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THE BIOSYNTHESIS OF LUNARINE IN SEEDS OF *LUNARIA ANNUA*

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Key Word Index—*Lunaria annua*; Cruciferae; biosynthesis; alkaloid; Pummerer-ketone; phenol-oxidative coupling; cytochrome P-450; lunarine; lunaridine.

Abstract—Using an optimized precursor feeding system, the biosynthesis of the alkaloid lunarine in *Lunaria annua* seeds was investigated. The synthesis of radioactively labelled precursors for the application experiments is described. Lunarine was shown to be synthesized by stereo-selective phenol-oxidative coupling of N^1,N^{10}-bis(*p*-coumaroyl)spermidine. *p*-Coumaric acid for the biosynthesis of this dimer is formed from L-phenylalanine via *trans*-cinnamic acid. The polyamine moiety of lunarine, spermidine, and its immediate precursor putrescine are preferentially synthesized from arginine. Enzyme studies with microsomes of *Lunaria annua* seeds indicated that a cytochrome P-450 enzyme might be responsible for the phenol-oxidative coupling of N^1,N^{10}-bis (*p*-coumaroyl)spermidine yielding the hexahydrodibenzofuran ring of lunarine.

INTRODUCTION

More than 70 years ago, Pummerer *et al.* [1] observed that oxidation of *p*-cresol affords phenolic radicals which in turn lead to an *ortho-para* coupled racemic product the Pummerer-ketone, the structure of which was later revised [2]. Pummerer *et al.* already noticed that their newly discovered oxidative phenol-coupling reaction might be a clue for the elucidation of plant phenol biosynthesis. This reflection was further extended by Barton and Cohen who postulated the occurrence of this radical mechanism for the formation of an overwhelming number of phenol-coupled natural products in higher plants [3]. The demonstration of highly specific cytochrome P-450 enzymes as biocatalysts responsible for the phenol-coupling reaction have confirmed this concept recently [4–7].

An example for a natural product which is presumably synthesized via a phenol-coupling reaction is the alkaloid lunarine from *Lunaria annua* L. This substance has a striking similarity to the Pummerer-ketone and was the first plant alkaloid to be shown to contain spermidine [8]. The macrocyclic structure of lunarine was verified by NMR spectroscopy as well as degradation experiments [9], while the correct stereochemistry was elucidated by X-ray crystallography [10, 11]. Similar to the phenol-coupling reaction during Pummerer-ketone synthesis, lunarine was proposed to be synthesized from two molecules of *p*-coumaric acid via oxidative phenol-coupling and previous or subsequent addition of spermidine [12].

In first biosynthetic experiments radioactively labelled potential precursors of lunarine such as phenylalanine and tyrosine were administered to twigs of *L. annua* in the stage of seed ripening [13]. While phenylalanine was incorporated up to 0.03%, the incorporation of tyrosine into lunarine was negligible [13]. Subsequent degradation experiments of the labelled lunarine confirmed the specific incorporation of phenylalanine into the hexahydrodibenzofuran ring [13]. These experiments indicated that in *Lunaria* phenylalanine is transformed via cinnamic acid into *p*-coumaric acid, the immediate precursor for the biosynthesis of lunarine.

Two possibilities now exist for the further steps of the lunarine pathway. Two molecules of *p*-coumaric acid may be first phenol-coupled and then added to spermidine or spermidine at atom N^1 and N^{10} may first be acylated by two molecules of *p*-coumaric acid and this dimer subsequently coupled to yield the hexahydrodibenzofuran ring of lunarine. Since bis (*p*-coumaroyl)spermidine has been favoured in biomimetic approaches towards lunarine [14], the latter possibility seems to be more likely. Moreover, bis (*p*-coumaroyl)spermidine has been shown to occur as a natural product in higher plants [15, 16].

In order to discover which of these possibilities is verified in the biosynthesis of lunarine, radioactively labelled potential precursors of the alkaloid under investigation where synthesized and applied to *L.*

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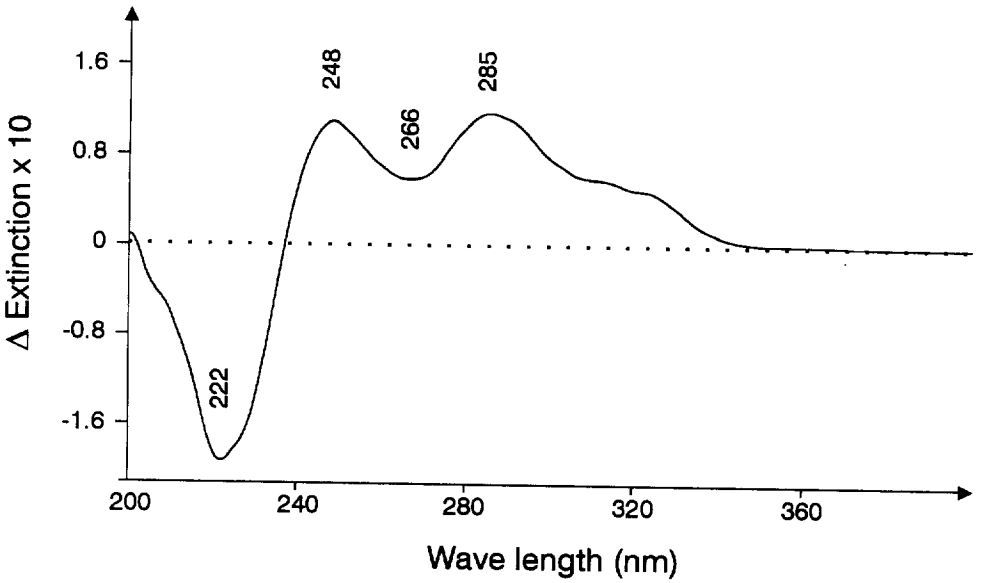


Fig. 1. The circular dichroism spectrum of lunarine at a concentration of 0.16 mg ml^{-1} in MeOH.

annua plant material. Feeding of ^{13}C-labelled precursors subsequently proved the postulated biosynthetic route. Prior to these experiments the physiological optimization of the application system was developed with regard to incorporation rates.

RESULTS

Lunarine properties

The striking similarity of the Pummerer-ketone to lunarine was the first indication that lunarine in the plant may be synthesized by an oxidative phenol-coupling reaction. While the chemical synthesis of the Pummerer-ketone as well as the enzymic synthesis by the unspecific enzyme horseradish peroxidase both starting from *p*-cresol lead to a racemic product [17], the biosynthesis of lunarine in the plant is expected to proceed stereo-selectively thus yielding the optical active alkaloid. This assumption was examined by recording a CD spectrum of lunarine at a concentration of 0.16 mg ml^{-1} in methanol. As depicted in Fig. 1, the CD spectrum measured in the range of 200–400 nm showed typical CD values at 222, 248, 266, and 285 nm. The optical rotation of lunarine was determined to be $[\alpha]_D^{20} + 295°$ ($CHCl_3$; *c* 0.39 mg ml^{-1}) and $[\alpha]_D^{20} + 275°$ (MeOH; *c* 0.59 mg ml^{-1}), respectively. The CD spectrum as well as the determined optical characteristics of lunarine coincided with data given in the literature [18] thus proving that the alkaloid under study is optical active. The phenol-coupling reaction for the biosynthesis of lunarine, therefore, must be catalysed by a specific and stereo-selective enzyme.

In previous investigations, lunarine was found in the seeds of *Lunaria* [19]. Our own studies now demonstrate that neither in roots, stems, leaves nor in seed pods could traces of the alkaloid under investigation be detected. In addition, cell cultures of *L. annua* were examined and were totally devoid of alkaloids of this type. A significant content of lunarine was exclusively found in the seeds thus demonstrating that the occurrence of lunarine in the *Lunaria* plant is restricted to this organ.

Besides lunarine, the seeds of *L. annua* mainly contain lunaridine, an isomer which differs from lunarine only in the orientation of its spermidine chain and, therefore, has almost similar characteristics. Since our investigations could confirm that the seeds are the only promising plant parts for biosynthetic studies, we now set out to optimize the conditions for precursor feeding experiments.

