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Bis(p-Coumaroyl)spermidine, the Penultimate Precursor of the Alkaloid Lunarine

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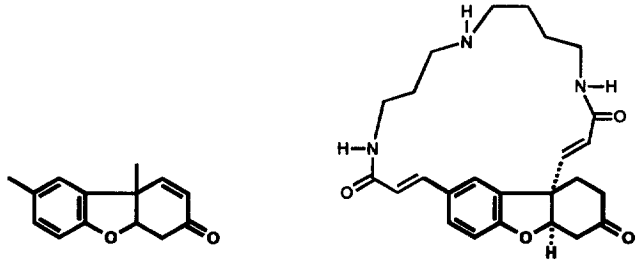
Abstract: The alkaloid lunarine occurring in *Lunaria annua* seeds was shown to be synthesized by stereoselective phenoloxidative coupling from synthetic bis(p-coumaroyl)spermidine. © 1997 Elsevier Science Ltd.

An overwhelming number of phenol-coupled natural products are found in higher plants and the concept of a radical mechanism leading to their formation has been put forward early on.[1] Recently, this concept has been confirmed by the demonstration that highly specific cytochrome P-450 enzymes are the biocatalysts responsible for this phenol-coupling reaction, which proceeds by radical pairing leading to new C-C or C-O bonds either intra- or intermolecularily, without the introduction of oxygen into the final product.[2-5] The concept of Barton and Cohen[1] was based on the observation of Pummerer *et al.*[6] 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 (Fig. 1).[1] This Pummerer ketone has a striking similarity to the alkaloid lunarine from *Lunaria annua*. The structure and absolute configuration of this first alkaloid that was shown to contain spermidine[7] was clarified by X-ray analysis (Fig. 1).[8,9] Initial biosynthetic experiments towards this phenol-coupled spermidine alkaloid were conducted by feeding potential precursors such as L-[7-^{14}C]phenylalanine to *L. annua* plants, yielding a non-random incorporation of up to 0.03%.[10]



Pummerer Ketone (revised)

Lunarine

Fig. 1 Similarities between synthetic Pummerer ketone and the alkaloid lunarine from *Lunaria annua* seeds.

This experiment led to the assumption that phenylalanine is transformed via cinnamic acid into p-coumaric acid, the immediate precursor of the target alkaloid. The question remaining was: Is p-coumaric acid first phenol-

coupled in a mechanism similar to the structural elements of lignin, and then added to spermidine or is spermidine first acylated by two molecules of p-coumaric acid at atom N^1 and N^{10} and this dimer subsequently coupled to yield the hexahydrodibenzofuran system in lunarine? The latter possibility seemed more likely since bis(p-coumaroyl)spermidine has been favored in biomimetic approaches towards lunarine[11] and this amide has been shown to be a natural product in higher plants.[12,13]

Physiological optimization of the application system was first attempted. *L. annua* plants produce lunarine only in their seeds, all other organs of this plant are devoid of this alkaloid. Cell cultures developed from germinating seeds did not synthesize lunarine. Therefore, developing seeds of different stages of maturity isolated from their seed pot were exposed to an aqueous solution of potential precursors and their incorporation into lunarine checked. A seed stage could be found (seed 6 mm in length; 63% water content, 0.75% lunarine on dwt. basis) where [1,4-^{14}C]spermidine was incorporated up to 20%, L-[U-^{14}C]phenylalanine to 14%, and [ring-^{14}C]p-coumaric acid to 4%. Since highly radioactive, double labeled bis(p-coumaroyl)spermidine was needed, the synthesis had to be conducted on less than 1 mg. The method finally adopted used 2-hydroxy-5-nitro-α-toluolsulfonic acid sultone as coupling reagent.[14] The 4-OH-group of p-coumaric acid was protected prior to coupling as the *tert*-butyl demethylsilyl (TBDMS) derivative.[15]

The [^{14}C,^{3}H] labeled N^1,N^{10}-bis([3,5-^{3}H]p-coumaroyl)[1,4-^{14}C]spermidine synthesis was achieved as follows: 1.9 µmol TBDMS-[3,5-^{3}H]p-coumaric acid (88 µCi), 2.8 µmol 2-hydroxy-5-nitro-α-toluolsulfonic acid sultone, 2.5 µmol triethylamine, and 10 µl tetrahydrofuran (THF) were added in a micro vial and incubated at 20°C overnight, after which [1,4-^{14}C]spermidine (8.8 µCi) were dissolved in 5 µl THF and added (theoretical ratio ^{3}H : ^{14}C = 10). After 4 hr of incubation, 7.6 µmol tetrabutylammonium fluoride dissolved in 10 µl THF were added for deprotection and the mixture shaken for 1 hr at 20°C. The crude mixture was resolved by TLC (solvent: $CHCl_3$: MeOH : $HN(C_2H_5)$ = 65:25:5). The band with Rf 0.30 was eluted and re-chromatographed (ethyl acetate : ETOH : NH_3 = 10 : 10 : 1) and the radioactive band (Rf 0.23) was eluted with MeOH. Radiochemical yield 23%; ^{3}H : ^{14}C = 10.8.

