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SOLUBILIZATION AND CHARACTERIZATION OF A SENECIONINE *N*-OXYGENASE FROM *CROTALARIA SCASSELLATII* SEEDLINGS

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Key Word Index—*Crotalaria scassellatii*; Fabaceae; pyrrolizidine alkaloids; *N*-oxidation; senecionine *N*-oxygenase.

Abstract—Seeds of *Crotalaria scassellatii* (Fabaceae) store pyrrolizidine alkaloids as tertiary amines. During the beginning of seed germination the tertiary alkaloids are rapidly converted into the respective alkaloid *N*-oxides which are the ultimate forms of alkaloid transport, metabolism and storage in vegetative plant organs. The enzyme catalyzing the *N*-oxidation was isolated from 2-day old seedling, partially purified and characterized. It is a membrane-bound, but not microsomal enzyme sedimenting in the 39 000*g* fraction. The particulate enzyme was solubilized in the presence of 0.4% CHAPS and 0.4 M NaCl. The solubilized enzyme was partially purified (228-fold) by means of 70% ammonium sulfate precipitation and monocrotaline affinity chromatography. Inhibitor experiments, temperature sensitivity and lack of a carboxy ferrocytochrome absorption maximum at 450 nm strongly indicate SNO to be a flavin dependent enzyme. It is a mixed function monooxygenase that specifically *N*-oxidizes a number of structurally related pyrrolizidine alkaloids including the alkaloids of *Crotalaria*. A great variety of related alkaloids and xenobiotics were tested as substrates, none was accepted. The apparent K_m values of senecionine, monocrotaline and heliotrine representing the three major types of pyrrolizidine alkaloids, are 12.4, 40.1 and 370.9 μM, respectively. Senecionine is the best substrate, consequently the enzyme was named senecionine *N*-oxygenase (SNO). The substrate specificity of SNO is almost identical with that of a soluble insect SNO recently characterized from the haemolymph of arctiid larvae [Lindigkeit, R., Biller, A., Buch, M., Schiebel, H.-M., Boppré, M. and Hartmann, T., *European Journal of Biochemistry*, 1997, **245**, 626].

INTRODUCTION

Pyrrolizidine alkaloids are a class of typical plant secondary compounds. They encompass a diverse group of about 360 structures with restricted occurrence in certain higher plant taxa such as the genera *Senecio* (Asteraceae tribe Senecioneae), *Eupatorium* (Asteraceae, tribe Eupatorieae), *Heliotropium* (Boraginaceae) and *Crotalaria* (Fabaceae) [1]. Pyrrolizidine alkaloids are important components in chemical defence of the producing species, which are usually avoided by herbivores. Pyrrolizidine alkaloids are strong feeding deterrents and per se non-toxic. But they are pro-toxins that are readily transformed into reactive pyrrolic intermediates by action of microsomal cytochrome *P*-450 oxidases (EC 1.14.14.1) which are part of xenobiotic-metabolism in vertebrates and insects. Because of this bioactivation pyrrolizidine alkaloids are strongly hepatotoxic and pneumotoxic to vertebrates [2, 3] and genotoxic to insects [4]. One mechanism that counteracts bioactivation is *N*-oxidation, a reaction by which the potentially toxic tertiary amine is converted into the polar pyrrolizidine alkaloid *N*-oxide which no longer can be transformed into a pyrrolic toxin. For example, guinea pigs possess a reactive microsomal multisubstrate flavin monooxygenase (EC 1.14.13.8) which catalyzes efficiently the conversion of ingested pyrrolizidine alkaloids into their *N*-oxides [5]. This explains the resistance of guinea pigs to poisoning by pyrrolizidine alkaloids.

