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# INCORPORATION OF CHIRALLY DEUTERATED PUTRESCINES INTO PYRROLIZIDINE ALKALOIDS: A REINVESTIGATION

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**Key Word Index**—*Senecio vulgaris*; Asteraceae; root cultures; pyrrolizidine alkaloids; biosynthesis; retronecine; homospermidine; deuterated putrescines.

**Abstract**—Based on previous tracer work and recent enzymatic studies it can be predicted that incorporation of (*S*)-1-[$^2$H]putrescine via the symmetrical intermediate homospermidine into the necine base moiety of pyrrolizidine alkaloids (PAs) should proceed with 50% retention of deuterium. However, values of only 34 to 34.5% retention had been found independently in two laboratories in the past. These results were confirmed in this study. Deuterium isotope effects during homospermidine formation as a reason for the low retention could be excluded by GC mass spectral studies. Doubly-labelled [$^2$H-$^{14}$C]putrescine was fed to *Senecio vulgaris* root cultures and by means of quantitative GC mass spectrometry the specific $^2$H-retention was established for various intermediates of PA-biosynthesis such as putrescine, spermidine and homospermidine. The results clearly indicate that $^2$H is stereoselectively lost from (*S*)-[1-$^2$H]-labelled putrescine during its reversible interconversion with spermidine. This loss corresponds precisely to the above mentioned difference between measured and predicted $^2$H-retention. Since (*S*)-[1-$^2$H]-labelled putrescine is incorporated into spermidine with deuterium retention, it is most likely the $^2$H is lost during the conversion of spermidine into putrescine. The mechanism of this unusual reaction which is insensitive to β-hydroxyethylhydrazine (a potent diamine oxidase inhibitor) needs to be elucidated. 

## INTRODUCTION

The biosynthesis of retronecine, the necine base of most pyrrolizidine alkaloids (PAs), was independently studied in the eighties by the groups of Spenser using *Senecio vulgaris* and Robins using *Senecio isatideus*. Extensive tracer studies carried out in the two laboratories confirmed that retronecine is derived from putrescine via a symmetrical intermediate such as symhomospermidine [1–5]. In the course of these experiments (*R*)[1-$^2$H]putrescine and (*S*)[1-$^2$H]putrescine were fed to the *Senecio* species and the deuterium label was localized by $^2$H NMR spectroscopy in the retronecine moiety of the alkaloids. The labelling patterns established in the retronecine moiety showed that the two oxidation steps at the primary amino groups of homospermidine (corresponding to C-atoms 8 and 9 of retronecine) occur with stereoselective loss of the *pro-S* hydrogen of putrescine (Fig. 1). This behaviour is consistent with the known stereochemistry of diamine oxidase in which the *pro-*

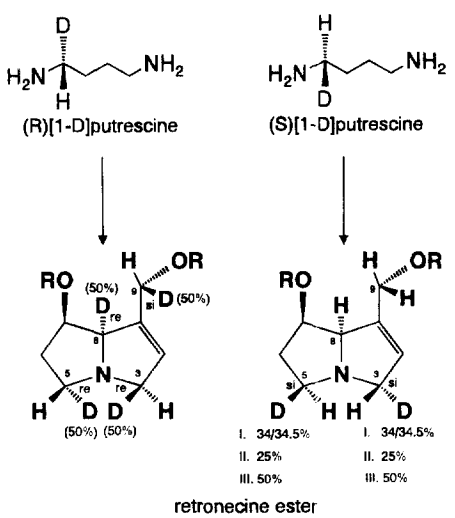


Fig. 1. Incorporation of chirally C1-deuterated putrescine into retronecine. (I): percent incorporation experimentally found according to ref. [9] and [10], respectively; (II): percent incorporation expected according to ref. [9 and 10]; (III): percent incorporation expected according to ref. [14].

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*R* hydrogen is retained in the resulting aldehyde [6, 7]. The stereochemistry of the third oxidation step affecting the primary amino group of one of the putrescine moieties during homospermidine formation (corresponding to C-atoms 3 and 5 of retronecine) is not as clear. In tracer experiments with (*S*)-[1-$^2$H]putrescine carried out in the two laboratories almost identical deuterium retentions of 34% [8, 9] and 34.5% [10, 11] were found for each of the two C-atoms, instead of 25% for the assumed involvement of diamine oxidase in homospermidine formation [Fig. 1, (I and II)]. As a possible explanation for this apparent discrepancy an intramolecular deuterium isotope effect in the enzymatic oxidation was suggested.

More recently the enzymatic formation of homospermidine was shown to be catalysed by homospermidine synthase (EC 2.5.1.44) [12, 13]. In an $NAD^+$-dependent reaction, this enzyme combines an aminobutyl group derived either from putrescine or from spermidine with a second mole of putrescine [14–16]. In a stoichiometric manner, $NAD^+$ functions as hydride acceptor in the oxidative deamination and as hydride donor in the reduction of the assumed imine intermediate. Participation of diamine oxidase in homospermidine formation or the role of free $\Delta_1$-pyrroline as biogenetic intermediate was excluded [12]. This predicts that in tracer experiments with (*S*)[1-$^2$H]putrescine the deuterium at the C-atoms 3 and 5 should be retained. Thus 50% deuterium would be expected at each of the carbons 3 and 5 instead of 34% [Fig. 1(III vs I)].