Development of an application system

In foregoing studies, Poupat and Kunesch found only very little incorporation of ^{14}C-labelled phenylalanine when they fed this potential precursor of lunarine to fruit-forming twigs of *L. annua* [13]. Therefore it was at first necessary to optimize the application system thus improving the rate of incorporation of potential precursors. For these studies, L-phenylalanine was chosen for application since it was already demonstrated that this substance is incorporated into lunarine [13].

Avoiding the twigs, we used isolated seed pods of *Lunaria* which were fed through the fruit stems. The application of 0.5 μCi L-[*U*-^{14}C]phenylalanine in doubly-water for 24 hr resulted in 0.01% incorporation into lunarine which was in the same range as the results of Poupat and Kunesch [13] and, therefore, too low for further feeding experiments.

Next, we tried to apply the precursor directly to isolated seeds. This procedure could enhance the uptake of radioactivity to 65% and at least 0.7% were incorporated into lunarine. Since the stage of ripening

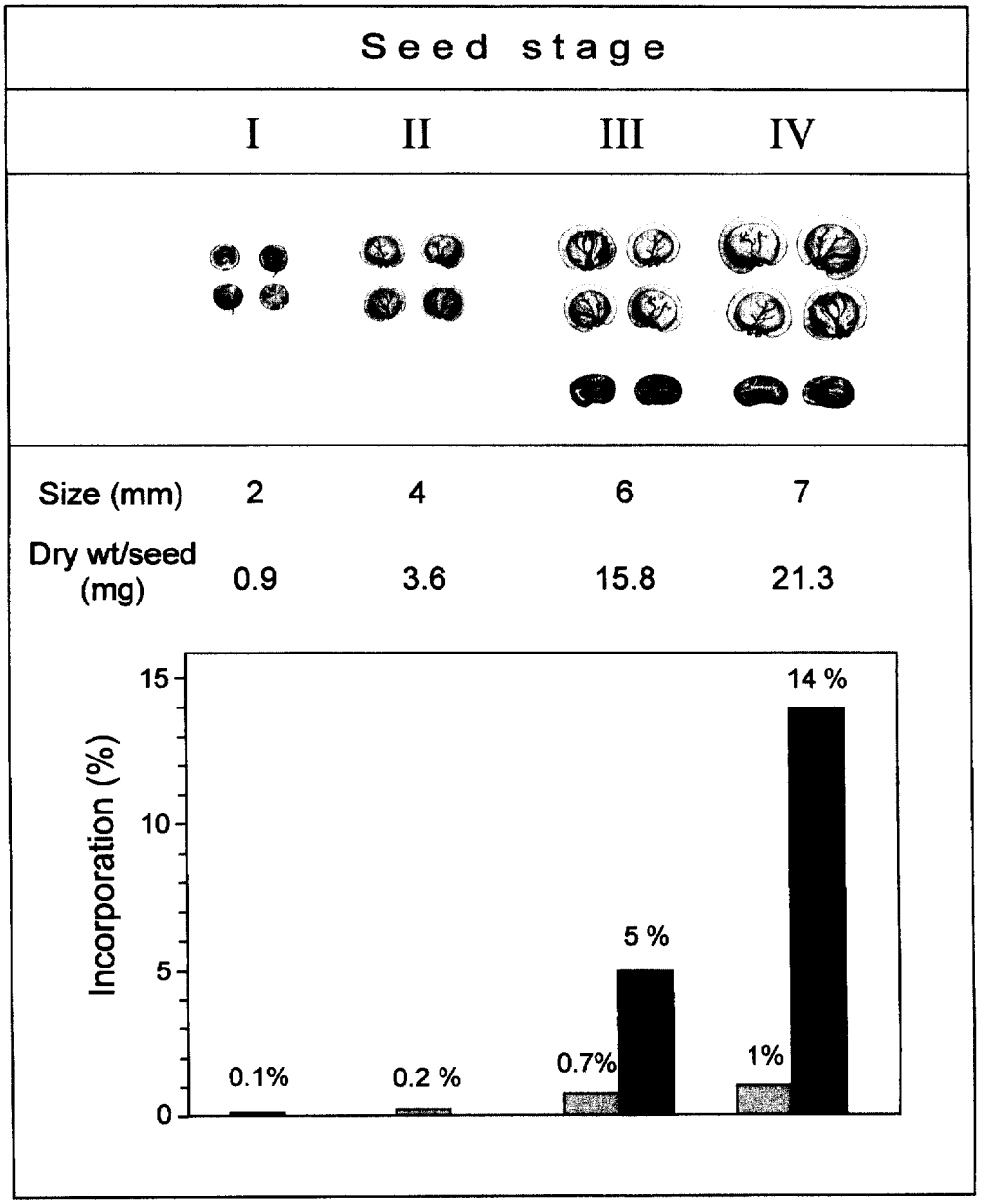


Fig. 2. Stages of maturity of *Lunaria annua* seeds and incorporation of applied L-[*U*-^{14}C]phenylalanine into lunarine. Seeds of stage III and IV were used without seed coat as well (black columns).

may influence the extent to which lunarine is synthesized, whole seeds in different stages of maturity were now used for incubation with labelled phenylalanine. For this purpose, four seed stages with increasing size and dry weight were chosen (Fig. 2) and the seeds incubated separately with the labelled precursor. At the end of incubation it was noticed, that the uptake of radioactivity increased with increasing maturity of the seeds. The analysis of the labelling of lunarine confirmed that the highest amount of radioactivity was found in lunarine from seeds in stage IV, where 1.0% incorporation was found (Fig. 2). A further dramatic increase in incorporation rate could be achieved, when the coat of seeds in stage III and IV was removed prior to precursor application. In that case, 5 and 14% incorporation was determined in seeds from stage III and IV, respectively.

According to the results depicted in Fig. 2 the seeds of stage IV (size: 7 mm; dry wt/seed: 21.3 mg) and the following conditions were chosen for an optimal application system: three seeds of *L. annua* from stage IV with their seed coat removed (*ca* 60 mg dry wt) were exposed to 0.5 ml aqueous solution of the applied precursor (0.5–1.5 μCi) in a well of a multiwell plate

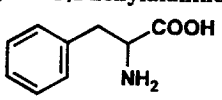
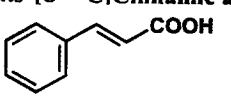
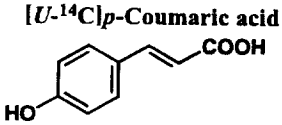
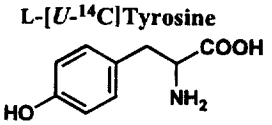
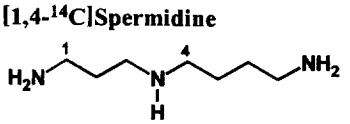
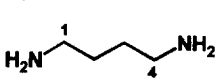
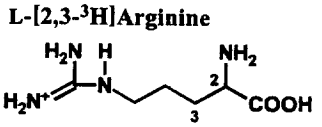
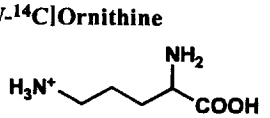
at 22° at 100 rpm in continuous light for 24 hr. Using this optimized application method, the incorporation of L-[*U*-^{14}C]phenylalanine into lunarine could be increased 470-fold over the results obtained previously [13].

Application of potential precursors

With the newly developed application system in hand, different radioactively labelled potential precursors of lunarine were now administered to *L. annua* seeds. Since it was assumed that lunarine is synthesized from *p*-coumaric acid and spermidine, these substances and, in addition, their respective precursors were chosen for feeding experiments. After incubating for 24 hr the *Lunaria* seeds were worked up and the extracts analysed as described in the Experimental. The results of this investigation are shown in Table 1.

The applied precursors were taken up very efficiently. In almost all cases a rate of uptake of more than 90% was found with only one exception that is *p*-coumaric acid from which only 85% of the total radioactivity was taken up from the seeds in 24 hr. A significant labelling of lunarine was found when L-phenylalanine, *trans*-cinnamic acid and *p*-coumaric acid were fed to the seeds yielding incorporation rates of 14, 9 and 4%, respectively. Tyrosine was not incor-

Table 1. Incorporation of radioactively labelled potential precursors into lunarine

Compound	Addition (nmol)	Rate of uptake (%)	Rate of incorporation (%)
L-[*U*-^{14}C]Phenylalanine 	1.1	99	14
***trans*-[*U*-^{14}C]Cinnamic acid** 	1.1	99	9
[*U*-^{14}C]*p*-Coumaric acid 	1.0	85	4
L-[*U*-^{14}C]Tyrosine 	1.0	99	0
[1,4-^{14}C]Spermidine 	4.4	99	20
[1,4-^{14}C]Putrescine 	4.4	97	19
L-[2,3-^{3}H]Arginine 	0.03	97	3
L-[*U*-^{14}C]Ornithine 	2.1	99	2

porated at all into lunarine while spermidine and putrescine gave the highest incorporation rates of 20 and 19%, respectively. In contrast, the early precursors L-ornithine and L-arginine were incorporated to only low extents.