A number of insect species from different taxa have evolved adaptations to acquire plant pyrrolizidine alkaloids and use them for their own defence

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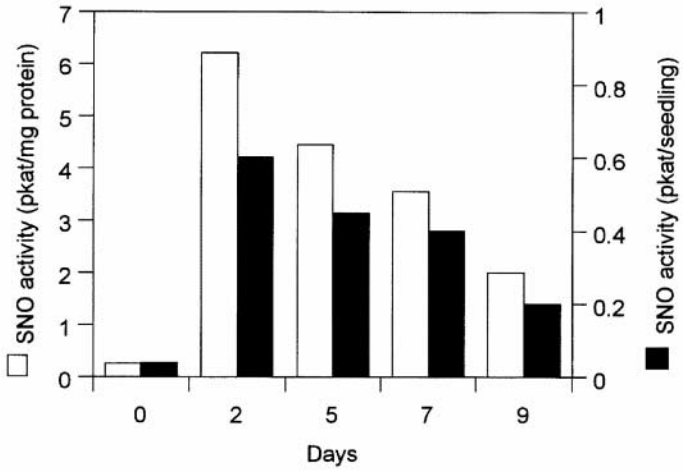

Fig. 1. Changes in specific and absolute activities of particulate senecionine *N*-oxidase (SNO) during the beginning of germination of seeds from *Crotalaria scassellatii*. Day 0 = Appearance of the radicle (*ca.* 48 h after beginning of seed imbibition in tap water).

against insectivores [1, 6–8]. All these species belonging to unrelated taxa such as Lepidoptera, Orthoptera and Chrysomelidae sequester pyrrolizidine alkaloids as *N*-oxides. Recently we isolated and purified a soluble flavin monooxygenase from the haemolymph of three arctiids (Lepidoptera). The enzyme catalyzes exclusively the oxidation of pyrrolizidine alkaloids with a hepatotoxic and genotoxic potential [9]. The enzyme is highly specific for pyrrolizidine alkaloids. It must have been evolved in the course of coadaptation of alkaloid sequestering insects to pyrrolizidine alkaloid producing plants as a means to keep the alkaloids in a nontoxic, metabolically safe state.

However, not only vertebrates and insects detoxify pyrrolizidine alkaloid by *N*-oxidation, most plants synthesize them as *N*-oxides. In this respect pyrrolizidine alkaloids are unique. In *Senecio* species, for instance, pyrrolizidine alkaloids are synthesized, allocated and stored as *N*-oxides [10, 11]. A membrane carrier has been identified which selectively translocate pyrrolizidine alkaloid *N*-oxides via the tonoplast into vacuoles [12].

The present study was undertaken to identify the enzyme responsible for pyrrolizidine alkaloid *N*-oxidation in plants. So far, there are no *N*-oxidating enzymes known from plants. Germinating seeds of the West African legume *Crotalaria scassellatii* were chosen as promising enzyme source. In all vegetative tissues of the plant pyrrolizidine alkaloids are stored as *N*-oxides. Dry seeds, however, contain the alkaloids as lipophilic tertiary amines, which in a desiccated tissue are assumed to be a well suited storage form. With beginning of germination there is a rapid *N*-oxidation of the seed alkaloids [13]. Here we describe the characterization of the enzyme responsible for this *N*-oxidation.

RESULTS

Detection of particulate senecionine N-oxygenase (SNO) activity in Crotalaria scassellatii seedlings

Dry seeds of *Crotalaria scassellatii* accumulate approximately 2% of pyrrolizidine alkaloids of the monocrotaline type such as axillaridine, axillarine and desoxyaxillarine [14]. These alkaloids are exclusively stored as tertiary amines. However, during seed germination these tertiary alkaloids are rapidly *N*-oxidized [13]. Using [^{14}C]senecionine as a substrate *N*-oxidation activity was detected in a particulate fraction obtained from young seedlings. Highest specific and absolute activities were found in very young seedlings just 48 h after appearance of the radicle (Fig. 1). Therefore, two-days old seedling were used as enzyme source in all subsequent studies. The enzyme responsible for the *N*-oxygenation was identified as a particulate mixed function monooxygenase catalyzing the reaction illustrated in Fig. 2. Its product senecionine *N*-oxide was identified by fast-atom-bombardment mass spectrometry (FAB-MS) [9]. The reaction is dependent on O_2 and NADPH. Since in subsequent studies senecionine turned out to be the best substrate we named the enzyme senecionine *N*-oxygenase (SNO). Differential centrifugation revealed $>90\%$ of particulate SNO activity in the 39 000*g* sediment. This suggests that SNO is not a typical microsomal enzyme, since microsomes generally sediment at much higher speeds, i.e. 100 000*g* [15].

