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New reagent for oxidative phenol coupling. The transformation of the monocyclic spermine base (*S*)-dihydroxyverbacine to the bicyclic alkaloid (*S*,*S*,*S*)-aphelandrine by cell free extract of barley seedlings

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Abstract—The soluble protein fraction of barley seedlings (*Hordeum vulgare*, Gramineae) in the presence of O_2 stereoselectively catalyzes the intramolecular phenol coupling of the monocyclic spermine base (*S*)-dihydroxyverbacine to the bicyclic aphelandrine in preparative yield. The expected microsomal-bond cytochrome P-450 enzyme system does not contribute to this reaction. The nature of the involved biocatalyst still remains uncertain. The catalytic potential of the cell free extract of barley seedlings suggests its possible use as an efficient tool for large scale stereoselective chemoenzymatic synthesis of aphelandrine type alkaloids.

The oxidative phenol coupling contributes to the biogenesis of a number of natural compounds namely alkaloids (benzylisoquinolines, Amarylidaceae alkaloids, colchicine etc.), flavonoids, lignins, antibiotics (vancomycine) etc. This reaction involves oxidative generation of phenolate radicals and their further intra- or intermolecular recombination to form new C–O and/or C–C bonds.[1] Cytochrome P-450 enzymes often participate in these coupling reactions as highly stereo- and regioselective biocatalysts[2–4] but other oxidases, peroxidases or laccases are also catalysts in certain cases.[5–7]

The polyamine alkaloid (+)-hordatine A (**4**), isolated from barley (*Hordeum vulgare L.*),[8] is a product of the phenol oxidation reaction. Obviously, (+)-hordatine A (**4**) is formed by intermolecular coupling of two coumaroylagmatine (**3**) molecules (Scheme 1). Until now, the enzyme involved in this reaction has not been well established. Hordatine A (**4**) has been enzymatically prepared from coumaroylagmatine (**3**) using cell free extracts from the shoots of barley seedlings.[9] The same reaction catalyzed by a horseradish peroxidase leads to the racemate.[8] This is indirect evidence that the specific peroxidases might be involved in this phenol coupling.[8,9]

The macrocyclic spermine alkaloid aphelandrine (**1**) is present in *Aphelandra* plants (Acanthaceae).[10–12] Recent studies confirmed that the last step in the aphelandrine (**1**) biosynthesis is the regioselective intramolecular phenol oxidative coupling of its terminal precursor (*S*)-dihydroxyverbacine (**2**) (Scheme 1).[13] It was demonstrated that in *Aphelandra* plants this reaction is catalyzed by a membrane-bound cytochrome P-450 enzyme, requiring NADPH and O_2.[14]

The similarities in the benzofuran moieties in both (+)-hordatine A (**4**) and aphelandrine (**1**) motivated us to introduce, instead of coumaroylagmatine (**3**), (*S*)-dihydroxyverbacine (**2**) as a substrate for phenol coupling in the presence of the cell free extract of barley seedlings.

(−)-(*S*)-Dihydroxyverbacine (**2**), synthesized recently,[15] was used as a substrate for the enzymatic assays and three different enzyme fractions from barley seedlings were tested as biocatalysts. The enzyme fractions were prepared as follows: 8-day-old seedlings were washed (222 g fr. wt.) and frozen for 1 h at −20°C. Roots were taken away and the upper parts were cut into small pieces and homogenized in 300 ml of 0.1 M K/Na phosphate buffer (pH 7.4), containing 0.6 M mannitol, 10 mM mercaptoethanol, 5 mM EDTA, 1 mM PMSF and 4 g Amberlite XAD-4. The homogenate was filtrated through four layers of cheesecloth, and cen-

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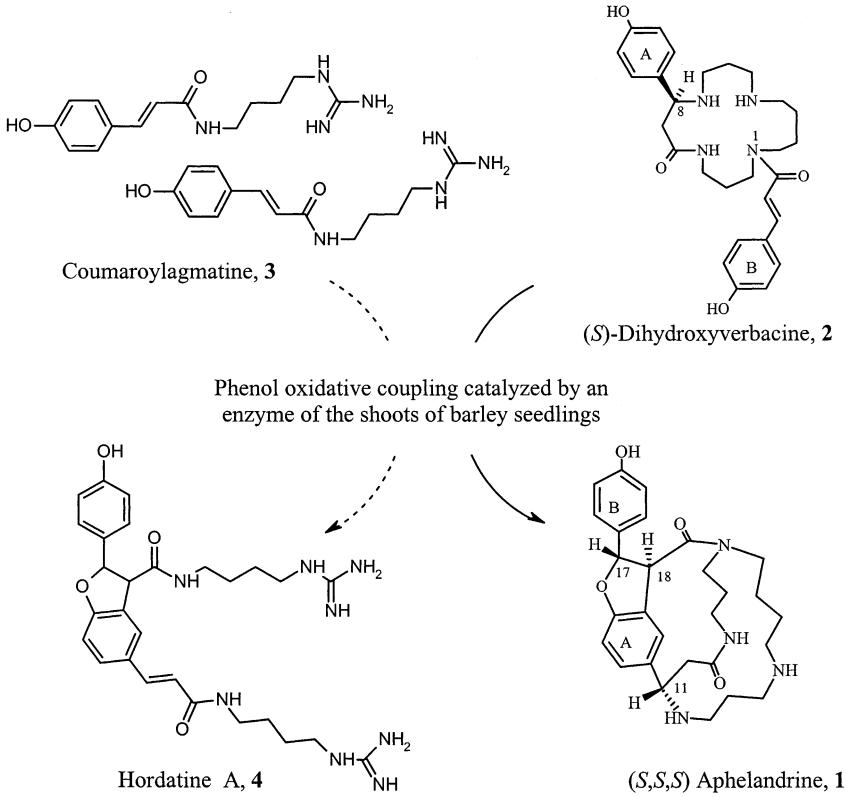