Separating the seed extracts after precursor application by TLC it was noticed that the radioactively labelled precursors were not only incorporated into lunarine. In addition, lunaridine (R_f 0.30) and two other alkaloids showing R_f values of 0.37 and 0.66, respectively, were labelled as well. Moreover, the application of L-phenylalanine and spermidine always yielded a totally coinciding incorporation pattern (Fig. 3) with predominant peaks at the position of lunarine (R_f 0.82).

The precursor feeding experiments described above together with the results of Poupat and Kunesch [13] could demonstrate the biosynthetic sequence up to the intermediate *p*-coumaric acid. Since L-tyrosine was not incorporated at all into lunarine, a deamination of L-tyrosine directly yielding *p*-coumaric acid could be excluded. In *L. annua* seeds, however, the biosynthesis of lunarine is initiated from L-phenylalanine, deamination to *trans*-cinnamic acid and subsequent hydroxylation to *p*-coumaric acid.

Feeding of ^{13}C*-labelled* p*-coumaric acid*

To further verify this pathway with regard to a random or specific incorporation of labelling and to

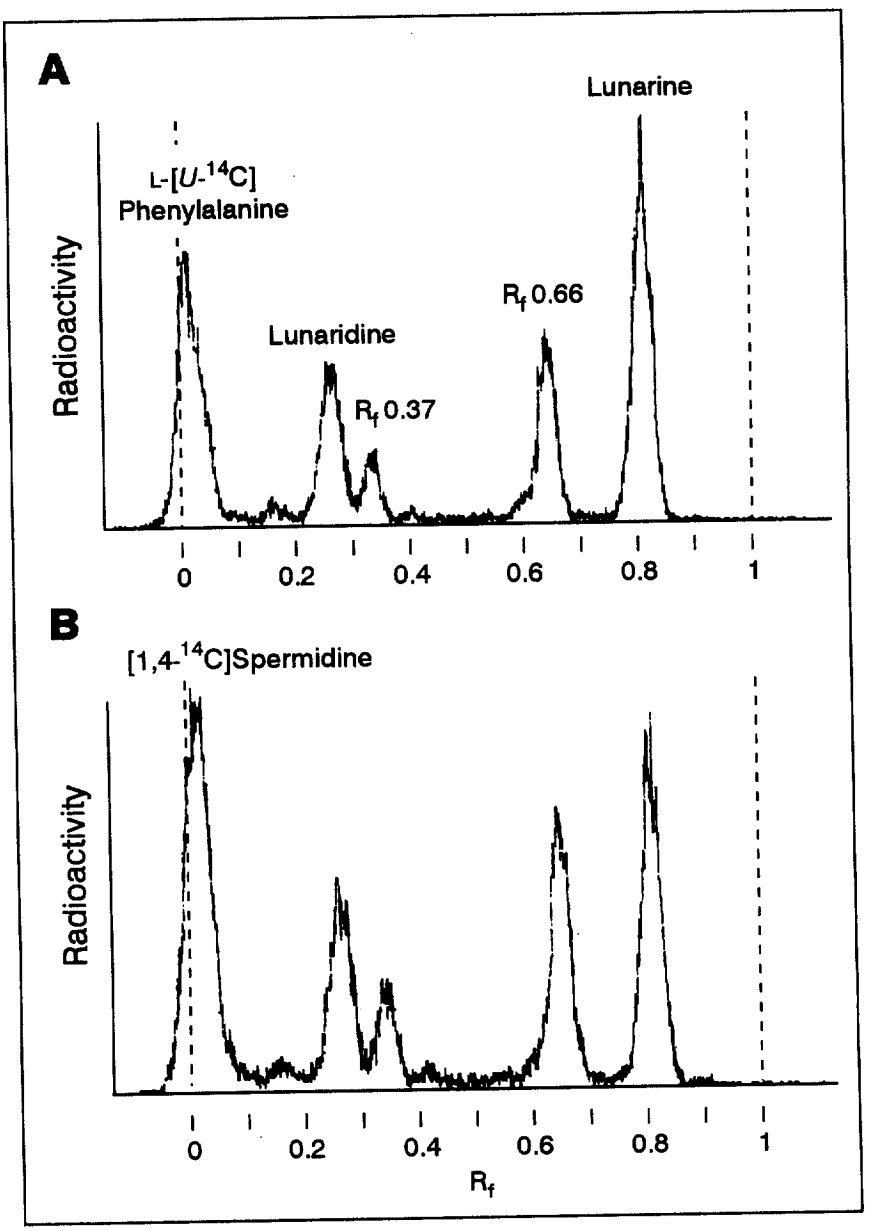


Fig. 3. Radioscan of *Lunaria* seed extracts after TLC separation from application experiments with (A) L-[U-^{14}C]phenylalanine and (B) [1,4-^{14}C]spermidine.

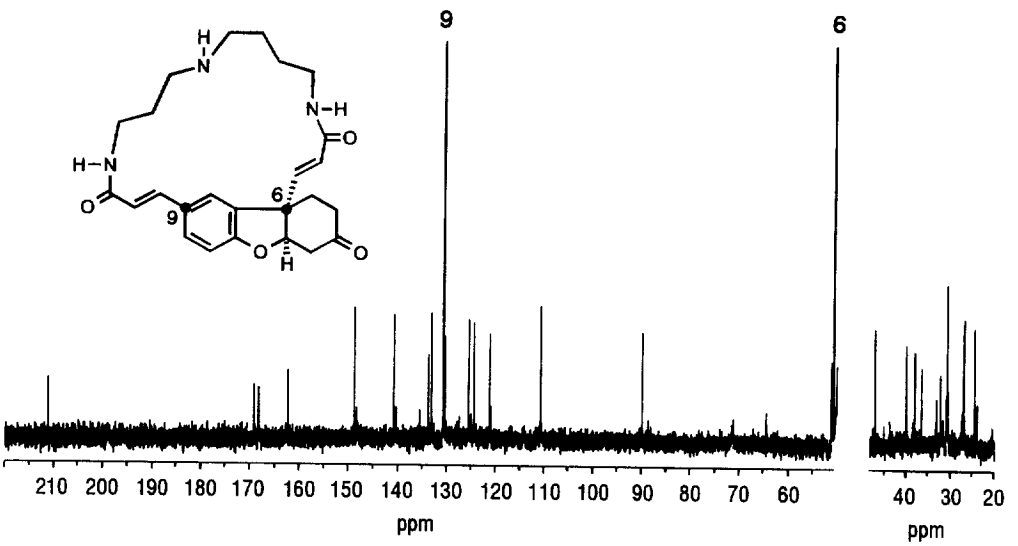


Fig. 4. Partial proton-decoupled ^{13}C NMR spectrum of lunarine after incorporation of [1-^{13}C]*p*-coumaric acid.

avoid degradation of lunarine, ^{13}C-labelled *p*-coumaric acid was fed to *Lunaria* seeds and the resulting lunarine analysed by NMR spectroscopy. For this purpose, [1-^{13}C]*p*-coumaric acid was enzymically synthesized from L-[1-^{13}C]tyrosine showing a ^{13}C NMR spectrum with an increased signal at 127.2 ppm. This ^{13}C-labelled *p*-coumaric acid was now exposed to *Lunaria* seeds in aqueous solution at a concentration of 1.4 mM. The resulting alkaloids were separated by solid phase extraction with subsequent TLC as described in the Experimental. After purification of lunarine it was determined that 6% of the added substrate had been incorporated into this alkaloid which was subsequently subjected to NMR spectroscopy.