Solubilization and partial purification of SNO

Treatment of the 39 000*g* sediment with a number of detergents revealed efficient solubilization of SNO activity only with CHAPS (Table 1). Optimal solubilization was obtained at 0.4% CHAPS in the

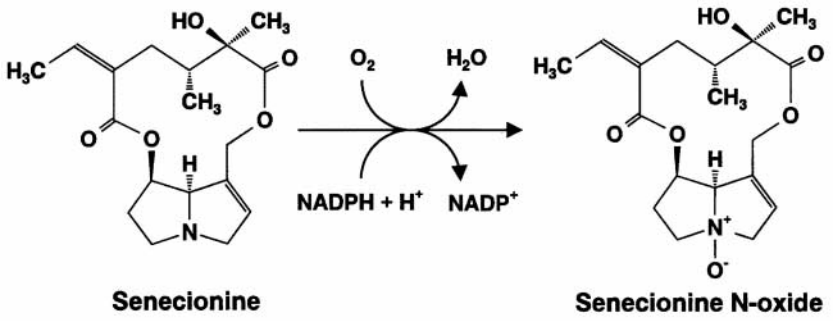

Fig. 2. Reaction catalyzed by senecionine *N*-oxygenase (SNO).

presence of 0.4 M NaCl to decrease the critical micellar concentration of the detergent [16]. All attempts to purify solubilized SNO with chromatographic standard procedures such as hydroxyapatite, anion-exchange, gel filtration, hydrophobic interaction or Cibacron Blue Sepharose failed or gave poor results under various conditions. Efficient purification was obtained only with monocrotaline affinity chromatography. The protein eluted with standard buffer containing 1 mM monocrotaline showed a 72-fold increase in SNO specific activity (Table 2). SNO was purified 228-fold over the particulate fraction.

General properties of SNO

The solubilized partially purified enzyme shows a pH optimum of 7.5 in standard buffer. The pH optimum of the particulate enzyme is in the range of 8.0 to 8.5 and thus considerably higher. SNO has its maximal activity at temperatures of 35–40°C (particulate SNO) and 40–45°C (soluble SNO); the activation energy (E_a) was determined over a range of 10 to 30°C and was found to be 37.5 and 36.1 kJ/mol, respectively. Different lines of evidence indicate that SNO is not dependent on cytochrome *P*-450. The carbon monoxide differential spectrum of the Na-dithionite reduced particulate SNO did not show the typical absorption maximum of cytochrome *P*-450 [17, 18]. Typical cytochrome *P*-450 inhibitors such as SKF 525A, metyrapone, *n*-octylamine, guanethidine, cyanides and azides did not significantly affect SNO activity (Table 3) [19]. Cytochrome c, another potent *P*-450 inhibitor also reduces sensitively SNO activity. The *P*-450 inhibitors *n*-octylamine and to a lesser extent metyrapone and guanethidine enhance SNO activity. Activation, particularly by *n*-octylamine, has been reported for a number of flavin-containing monooxygenases [20–23]. As the activity of solubilized SNO is 1.6-fold increased by 0.5 mM *n*-octylamine; it was routinely included as an activator in the photometric assay of enzyme activity. Methimazole which is an alternative substrate and inhibitor of FMO [20] slightly inhibits SNO. Many FAD-containing monooxygenases are extremely sensitive to thermal inactivation in the absence of NADPH while *P*-450 enzymes remain stable [5, 21, 23, 24]. This thermal inactivation was also found with SNO (Table 4). Particularly, the solubilized enzyme is completely inactivated by preincubation at 37°C for 20 min in absence of NADPH, while preincubation at 37°C in the presence of NADPH has no effect at all.

Table 1. Effect of various detergents on the solubilization of SNO from the 39 000 *g* sediment of *C. scassellatii* seedlings

Detergent	Solubilized SNO activity (%)*
Particulate fraction	100
No addition	< 1
CHAPS	44
CHAPS + 0.4 M NaCl	107
Emulgen 911	2.4
Emulgen 913	3.4
Synperonic	< 1
Tween 20	8.3
Tween 80	9.7
Triton x-100	2.4
n-Octylglucoside	< 1

The detergents were applied to the suspended 39 000*g* sediment at concentrations of 0.4% (w/v). The suspension was gently stirred for 1 h and centrifuged at 100 000*g* for 90 min. Enzyme Assay: standard conditions, toluene method.