The synthesis was conducted several times with unlabeled material, and the following spectroscopic data were obtained: 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 (2d, J = 8.4 Hz, 4H, H-3/5/22/24), 7.21/7.23 (2d, J = 8.4 Hz, 4H, H-2/6/21/25), 7.24-7.30 (m, 2H, H-7/19). The spectral data are identical with the natural product.[13]

The double-labeled potential intermediate was supplied in aqueous solution (each 3 seeds in 0.5 ml solvent containing 12.8 nmol of the amide and incubated for 24 hrs at 20°C). The seeds, which had taken up 94% of the radioactivity, were extracted with ETOH. The rate of incorporation of the double-labeled amide into lunarine was 6%. The ^{3}H : ^{14}C ratio in that alkaloid dropped from an original 10.8 to 2.8. The removal of tritium from C-7 of lunarine through the phenol-coupling reaction in addition to the complete removal of the tritium atoms at C-2 and C-4 of the hexahydrodibenzofuran ring by keto-enol tautomerism led to a loss of 3/4 of the tritium atoms supplied by positions 3, 5 and 3', 5' of the phenolic rings of N^1, N^{10}-bis ([3,5-^{3}H]p-coumaroyl)[1,4-^{14}C]spermidine. This keto-enol tautomerism of lunarine has been observed already by Poupat and Kunesch[10] and could here be verified again by ^{1}H NMR spectroscopy of lunarine in D_2O that led to a quantitative labeling of position 2 and 4 of lunarine by deuterium. Considering this tautomerism and the removal of tritium at position 7 due to the phenoloxidative coupling reaction, a theoretical drop of the ratio of ^{3}H : ^{14}C in

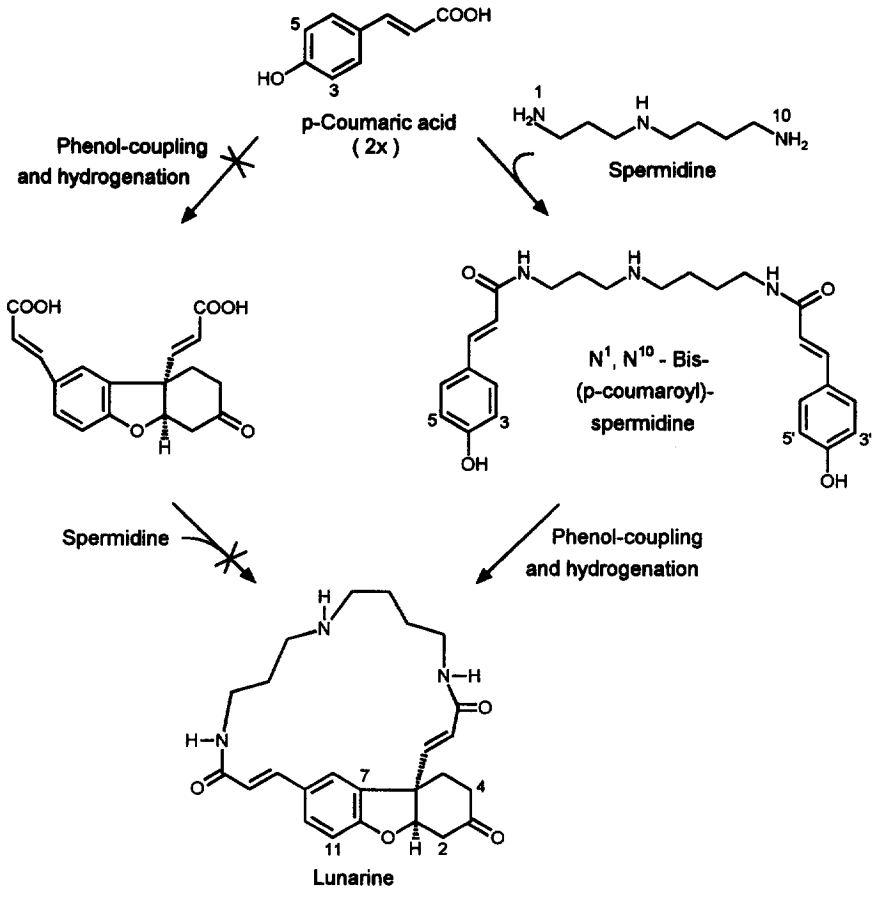


Fig. 2 Biosynthesis of lunarine occurs by phenoloxidative coupling of the intermediate bis(p-coumaroyl)spermidine and not through a p-coumaroyl dimer.

the precursor from 10.8 to 2.7 in lunarine is expected, which is in good agreement with the observed data of 2.8. [ring-3,5-^{13}C]p-Coumaric acid proved to label positions 2, 4, 7 and 11 of lunarine (rate of incorporation 5%). p-Coumaric acid is certainly first activated by forming the known p-coumaroyl-CoA thioester, which will subsequently acylate spermidine to form bis(p-coumaroyl)spermidine in a manner that is reminiscent of other higher plant amides.[16,17] The bis-amide formed is presumed to undergo oxidative coupling. Since this coupling reaction is highly stereoselective, we assume that as in other phenol coupling reactions[2-5] a cytochrome P-450 enzyme might be involved. The biosynthesis of lunarine as it now stands , is depicted in Fig. 2.

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