The ^{13}C NMR spectrum of lunarine after incorporation of [1-^{13}C]*p*-coumaric acid as shown in Fig. 4 confirmed the incorporation of the applied compound. As expected, the primary increased signal at 127.2 ppm had disappeared while the signals of C-6 and C-9 of lunarine at 50.9 ppm and 130.5 ppm, respectively, were clearly enlarged resulting from the incorporation of [1-^{13}C]-labelled *p*-coumaric acid.

Furthermore, an even more distinct shift of the increased NMR signals should be expected after feeding of [3,5-^{13}C]-labelled *p*-coumaric acid. For this purpose, [3,5-^{13}C]*p*-coumaric acid was synthesized (from L-[3,5-^{13}C]tyrosine by catalysis of phenylalanine ammonia lyase) and exposed to *Lunaria* seeds at 1.4 mM concentration which yielded in an incorporation rate of 5%. The ^{13}C NMR spectrum of the resulting lunarine is depicted in Fig. 5. The distinct shift from the primary increased signal at 116.8 ppm of [3,5-^{13}C]*p*-coumaric acid to 36.1 ppm (C-2/4) as well as 110.7 ppm (C-11) and 134.0 ppm (C-7) of lunarine clearly demonstrated the incorporation of this ^{13}C-labelled precursor into lunarine. Therefore, the application of ^{13}C-labelled *p*-coumaric acid to *L. annua* seeds confirms without doubt that the hexahydrodibenzofuran ring of lunarine is composed of two molecules of *p*-coumaric acid.

Synthesis of radioactively labelled N^1,N^{10}-*bis*(p-*coumaroyl*)*spermidine*

The biosynthetic pathway to lunarine may proceed by two possible routes. Two molecules of *p*-coumaric acid may first be phenol-coupled and then added to spermidine or spermidine at atom N^1 and N^{10} may first be acylated by two molecules of *p*-coumaric acid to yield N^1,N^{10}-bis(*p*-coumaroyl)spermidine which subsequently may be phenol-coupled. In order to prove which of these possibilities is verified in lunarine biosynthesis, doubly labelled N^1,N^{10}-bis(*p*-coumaroyl)spermidine had to be synthesized in the milligram and submilligram range for further feeding experiments.

A classical coupling agent for the formation of a peptide bond is *N*,*N*′-dicyclohexylcarbodiimide (DCC) [20], however, it was not possible to condense *p*-coumaric acid with spermidine under the addition of DCC. Neither the protection of the hydroxyl group of *p*-coumaric acid nor the employment of the water soluble modified 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was successful in this regard. Corresponding to the synthesis of N^1,N^{10}-bis(*trans*-cinnamoyl)spermidine (maytenine) [21] we next tried to couple *p*-coumaroylchloride with spermidine, but also without success. As further possibilities, the synthesis under addition of formaldehyde protected spermidine (hexahydropyrimidine) [22] and the selective aminolysis of 3-coumaroyl-1,3-thiazolidine-2-thione [23] were tested but neither method yielded the desired product bis(*p*-coumaroyl)spermidine. In addition, an adopted procedure similar to the formation of maytenine under application of *N*-methoxydicinnamamide [24] did not lead to the respective

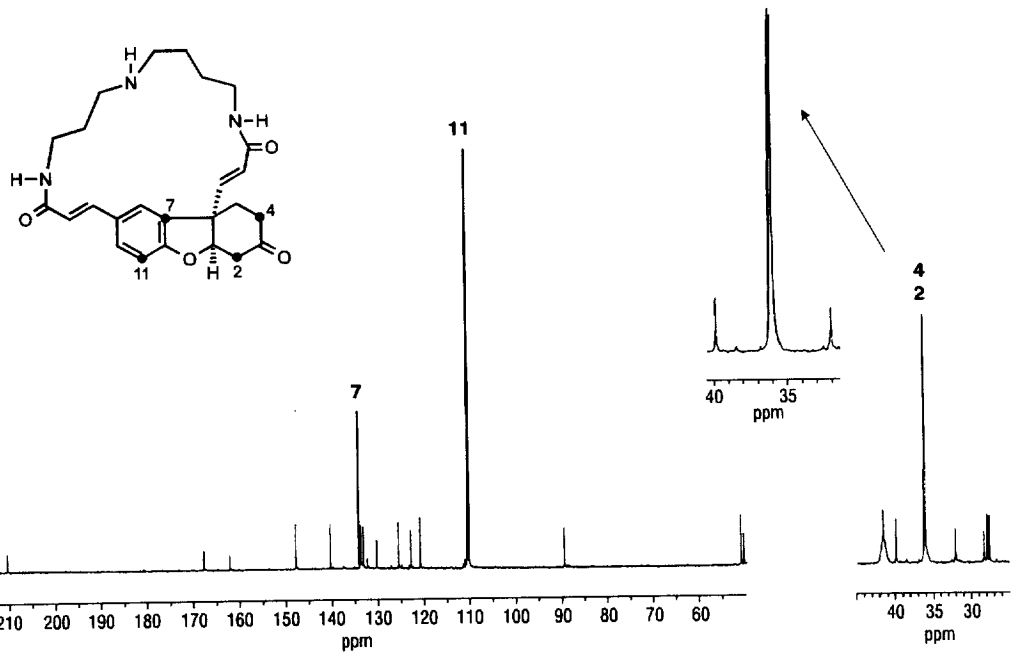


Fig. 5. Partial proton-decoupled ^{13}C NMR spectrum of lunarine after incorporation of [3,5-^{13}C]*p*-coumaric acid. C-2 and C-4 of lunarine are enlarged.

coupling product. A further method for the synthesis of maytenine [25] was then adopted and finally succeeded in the synthesis of N^1,N^{10}-bis(*p*-coumaroyl) spermidine. For this purpose, 2-hydroxy-5-nitro-α-toluene sulphonic acid sultone was used as coupling agent which was synthesized by a modified method according to Ref. [26]. The 4-OH group of *p*-coumaric acid had to be protected prior to coupling as the *tert*-butyldimethylsilyl (TBDMS) derivative [27].

The synthesis of [^3H,^{14}C] doubly labelled N^1,N^{10}-bis([3,5-^3H]*p*-coumaroyl)[1,4-^{14}C]spermidine was now achieved as follows: 1.9 μmol TBDMS-[3,5-^3H] *p*-coumaric acid (88 μCi), 2.8 μmol 2-hydroxy-5-nitro-α-toluene sulphonic acid sultone, 2.5 μmol triethylamine, and 10 μl tetrahydrofuran (THF) were added in a micro vial and left shaking at 20° overnight. Then [1,4-^{14}C]spermidine (8.8 μCi) was dissolved in 5 μl THF and added (theoretical ratio $^3H:^{14}C = 10$). After 4 hr of reaction, 7.6 μmol tetrabutylammonium fluoride dissolved in 10 μl THF were added for deprotection and the mixture shaken for 1 hr at 20°. The mixture was then subjected to TLC and the band corresponding to the desired coupling product eluted with methanol. With this procedure a total of 0.09 mg (0.2 μmol) N^1,N^{10}-bis([3,5-^3H]*p*-coumaroyl)[1,4-^{14}C] spermidine was obtained at a yield of 23% with a ratio of $^3H:^{14}C = 10.8$.

Feeding of N^1,N^{10}-*bis*([3,5-^3H]p-*coumaroyl*)[1,4^{14}C] *spermidine*

The doubly labelled bis-(*p*-coumaroyl)spermidine (0.013 μmol) was supplied in 0.5 ml aqueous solution to three seeds of *L. annua* at stage IV. After 24 hr of incubation at 20° the seeds had taken up 94% of the radioactivity and then were extracted with ethanol. Lunarine was separated by TLC and showed a 6% incorporation of the doubly labelled compound supplied, but the $^3H:^{14}C$ ratio had dropped from an initial 10.8 of the applied precursor to 2.8 of lunarine. Considering the removal of tritium from C-7 of lunarine through the phenol-coupling reaction, a tritium loss of only 1/4 would have been expected. The additional loss of tritium up to almost exactly 3/4 of the total tritium supplied can be explained by keto-enol tautomerism leading to the removal of tritium from carbon C-2 and C-4 of the hexahydrodibenzofuran ring. This keto-enol tautomerism had been suggested previously [13] after feeding of doubly labelled L-phenylalanine to *L. annua*.

