PERGAMON

Phytochemistry 62 (2003) 339–344

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

# Evidence for general occurrence of homospermidine in plants and its supposed origin as by-product of deoxyhypusine synthase

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Received 22 July 2002; received in revised form 5 September 2002

Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday

**Abstract**

Deoxyhypusine synthase (DHS) is involved in the post-translational activation of the eukaryotic initiation factor 5A (eIF5A) and, as a side-reaction, catalyzes the formation of homospermidine if its substrate, the eIF5A precursor protein, is replaced by putrescine. Plant homospermidine synthase is assumed to be phylogenetically derived from DHS; it represents a DHS having lost its intrinsic activity. The enzyme is expressed in plants producing pyrrolizidine alkaloids where it catalyzes the formation of homospermidine the unique precursor of pyrrolizidine alkaloids. Here we show that 29 species randomly selected from 18 angiosperm families as well as a few other terrestrial plant species, all were able to produce small amounts of homospermidine. Basing on these results and in the context of literature on the occurrence of homospermidine in the organismic kingdoms, a universal occurrence of homospermidine is assumed and ubiquitous DHS is suggested to be responsible for its formation. The synthesis of homospermidine as an enzymatic by-product of an essential enzyme is discussed in respect to the evolutionary origin of homospermidine synthase and the biosynthetic pathway of pyrrolizidine alkaloids.


*Keywords:* Deoxyhypusine synthase; Homospermidine synthase; Polyamine; Homospermidine; Pyrrolizidine alkaloids; Evolution of secondary metabolism

## 1. Introduction

Homospermidine is the unique biosynthetic precursor of the necine base moiety (e.g., retronecine) of pyrrolizidine alkaloids (Khan and Robins, 1985; Spenser, 1985). Its formation is catalyzed by plant homospermidine synthase (spermidine-specific; EC 2.5.1.45; plant HSS), an enzyme that was identified and characterized from *Senecio* root cultures (Böttcher et al., 1993, 1994) and later cloned and actively expressed in *Escherichia coli* (Ober and Hartmann, 1999b; Ober et al., 2000). The enzyme catalyzes in an $NAD^+$-dependent reaction the transfer of the aminobutyl moiety of spermidine to putrescine yielding homospermidine (Fig. 1B).

The molecular data about plant HSS provided conclusive evidence for a close phylogenetic relation to deoxyhypusine synthase (EC 2.5.1.46; DHS) an enzyme involved in the post-translational activation of the eukaryotic initiation factor 5A (eIF5A). DHS catalyzes the transfer of the aminobutyl moiety of spermidine to the terminal amino group of a specific lysine residue of the eIF5A precursor protein, yielding a deoxyhypusine residue (Fig. 1A). The activation of eIF5A is completed by a hydroxylation of the deoxyhypusine group generating the unique hypusine residue. eIF5A was originally classified as an initiation factor of protein synthesis (Kemper et al., 1976). However, soon doubts arose about the main function of eIF5A. Although the precise function of the eIF5A is still unknown, it most likely functions as a carrier for the export of specific RNAs from the nucleus into the cytosol (Lipowsky et al., 2000; Rosorius et al., 1999). Deoxyhypusine synthase and eIF5A appear to be highly conserved among eukaryotes (Gordon et al., 1987) and archaebacteria (Bartig et al., 1990). Recently the same mechanism of activation was

*Abbreviations:* DHS, deoxyhypusine synthase (EC 2.5.1.46); plant HSS, homospermidine synthase (spermidine-specific EC 2.5.1.45); bacterial HSS, homospermidine synthase (EC 2.1.5.44).