*Activity of the non-treated particulate fraction was set 100%.

Substrate specificity and substrate kinetics of SNO

A total of 23 pyrrolizidine alkaloids (Fig. 3) were tested as substrate of solubilized SNO (Table 5). The alkaloids represent a selection of compounds from the five structural types of pyrrolizidine alkaloids according to the classification given in Ref. [1]. Not all of them are substrates. The unesterified necine bases are not *N*-oxidized, as well as monoesters lacking a hydroxyl group at the C-7 (i.e. supinine and phalaenopsine). With the exception of senkirkine, all macrocyclic pyrrolizidines, open-chain diesters and monoesters with a free hydroxyl group at the C-7 are substrates of SNO. Senkirkine, the otonecine analogue of senecionine, is not *N*-oxidized. This is not surprising, plant otonecine esters in contrast to the respective retronecine esters have never been described as *N*-oxides.

In addition to pyrrolizidine alkaloids more than 35 structurally related compound and potential substrates of multisubstrate FMOs were tested as sub-

Table 2. Partial purification of senecionine *N*-oxygenase (SNO) from seedlings of *C. scassellatii*

Purification step	Protein (mg)	Total activity (pkat)	Specific activity (pkat mg^{-1})	Yield (%)	Purification (-fold)
Particulate fraction	126.8	37.8	0.40	100	1
Solubilized fraction	70.95	56.1	0.79	148	1.9
Ammonium sulfate	30.45	38.5	1.26	102	3.2
Monocrotaline sepharose	0.253	10.2	91.06	27	227.6

The particulate fraction (39 000 *g* sediment) was prepared from 600 seedlings.

strates. None of these compounds, which include tropane alkaloids, quinolizidine alkaloids, indole alkaloids, isoquinoline alkaloids, and several synthetic heterocyclic tertiary amines were found to be substrates of SNO.

Substrate kinetics were established for three selected alkaloids representing the three major types of pyrrolizidine alkaloids (Table 6). It is remarkable that senecionine shows a lower apparent K_m' value and a higher relative efficiency as substrate than monocrotaline, which represents the alkaloid type occurring in the genus *Crotalaria*. Heliotrine a monoester of the lycopsamine type (Fig. 3) is much less efficiently *N*-oxidized than the two macrocyclic diesters.

The SNO-catalyzed reaction is NADPH-dependent. However, although much less efficiently, NADH can replace NADPH as cosubstrate (Table 6). This is only true for the solubilized enzyme. With particulate SNO, no activity could be recorded with NADH as hydrid donor (data not shown).

DISCUSSION

The mixed function monooxygenase isolated from seedling of *Crotalaria scassellatii* specifically *N*-oxidizes a number of pyrrolizidine alkaloids. In this respect it is very similar to a mixed function monooxygenase which recently was isolated from the haemolymph of specialized insects (i.e. Arctiids, Lepidoptera) which sequester plant acquired pyrrolizidine alkaloids [9]. For the two enzymes senecionine was found to be the best substrate, consequently the enzymes were named senecionine *N*-oxygenase (SNO). They will subsequently be referred to as "plant SNO" and "insect SNO". Insect SNO is a soluble flavin-dependent enzyme which efficiently keeps sequestered pyrrolizidine alkaloids in the non-toxic *N*-oxide state. It should be mentioned, that in alkaloid sequestering lepidopterans the plant derived alkaloid *N*-oxides are reduced in the guts and are taken up passively as tertiary alkaloids [9]. In germinating *C. scassellatii* seeds plant SNO has the function to convert the seed-stored tertiary alkaloids into the more polar soluble *N*-oxides, the genuine form of alkaloid transport, metabolism and vacuolar storage in plants. The occurrence of SNOs with almost identical substrate specificity in plants and insects is striking. The only marked difference is that the insect SNO does not accept the 1,2-saturated sarracine (Fig. 3) as a substrate, while plant SNO does. With the exception of this compound the enzymes *N*-oxidize alkaloids with structural features of hepatotoxic [25] and genotoxic [4] pyrrolizidine alkaloids, i.e. presence of an 1,2-double bond, an allylic esterified hydroxyl group (at C-9), a free or esterified second hydroxyl group at C-7 (Fig. 3) [9]. This might indicate that not only animals but also plants keep pyrrolizidine alkaloids in the *N*-oxidized state in order to prevent self-poisoning.

