Phytochemistry, Vol. 26, No. 6, pp. 1639–1643, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00
Pergamon Journals Ltd.

SENECIONINE N-OXIDE, THE PRIMARY PRODUCT OF PYRROLIZIDINE ALKALOID BIOSYNTHESIS IN ROOT CULTURES OF *SENECIO VULGARIS*

THOMAS HARTMANN and GERD TOPPEL

Institut für Pharmazeutische Biologie der Technischen Universität, D-3300 Braunschweig, F.R.G.

(*Received* 19 *August* 1986)

Key Word Index—*Senecio vulgaris*; Asteraceae; root cultures; biosynthesis; pyrrolizidine alkaloids; alkaloid *N*-oxides.

Abstract—Root cultures of *Senecio vulgaris* synthesize pyrrolizidine alkaloids which are accumulated in the form of their *N*-oxides. The cultures incorporate biosynthetic precursors, such as arginine, ornithine, isoleucine, putrescine and spermidine, with high efficiency into the alkaloids. Senecionine *N*-oxide is found to be the primary product of biosynthesis. With putrescine and spermidine incorporation rates of 20–30% are obtained. The *N*-oxide synthesized does not appear to undergo significant turnover. Tertiary pyrrolizidine alkaloids, if found at all, occur in small amounts in old tissues only. They are derived from the corresponding oxides, and are easily formed spontaneously during alkaloid extraction. The suitability of *N*-oxides in alkaloid storage is discussed.

INTRODUCTION

The pyrrolizidine alkaloids (PAs) are a fascinating group of secondary plant constituents which presumably serve as protective chemicals for the plant [1, 2]. Some insects can store PAs for their own chemical defence [3] or use them as pheromone precursors [4]. The various structures of PAs and their distribution in the plant kingdom are well established [5, 6]. Recent tracer studies clarified the biosynthetic origin of the PA carbon skeleton and revealed that the necine moiety is derived from ornithine or arginine via putrescine and homospermidine as symmetrical intermediates [7–12]. In spite of intense investigations on PAs, their metabolism and functional integration into plant metabolism is still poorly understood. The naturally occurring PAs, for instance, are probably the best known examples of alkaloid *N*-oxides [13, 14], but the significance of *N*-oxide formation is still unknown.

In this communication we introduce root cultures of *Senecio vulgaris* as an efficient biosynthetic system and present evidence that the *N*-oxides of the pyrrolizidine alkaloids (PA *N*-oxides) are the primary products of biosynthesis. The possible function of PA *N*-oxides is discussed against the background of earlier publications [15–17].

RESULTS

Spontaneous reduction of PA N-*oxides*

PA *N*-oxides are easily reduced to the corresponding tertiary alkaloids under various experimental conditions. Some typical examples are given in Table 1. Prolonged refluxing with MeOH (Soxhlet extraction), a method commonly used for PA extraction, causes a reduction of almost 50% of PA *N*-oxides present in the initial material (Table 1). Monocrotaline *N*-oxide is reduced to some extent in pure methanol. However, reduction is increased in the presence of plant material, e.g. *Lycopersicon esculentum* (Table 1). Even in acid macerates a spontaneous reduction occurs which can be prevented when the residual plant material is removed (Table 1). Obviously naturally occurring reducing agents are responsible for this phenomenon. Among various compounds tested, thiols appear to be the most likely candidates. Small amounts of cysteine effectively reduce monocrotaline *N*-oxide; glutathione and reducing sugars are ineffective.

Thus the contradictory reports of the occurrence of PA *N*-oxides frequently depend on the extraction procedure used. Although the importance of PA *N*-oxides as genuine constituents of *Senecio* species has been recognized for a long time [see 13–15] it has been greatly neglected. Most phytochemical reports on PAs are inadequate with respect to PA *N*-oxides.

Table 1. Spontaneous reduction of PA *N*-oxides

Conditions/treatment	Percentage of total PA as tertiary alkaloid
Oven-dried (45°) S. vulgaris	
Control (standard extraction)	5
Soxhlet extraction (48 hr)	44
Freeze-dried S. vulgaris	
Control (standard extraction)	tr
Acid macerate (0.25 M H_2SO_4) 24 hr at 50°	30
Supernatant of acid macerate 24 hr at 50°	tr
Monocrotaline N-*oxide*	
Control (standard extraction)	tr
Soxhlet extraction (48 hr)	22
Soxhlet extraction (48 hr) + plant dry powder*	46
24 hr incubation with 1 mg/ml cysteine at 25°	15
24 hr incubation with 1 mg/ml cysteine at 55°	43

*Freeze-dried leaves of *Lycopersicon esculentum*, a species which does not contain PAs, were mixed with monocrotaline *N*-oxide (10 g dry powder + 10.4 mg alkaloid *N*-oxide).