To finally confirm the expected hydrogen exchange of lunarine, the alkaloid was dissolved in D_2O–HCl and the ^1H NMR spectrum recorded showing the signal of the H-atoms at position 4 at 2.71 ppm slightly shifted with regard to the lunarine spectrum in CD_3OD–HCl [Fig. 6(A)]. After standing in D_2O for 21 days, the signal of H-4 was almost eliminated [Fig. 6(B)] based on the exchange of hydrogen by deuterium. With this experiment it was verified that a keto-enol tautomerism does occur at the carbonyl group in position C-3 of lunarine.

Considering this tautomerism and the removal of tritium at position 7 due to the phenol-oxidative-coupling reaction, the determined $^3H:^{14}C$ ratio of 2.8 measured after feeding of doubly labelled bis(*p*-coumaroyl)spermidine, therefore, agreed with the theoretically expected drop of double label to 2.7.

This precursor feeding experiments using doubly

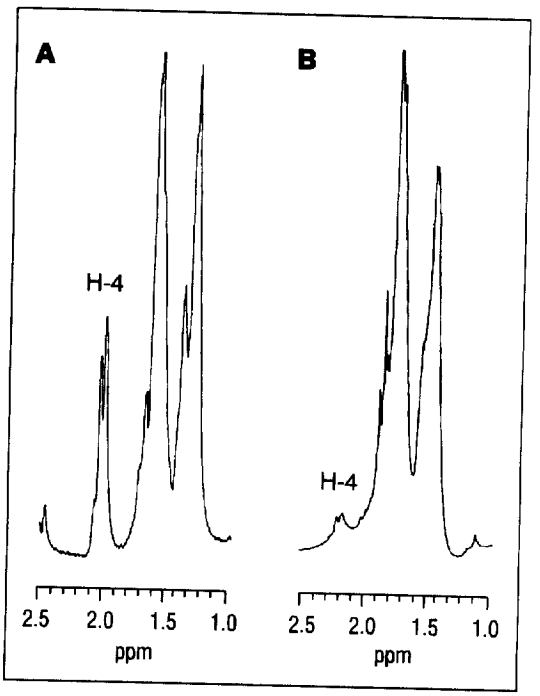


Fig. 6. Partial proton-decoupled ^{1}H NMR spectrum of lunarine in CD_3OD–HCl (A) reference sample, (B) sample after incubation in D_2O–HCl for 21 days.

labelled N^1,N^{10}-bis([3,5-^{3}H]*p*-coumaroyl)[1,4-^{14}C] spermidine clarified the key step in the biosynthesis of lunarine. Two molecules of *p*-coumaric acid, certainly almost first activated by forming the CoA thioesters subsequently acylate spermidine in position N^1 and N^{10} to form bis(*p*-coumaroyl)spermidine. This intermediate will then be phenol-coupled to form the hexahydrodibenzofuran ring of lunarine. Finally, we wanted to investigate the enzyme that is responsible for this closure of the lunarine ring system.

Enzyme studies

Since the coupling reaction yielding the hexahydrodibenzofuran ring of lunarine must be highly stereo-selective, the participation of a peroxidase is unlikely since coupling by such an enzyme would yield a racemic product [17]. In contrast, highly stereo- and regio-selective phenol-coupling reactions in benzylisoquinoline alkaloid synthesis were already shown to be catalysed by membrane-bound cytochrome P-450 enzymes [4–7]. To prove, whether this type of enzyme, indeed, is responsible for the closure of the lunarine ring, microsomes were prepared from *Lunaria* seeds of stage IV and incubated with the labelled precursor N^1,N^{10}-bis([3,5-^{3}H]*p*-coumaroyl)spermidine under optimized conditions. After incubation at 40° for 20 min, the incubation mixture was separated by TLC and the distribution of radioactivity on the plates determined (Fig. 7). In the presence of NADPH or H_2O_2, the radioactively labelled precursor was converted into four products showing R_f values of 0.15, 0.20, 0.30 and 0.40, respectively [Fig. 7(A)] none of which was identical with lunarine. When NADPH was omitted from the incubation mixture [Fig. 7(B)] these substances were not produced at all but a product with R_f 0.08 was. This was also spontaneously formed in control assays without enzyme addition.

Attempts to identify the reaction products, which were formed enzymically from N^1,N^{10}-bis([3,5-^{3}H]*p*-coumaroyl)spermidine failed because of their low concentration. We assume that the products are intermediates of the lunarine biosynthesis that are already phenol-coupled but are still bearing the double bond at C-4. In spite of the presence of NADPH, microsomes may lack the enzymes for hydrogenation. These possibly are localized in the cytosol.

Spermidine biosynthesis

Spermidine may be synthesized in the plant by different routes. Putrescine, the immediate precursor of spermidine, may be formed from ornithine by decarboxylation or from arginine via agmatine and *N*-carbamoylputrescine [28]. The route by which spermidine is synthesized in *Lunaria* is, however, unknown. The feeding experiments described above had demonstrated that the spermidine precursor [1,4-^{14}C]putrescine was incorporated into lunarine with a rate of 19% while the more distant precursors L-[*U*-^{14}C]ornithine and L-[2,3-^{3}H]arginine showed only low incorporation rates of 2 and 3%, respectively. A simultaneous feeding of L-[*U*-^{14}C]ornithine and L-[2,3-^{3}H]arginine could possibly indicate whether one of the possible routes for spermidine synthesis may be favoured in *Lunaria*. For this purpose, the precursors were administered in a ^{3}H:^{14}C ratio of 10:1. The ratio in lunarine should increase, when [^{3}H]arginine and decrease when [^{14}C]ornithine is the preferred source.

Three *Lunaria* seeds of stage IV (53 mg dry wt) were incubated in 0.5 ml aqueous solution with 0.08 nmol L-[2,3-^{3}H]arginine, 1.45 nmol L-[*U*-^{14}C]ornithine hydrochloride and 1.37 nmol L-arginine hydrochloride for 24 hr. Thereafter, the seeds were extracted with EtOH and the extracts separated by TLC. The radioactively labelled precursors were incorporated into lunarine and lunaridine to 4 and 2%, respectively. The original ^{3}H:^{14}C ratio of 10, thereby, was raised in lunarine to 16 and in lunaridine to 19.3. This result demonstrated that in *Lunaria* arginine is preferentially used for lunarine biosynthesis, indicating that spermidine and its immediate precursor putrescine may not directly be synthesized from ornithine but are produced via decarboxylation and deamination of arginine.

DISCUSSION

The early feeding experiments of Poupat and Kunesch [13] who found 0.03% incorporation of radioactively labelled phenylalanine into lunarine in *L. annua* were

