-dried at 50°, weighed again and ground. Non-labelled PAs were reduced with Zn/H^+ and purified over Extrelut [36]. Individual PAs were identified and quantified by GLC and GC-MS, as described in ref. [3]. PAs were identified by their fragmentation pattern and typical RI (refractive index) in comparison to authentic samples [3]. Total PA content of material sampled in The Netherlands was determined by a spectrophotometric colour reaction [37].

Biosynthesis in isolated roots. Roots of 4-week-old plants were placed in Al-covered glass containers. Roots were allowed to take up 66 kBq of [1,4-^{14}C] putrescine (4 GBq mmol^{-1}, Amersham) from 20 ml medium (100 μM putrescine in Steiner soln). To avoid rotting of roots, the medium was aerated. After 26 hr the tracer was taken up completely and roots transferred to fr. Steiner soln. Roots were harvested in duplicate, 27 and 72 hr after incubation.

Biosynthesis in isolated shoots. Shoots of 4-week-old plants were cut off just above the root crown and placed in cut Eppendorf cups containing 200 μl medium (100 μM putrescine in H_2O). Shoots were allowed to take up 74 kBq of ^{14}C-labelled putrescine. To avoid wilting, cups were placed in a plastic tray with a transparent cover. After 19 hr the medium with tracer was taken up completely and shoots transferred to Eppendorf cups with fresh H_2O. Shoots were harvested in duplicate at 27 and 81 hr after incubation. Single leaves, inflorescences and stem pieces were also tested for their capacity to produce PAs from labelled putrescine. They were treated as described above, but fed with 34 kBq of ^{14}C-labelled putrescine and harvested 47 hr after incubation.

Biosynthesis and transport in intact plants. Five 24-day-old hydroponically grown plants were incubated with 269 kBq ^{14}C-labelled putrescine in 40 ml medium (100 μM putrescine in Steiner soln). The medium was aerated to avoid rotting of roots. After 46 hr the label had been completely taken up and plants were transferred to

1.2 l pots, containing quarter strength Steiner soln. The concn of nutrient soln was gradually increased to full strength, according to the following scheme: week 2: half/strength; weeks 3, 4 and 5: full strength. Plants were harvested at 4, 11, 18, 25 and 32 days after incubation. Roots, cotyledons, the youngest three leaves and older leaves were extracted separately.

Extraction, detection and identification of labelled PAs. Plants parts were weighed, cut and extracted ×3 with MeOH. The combined extracts were analysed for total radioactivity by liquid scintillation counting. The ^{14}C-labelled alkaloids were sepd and detected on TLC as described in ref. [38]. An aliquot of the extract was reduced with Zn in 1 N HCl, purified over Extrelut (Merck) and again analysed by TLC. The spots of a few extracts, which contained sufficient label, were recovered from the silica gel with MeOH and analysed by GC-MS in order to relate R_f values to PAs. Since the selected samples did not contain enough heliosupine for unequivocal GC-MS analysis, this PA was identified on the basis of the R_f value of a purified heliosupine ref. sample (for purification method see ref. [7]).

Residual radioactivity measurements. Plant material that had been extracted with MeOH was incubated in 5-ml containers with 0.2 ml H_2O and 1 ml Lumasolv (Baker). Samples were mixed daily on a vortex mixer to enhance the destruction of plant material. After one week plant material was totally destroyed. After addition of 1 ml Lipoluma, an aliquot was taken to measure radioactivity.

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