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0031-9422/03/$ - see front matter © 2003 Published by Elsevier Science Ltd.
PII: S0031-9422(02)00553-8

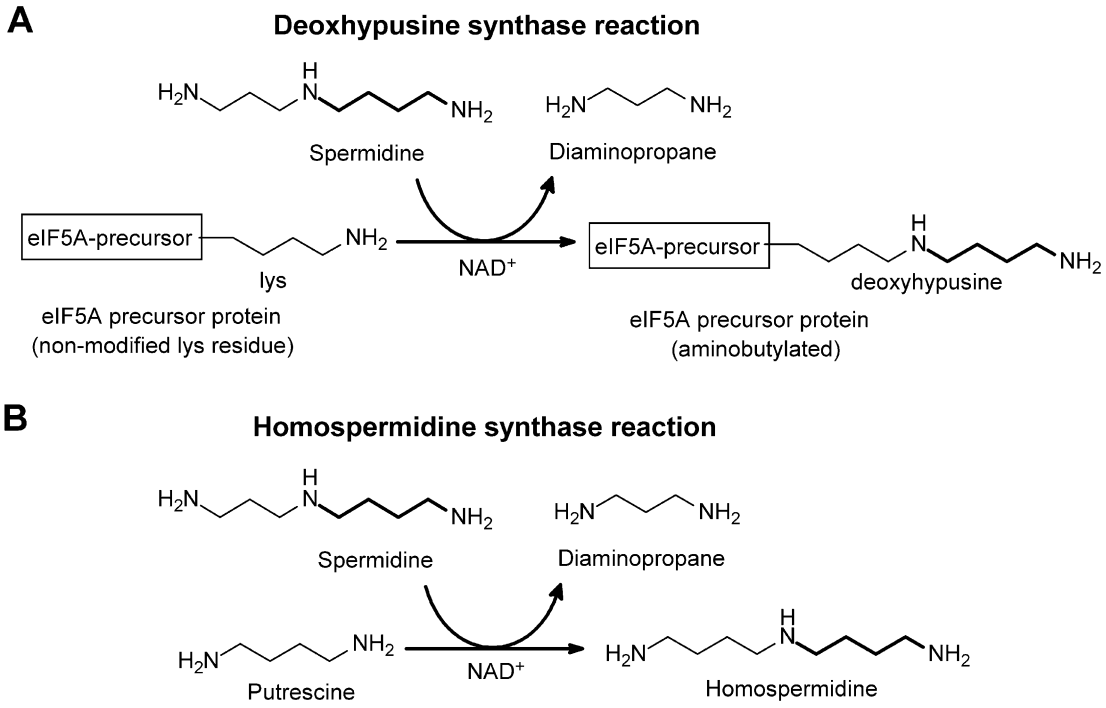


Fig. 1. Deoxyhypusine synthase (DHS) catalyzes the first reaction in the activation of the essential eukaryotic initiation factor 5A (eIF5A); deoxyhypusine synthase reaction (A). DHS is capable of catalyzing as side-reaction the formation of homospermidine; homospermidine synthase reaction (B). Homospermidine synthase (HSS) catalyzes the first pathway-specific reaction in the biosynthesis of pyrrolizidine alkaloids; HSS originated from plant DHS by gene duplication, loss of its intrinsic activity and retention of its side-activity.

confirmed in plants by cloning and functional expression of deoxyhypusine synthase and its substrate eIF5A from tobacco (Ober and Hartmann, 1999a) and *Senecio vernalis* (Ober and Hartmann, 1999b).

Due to their sequence homology, there is no doubt that the HSS originated from DHS by gene duplication and recruitment for pyrrolizidine alkaloid biosynthesis (Ober and Hartmann, 1999b, 2000). The two enzymes share the same reaction mechanism and DHS, in addition to its intrinsic activity, already catalyzes the aminobutylation of putrescine yielding homospermidine. A detailed comparison of the substrate specificity and kinetic properties of HSS and DHS revealed identical properties for the two enzymes, except for the loss of the ability of HSS to bind the eIF5A precursor protein to its surface. Thus, HSS has to be considered a DHS which lost its intrinsic activity; in HSS the "silent" activity of DHS now became the intrinsic activity without further modification of its kinetic properties (Ober et al., submitted for publication).

It is an open question whether the potential ability of universal DHS to synthesize homospermidine is maintained silent in most plants. Homospermidine is considered an uncommon polyamine, since it has been detected as free amine only in a restricted number of species (see Discussion). In the present study we screened randomly selected plant species for the occurrence of homospermidine applying a method that allows the unequivocal detection of small amounts of the polyamine.