The following studies were undertaken to clarify the contradiction between predicted and experimentally established deuterium retention in the necine base moiety of PAs upon feeding of (*S*)[1-$^2$H]putrescine. Root cultures of *Senecio vulgaris* were used as the biological system.

## RESULTS AND DISCUSSION

### *Incorporation of chirally deuterated putrescines into pyrrolizidine alkaloids*

Root cultures of *Senecio vulgaris* were allowed to incorporate (*R*)[1-$^2$H]putrescine and (*S*)[1-$^2$H]putrescine into PAs. The alkaloids (a mixture of 90% senecionine *N*-oxide and its *E*-isomer, integerrimine *N*-oxide) were isolated, reduced to the respective tertiary amines, purified, and analysed by $^2$H NMR spectroscopy. The respective NMR spectra are shown in Fig. 2. They clearly confirm the results of the groups of Robins [8, 9] and Spenser [10, 11] summarized in Fig. 1, concerning retention and loss of deuterium at the respective four positions.

Evaluation of the specificity of $^2$H-incorporation was performed by means of GC mass spectrometry by measuring the relative intensities of the respective cluster of molecular ions. This was simple to perform and did not require milligram quantities of purified alkaloid as required for $^2$H NMR spectrometry. Senecionine synthesized from doubley-labelled (*R*)[1-$^2$H][1,4-$^{14}$C]putrescine and (*S*)[1-$^2$H][1,4-$^{14}$C]putrescine fed to root cultures of *S. vulgaris* was analysed as described in the experimental section. The results are summarized in Table 1. As expected, the (*R*)-enantiomer is incorporated into senecionine with complete $^2$H-retention whereas the (*S*)-enantiomer resulted in a $^2$H retention of 32.4% per putrescine unit. This confirms the results of earlier tracer studies [Fig. 1(I)] The values established experimentally independently in three laboratories are almost identical (i.e., 34, 34.5 and now 32.4%) and diverge considerably from the predicted value of 50% $^2$H retention [Fig. 1(III)]. One plausible explanation could be an $^1$H/$^2$H isotope effect during homospermidine formation, i.e. a preferential enzymatic oxidation of putrescine at its non-labelled terminal carbon. As a consequence the alkaloid would have a lower retention of $^2$H in comparison to $^{14}$C.

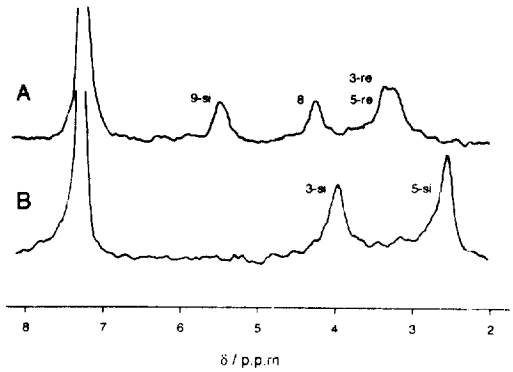


Fig. 2. 30.72 MHz $^2$H NMR spectra of senecionine in chloroform at 60 . A = alkaloid sample derived from (*R*)-[1-$^2$H]putrescine; B = alkaloid sample derived from (*S*)-[1-$^2$H]putrescine. $^2$H signals: A, at $\delta$ 3.40; 4.27; 5.50. B, at $\delta$ 2.57 and 3.96. Natural abundance $^2$H signal for $CHCl_3$ at $\delta$ 7.25. See Fig. 1 for signal assignment.

### *Incorporation of chirally deuterated putrescines into homospermidine*

The incorporation of (*R*)[1-$^2$H]putrescine and (*S*)[1-$^2$H]putrescine into homospermidine in *S. vulgaris* root cultures was achieved in the presence of $\beta$-hydroxyethylhydrazine (HEH), a potent diamine oxidase inhibitor, which completely blocks the further conversion of homospermidine into PAs (Fig. 3). Consequently homospermidine accumulates in the root tissue and can easily be isolated [12, 16]. In addition to *in vivo* homospermidine formation in root cultures, enzymatically synthesized homospermidine was included in the study prepared from (*S*)[1-$^2$H]putrescine with bacterial homospermidine synthase [13–15]. The enzymatically produced homospermidine was analysed by GC mass spectrometry as the methoxycarbonyl (MOC)-derivative [17] and the trimethylsilyl(TMS)-derivative [18]. The fragmentation patterns allow one to distinguish between total $^2$H-retention and $^2$H-retention at the outer carbons (i.e.,

Table 1. Incorporation of chirally labelled putrescines into senecionine by root cultures of *Senecio vulgaris*

| Tracer fed | Specific incorporation per $C_4$ unit of senecionine (%) | | $^2H$ retention (%) |
|---|---|---|---|
| | $^2H$ | $^4C$ | $(^2H/^{14}C) \cdot 100$ |
| (*R*)-[1-$^2$H][1,4-$^{14}$C]putrescine | 19.8 ± 0.33* | 19.6 ± 1.06 | 101.0 ± 7.17 |
| (*S*)-[1-$^2$H][1,4-$^{14}$C]putrescine | 12.3 ± 0.21 | 38.0 ± 2.05 | 32.4 ± 2.30 |

* s.d.