In contrast to insect SNO, the genuine plant enzyme is particulate and at least after solubilization it accepts NADH, although less efficient, as

Table 3. Effects of potential inhibitors of cytochrome *P*-450 and flavin dependent monooxygenase on SNO activity

Inhibitor	Concentration (mM)	Particulate SNO (activity, %)	Soluble SNO (activity, %)
Control		100	100
SFK 525A	0.5	100	106
	1.0	88	94
Metyrapone	0.5	109	113
	1.0	109	107
Cytochrome c	0.005	53	65
	0.025	28	56
n-Octylamine	0.5	106	161
	1.0	121	122
Guanethidine	0.1	100	
	0.5	116	120
KCN	2.0	97	103
	3.0	90	95
NaN_3	1.0	99	106
	3.0	97	104
Methimazole	0.1	86	
	0.5	76	78

Assays were preincubated with the inhibitors for 5 min before the reaction was started by addition of NADPH. Standard assay conditions with senecionine as substrate; toluene method.

Table 4. *N*-Oxidation of senecionine by particulate and solubilized SNO from *C. scassellatii* seedlings at 37°C in the presence and absence of NADPH

Preincubation 20 min; 37°C	Particulate SNO (activity, %)*	Solubilized SNO (activity, %)*
None (control)	100	100
With NADPH	82	108
Without NADPH	28	0

SNO was preincubated in standard assay mixture with or without NADPH as indicated. Reactions were started by addition of senecionine and senecionine plus NADPH, respectively.

*Non-treated control was set 100%.