With our standard procedure of PA extraction and analysis, generally more than 90% of total PAs in *S. vulgaris* and many other species so far analysed were present as *N*-oxides. Spontaneous oxidation of tertiary alkaloids has never been observed.

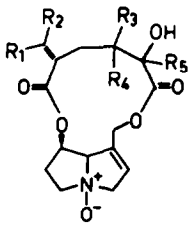
Alkaloid pattern of root cultures

Root cultures of *S. vulgaris* show the same alkaloid pattern as that found in the respective parent plants (Fig. 1, Table 2). *S. vulgaris* as a self-pollinating species displays great variation in the alkaloid composition of specimens collected at different locations. This is particularly true of the relative abundancies of senecionine and seneciphylline [15 and refs quoted therein]. The root culture originated from a plant population characterized by senecionine *N*-oxide as the dominating alkaloid. In young root cultures this alkaloid and its geometrical isomer integerrimine *N*-oxide account for more than 95% of total alkaloids. The *N*-oxides of seneciphylline and retrorsine are found in older root cultures in a similar proportion to that found in roots of intact plants. This indicates that they may be formed from senecionine by dehydrogenation and hydroxylation, respectively.

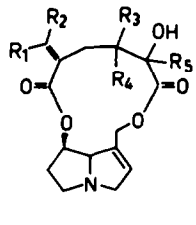
In old root tissues low but significant amounts of tertiary alkaloids are detectable, whereas only trace amounts are found in young tissues (Table 2). No alkaloids could be identified from the culture medium. The alkaloid concentration in cultured roots is always higher than in the roots of intact plants at various stages of development (Table 2) [15]. One reason for this discrepancy is that it is impossible to harvest the roots from intact plants without some loss of the alkaloid rich fine roots.

Biosynthesis of senecionine N-*oxide in root cultures*

Feeding *S. vulgaris* root cultures with [^{14}C]putrescine resulted in an unexpectedly high incorporation of radioactivity into the fraction containing PA *N*-oxides. The percentage of ^{14}C incorporation was generally between 20 and 30%, but reached 50% in some experiments. HPLC separation of the PA *N*-oxide fraction revealed only one labelled peak which coincided with authentic senecionine *N*-oxide. No other labelled PAs could be detected. Senecionine *N*-oxide can easily be reduced to the corresponding tertiary senecionine which, however, was not detected as a product of biosynthesis.



PA-N-oxide



tertiary PA

		R_1	R_2	R_3	R_4	R_5
1	Senecionine N-oxide	Me	H	Me	H	Me
2	Integerrimine N-oxide	H	Me	Me	H	Me
3	Seneciphylline N-oxide	Me	H	$=CH_2$		Me
4	Retrorsine N-oxide	Me	H	Me	H	CH_2OH

Fig. 1. The PAs known to be present in *S. vulgaris* plants.

In addition to putrescine, several labelled substrates known to be percursors of PAs were tested. With the exception of spermine, all were effectively incorporated into senecionine *N*-oxide (Table 3). The incorporation of spermidine was almost as high as that for putrescine. Arginine and ornithine behave identically and are incorporated at similar rates as isoleucine which is a precursor of the senecic acid moiety of the alkaloid [18–21]. All precursors were taken up almost completely into the roots and incorporated most efficiently at concentrations of *ca* 0.1 mM.

S. vulgaris root cultures thus provide an excellent system for the synthesis of isotopically labelled PAs. Using [^{14}C]putrescine as precursor labelled senecionine *N*-oxide could be prepared with a radiochemical yield of *ca* 20%.