$C_1$ and $C_8$; Fig. 3). The results summarized in Table 2 clearly show that the $^2$H-label is equally distributed over the terminal ($C_1$ and $C_8$) and inner ($C_4$ and $C_5$) carbons of the compound. Since for the MOC-derivatives an interference by McLafferty rearrangement of the secondary amino group could not be excluded *a priori*, the same analysis was done with the TMS-derivatives which clearly allow one to distinguish between primary (two TMS residues) and secondary (one TMS residue) amino groups. The TMS-derivatives gave the same results. Thus, any significant isotope effect during the course of *in vivo* or enzymatic homospermidine formation from [1-$^2$H]putrescine can be excluded.

In addition, the incorporation of $^{14}$C-,$^2$H-doubly-labelled homospermidine into senecionine was studied. The labelled homospermidine had been prepared from (*S*)[1-$^2$H][1,4-$^{14}$C]putrescine biosynthetically by *S. vulgaris* root cultures and enzymatically with bacterial homospermidine synthase. The results show, as expected, that homospermidine is incorporated into senecionine with loss of 50% of the label (Table 3). This again proves that the deuterium must be equally distributed over the four positions in homospermidine.

The result presented so far indicate that the discrepancy between expected and experimentally found loss of $^2$H from (*S*)[1-$^2$H]putrescine in the course of its incorporation into senecionine cannot be explained by isotope effects during the formation of homospermidine or subsequent reactions. Furthermore, since the incorporation of [1,4,5,8-$^2H_4$]homospermidine occurs with 50% $^2$H-retention, as expected, the loss of label is most likely to happen at the very beginning of the biosynthetic pathway, i.e. the interconversion of putrescine and spermidine.

*Specific loss of $^2H$ from* (S)[1-$^2H$]*putrescine during metabolism in root cultures*

In a preliminary experiment the specific incorporation of (*S*)-[1-$^2$H][1,4-$^{14}$C]putrescine into homospermidine was studied. The result revealed a $^2$H-retention at 66.7% per $C_4$-unit instead of 100%. Since the homospermidine synthase reaction proceeds with stoichiometric retention of $^2$H [14], the loss must have occurred at the putrescine/spermidine level. Consequently, short term (22 to 54 hr) feeding experiments with (*S*)[1-$^2$H][1,4-$^{14}$C]putrescine were performed in the presence of HEH and the specific tracer incorporations were analysed for the intracellular pools of putrescine, spermidine and homospermidine. Roots were harvested at times when 95% of the tracer had just been taken up by the roots. The results are summarized in Table 4. The data clearly show that intracellular putrescine, spermidine and homospermidine have lost 30 to 35% of their $^2$H-label in comparison to the $^4$C-label. This proportion did not change when the tracer concentrations were lowered from 200 to 100 or 50 $\mu$M and the harvest times were reduced to 38 and 22 hr, respectively (data not shown). As a control, bacterial homospermidine synthase was included. As expected, the enzymatically-produced homospermidine retained its $^2$H quantitatively (Table 4).

*Senecio* root cultures dynamically interconvert putrescine and spermidine [16, 19] and both are direct donors of the aminobutyl group in homospermidine formation (Fig. 3) [16]. The formation of spermidine by transfer of an aminopropyl group from decarboxy-*S*-adenosylmethionine (dSAM) to a terminal amino group of putrescine catalysed by spermidine synthase (EC 2.5.1.16) is well understood [20, 21] (Fig. 3). It seems unlikely that this reaction proceeds with a stereoselective elimination of a *pro-S* hydrogen from putrescine. To obtain definitive proof, chirally deuterated putrescine was fed to *S. vulgaris* root cultures and the labelled spermidine was analysed subsequently by GC mass spectrometry. The results summarized in Table 5 indicate that the putrescine-derived aminobutyl moiety of spermidine contains 50% of its $^2$H-label at C-4. The same was found with spermidine synthesized enzymatically from (*S*)[1-$^2$H]putrescine with bacterial spermidine synthase. As expected, the *pro-S* hydrogen of putrescine is retained during spermidine synthesis.

The only process which remains to be analysed is the conversion of spermidine into putrescine. The formation of putrescine from spermidine has also been reported for other plants [22, 23] but the enzymatic nature of the reaction is still obscure. Well known plant diamine oxidases usually convert spermidine into $\Delta_1$-pyrroline and diaminopropane and not aminopropanal and putrescine as expected in our case.