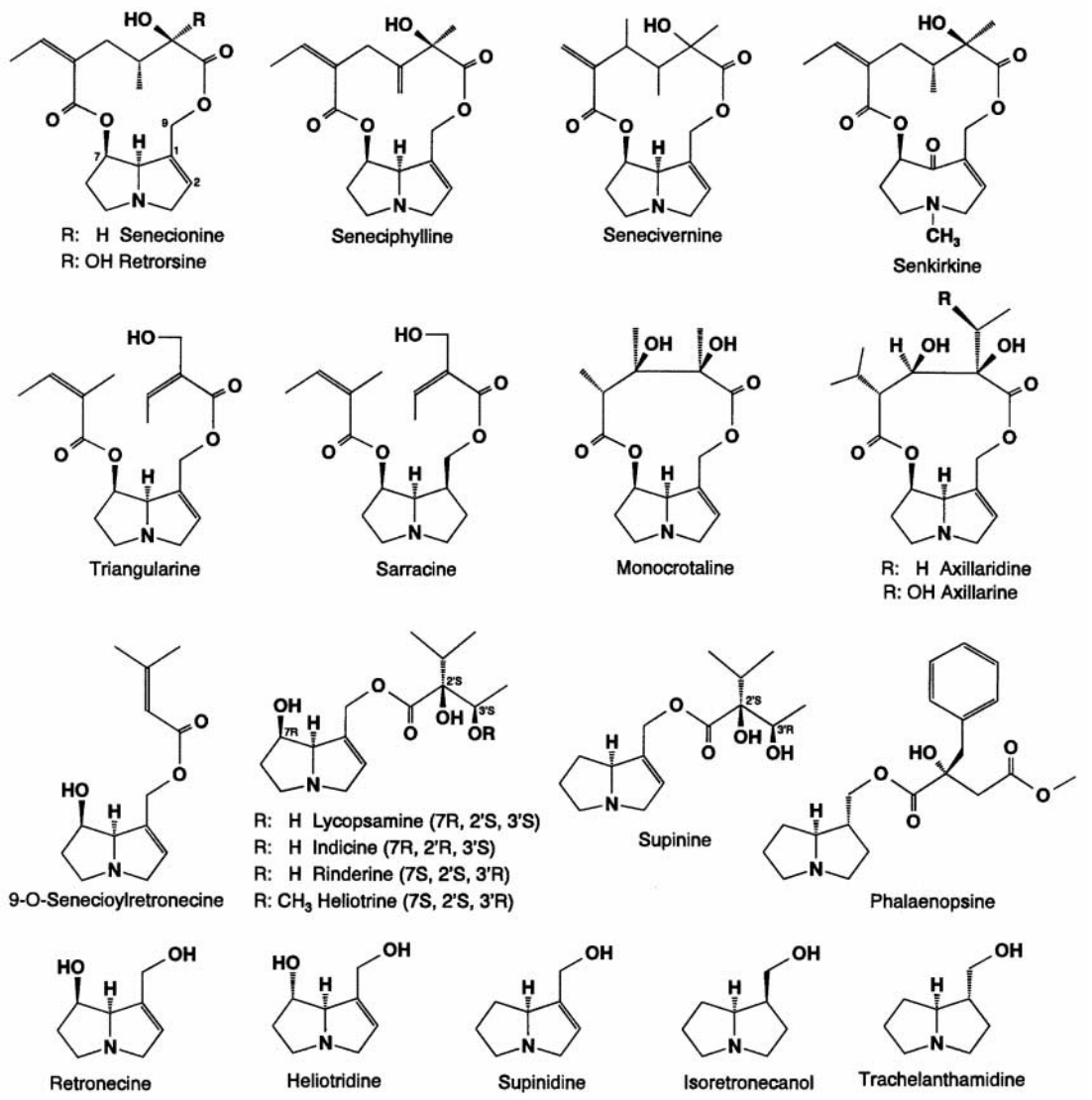

Fig. 3. The structures of pyrrolizidine alkaloids applied as potential substrates of senecionine *N*-oxygenase (SNO).

alternative hydrid donor, while insect SNO does not. The results of the inhibitor experiments, lack of the typical carboxy ferrocytochrome absorption maximum at 450 nm [18] and the temperature sensitivity in the absence of NADPH (Table 4) strongly indicate that plant SNO like insect SNO is flavin-dependent and not a cytochrome *P*-450 enzyme. In this respect the two SNOs share the flavine dependency and ability to *N*-oxidize pyrrolizidine alkaloids with the mammalian microsomal multisubstrate flavine monooxygenase (FMO), which has been purified from liver, lung and kidney [26–28]. FMO plays a major role in xenobiotic metabolism and shows an extraordinary broad substrate specificity and oxidizes a variety of nucleophilic organic nitrogen compounds [29]. In contrast to FMO the two SNOs are highly specific for pyrrolizidine alkaloids with certain structural features. No other substrate outside the pyrrolizidine alkaloids have been found so far. During recent years a number of highly substrate specific *P*-450 enzymes have been detected that are integrated in biosynthetic pathways of secondary compounds, e.g. cyanogenic glycosides [30] or alkaloids [31]. These enzymes are clearly distinct from microsomal *P*-450 (unspecific xenobiotic) monooxygenase (EC 1.14.14.1). In a comparable way the two SNOs, which specifically participate in pyrrolizidine alkaloid metabolism, are distinct from multisubstrate FMO.

Table 5. Substrate specificity of solubilized SNO from *C. scassellatii*

Substrates	Relative SNO activity (%)
Senecionine type	
Senecionine	100
Seneciphylline	97
Retrorsine	72
Senecivernine	94
Senkirkine	0
Triangularine type	
Triangularine	37
Sarracine	33
9-*O*-Senecioylretronecine	36
Monocrotaline type	
Monocrotaline	69
Axillarine	65
Axillaridine	87
Lycopsamine type	
Lycopsamine	20
Intermedine	23
Rinderine	16
Indicine	10
Heliotrine	39
Supinine	0
Phalaenopsine type	
Phalaenopsine	0
Necines bases	
Retronecine	0
Heliotridine	0
Supinidine	0
Isoretronecanol	0
Trachelanthamidine	0

Triangularine, supinine, phalaenopsine, supinidine, isoretronecanol and trachelanthamidine were available only as ^{14}C-labelled substrates and were assayed in comparison to [^{14}C]senecionine with the TLC-method. All other compounds were assayed photometrically under standard assay conditions in the presence 150 μM substrate. See Fig. 3 for chemical structures.