Stability of newly synthesized senecionine N-*oxide*

The observed high incorporation of precursors into senecionine *N*-oxide led us to examine the turnover or stability of the root alkaloids. Root cultures were allowed to metabolize [^{14}C]putrescine for 24 hr and were then transferred into normal culture medium and kept under normal growth conditions for 10 days. The results are summarized in Table 4. During the growth period (10 days) both the fresh weight and the total alkaloid content

Table 2. Alkaloids of root cultures and roots of intact plants

	Alkaloid abundance (area %)				Total alkaloids (mg/g fr.wt)	Percentage of total alkaloid as tertiary PA
	Senecionine *N*-oxide	Integerrimine *N*-oxide	Seneciphylline *N*-oxide	Retrorsine *N*-oxide		
Root culture						
14-days-old	86	14	tr	tr	0.66	<5
22-days-old						
Fine roots	74	9	12	5	0.76	<2
Coarse roots	72	12	13	3	0.36	16
Roots from intact plants*	66	17	14	3	0.12	15

*The same population of plants from which the root culture was derived.

increased *ca* seven-fold. However, the amount of radioactive alkaloid synthesized during the initial 24 hr incubation period, not only remained unchanged, but actually increased at the expense of other labelled compounds (presumably polyamines). Senecionine *N*-oxide behaves as a metabolic end product which is continuously synthesized during root growth. Autoradiographic studies revealed that no translocation of previously synthesized alkaloid takes place into newly grown roots.

DISCUSSION

On the basis of the current literature on PAs our studies revealed two unexpected results. (a) Roots of *Senecio* were found to be the site of highly effective PA synthesis. (b) The PA *N*-oxides were found to be the primary products of PA biosynthesis.

There are only two previous reports related to the sites of PA biosynthesis. Excised shoots of *Crotalaria spectabilis*, fed via the transpiration stream, incorporated radioactivity from [^{14}C]ornithine and [^{14}C]acetate with an efficiency of *ca* 0.35% into PAs [22]. Shoots of young plants of *Heliotropium spathulatum* exposed to pulse labelling with $^{14}CO_2$ synthesized labelled PAs [23]. Removal of roots did not affect the efficiency of this process. These results are in favour of the leaves as preferred organs of PA synthesis. Furthermore, labelled precursors used in biosynthetic studies are usually applied by either the wick method [11, 12], hydroponically to growing seedlings [19, 24], or, most efficiently, via the

Table 3. Incorporation of ^{14}C-labelled precursors into senecionine *N*-oxide by root cultures of *S. vulgaris*

Precursor	Assay concentration (mM)	Uptake into roots (%)	Total incorporation into senecionine *N*-oxide (%)
L-[U-^{14}C]Arginine	0.01	98	6.2*
	0.1	96	9.2*
	1.0	89	4.1*
L-[U-^{14}C]Ornithine	0.01	97	8.7*
	0.1	96	9.0*
	1.0	94	7.1*
[1,4-^{14}C]Putrescine	0.01	95	27.8
	0.1	94	24.5
	1.0	85	2.4
[^{14}C]Spermidine†	0.1	95	23.3
	1.0	49	10.0
[^{14}C]Spermine‡	0.1	72	tr
L-[U-^{14}C]Isoleucine	0.1	96	8.9*
	1.0	94	3.1*

Incubation: growing roots, 0.7 g fr.wt in 5 ml culture medium plus tracer (0.5–1.0 μCi each), 24 hr at 25°.

*Corrected for loss of labelled C-atoms during biosynthesis.

†*N*-(3-aminopropyl)-[1,4-^{14}C]tetramethylene-1,4-diamine.

‡*N*,*N*′-bis-(3-aminopropyl)-[1,4-^{14}C]tetramethylene-1,4-diamine.

Table 4. Stability of [^{14}C]senecionine *N*-oxide, newly synthesized from [^{14}C]putrescine in growing root cultures of *S. vulgaris*

	Exp. No.	Fr.wt of roots at harvest (g)	Total root alkaloids (mg)	Total root alkaloids (mg/g fr.wt)	Tracer uptake into roots (%)*	^{14}C-Activity in MeOH extracts (10^{-3}, cpm)	^{14}C-Activity in MeOH extracts Percentage as senecionine *N*-oxide
Incubation with 0.1 mM	Ia	0.89	—	—	52	381	36
[^{14}C]putrescine for 24 hr	Ib	0.91	0.429	0.471	45	440	26
As Exp. I, but growth in 'cold'	IIa	5.48	2.992	0.546	41	439	57
culture medium for further 10 days	IIb	6.53	—	—	52	384	57

*Calculated from decrease of radioactivity in the medium.