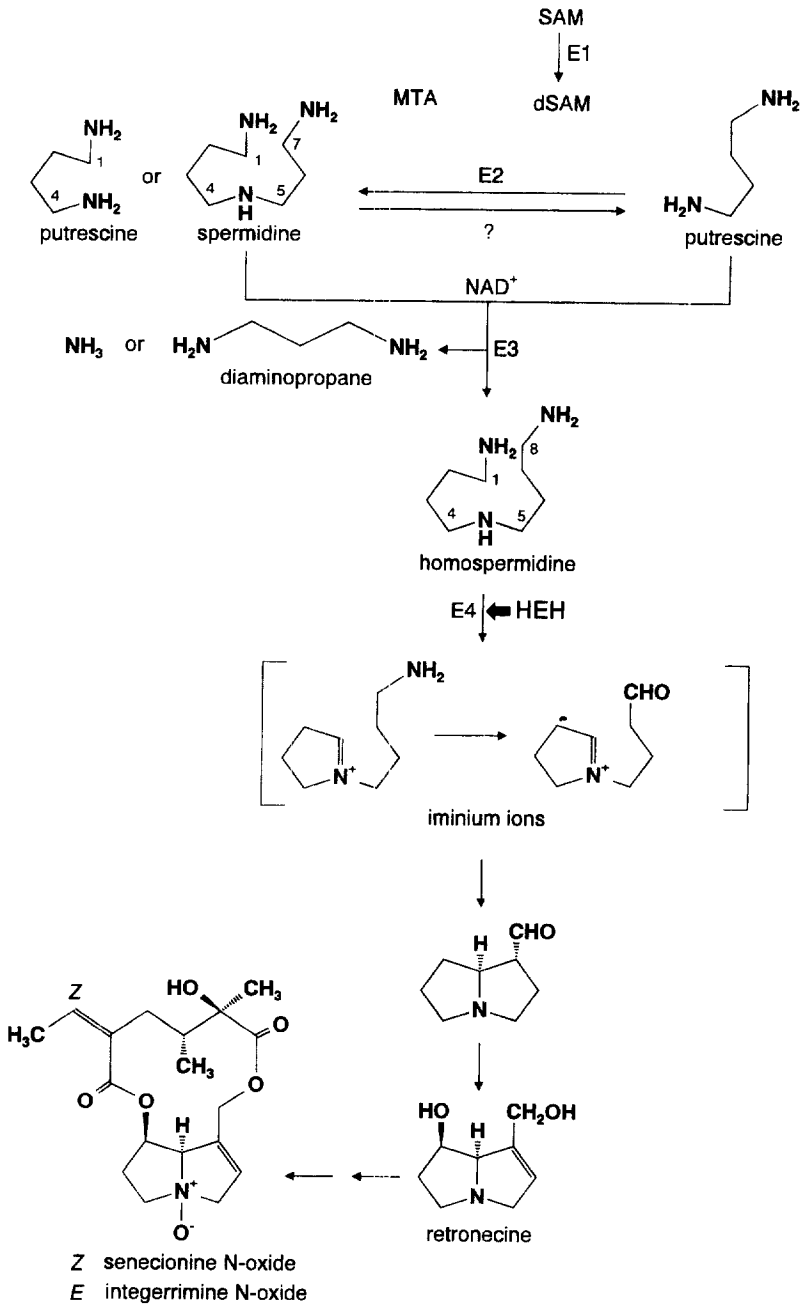


Fig. 3. Enzymatic links between putrescine, spermidine and homospermidine and further fate of homospermidine in PA biosynthesis. SAM: *S*-adenosylmethionine, dSAM: decarboxylated SAM, MTA: 5′-methylthioadenosine, HEH: β-hydroxyethylhydrazine. E1: SAM decarboxylase (EC 4.1.1.50), E2: spermidine synthase (EC 2.5.1.16), ?: putrescine producing enzyme activity (HEH insensitive), E3: homospermidine synthase (EC 2.5.1.44), E4: PA biosynthetic oxidase activity (HEH sensitive).

Furthermore, the *Senecio* enzyme cannot be a typical diamine oxidase as the conversion of spermidine to putrescine is completely insensitive to HEH which, however, efficiently blocks the oxidation of homospermidine [12, 16] in the course of PA biosynthesis as well as the oxidation of putrescine to $\Delta_1$ pyrroline [12]. Due to the rapid reversible interconversion of the two polyamines [16, 19] tracer studies would not provide a conclusive answer.

## CONCLUSIONS

The discrepancy between the predicted and experimentally found specific incorporation of deuterium from (*S*)[1-$^2$H][1,4-$^{14}$C]putrescine into the necine base of PAs has been elucidated. The final conclusion can be summarized as follows (Table 6):

- The $^2$H is incorporated into homospermidine without significant isotope effects; the specific

Table 2. Deuterium distribution in homospermidine (hspd) synthesized from (*R*)-[1-$^2$H] putrescine and (*S*)-[1-$^2$H]putrescine by root cultures of *Senecio vulgaris* and bacterial homospermidine synthase, respectively (see Fig. 3 for carbon numbering of hspd). Analysis by GC-MS: evaluation of the relative intensities of the $[M]^+$ cluster and a fragment cluster containing only terminal carbons (C-1 or C-8)

| Homospermidine analysed (source of synthesis) | % $^2$H at carbons 1,4,5,8 | % $^2$H at carbons 1 and 8 | % of total $^2$H at carbons 1 and 8 |
|---|---|---|---|
| A. MOC-derivatives | | | |
| From (*R*)-[$^2$H]putrescine | | | |
| [1,4,5,8-$^2H_1$]hspd (plant) | 62.5 | 31.0 | 51.2 |
| From (*S*)-[$^2$H]putrescine | | | |
| [1,4,5,8-$^2H_1$]hspd (plant) | 55.5 | 28.1 | 50.6 |
| [1,4,5,8-$^2H_1$]hspd (bacterial) | 75.2 | 39.7 | 52.8 |
| B. TMS-derivatives | | | |
| From (*R*)-[$^2$H]putrescine | | | |
| [1,4,5,8-$^2H_1$]hspd (plant) | 84.0 | 40.8 | 48.6 |
| From (*S*)-[$^2$H]putrescine | | | |
| [1,4,5,8-$^2H_1$]hspd (plant) | 75.2 | 35.4 | 47.1 |
| [1,4,5,8-$^2H_1$]hspd (bacterial) | 85.3 | 45.6 | 53.3 |