*Enzyme activity with senecionine as substrate was set 100%.

EXPERIMENTAL

Plant material

Seeds of *Crotalaria scassellatii* Chiov. were collected in Kenya. To facilitate seed imbibition a small part of the hard seed-coat was carefully clipped off with a small pair of pincers. Then the seeds were soaked in aerated tap water for 48 h and subsequently were germinated on moist sand at 25° for 2 days.

Table 6. Substrate kinetics (apparent K_m values) for solubilized SNO from *C. scassellatii*

Substrate	K_m' (μM)	V (pkat mg^{-1})	V/K_m' (%)
Senecionine	12.4	326	100
Monocrotaline	40.1	161	15.2
Heliotrine	370.9	194	2.0
Cosubstrate			
NADPH	31.6	10.9	100
NADH	340.2	19.2	16.0

The three substrates represent examples of each of the three major types of pyrrolizidine alkaloids. Cosubstrate kinetics were determined in the presence of senecionine as substrate. Standard assay conditions (optical assay).

Crude particulate enzyme preparations

All operations were carried out at 4°. Fifty 2-day-old seedlings (*ca.* 5.3 g fr. wt; seed-coat removed) were homogenized in a mortar with sea-sand and 20 ml isolation buffer [50 mM Na–Pi, pH 8.0; 2 mM dithioerythritol; 1 mM EDTA and 20% (v/v) glycerol]. The crude homogenate was filtered through nylon cloth (60 μm) and the cell debris were again extracted with isolation buffer up to a total volume of 35 ml. Cell debris were removed by centrifugation at 1000*g* for 20 min and from the resulting supernatant a particulate fraction was recovered by centrifugation at 39 000*g* for 1 h, which contained >90% of total particulate SNO activity. The pellet could be stored at −18° for several months without significant loss of enzyme activity.

Enzyme solubilization

The 39 000*g* fraction (prepared from 50 seedlings) was suspended in 2 ml standard buffer (50 mM K–Pi, pH 7.3; 2 mM dithioerythritol; 1 mM EDTA and 20% (v/v) glycerol). Then the protein concentration was adjusted to 4.0 mg ml^{-1} and 0.4% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate (CHAPS) and 0.4 M NaCl (final concentration) were added. The protein/detergent ratio should be 1:1 (w/w). The mixture was gently stirred at 4° for 1 h and then centrifuged at 100 000*g* for 90 min. The supernatant was desalted via Sephadex G25 (PD-10 columns, Pharmacia). It could be stored at −80°C without significant loss of activity for several months.

Partial purification of SNO

The particulate fraction prepared from 600 *C. scassellatii* seedlings was solubilized as described above. The desalted enzyme solution was treated with $(NH_4)_2SO_4$ up to 70% saturation. After centrifugation at 27 000*g* for 20 min the pellicle at the top of the solution was resuspended in a small volume (<10 ml) standard buffer, desalted via PD-10 and directly applied to a monocrotaline-Sepharose column (bed volume 3 ml; 1.7 cm length, 1.5 cm i.d.) pre-equilibrated with 20 ml standard buffer. The column was washed with 18 ml of the same buffer and enzyme activity was eluted with 20 ml 1 mM monocrotaline in standard buffer, followed by a linear gradient of 30 ml from 0–1 M NaCl in standard buffer (flow rate: 0.1 ml min^{-1}, fraction size: 2.5 ml). SNO activity was mainly found in fractions 13–16. Some additional activity which was not completely eluted by monocrotaline containing buffer could be recovered by elution with the NaCl. Prior to activity determination the

monocrotaline was removed by desalting of the fractions on PD-10 columns.