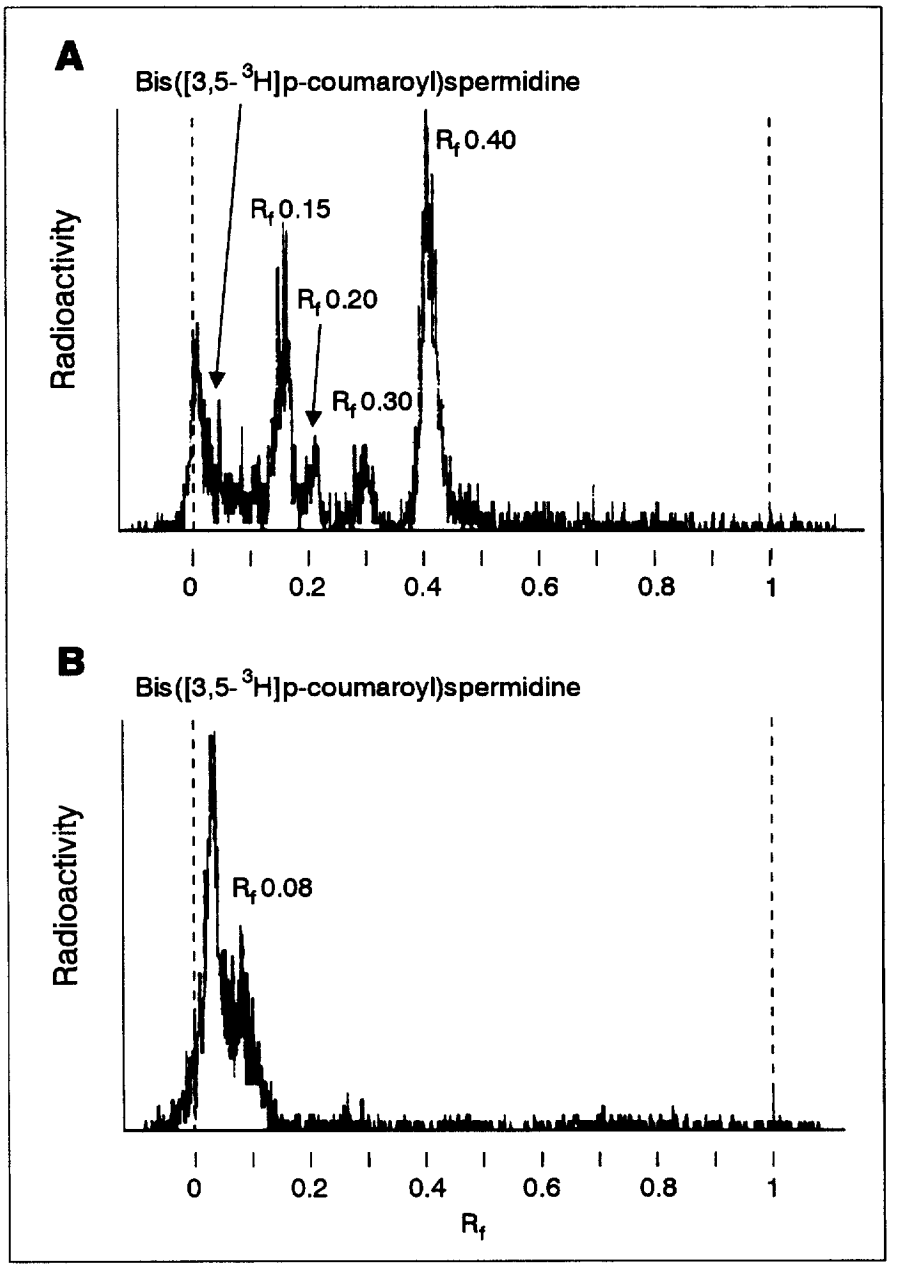


Fig. 7. Radioscan of the conversion of bis([3,5-^{3}H]*p*-coumaroyl)spermidine by a microsomal preparation of *Lunaria* seeds after TLC separation. (A) incubation at optimal conditions, (B) control without NADPH.

verified and extended. The application of potential precursors succeeded in the incorporations rates found here (see Table 1): L-[*U*-^{14}C]phenylalanine (14%), *trans*-[*U*-^{14}C]cinnamic acid (9%), [*U*-^{14}C]*p*-coumaric acid (4%), L-[*U*-^{14}C]tyrosine (0%), [1,4-^{14}C]spermidine (20%), [1,4-^{14}C]putrescine (19%), L-[2,3-^{3}H]arginine (3%) and L-[*U*-^{14}C]ornithine (2%). Tyrosine was the only applied substance which was not incorporated at all into lunarine.

These high incorporation rates were rendered possible by use of an optimized system applying *L. annua* seeds of a certain and defined stage of maturity which were, in addition, freed from their seed coat prior to precursor feeding. Therefore, the seed coat proved to be a considerable barrier for the uptake of potential precursors. Compared with earlier results [13] using fruit-forming twigs of *L. annua* for feeding experiments, the incorporation rates achieved with the optimized method described here could be improved almost 500-fold (Table 1).

The application of ^{13}C-labelled *p*-coumaric acid with subsequent NMR spectroscopy of the labelled lunarine clearly verified that the hexahydrodibenzofuran ring of lunarine is derived from two molecules of *p*-coumaric acid (Fig. 4, Fig. 5).

Our attempt, therefore, was to investigate the way

on which the lunarine component, *p*-coumaric acid, is synthesized in *Lunaria*. The incorporation rates determined in our studies (see Table 1) showed phenylalanine, cinnamic acid and *p*-coumaric acid to be excellent precursors for this alkaloid.

The polyamine part of the lunarine molecule, spermidine, its immediate precursor, putrescine, as well as the potential precursors ornithine and arginine were incorporated into lunarine too, demonstrating that the biosynthesis of spermidine takes place in the seeds of *L. annua* as well. Simultaneous feeding of [^{3}H]arginine and [^{14}C]ornithine showed that the incorporation rate of arginine was 1.5–2 times higher than that of ornithine. This result indicated that putrescine and therewith spermidine in *L. annua* seeds is preferentially synthesized from arginine. Since in the plant ornithine and arginine can be converted into each other via the urea cycle [28], one cannot totally exclude the participation of ornithine in the synthesis of spermidine as well.

Next we investigated whether two molecules of *p*-coumaric acid or bis(*p*-coumaroyl)spermidine are used for the phenol-oxidative coupling in lunarine biosynthesis. The participation of the naturally occurring bis(*p*-coumaroyl)spermidine [15, 16] was favoured, since it would perfectly explain the formation of two optical active molecules, lunarine and lunaridine, which differ only in the orientation of the spermidine chain [14]. Therefore, bis(*p*-coumaroyl) spermidine was chosen as substrate for further investigations.

The synthesis of doubly labelled bis(*p*-coumaroyl) spermidine was finally achieved at the submilligram level when the method of Acher and Wakselman [25] was applied using 2-hydroxy-5-nitro-α-toluene sulphonic acid sultone as coupling reagent [26].

Application of doubly labelled N^1,N^{10}-bis([3,5-^{3}H]*p*-coumaroyl)[1,4-^{14}C]spermidine confirmed the incorporation of this intermediate and the phenol coupling to lunarine. Moreover, the drop of the original ^{3}H:^{14}C ratio in the labelled bis(*p*-coumaroyl)spermidine of 10.8 to 2.8 in lunarine indicated the occurrence of a keto-enol tautomerism which could be verified by ^{1}H spectroscopy of lunarine in D_2O leading to a quantitative labelling of position 2 and 4 of lunarine (Fig. 6).

The biosynthesis of lunarine as it stands now is depicted in Fig. 8. L-Phenylalanine is deaminated to yield cinnamic acid which is converted to *p*-coumaric acid by hydroxylation. Spermidine which is formed preferentially from arginine via putrescine is acylated by two molecules of activated *p*-coumaric acid in position N^1 and N^{10} to form bis(*p*-coumaroyl)spermidine. This intermediate will then be phenol-coupled to form the hexahydrodibenzofuran ring of lunarine.

Although soluble enzymes like peroxidases are able to catalyse the coupling of phenols *in vitro*, these reactions, however, proceed non-stereospecifically thus yielding a racemic product. Nevertheless in some cases, enzymes like peroxidases, laccases, tyrosinases

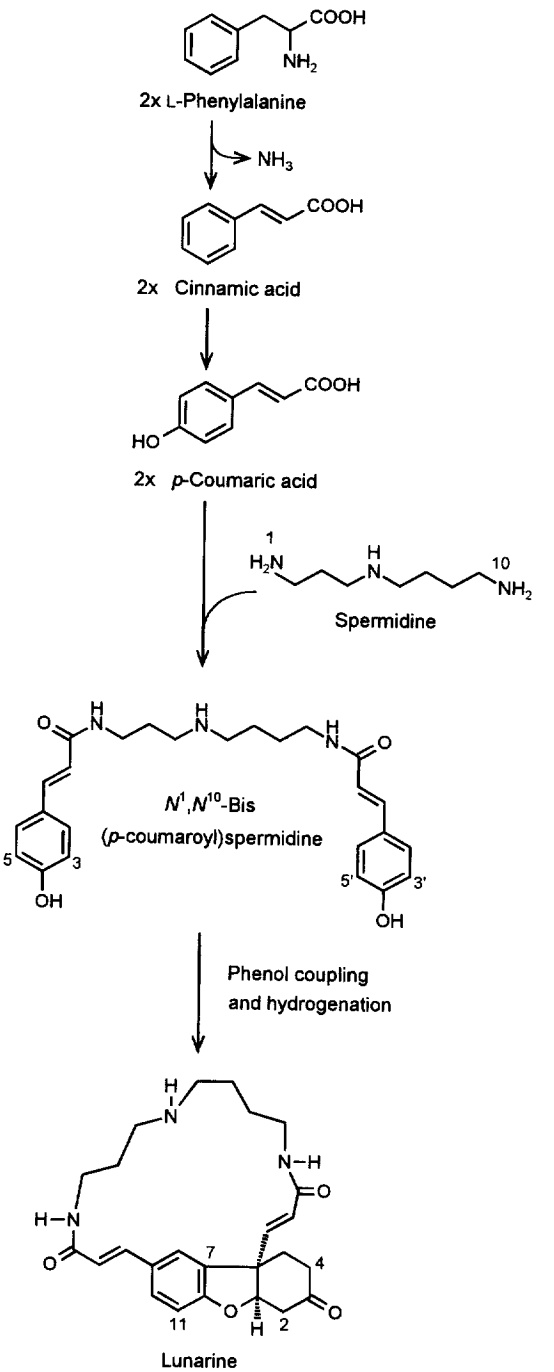


Fig. 8. Reaction sequence in *Lunaria annua* seeds leading from L-phenylalanine via cinnamic acid and *p*-coumaric acid to N^1,N^{10}-bis(*p*-coumaroyl)spermidine which is subsequently phenol-coupled to yield the hexahydrodibenzofuran ring of lunarine.