Table 3. Incorporation of [1,4,5,8-$^2H_1$][1,4-$^{14}$C]homospermidine into senecionine by root cultures of *Senecio vulgaris*. The labelled homospermidine was synthesized from (*S*)-[1-$^2$H][1,4-$^{14}$C]putrescine either by *Senecio vulgaris* root cultures or with bacterial homospermidine synthase

| Tracer fed | Specific incorporation per $C_4$ unit of senecionine (%) | | $^2$H retention (%) ($^2$H/$^{14}$C) · 100 |
|---|---|---|---|
| | $^2$H | $^{14}$C | |
| Plant origin (biosynthetically prepared) [1,4,5,8-$^2H_1$][1,4-$^{14}$C]homospermidine | 10.0 ± 0.18* | 20.7 ± 1.12 | 48.3 ± 3.48 |
| Bacterial origin (enzymatically prepared) [1,4,5,8-$^2H_1$][1,4-$^{14}$C]homospermidine | 9.3 ± 0.21 | 20.0 ± 1.08 | 46.7 ± 3.59 |

* s.d.

Table 4. Loss of $^2$H-label from (*S*)-[1-$^2$H][1,4-$^{14}$C]putrescine during metabolism in root cultures of *Senecio vulgaris*. Incubation with 200 μM tracer for 54 hr in the presence of 2 mM HEH

| Labelled intermediate analysed | Specific incorporation per $C_4$ unit | | $^2$H retention (%) ($^2$H/$^{14}$C) · 100 |
|---|---|---|---|
| | $^2$H | $^{14}$C | |
| Putrescine | 37 ± 0.67* | 52 ± 2.70 | 71 ± 5.0 |
| Spermidine | 43 ± 0.73 | 68 ± 3.67 | 63 ± 4.5 |
| Homospermidine | 49 ± 0.83 | 73 ± 3.72 | 67 ± 4.6 |
| Homospermidine† | 76 ± 1.37 | 78 ± 4.05 | 97 ± 6.8 |

* s.d.

† produced enzymatically from (*S*)-[1-$^2$H][1,4-$^{14}$C]putrescine by action of bacterial homospermidine synthase.

label is equally distributed over the labelled C-atoms of homospermidine.

- However, 34% of the $^2$H is lost during the course of homospermidine formation from putrescine (i.e. only 66% $^2$H is recovered in comparison to $^{14}$C) (Table 6).
- This loss of $^2$H must occur during the dynamic interconversion of putrescine and spermidine, most likely during the conversion of spermidine into putrescine.
- This loss is stereoselective and reflects a well balanced dynamic equilibrium between putrescine

Table 5. Incorporation of (*R*-[$^2$H]putrescine and (*S*)-[$^2$H]putrescine into spermidine by root cultures of *Senecio vulgaris* and spermidine synthase isolated from *Escherichia coli*, respectively. Spermidine is labelled at $C_1$ and $C_4$ (see Fig. 3 for carbon numbering). Analysis of the MOC derivatives by GC-MS; evaluation of the relative intensities of the $[M]^+$ cluster (i.e. *m/z* 319 [unlabelled], 320 [+1 $^2$H] and the fragment cluster *m/z* 203 and 204 containing only carbon 4

| Substrate fed | Product (producing organism) | % $^2$H at carbons 1 and 4 | % $^2$H at carbon 4 | % of total $^2$H at carbon 4 |
|---|---|---|---|---|
| (*R*)-[1-$^2$H]putrescine | spermidine (plant) | 72 | 34 | 47 |
| (*S*)-[1-$^2$H]putrescine | spermidine (plant) | 86 | 44 | 51 |
| (*S*)-[1-$^2$H]putrescine | spermidine (bacterial) | 72 | 36 | 50 |

Table 6. Retention of $^2$H in A: homospermidine (hspd) and B: senecionine (sen) isolated from root cultures of *Senecio vulgaris* incubated with (*R*)- and (*S*)-[1-$^2$H]putrescine. Summary of the predicted and experimentally evaluated results.