## 2. Results

Twenty nine randomly selected species out of 18 families of the angiosperms were screened for the presence of homospermidine. For a quantitative comparison the two generally occurring amines putrescine and spermidine were included. The screening was supplemented by a gymnosperm (*Ginkgo*), a fern (male fern) and a sphagnum species. Since we expected, if any, very low concentrations of homospermidine, the extraction procedure was adapted accordingly. Prolonged acidic aqueous extraction and enrichment of the polyamine fraction by cationic ion exchange chromatography was found a suitable method. The final polyamine fraction was pure enough to allow the detection and quantification of homospermidine as benzoyl derivative by HPLC even in more than 100-fold lower concentrations than those of putrescine or spermidine in the extracts. The unequivocal identification of homospermidine detected by HPLC was achieved by GC–MS analysis of the polyamine fraction as their methoxycarbonyl (MOC)-derivatives. The results of the polyamine screening, summarized in Table 1, revealed the presence of homospermidine in all species tested. There was not a single exception of a species in which homospermidine could not be detected by GC–MS. Because of the often very small quantities special care was taken to exclude analytical memory effects. In most species, the concentrations of homospermidine are 10- to 100-fold

Table 1
Occurrence of homospermidine in randomly selected angiosperm species and a few other taxa

| Species | Plant organ | Polyamine (nmol g dry wt$^{-1}$) | | | Polyamine (relative abundance, %) | | | Ratio total amount Spd/Hspd | Plant containing pyrrolizidine alkaloids |
|---|---|---|---|---|---|---|---|---|---|
| | | Put | Spd | Hspd | Put | Spd | Hspd | | |
| Apiaceae | | | | | | | | | |
| *Angelica archangelica* | Roots | 145 | 50 | 1.3 | 73.9 | 25.5 | 0.7 | 39 | No |
| Apocynaceae | | | | | | | | | |
| *Vinca minor* | Leaves | 76 | 140 | 15[a] | | | | | No |
| Aquifoliacea | | | | | | | | | |
| *Ilex parguariensis* | Leaves | 101 | 62 | 1 | 61.6 | 37.8 | 0.6 | 63 | No |
| Asteraceae | | | | | | | | | |
| *Senecio vernalis* | Leaves | 76 | 122 | 4 | 37.6 | 60 | 2 | 31 | Yes |
| | Stems | 21 | 48 | 2 | 29.6 | 67.6 | 2.8 | 24 | |
| | Flowers | 143 | 140 | 10 | 48.8 | 47.8 | 3.4 | 14 | |
| | Roots | 45 | 71 | 13 | 34.9 | 55 | 10.1 | 6 | |
| *Senecio jacobaea* | Leaves | 88 | >147 | 1.7 | | | | >87 | Yes |
| *Taraxacum officinale* | Leaves | 129 | 160 | 2.2 | 44.3 | 54.9 | 0.8 | 73 | No |
| | Stems | 123 | 125 | | | | | | |
| | Flowers | >200 | 157 | 0.7 | 42.3 | 56.4 | 1.3 | 224 | |