or ascorbate oxidases are *in vivo* able to catalyse a stereospecific phenol-coupling by specifically binding the respective substrates at their active site. For instance, a soluble copper-containing enzyme was isolated from *Aspergillus terreus*, that has a striking similarity to ascorbate oxidases and laccases and is able to catalyse the regio- and stereo-specific phenol-coup-

ling of dihydrogeodin to yield (+)-geodin [29, 30]. In addition, a peroxidase-like enzyme belonging to the lignan biosynthetic pathway could be isolated from stems of *Forsythia suspensa*, that was able to stereo specifically dimerize coniferyl alcohol into (+)-pinoresinol [31]. Recently, a cytochrome P-450 enzyme from yeast was described, that catalyses the coupling of *N*-formyl-tyrosine [32]. Since microsomal-bound cytochrome P-450 enzymes are already known to participate in highly regio- and stereo-selective phenol-coupling reactions in benzylisoquinoline alkaloid biosynthesis [4, 6] it was expected that an enzyme of this type might be responsible for the phenol-coupling of bis(*p*-coumaroyl)spermidine in *Lunaria*.

The incubation of N^1,N^{10}-bis([3,5-^{3}H]*p*-coumaroyl)spermidine with a microsomal preparation of *Lunaria* seeds, however, failed to form lunarine. The substrate was strictly NADPH-dependently converted into four products none identical with lunarine. Although it was not possible to identify these substances because of their low concentrations we assume that they are already phenol-coupled intermediates of lunarine biosynthesis whose further conversion might be blocked because soluble enzymes and/or cofactors necessary for further conversion are missing from the microsomal preparation.

A complete elucidation of the pathway leading to lunarine type alkaloids in the genus *Lunaria* will only be possible at the enzyme level.

EXPERIMENTAL

General. All NMR spectra were obtained on a Bruker AM 360 spectrometer. CD_3OD was used as an int. standard. ^{1}H NMR and ^{13}C NMR spectra at 360.166 and 90.56 MHz, respectively. Mass spectra were recorded with a Finnigan-MAT SSQ 700 in the CI mode (iso-butane). FAB spectra were obtained on a Kratos MS 80 RFA instrument. The CD spectrum was measured in MeOH with a CD-6 (Jobin-Yvon) instrument. Optical rotations were recorded with a Perkin Elmer polarimeter 241. Distribution of radioactivity on TLC plates was monitored with a Berthold linear analyser Tracemaster 20.

Plant material. Seeds of *Lunaria annua* L. were germinated under greenhouse conditions, the plants were further cultivated in the greenhouse or outdoors, either, and 10–12-month-old plants were used for the experiments.

Plant cell cultures were provided by the cell culture laboratory of his department.

Chemicals. All solvents and reagents were of the highest purity commercially available. The reference alkaloid lunarine was a kind gift of Drs C. Poupat and G. Kunesch (Gif-sur-Yvette) as well as Prof. S. Huneck (Halle). Radioactively labelled substances were provided by Amersham Buchler and NEN DuPont. *trans*-[*U*-^{14}C]Cinnamic acid was synthesized from L-[*U*-^{14}C]phenylalanine (472 mCi mmol^{-1}) using phenylalanine ammonia lyase (Sigma). The product formed was purified by TLC (Polygram Sil G/UV254, Macherey and Nagel, toluene–HOAc, 8:2, R_f 0.65). The isolated *trans*-[*U*-^{14}C]cinnamic acid had a sp. act. of 472 mCi mmol^{-1}. Analogously, [*U*-^{14}C]*p*-coumaric acid was obtained from L-[*U*-^{14}C]tyrosine (497 mCi mmol^{-1}), and [3,5-^{3}H]*p*-coumaric acid from L-[3,5-^{3}H]tyrosine (48 mCi μmol^{-1}) purified in solvent system $CHCl_3$–MeOH–diethylamine (13:5:1, R_f 0.55) with a sp. act. of 497 mCi mmol^{-1} and 48 mCi μmol^{-1}, respectively. ^{13}C-Labelled *p*-coumaric acid was obtained from the respectively labelled tyrosine as described above. Specific activities: [1-^{13}C]*p*-coumaric acid, 72.9 μCi mmol^{-1}; [3,5-^{13}C]*p*-coumaric acid, 183.2 μCi mmol^{-1}.

Synthesis of N^1,N^{10}-*bis*(p-*coumaroyl*)*spermidine.* The synthesis of maytenine as described by [25] was adopted for this purpose. The coupling agent 2-hydroxy-5-nitro-α-toluene sulphonic acid sultone [26] was synthesized as follows: 5 g (21.55 mmol) 2-hydroxy-5-nitrobenzyl bromide (Aldrich) and 3 g (15.78 mmol) $Na_2S_2O_5$ were dissolved in 125 ml doubly-H_2O and left at 115° for 8 hr under reflux. The cooled soln was evapd to dryness, the residue dried for 2 hr at 60°, then extracted × 3 with a total of 200 ml EtOH. The extracts were combined, evapd to dryness and dried at 60° overnight yielding 4.9 g (19.27 mmol) Na 2-hydroxy-5-nitro-α-toluene sulphonate. This product was mixed with 25 ml (265 mmol) phosphorous oxychloride, gently heated to 125° and kept at this temp. for 90 min. Thereafter, the solvent was evapd and the residue combined with 100 ml ice H_2O and stirred for 30 min. The ppt. was sepd by suction filtration, washed with ice-cold doubly-H_2O until neutralised and dried over P_2O_5 affording 0.9 g (4.17 mmol) 2-hydroxy-5-nitro-α-toluene sulphonic acid sultone. ^{1}H NMR (CD_3OD): δ (ppm) = 3.17 (*s*, 2H, H-7), 7.23 (*d*, J = 8.9 Hz, 1H, H-3), 8.23 (*d*, J = 18.8 Hz, 1H, H-6), 8.23 (*dd*, $J_{H\text{-}5/H\text{-}6}$ = 12.1 Hz, $J_{H\text{-}5/H\text{-}3}$ = 8.8 Hz, 1H, H-5). ^{13}C NMR (CD_3OD): δ (ppm) = 50.9 (C-7), 113.8 (C-3), 123.3 (C-2), 123.4 (C-5), 127.3 (C-6), 145.8 (C-4), 156.2 (C-1).

Prior to coupling with spermidine, the 4-OH group of *p*-coumaric acid had to be protected. This was achieved with the modified method of [27]: 345 mg (2.1 mmol) *p*-coumaric acid and 760 mg (5.04 mmol) *tert*-butyldimethylsilyl chloride (TBDMS, Fluka) were dissolved in 5 ml purified DMF, 715 mg (10.5 mmol) imidazole were added and the mixt. stirred for 90 min at room temp. Thereafter, the reaction mixt. was poured into 70 ml doubly-H_2O and extracted × 3 with a total of 150 ml CH_2Cl_2. The extracts were combined, washed with doubly-H_2O, dried over Na_2SO_4 and the solvent evapd yielding 0.556 (2 mmol) TBDMS-*p*-coumaric acid. CI MS: *m/z* (rel. int.): 279 ([M+H]$^+$, 100).