Assay of SNO activity (*tracer assays*)

Enzyme activity was determined by measuring the formation of radioactively labelled senecionine-*N*-oxide from [^{14}C]senecionine as a substrate. Radioactivity was detected by radio thin-layer-chromatography (TLC method) or by measuring the remaining [^{14}C]senecionine due to its selective solubility in toluene (toluene method) as described in Ref. [9]. The reaction mixture contained in a total volume of 600 μl: 50 mM K–Pi, pH 7.3, 2 mM dithioerythritol, 1 mM EDTA, 20% (v/v) glycerol (only toluene method), 100 μM [^{14}C]senecionine (2×10^4 cpm/assay) and 480 μl enzyme solution (particulate fraction and solubilized enzyme). The reaction was started by addition of 60 μl 1 mM NADPH and generally allowed to proceed 15 to 45 min at 35°. The reaction was terminated by addition of 100 μl 2 M HCl.

Toluene method. The enzyme assay as described above was performed in 4-ml-scintillation-vials. The terminated assay was mixed with 3 ml toluene/Lipoluma (Baker) (1:1, by vol.). After addition of 100 μl 5 M NaOH the mixture was shaken, centrifuged and without separation the unreacted [^{14}C]senecionine was directly analyzed in a scintillation counter. The polar [^{14}C]senecionine *N*-oxide remained quantitatively in the aqueous layer and does not interfere with scintillation counting.

TLC method. The reaction mixture, which did not contain glycerol, was evaporated under air stream, redissolved in 50 μl methanol, centrifuged and the supernatant was subjected to radioactive TLC analysis. Enzyme activity was calculated from the substrate (senecionine)/product (senecionine *N*-oxide) ratio.

Assay of SNO activity (optical assay)

The reaction mixture contained in a final volume of 0.6 ml: 50 mM K–Pi, pH 7.3, 2 mM dithioerythritol, 1 mM EDTA, 0.5 mM *n*-octylamine, 0.1 mM NADPH and 480 μl solubilized enzyme (desalted 70% ammonium sulfate precipitate). Cuvettes were preincubated at 35° for 2 min and then monitored for 2 to 3 min to record endogenous NADPH oxidation. Reaction was started by addition of 10 μl 0.1 to 0.2 mM substrate and the decrease of absorption (NADPH) was continuously followed at 340 nm (Pye Unicam UV/VIS) at 35°.

Preparation of monocrotaline Sepharose 6B

Coupling of monocrotaline to the gel matrix was performed according to the manufacturer's instructions. Ligand coupling was achieved by mixing 1.0 g epoxy-activated Sepharose 6B (*Pharmacia*) with 20 mM monocrotaline in 15 ml coupling solution under gentle stirring at room temperature for 48 h. Due to the basic and hydrophobic properties of monocrotaline the coupling reaction must be performed at pH 7.5 instead of pH 9–13 as recommended. Therefore the reaction time had to be increased from 16 to 48 h.

Pyrrolizidine alkaloids used as SNO substrates

Seneciphylline and retrorsine were obtained from Roth (Karlsruhe), monocrotaline from Aldrich (Steinheim), heliotrine from Corkwood Enterpr., (Blakehurst, Australia). The other pyrrolizidine alkaloids were isolated and purified from respective plant sources: senecionine, senecivernine and senkirkine from *Senecio vernalis* inflorescences according to Ref. [32], axillaridine and axillarine from seeds of *Crotalaria scassellatii* according to Ref. [14]; triangularine and sarracine were isolated from *S. silvaticus*, indicine was isolated from flowers and lycopsamine from seeds of *Heliotropium indicum*, rinderine from flowers of *Eupatorium laevigatum* and phalaenopsine from flowers of *Phalaenopsis*-hybrides. Purity and identity of the structures was verified by GC-MS and if necessary NMR analysis. All pyrrolizidine alkaloids were purified as tertiary alkaloids. The respective *N*-oxides were prepared according to Ref. [33]. Retronecine and heliotridine were prepared by alkaline hydrolysis of monocrotaline and heliotrine, respectively [34]. ^{14}C-Labelled pyrrolizidine alkaloids were isolated from the respective root-cultures after feeding of labelled putrescine as biosynthetic precursor [35]. ^{14}C-Labelled pyrrolizidine alkaloids were isolated from the respective root-cultures after feeding of labelled putrescine as biosynthetic precursor [35].

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