| | Roots | 54 | 72 | 1.7 | | | | 42 | |
| *Matricaria recutita* | Flower heads | >231 | 123 | 4 | | | | 31 | No |
| *Tussilago farfara* | Leaves | 52 | 45 | 0.8 | 53.2 | 46 | 0.8 | 56 | Yes |
| *Arthemisia absinthium* | Herb | 75 | 26 | 2.4 | 72.5 | 25.1 | 2.2 | 11 | No |
| Betulaceae | | | | | | | | | |
| *Betula pubescens* | Leaves | 62 | 33 | 0.3 | 65.1 | 34.6 | 0.3 | 110 | No |
| Boraginaceae | | | | | | | | | |
| *Cynoglossum officinale* | Leaves | 40 | 82 | 43 | 42.2 | 49.7 | 26.1 | 1.9 | Yes |
| *Heliotropium indicum* | Leaves | 25 | 69 | 41 | 18.5 | 51.1 | 30.4 | 1.7 | Yes |
| Caesalpiniaceae | | | | | | | | | |
| *Cassia senna* | Leaves | 38 | 52 | 0.9 | 41.8 | 57.2 | 1 | 58 | No |
| Cannabaceae | | | | | | | | | |
| *Humulus lupulus* | Female flowers | 59 | 61 | 1.9 | 48.4 | 50 | 1.6 | 32 | No |
| Convallariaceae | | | | | | | | | |
| *Conavallaria majalis* | Herb | >217 | 68 | 1 | | | | 68 | No |
| Fabaceae | | | | | | | | | |
| *Ononis spinosa* | Roots | 75 | 21 | 1.7 | 76.8 | 21.5 | 1.7 | 12 | No |
| *Pisum sativum* | Leaves | 70 | >200 | 2.1 | | | | >95 | No |
| Hypericaceae | | | | | | | | | |
| *Hypericum perforatum* | Herb | 89 | 43 | 0.9 | 67 | 32.4 | 0.7 | 48 | No |
| Lamiaceae | | | | | | | | | |
| *Melissa officinalis* | Leaves | 58 | 4 | 0.3 | 93.1 | 6.4 | 0.5 | 13 | No |
| *Rosmarinus officinalis* | Leaves | 24 | 9 | 0.4 | 71.9 | 26.9 | 1.2 | 23 | No |
| *Orthosiphon aristatus* | Leaves | 16 | 2 | 0.2 | 87.9 | 11 | 1.1 | 10 | No |
| *Mentha x piperita* | Leaves | 69 | 14 | 0.6 | 82.5 | 16.7 | 0.7 | 23 | No |
| Ranunculaceae | | | | | | | | | |
| *Ranunculus ficaria* | Leaves | >179 | 112 | 6.4 | | | | 18 | No |
| Rosaceae | | | | | | | | | |
| *Filipendula ulmaria* | Flowers | 91 | 36 | 1.9 | 70.6 | 27.9 | 1.5 | 19 | No |
| Santalaceae | | | | | | | | | |
| *Santalum album* | Leaves | 130 | ?[b] | 1 | | | | | No |
| Solanaceae | | | | | | | | | |
| *Datura stramonium* | Leaves | 73 | 64 | 0.4 | 53.1 | 46.6 | 0.3 | 160 | No |
| *Nicotiana tabacum* | Leaves | 88 | 58 | 5.5 | 58.1 | 38.3 | 3.6 | 11 | No |
| Tiliaceae | | | | | | | | | |
| *Tilia cordata* | Flowers | 17 | 64 | 1.8 | 20.5 | 77.3 | 2.2 | 36 | No |
| Urticaceae | | | | | | | | | |
| *Urtica dioica* | Roots | 87 | 51 | 1.8 | 62.2 | 36.5 | 1.3 | 28 | No |
| Ginkoopsida | | | | | | | | | |
| *Ginkgo biloba* | Leaves | >155 | 128 | 0.8 | | | | 160 | No |
| Pteridophyta | | | | | | | | | |
| *Dryopteris filix-mas* | Leaves | 89 | 137 | 15 | 36.9 | 56.8 | 6.2 | 9 | No |
| Bryophyta | | | | | | | | | |
| *Sphagnum* spec. | Whole plant | 69 | 72 | 27 | 41.1 | 42.9 | 16.1 | 3 | No |