| **A Homospermidine** | $^2$H-retention in hspd (%)* | | | | |
|---|---|---|---|---|---|
| Tracer applied (incorporation) | C-1 | C-4 | C-5 | C-8 | Total retention per $C_4$-unit (%) |
| (*S*)-[1-$^2$H]putrescine (predicted) | 50 | 50 | 50 | 50 | 100 |
| (*S*)-[1-$^2$H]putrescine (found) | 33 | 33 | 33 | 33 | 66 |
| **B Senecionine** | $^2$H-retention in sen (%)* | | | | |
| Tracer applied (incorporation) | C-8 | C-5 | C-3 | C-9 | Total retention per $C_4$-unit (%) |
| (*R*)-[1-$^2$H]putrescine (predicted) | 50 | 50 | 50 | 50 | 100 |
| (*R*)-[1-$^2$H]putrescine (found) | 50 | 50 | 50 | 50 | 100 |
| (*S*)-[1-$^2$H]putrescine (predicted) | 0 | 50 | 50 | 0 | 50 |
| (*S*)-[1-$^2$H]putrescine (found) | 0 | 32.4 | 32.4 | 0 | 32.4 |
| (*S*)-[1-$^2$H]putrescine (predicted from label in homospermidine, see A) | 0 | 33 | 33 | 0 | 33 |
| (*S*)-[1-$^2$H]putrescine (found) | 0 | 32.4 | 32.4 | 0 | 32.4 |

* Each symmetrical $C_4$-unit of putrescine is assumed to contain 100% $^2$H (50% each at the terminal carbons 1 and 5).

and spermidine since under various conditions it is always the same magnitude.

- Homospermidine is synthesized without loss of $^2$H and is incorporated into PAs with quantitative retention of $^2$H at the C-atoms 5 and 3.

## EXPERIMENTAL

*Plant material.* Root cultures of *Senecio vulgaris* L. (Asteraceae), established in 1993, were routinely grown in 25 ml conical flasks containing 75 ml Murashige–Skoog (MS) medium [24] with 4% sucrose and the phytohormones omitted [25]. Cultures were kept on gyratory shakers (120 rpm) at 25° in the dark and transferred every 2 weeks.

*Incorporation of chirally labelled putrescine into senecionine.* 50 ml conical flasks were each inoculated with *ca* 3 g (fr. wt) growing roots (5-day-old cultures) of *S. vulgaris* in 10 ml MS medium. Exp. A: a total of 46 μmol (*R*)-[1-$^2$H]putrescine (>98.5 atom% $^2$H) containing 10 μCi [1,4-$^{14}$C]putrescine was added to 2 flasks. Exp. B: a total of 95.8 μmol (*S*)-[1-$^2$H]putrescine (>98.5 atom% $^2$H) containing 10 μCi [1,4-$^{14}$C]putrescine was added to 4 flasks. Roots were allowed to grow for 11 days.

During this period *ca* 70% of the tracers had been taken up by the roots. Then the roots were harvested, washed with tap $H_2O$ and blotted between sheets of filter paper. Roots were homogenized in a mortar in acidic MeOH (1% HCl); after centrifugation the supernatant was evapd and the residue dissolved in 2 ml 0.5 M $H_2SO_4$, mixed with excess Zn dust and stirred for 5 hr at room temp. to reduce the PA *N*-oxides. After centrifugation, the soln was extracted ×3 with $Et_2O$. The aq. soln was made basic with $NH_4OH$ and applied to an Extrelut (Merck) column (1.4 ml $g^{-1}$ Extrelut). Tertiary PAs were eluted with $CH_2Cl_2$ (6 ml $g^{-1}$ Extrelut). Exp. A and B yielded 6.4 and 6.6 mg PA, respectively. GC-MS revealed pure senecionine contaminated by 10% of its *E*-isomer intergerrimine (Fig. 3). Samples were subjected to $^2$H NMR spectroscopy.

*Quantitative evaluation of specific tracer incorporation into senecionine.* Specific $^2$H-incorporation was achieved by means of GC-MS by measuring the intensities of the cluster of respective molecular ions

$[M]^+$ of senecionine, i.e. *m/z* 335 (unlabelled), *m/z* 336 [+1 $^2H$] and *m/z* 337 [+2 $^2H$]. Calculations are exemplified by the incorporation of (*S*)[1-$^2H$]putrescine into senecionine: Tracer fed: (*S*)[1-$^2H$][1,4-$^{14}C$]putrescine (98.5 atom% $^2H$; specific radioactivity $2.54 \cdot 10^5$ cpm · $\mu$mol$^{-1}$). GC-MS of isolated senecionine: relative intensities of the molecular ions (corrected for natural $^{13}C$-abundance), *m/z* 335 = 79.05%; *m/z* 336 = 17.85%; *m/z* 337 = 3.36%. Specific radioactivity of senecionine: $1.93 \cdot 10^5$ cpm · $\mu$mol$^{-1}$.

*Specific $^{14}C$-incorporation per $C_4$ unit of senecionine* (%):

$$\frac{\text{(spec. radioact. [}^{14}\text{C]senecionine)} \cdot 0.5}{\text{spec. radioact. [}^{14}\text{C]putrescine}} \cdot 100;$$

$$\frac{0.193 \cdot 10^6 \cdot 0.5}{0.254 \cdot 10^6} \cdot 100 = 38.0\%$$

*Specific $^2H$-incorporation per $C_4$ unit of senecionine* (%):

$$\{m/z\ 336 + (m/z\ 337 \cdot 2)\} \cdot 0.5;$$

$$(17.85 + 6.72) \cdot 0.5 = 12.3\%$$

*$^2H$-retention per $C_4$ unit of senecionine* (%):

$$\frac{\text{Spec. }^2\text{H-incorp. (\%)}}{\text{Spec. }^{14}\text{C-incorp. (\%)}} \cdot 100; \quad \frac{12.3}{38.0} \cdot 100 = 32.4\%$$