Incubation: 4 × 100 ml flasks each containing 20 ml MS-medium and 0.1 mM [^{14}C]putrescine (*ca* 1.5 μCi) were inoculated with 0.7 g fresh roots. Two flasks (exp. no. I) were harvested after 24 hr. The roots of the two remaining flasks were removed, thoroughly washed aseptically, transferred into 300 ml flasks with 100 ml MS-medium, and allowed to grow. After 10 days they were harvested (exp. no. II).

stem puncture method [8–10, 25]. With these methods incorporations of *ca* 0.3–3% have been obtained, depending on the kind of precursor and time of exposure which often exceeded 1 week. Our data show that root cultures of *S. vulgaris* incorporate various precursors into PA *N*-oxides with an efficiency which is by far the highest ever observed for PA biosynthesis. The relative efficiencies by which the precursors are incorporated into the alkaloids (see Table 3) are comparable to those described for long-term experiments. The only exception is spermine. Spermidine and spermine have been shown to be efficient PA precursors [26, 27]. We found spermidine as a precursor to be as effective as putrescine, whereas spermine was ineffective. In *S. vulgaris* root cultures PA synthesis appears to be closely related to polyamine metabolism and depends on conditions of active growth [Hartmann and Toppel, unpublished work]. The efficient channelling of radioactivity from the various precursors into the PA carbon skeleton can only be understood if we assume the presence of a highly specific biosynthetic sequence functionally integrated into primary cellular metabolism. In addition the high incorporation rates may be partially explained by the fact that the PA *N*-oxides in root cultures appear not to undergo significant metabolic changes. Very low (if any) turnover of PAs has been recently reported for *Heliotropium* [23, 28]. The observed synthesis of PA *N*-oxides in root cultures is not unique to *S. vulgaris*. At present we have root cultures of four *Senecio* species, each of which synthesizes PA *N*-oxides characteristic to the parent plant [Hartmann, Toppel and v. Borstel, unpublished work].

Our biosynthetic experiments revealed that senecionine is synthesized exclusively in the form of its *N*-oxide. This is the first time that an alkaloid *N*-oxide has been identified as a primary product of biosynthesis. Cultured roots of *S. vulgaris*, as well as the various organs of intact plants [15], accumulate PAs as *N*-oxides. The corresponding tertiary PAs, which are present in small amounts only, if at all, can be regarded as degradation products of the PA *N*-oxides. They are easily formed spontaneously; whether in addition an enzymatic reduction is involved remains to be established. PA *N*-oxides and their respective tertiary PAs display very different properties. The *N*-oxides no longer behave like typical alkaloids, but instead are very polar, salt-like compounds which are readily soluble in water and insoluble in most organic solvents. They are not able to non-specifically permeate biological membranes like typical tertiary alkaloids in their unprotonated form. Recently we discovered that cell suspension cultures of PA-producing plants are able to take up PA *N*-oxides, whereas cultures of non PA-producing plants do not [16]. Uptake studies with ^{14}C-labelled senecionine *N*-oxide proved that the *N*-oxides are taken up by and stored in the vacuoles [17]. Taking all these facts together we suggest that *N*-oxidation creates a molecular species which can be translocated and stored in the cell in a much safer way than the respective tertiary alkaloid. In this respect *N*-oxidation of an alkaloid may be compared to glycosylation or acylation of some vacuole-stored secondary compounds. Regarding the function of PAs as powerful protective agents, maintenance of high concentrations and safe storage of PA must be a functional prerequisite to fulfill this function.

EXPERIMENTAL

Plant material. Root cultures were established in December 1985 from aseptically grown seedlings of *Senecio vulgaris* L. Plants were collected in 1985 from a wild population (vicinity Braunschweig). The achenes were washed with detergent and EtOH and surface sterilized in 15% H_2O_2 for 15 min and then kept in sterile H_2O for 1 day. The fruits were germinated on agar plates in Murashige–Skoog medium (MS-medium) [29] with sugar and phytohormones omitted. Roots (length *ca* 1 cm) from 3 to 4 seedlings were excised and transferred into 10 ml MS-medium, pH7, containing 4% sucrose and no phytohormones. To initiate root growth it was important to start with a small vol. of liquid medium and more than one root. As soon as the culture was established it was subcultured in 300 or 500 ml flasks containing 50 or 100 ml culture medium, respectively. The cultures were routinely transferred every 14 days and kept in the dark on a gyratory shaker (120 rpm at 25°).