Put, putrescine; Spd, spermidine; Hspd, homospermidine.

[a] Value too high due to overlapping with an unknown metabolite; GC–MS analysis confirmed the presence trace amounts of homospermidine.

[b] Due to overlapping with the huge amount of homospermidine quantification of spermidine was not possible.

lower than those of putrescine or spermidine (Table 1, ratio Spd/Hspd). Within the angiosperms, the only species with a spermidine/homospermidine ratio below 10 are among those who contain pyrrolizidine alkaloids, i.e. the two species of the Boraginaceae and, within the Asteraceae, *S. vernalis* roots, but not shoot tissues of *Senecio* and *Tussilago*. In the latter case it must be recalled that in Asteraceae the pyrrolizidine alkaloids are synthesized exclusively in the roots (Hartmann et al., 1989; Moll et al., 2002). Comparatively high levels of homospermidine are also detected in the randomly chosen fern and moss.

## 3. Discussion

The present study revealed an occurrence of low but substantial amounts of homospermidine in all randomly tested plant species, indicating a general distribution of this polyamine in plants. This is consistent with several reports on the occurrence of homospermidine in plants. Homospermidine was found in a number of gramineous species (Hamana et al., 1994) and aquatic plants (Hamana et al., 1998, 2000), but also occasionally in mosses and ferns (Hamana and Matsuzaki, 1985a) and in eukaryotic algae (Hamana and Matsuzaki, 1985b). Even outside the plant kingdom homospermidine was detected in arthropods (Hamana et al., 1989, 1991a), marine invertebrates (Hamana et al., 1991b) and various worms belonging to the Platyhelminthes, Nematoda and Anelida (Hamana et al., 1995). In vertebrates homospermidine was found in amphibian testes (Hamana and Matsuzaki, 1979), hamster epididymis (Matsuzaki et al., 1987) and human neoplastic tissue (Matsuzaki et al., 1993). Considering all these data, an universal distribution of homospermidine in plants and perhaps all eukaryotic organisms appears likely.

We have three enzymes which may be responsible for the formation of homospermidine: (1) DHS (EC 2.5.1.46) universal among eukaryotes and archaebacteria; (2) plant HSS (spermidine-specific EC 2.5.1.45) evolved from DHS and so far only detected in plants producing pyrrolizidine alkaloids and (3) bacterial HSS (EC 2.5.1.44) present in certain taxa of the eubacteria and cyanobacteria. Bacterial HSS has been cloned from *Rhodopseudomonas viridis* (Tholl et al., 1996), it shares no sequence similarity with plant HSS. In contrast to plant HSS the bacterial enzyme uses putrescine not only as aminobutyl acceptor but also as major aminobutyl donor (Ober et al., 1996). Within the bacteria homospermidine occurs in certain taxa of nitrogen-fixing cyanobacteria (Hamana and Miyagawa, 1983) and the genera of the α-2 subclass of the Proteobacteria (Busse and Auling, 1988). This subclass covers also the symbiotic root-nodule bacteria of legumes (e.g. *Rhizobium*) (Stackebrandt et al., 1988). The occurrence of homospermidine in legumes is long known and has often been associated with the bacterial symbionts (Fujihara et al., 1986, 1994; Hamana et al., 1992, 1996). It remains open to what extent bacterial HSS and plant DHS are responsible for the formation of homospermidine in legumes. Taking all evidence together, is appears reasonable to assume that in most plants the side-activity of universal DHS accounts for the trace amounts of detectable homospermidine. The only exceptions may be plants containing pyrrolizidine alkaloids and thus possess a spermidine-dependent HSS (Hartmann and Ober, 2000) as well as legumes were a participation of bacterial HSS cannot be excluded.

Homospermidine is often accompanied by other uncommon polyamines; some of these may be also regarded as by-products of DHS. A detailed analysis of the substrate specificity of DHS revealed the ability of the enzyme to aminobutylate cadaverine and even spermidine and homospermidine yielding aminobutylcadaverine and the tetraamines aminobutylspermidine (canavalmine) and aminobutylhomospermidine, respectively (Ober et al., submitted for publication). Besides homospermidine the two tetraamines were detected in a variety of plants (Fujihara et al., 1982, 1986; Hamana et al., 1994, 1998, 2000). Aminobutylcadaverine, isolated from root-nodules of a legume (Fujihara et al., 1995) could be either derived from DHS or bacterial HSS which also was shown to produce this amine as a by-product (Ober et al., 1996; Yamamoto et al., 1993).

In the past, the immense diversity of plants and microbial secondary metabolism has often been regarded as the "playground of evolution" (Haslam, 1986; Zähner, 1979). Today it is generally accepted that secondary metabolism evolved under the selection pressure of a mostly hostile environment and plays an important functional role in all facets of chemical interactions between plants and their environment (Hartmann, 1996). However, the genesis of a new metabolic pathway needs an "evolutionary playground" on which selective forces may act. In this regard, formation of homospermidine as by-product of DHS might be a appropriate example. Homospermidine itself, formed in tiny amounts, is probably without any functional importance for the plant, but it could provide the initiation for an evolutionary process, as for instance the development of the biosynthetic pathway of pyrrolizidine alkaloids or the formation of simple *N*-substituted polyamines, sometimes named polyamine alkaloids, which are found in certain species of the family Solanaceae (Guggisberg and Hesse, 1983). In this context it is worth mentioning that trachelanthamidine, a simple necine base, was obtained biomimetically from homospermidine in the presence of pea diamine oxidase and alcohol dehydrogenase (Robins, 1982). In an evolutionary scenario the first step would be an increased production of homospermidine as it has been observed