*Incorporation of chirally deuterated putrescines into homospermidine.* (1) *Senecio vulgaris*: Conical flasks with 8 ml MS medium and addition of 2 mM $\beta$-hydroxyethylhydrazine (HEH), 10 $\mu$mol (*S*)[1-$^2H$]putrescine (>98.5 atom% $^2H$) or (*R*)[1-$^2H$]putrescine (>98.5 atom% $^2H$), respectively, were incubated with 1.2 g (fr. wt) roots and allowed to grow for 5 days. The roots were harvested and extracted ×2 in a mortar with 8 ml acidic (1% HCl) MeOH under sonication. The homospermidine was purified and sepd from putrescine by ion exchange chromatography modified according to ref. [26]: a 8 ml column ($\phi$ 1.2 cm) containing 3 ml Dowex-50X8–200, cross-linkage 8%, 1.7 meq ml$^{-1}$, $H^+$-form (Sigma) was used. Putrescine was eluted with 200 ml 1.65 M HCl, homospermidine with 50 ml 2.6 M HCl. The solvent was evapd and the sample subjected to derivatization for GC-MS. (2) Homospermidine synthase from *Rhodopseudomonas viridis*: $Me_2CO$ dry powder (1 g) prepd from *R. viridis* cells [14] was incubated in 10 ml 50 mM K-Pi-buffer pH 8.5, containing 2 mM dithioerythritol for 1 hr. After centrifugation the supernatant was desalted via Sephadex PD-10. The crude enzyme extract (10 ml) was incubated with 10 $\mu$mol (*S*)[1-$^2H$]putrescine (>98.5 atom% $^2H$). The assay was incubated for 12 hr at 37°; subsequently the homospermidine was extracted and purified as described above for the plant sample.

*GC-MS of N-methoxycarbonyl (MOC) derivatives of polyamines.* The MOC-derivatives were prepd according to ref. [17]. The pre-purified dry sample was dissolved in 1.5 ml 0.05 M HCl, made basic with 1 M $Na_2CO_3$ and mixed with 20 $\mu$l methyl chloroformate and allowed to react for 15 min at room temp. The MOC-derivatives were extracted ×3 with $Et_2O$. The solvent was removed under a stream of $N_2$ and the sample dissolved in $CH_2Cl_2$ for GC-MS. The $^2H$-retention and distribution was analysed quantitatively by GC-MS. Homospermidine: Total $^2H$-content was evaluated by measuring the relative intensities of the $[M]^+$ cluster, i.e. *m/z* 333 [unlabelled], 334 [+1 $^2H$], 335 [+2 $^2H$]; the relative intensities, corrected for natural abundance of $^{13}C$, of the fragments *m/z* 88 (i.e. [MOC-NH-$C^1H_2$]$^+$) and *m/z* 89 (i.e. [MOC-NH-$C^1H^2H$]$^+$) were chosen for selective calculation of $^2H$-retention at $C_1/C_8$. Spermidine: Total $^2H$-content was evaluated by measuring of the relative intensities of the $[M]^+$ cluster, i.e. *m/z* 319 [unlabelled], 320 [+1 $^2H$]. The relative intensities, corrected for natural abundance of $^{13}C$, of the fragments *m/z* 203 (i.e. [MOC-NH-$C^1H_2$-$C^1H_2$-$C^1H_2$-N(MOC)-$C^1H_2$]$^+$) and *m/z* 204 (i.e. [MOC-NH-$C^1H_2$-$C^1H_2$-$C^1H_2$-N(MOC)-$C^1H^2H$]$^+$) were chosen for selective calculation of $^2H$-retention at $C_4$.

*GC-MS of homospermidine as its TMS-derivative.* The TMS derivatives were prepared according to ref. [18]. Total $^2H$-content was evaluated by measuring the relative intensities, corrected for natural abundance of $^{13}C$, of the $[M]^+$ cluster of the penta-TMS-derivative, i.e. *m/z* 447 [unlabelled], 448 [+1 $^2H$], 449 [+2 $^2H$]. Selective $^2H$-retention at $C_1/C_8$ was evaluated by measuring the relative intensities of the fragments *m/z* 174 (i.e. [$(TMS)_2$-N-$C^1H_2$]$^+$) and *m/z* 175 (i.e. [$(TMS)_2$-N-$C^1H^2H$]$^+$).

*Capillary GC and GC-MS.* Sepn and quantification of PAs was achieved by capillary GC according to ref. [27]. For GC-MS the capillary column (30 m × 0.32 mm fused-silica; DB-1, J and W Scientific) was directly coupled to a quadrupole mass-spectrometer Finnigan MAT 4515, EI-MS were recorded at 40 eV. The temp. programme of GC for PAs and polyamine derivatives was: 150–300°, 6° min$^{-1}$. Correction for natural $^{13}C$-abundance were made according to ref. [28]. To prevent interference with concn-dependent protonation effects at the ion source, the analysis of the unlabelled control sample is recommended at the same concn as the labelled sample.