Freshly prepd TBDMS-*p*-coumaric acid (7 mg, 24.36 μmol) and 3.5 μl triethylamine were mixed with 25 μl purified THF and then 2-hydroxy-5-nitro-α-toluene sulphonic acid sultone (8 mg, 37.2 μmol) dissolved in 25 μl THF added. The soln was thoroughly mixed

and left to be shaken overnight in the dark. In the morning 5 μl triethylamine, then 1.8 mg (12.18 μmol) spermidine dissolved in 20 μl THF were added and the mixt. shaken for 4 hr at room temp. The production of N^1,N^{10}-bis(TBDMS-*p*-coumaroyl)spermidine was monitored by TLC (EtOAc–EtOH–NH_3, 10:10:1, R_f 0.55). Product yield: 3.7 mg (5.5 μmol). CI MS: *m/z* (rel. int.) 667 ($[M+H]^+$, 100), 279 (63), 336 (33). ^{1}H NMR (CD_3OD): δ (ppm) = 0.68–0.89 (*s*, 18H, 6 × CH_3), 1.14 (*s*, 12H, Si-CH_3), 1.50 (*m*, 6H, H-14/15), 1.68 (*m*, 2H, H-11), 2.61 (*m*, 4H, H-12/13), 3.16–3.25 (*m*, 2H, H-10/16), 6.34 (2*d*, *J* = 15.8 Hz, 2H, H-8/18), 6.71 (2*d*, *J* = 8.5 Hz, 4H, H-3/5/22/24), 7.32 (2*d*, *J* = 8.5 Hz, 4H, H-2/6/21/25), 7.31–7.36 (*m*, 2H, H-7/19). ^{13}C NMR (CD_3OD): δ (ppm) = 1.5, 19.1, 26.1, 26.8/28.0/29.5 (C-11/14/15), 30.5, 30.8, 33.1, 38.0/40.1 (C-12/13), 47.4 (C-10/16), 119.5/119.8 (C-8/18), 120.8/121.6 (C-3/5/22/24), 129.7 (C-1/20), 130.4/130.5/132.1 (C-2/6/21/25) 141.3/141.6 (C-7/19), 158.6/158.7 (C-4/23), 169.0/169.3 (C-9/17).

The deprotection of N^1,N^{10}-bis(TBDMS-*p*-coumaroyl)spermidine was now achieved with the modified method of [27] as follows: tetrabutylammoniafluoride (31 mg, 97.44 μmol) dissolved in 70 μl THF were added to the reaction mixt. described above and shaken for 1 hr at room temp. N^1,N^{10}-Bis(*p*-coumaroyl)spermidine was then purified by TLC in two subsequent steps (solvent system 1: $CHCl_3$–MeOH–diethylamine, 13:5:1, R_f 0.30; solvent system 2: EtOAc–EtOH–NH_3, 10:10:1, R_f 0.23) with a yield of 2 mg (4.66 μmol). FAB MS: *m/z* (rel. int.): 438 ($[M+H]^+$, 63), 147 (100). ^{1}H NMR (CD_3OD): δ (ppm) = 1.41–1.52 (*m*, 4H, H-14/15), 1.61–1.75 (*m*, 2H, H-11), 2.57–2.78 (*m*, 4H, H-12/13), 3.04–3.22 (*m*, 4H, H-10/16), 6.24 (*d*, *J* = 16.0 Hz, 2H, H-8/18), 6.56/6.60 (2*d*, *J* = 8.4 Hz, 4H, H-3/5/22/24), 7.21/7.23 (2*d*, *J* = 8.4 Hz, 4H, H-2/6/21/25), 7.24–7.30 (*m*, 2H, H-7/19). ^{13}C NMR (CD_3OD mixt. of several conformers: C-atoms yielding more than one signal): δ (ppm) = 25.3/25.6/27.4/27.7/28.2/28.5 (C-11/14/15), 37.3/37.4/39.6/39.7 (C-12/13), 46.7/46.9 (C-10/16), 116.0/116.8 (C-3/5/22/24), 117.8/118.3/121.2/121.4 (C-8/18), 127.4/127.5/127.9 (C-1/20), 130.6/130.7/132.3/132.4 (C-2/6/21/25), 138.3/138.8/141.9/142.3 (C-7/19), 159.4/159.5/160.8/160.9 (C-4/23), 169.4/169.9/170.5/170.0 (C-9/17).

Synthesis of radioactively labelled N^1,N^{10}-*bis*(p-*coumaroyl)spermidine.* Tritium-labelled *p*-coumaric acid was obtained by enzymic synthesis from [3,5-^{3}H]tyrosine and purified by crystallization. Then, 2.74 μmol (102.6 μCi) [3,5-^{3}H]*p*-coumaric acid (37.4 μCi μmol^{-1}) was combined with 4.4 mg *tert*-butyldimethylsilyl chloride (29.2 μmol) and dissolved in 5 μl DMF. Imidazole (4 mg, 58.8 μmol) was added and the mixt. shaken for 1 hr at room temp. in the dark. Thereafter, the reaction mixt. was supplemented with CH_2Cl_2 up to 0.5 ml, washed × 3 with 0.5 ml doubly-H_2O, the organic phase evapd with N_2 and the residue taken up in 1 ml MeOH. The organic phase was evapd again with N_2 and the remaining TBDMS-[3,5-^{3}H]*p*-coumaric acid dried over P_2O_5 affording 0.73 mg (97.47 μCi) of product at a sp. act. of 37.4 μCi μmol^{-1}.

The TBDMS-protected tritium-labelled *p*-coumaric acid was then used for coupling with spermidine as described above. After deprotection and purification by TLC, [3,5-^{3}H]*p*-coumaric acid was obtained at a yield of 0.2 mg (33.3 μCi) and a sp. act. of 75 μCi μmol^{-1}.

Doubly-labelled bis(*p*-coumaroyl)spermidine was analogously synthesized from TBDMS-[3,5-^{3}H]*p*-coumaric acid (88 μCi, 1.9 μmol, 46.7 μCi μmol^{-1}) and [1,4-^{14}C]spermidine (8.8 μCi, 0.08 μmol, 113 μCi μmol^{-1}) at a yield of 0.09 mg N^1,N^{10}-bis([3,5-^{3}H]*p*-coumaroyl)[1,4-^{14}C]spermidine (0.2 μmol, ^{3}H:^{14}C: 20.24/1.87 μCi = 10.8:1).

Application experiments. Precursor substances for feeding experiments (0.5–1.5 μCi) were dissolved in 0.5 ml H_2O and were then incubated together with the respective plant material in a well of a sealed multiwell plate (24 × 1 ml wells, Nunc) for 24 hr at 22° in continuous light (650 lux) under shaking at 100 rpm. Optimal incorporation rates were achieved with *L. annua* seeds of stage IV with their seed coat removed.

For a subsequent analysis, the plant material was removed from the incubation mixt., washed with H_2O, cut into small pieces, and extracted in 80% EtOH for 30 min under reflux. The extract was then evapd to dryness and the residue taken up in a defined vol. of MeOH. The extracts (50 μl, *ca* 20000 cpm) were subjected to TLC on Polygram Sil G/UV_{254} (Macherey and Nagel) with $CHCl_3$–EtOH–NH_3 (9:1:1) as solvent system. The products sepd were localized on the plates under UV light and by their respective radioactivity.

After feeding of ^{13}C-labelled substrates, the seed extracts had to be purified by solid phase extraction on a Chromabond SA column (Macherey and Nagel, 500 mg 3 ml^{-1}) prior to alkaloid determination. For this purpose, 0.9 ml extract were acidified with 1% HOAc and applied to the column. The column was then washed with 3 vols of MeOH and the bound alkaloids subsequently eluted with 5 vols of 5% NH_3 dissolved in MeOH. The alkanoids were separated by TLC (see above) and eluded with MeOH.

Microsome preparation. Seeds of *L. annua* (*ca* 55 g fr. wt) were shock frozen with liquid N_2, ground in a chilled mortar with a pestle, with 5.5 g PVP and 0.1 M Tricine buffer (pH 7.5, 2 ml g^{-1} seed material). The mixt. was stirred for 30 min until completely thawed, filtered through four layers of cheesecloth, and centrifuged for 1 hr at 105000 × *g*. The resulting pellet was resuspended in 2 ml 0.1 M Tricine buffer (pH 7.5) and homogenized using a Potter-Elvehjem system. Aliquots of the resulting microsomes were shock-frozen in liquid N_2 and stored at −80° until use.

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