in leaves of *Santalum rubrum* (Kuttan et al., 1971) (see also Table 1) and some aquatic plants (e.g. *Nelumbo nucifera*) (Hamana et al., 1994). The next steps, assumed for a pyrrolizidine alkaloid producing plant, would be (i) utilization of homospermidine as precursor of simple necines such as trachelanthamidine, (ii) selection of HSS derived from DHS by gene duplication as an independent source for homospermidine (Ober and Hartmann, 2000), (iii) mechanistic and regulatory integration of HSS into the newly evolving pathway and further improvement of the whole system under selective forces, e.g. of insect herbivory. A better understanding of the recruitment of homospermidine as unique precursor for pyrrolizidine alkaloid biosynthesis is a major goal for further studies.

## 4. Experimental

### 4.1. Plant material, extraction and sample preparation

Plants were collected in the Medicinal Plant Garden of the Institute and in the vicinity of Braunschweig. The various plant tissues were separated and immediately frozen at −20 °C and lyophilized. The dried material was minced in a ball mill and subsequently 4 g of the fine powder was extracted in 40 ml 0.1 M HCl for 16 h at room temperature on a gyratory shaker. After centrifugation and filtration the supernatant was extracted thrice with diethyl ether. The clear aqueous solution was further purified by ion exchange chromatography (Dowex-50 W $H^+$; 100–200 mesh; 1.8 meq $g^{-1}$). Diamines and polyamines were quantitatively eluted with 3 M HCl. The eluates were evaporated and kept dry until amine analysis by HPLC and GC–MS, respectively.

### 4.2. HPLC analysis of polyamines

Separation and quantification of the polyamines was achieved via their benzoyl derivatives (Flores and Galston, 1982). An RP-18 column (Nucleosil, 25 cm, 4 mm id.; Macherey & Nagel) was applied; elution: isocratically using the solvent system acetonitrile/$H_3PO_4$ (1.5%) (40/60); detection: photometrically at 230 nm. The retention times ($R_f$; min) for the polyamines are: putrescine, 5.1; cadaverine (internal standard), 6.1; spermidine 8.0; homospermidine, 9.2. In most cases quantification of the polyamines was achieved with calibration curves of putrescine and spermidine as external standards for diamines and polyamines, respectively. Putrescine and spermidine as reference compounds were purchased by Sigma (München), homospermidine was prepared enzymatically by using recombinant bacterial HSS (EC 2.5.1.44) from *R. viridis* overexpressed in *E. coli* (Graser et al., 1998; Tholl et al., 1996).

### 4.3. GC–MS of N-methoxycarbonyl derivatives (MOC derivatives) of polyamines

The MOC derivatives were prepared using a slightly modified procedure described previously (Husek et al., 1992). The pre-purified dry polyamine extract was dissolved in 0.2 ml aqua dest and made alkaline with 1 ml saturated solution of $Na_2CO_3$, then 20 μl methyl chloroformate were added and the mixture was allowed to react for 15 min at room temperature. The MOC derivatives were extracted thrice with each 0.5 ml ethyl acetate. The combined extracts were dried over $Na_2SO_4$, the solvent was evaporated and the residue was dissolved in 25 μl methanol and directly applied to GC–MS analysis. Capillary GC was performed as described elsewhere (Witte et al., 1993). For GC–MS the capillary column (30 m×0.32 mm fused-silica; DB-1, J & W Scientific) was directly coupled to a quadrupole mass-spectrometer Finnigan MAT 4515, EI-MS were recorded at 40 eV. The temperature program of GC for the polyamine derivatives was 150–300 °C, 10 °C $min^{-1}$. The retention indices, *RI* (Kovats index) and $M_r$s ($m/z$ [$M^+$]) of the respective MOC derivatives are: putrescine, *RI* 1622, [$M^+$] 204; spermidine, *IR* 2365, [$M^+$] 319; homospermidine, *IR* 2467, [$M^+$] 333.

## Acknowledgements

This work was supported by grants of the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

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