Radiochemicals. L-[U-^{14}C]Arginine, L-[U-^{14}C]ornithine, L-[U-^{14}C]isoleucine, [1,4-^{14}C]putrescine, [^{14}C]spermidine (*N*-(3-aminopropyl)-[1,4-^{14}C]tetramethylene-1,4-diamine), [^{14}C]-spermine (*N*,*N*'-bis-(3-aminopropyl)-[1,4-^{14}C]tetramethylene-1,4-diamine) were obtained from Amersham Int. All radiochemicals were provided as hydrochloride salts.

Alkaloids. Senecionine, integerrimine, seneciphylline and retrorsine were isolated as tertiary PAs from *S. vulgaris* or *S. vernalis* according to [15]. Monocrotaline was obtained (99% pure) from Aldrich Chem. Comp. The alkaloid *N*-oxides were prepared as described in [30].

Alkaloid extraction. Standard procedure: plant material, *ca* 6–10 g fr. wt/20 ml 0.05 M H_2SO_4, was homogenized for 3–4 min (Ultra-turrax) and the homogenate left to stand for 30 min at room temp. After centrifugation half of the supernatant was made basic with NH_4OH and applied to a Extrelut (Merck) column (1.4 ml aq. soln/g Extrelut). PAs were eluted with CH_2Cl_2 (6 ml/g Extrelut). After evapn of the eluent the residue was redissolved in a known vol. of MeOH. This soln contains all tertiary PAs. The remaining half of the acid supernatant was adjusted to 0.25 M H_2SO_4 and mixed with an excess of Zn dust. The mixture was stirred at room temp. for 5 hr unless PA *N*-oxides were to be reduced quantitatively. In some experiments Zn dust was replaced by $Na_2S_2O_4$; in 0.05 M H_2SO_4 quantitative reduction was obtained within 1 hr. The soln was made basic and further processed as given above; it constituted the total alkaloid fraction (PAs + PA *N*-oxides).

Soxhlet extraction. Dried and powdered plant material was extracted with MeOH for 48 hr. The solvent was evapd, the residue redissolved in 0.25 M H_2SO_4 and processed further as given for the standard procedure.

Capillary GC. Alkaloid extracts were separated and evaluated quantitatively on fused silica columns (WCOT, 25 m × 0.25 mm, DB-1, ICT). Conditions: injector 250°, temp. programme 120–290°, 8°/min; split ratio 1:50; injection vol. 1–2 μl; carrier gas He 0.7 bar; detectors: FID, PND.

HPLC separation of PA N-oxides. Freeze dried roots were extracted with MeOH (0.3 g dry wt/30 ml) for 3 min (Ultra-turrax). After centrifugation and evapn of the solvent, the residue was redissolved in 2 ml MeOH; 20 μl was injected into the HPLC. Separation was achieved by means of reverse-phase HPLC on a RP-18 column (μ-Bondapak, 30 cm × 3.9 mm) developed isocratically with MeOH–K-Pi buffer, 15 mM, pH 7.5 (1:2). Pressure: 15 MPa; flow: 1.5 ml/min: detection: UV at 209 nm. The R_t (min) values for the PA *N*-oxides were as follows: retrorsine *N*-oxide, 3.9; seneciphylline *N*-oxide, 6.1; senecionine *N*-oxide–integerrimine *N*-oxide, 8.0. Suitable separation of PAs was achieved by changing the solvent composition to MeOH–K-Pi buffer, 15 mM, pH 7.5 (1.5:1). Peaks were quantified by the external standard method with previously isolated and purified compounds.

TLC. PAs and PA *N*-oxides were separated on silica gel 60F254 (Merck). The solvent systems used were: A, $CHCl_3$–MeOH–NH_3 (91:15:3); B, toluene–EtOAc–diethylamine (5:3:2). The R_f values for senecionine *N*-oxide and senecionine were, solvent A: 37 and 93, respectively; solvent B: 2 and 66, respectively. The PA *N*-oxides were detected specifically according to [31]. Radioscans were performed by means of a TLC multichannel analyser (Berthold).

Tracer studies. Growing roots (0.6–0.7 g fr. wt) were incubated in 5 ml growth medium containing the appropriate tracer (0.5–1 μCi) under continuous shaking at 25° for 24 hr. The roots were washed with tap water and extracted with acid MeOH as given for PA *N*-oxide extraction for HPLC. The final extracts were analysed by scintillation counting and TLC-scanning.

Acknowledgements—Financial support by Fonds der Chemischen Industrie is gratefully acknowledged. We wish to thank Dr. M. Isman for linguistic help.

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