*HPLC analysis of polyamines.* Sepn and quantification of polyamines was achieved via their benzoyl derivatives according to ref. [29] as described in ref. [12]. All quantifications of radioactively labelled samples were performed by liquid scintillation counting.

*$^2H$ NMR measurements* were performed as given in ref. [6].

*Radiochemicals and deuterated putrescines.* [1,4-$^{14}C$]Putrescine (118 mCi mmol$^{-1}$), [1,4*n*-$^3H$]putrescine (22.5 Ci mmol$^{-1}$), *N*-(3-aminopropyl)-[1,4-$^{14}C$]tetramethylene-1,4-diamine (118 mCi mmol$^{-1}$) (=[1,4-$^{14}C$]spermidine) were purchased from Amersham, Braunschweig. (*R*)-[1-$^2H$]putrescine (>98.5% $^2H_1$ species) and (*S*)-[1-$^2H$]putrescine (>98.5% $^2H_1$ spec-

ies) were prepd according to the method described in ref. [7]. [1,4,5,8-$^{2}H_{1}$][1,4,5,8-$^{14}$C]homospermidine was prepd from labelled putrescine biosynthetically using root cultures of *Senecio vulgaris* [12] or enzymatically with homospermidine synthase prepd either directly from *Rhodopseudomonas viridis* [14] or from over-expressed *Escherichia coli* BL21 transformed with the homospermidine synthase gene from *R. viridis* [13, 15]. [1,4-$^{14}$C]Spermidine was prepared enzymatically from labelled [1,4-$^{14}$C]putrescine with spermidine synthase from *Escherichia coli* according to ref. [30].

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## REFERENCES

1. Khan, H. A. and Robins. D. J., *Journal of the Chemical Society, Chemical Communications*, 1981, 146.
2. Khan, H. A. and Robins D. J., *Journal of the Chemical Society, Chemical Communications*, 1981, 554.
3. Grue-Sorensen, G. and Spenser, I. D., *Journal of the American Chemical Society*, 1981, **103**, 3208.
4. Grue-Sorensen, G. and Spenser, I. D., *Canadian Journal of Chemistry*, 1982, **60**, 643.
5. Robins, D. J. *Chemical Society Reviews*, 1989, **18**, 375
6. Battersby, A. R., Staunton, J. and Summers, M. C., *Journal of the Chemical Society, Perkin Transactions I*, 1976, 1052.
7. Richards, R. C. and Spenser, I. D., *Tetrahedron*, 1983, **39**, 3549.
8. Rana, J. and Robins. D. J., *Journal of the Chemical Society, Chemical Communications*, 1984, 517.
9. Rana, J. and Robins. D. J., *Journal of the Chemical Society, Perkin Transactions I*, 1986, 983.
10. Grue-Sorensen, G. and Spenser, I. D., *Journal of the American Chemical Society*, 1983, **105**, 74.
11. Spenser, I. D., *Pure and Applied Chemistry*, 1985, **57**, 453.
12. Böttcher, F., Adolph, R. and Hartmann, T., *Phytochemistry*, 1993, **32**, 679.
13. Tholl, D., Ober, D., Martin, W., Kellermann, J. and Hartmann, T., *European Journal of Biochemistry*, 1996, **240**, 373.
14. Böttcher, F., Ober, D. and Hartmann, T., *Canadian Journal of Chemistry*, 1994, **72**, 80.
15. Ober, D., Tholl, D., Martin, W. and Hartmann, T., *Journal of General and Applied Microbiology*, 1996, **42**, 411.
16. Graser, G. and Hartmann, T., *Phytochemistry*, 1997, **45**, 1591.
17. Husek, P., *Analytica Chimica Acta*, 1992, **259**, 185.
18. Donike, M., *Journal of Chromatography*, 1969, **42**, 103.
19. Hartmann, T., Sander, H., Adolph, R and Toppel, G., *Planta*, 1988, **175**, 82.
20. Sindhu, R. K. and Cohen, S. S., *Plant Physiology*, 1984, **74**, 645.
21. Hirasawa, E. and Suzuki, Y., *Phytochemistry*, 1983, **22**, 103.
22. Santanen, A. and Simola, L. K., *Physiologia Plantarum*, 1994, **90**, 125.
23. de Agazio, M., Zacchini, M., Frederico, R. and Grego, S., *Plant Science*, 1995, **111**, 181.
24. Murashige, T. and Skoog, F., *Physiologia Plantarum*, 1962, **15**, 473.
25. Hartmann, T., in *Biotechnology in Agriculture and Forestry*, Vol. 26, *Medicinal and Aromatic Plants* VI. ed. Y. P. S. Bajaj. Springer, Berlin 1994, p. 339.
26. Inoue, H. and Mizutani, A., *Analytical Biochemistry*, 1973, **56**, 408.
27. Witte, L., Rubiolo, P., Bicchi, C. and Hartmann, T., *Phytochemistry*, 1993, **32**, 187.
28. Biemann, K., in *Mass Spectrometry, Organic Chemical Applications*, McGraw-Hill Book Company, New York, 1962, p. 204.
29. Flores, H. E. and Galston, A. W., *Plant Physiology*, 1982, **69**, 701.
30. Tabor, C. W., *Methods in Enzymology*, 1962, **5**, 761.