Scheme 1.

trifuged for 20 min at 20 000*g*. The pellet was separated from the supernatant, washed several times with buffer and used immediately or shock frozen in liquid N_2 and stored at −80°C until use (fraction 1). The supernatant was centrifuged for 90 min at 200 000g to obtain the microsomal pellet (fraction 2). After removing the microsomes, the supernatant was concentrated by ultrafiltration using 10K ultrafiltration membranes (soluble protein fraction 3).

All three fractions were used for enzyme assays. The enzyme assays for attempted phenol coupling reaction contained 0.99 mM **2**, 0.1 M K/Na phosphate buffer (pH 7.4) and 400 μl protein suspension in a total volume of 500 μl. In the case of microsomes (fraction 2) 5 mM NADPH was added into the incubation mixture. The mixtures were incubated for 1 h at 25°C with gentle shaking in the presence of air. The reactions were stopped by adding 100 μl AcOH in 20 ml MeOH and centrifuged for 10 min at 3000*g*. The supernatants were evaporated to dryness (30°C), the residues dissolved in 0.1 N aq. HCl and extracted ×5 with EtOAc. The water layers were adjusted to pH 9 (K_2CO_3) and extracted ×5 with $CHCl_3$. The combined organic extracts were dried (Na_2SO_4), evaporated and analyzed by on-line coupled HPLC–UV(DAD)–ESI-MS and by HPLC–UV(DAD)–ESI-MS/MS (Waters Symmetry® C_8 column 150×2 mm at 20°C; flow rate 0.2 ml min^{-1}; DAD detector setting at 280 and 309 nm; mobile phase, A: 1% AcOH in H_2O, solvent B: 1% AcOH in CH_3CN; linear gradient 97:3 (A:B) to 0:100 within 70 min; R_t (17*S*)-aphelandrine (**1**) = 11.3 min; R_t (17*R*)-orantine = 2.3 min). The ESI-MS detector (ion trap instrument) was interfaced directly to the output of the UV detector.

Based on the results obtained with the enzymes from *Aphelandra squarrosa* Nees roots,[14] the formation of aphelandrine (**1**) was expected in the microsomes (fraction 2). The analysis of the enzymatic assay with the microsomal fraction from the barley seedlings containing cytochrome P-450 did not show any formation of aphelandrine (**1**). However, in the HPLC-ESI-MS of the assay containing the soluble protein fraction 3 a $[M+H]^+$ quasi molecular peak at m/z 493 was detected showing the same retention time as the natural aphelandrine (**1**) (m/z 493). Additionally, the MS/MS fragmentation pattern was identical with that of the natural aphelandrine (**1**), used as a standard. This reaction was strictly dependent on the presence of the protein. Heat denatured enzyme did not yield any product formation. A barley variety, devoid of endogenous hordatine A (**4**) were also unable to produce (11*S*,17*S*,18*S*)-aphelandrine (**1**).

Thus, it was shown that, although being an unnatural substrate, (*S*)-dihydroxyverbacine (**2**) was transformed stereospecifically by the soluble protein fraction 3 into (11*S*,17*S*,18*S*)-aphelandrine (**1**) in a surprisingly high yield of 25% (determined by HPLC using aphelandrine (**1**) as external standard). No other products where detected in the enzymatic assay. The unchanged starting (*S*)-dihydroxyverbacine is recoverable from the reaction mixture.

Presumably both (*S*)-dihydroxyverbacine (**2**) and coumaroylagmatine (**3**) are substrates of one and the same enzyme, present in the soluble fraction of the barley homogenate. Thus, the transformation of (*S*)-dihydroxyverbacine (**2**) to (11*S*,17*S*,18*S*)-aphelandrine (**1**) is the

indirect evidence for the (*S*,*S*)-configuration of hordatine A (**4**). Unfortunately the absolute configuration of hordatine A (**4**) is so far unknown.

Besides aphelandrine (**1**, $R_t = 11.3$ min) the formation of its (11*S*,17*R*,18*R*)-diastereoisomer, namely orantine (ephedradine A, $R_t = 2.3$ min) was not observed by HPLC during this reaction. Orantine is easily available by a base-catalyzed isomerization of aphelandrine (**1**).[16]

The group of hordatines A, B and M are a subject of interest because of their antifungal activity.[8,9,17] The ephedradines A (orantine), B, C and D show interesting pharmacological properties as perspiratory, antitussive and anti-allergic agents.[18] A number of synthetically prepared analogues, containing differently substituted benzofuran moieties are under investigation because of their cytostatic activity.[19] It is known that the biomimetic chemical phenol oxidation reactions usually leads to low yields of the desired product and complex mixtures of by-products. A variety of chemical and enzymatic (peroxidases or laccases) oxidizing systems have been used for the oxidative coupling of a number of phenolic compounds. However, often these reagents have been deprived of regio- and/or stereoselectivity.[8,20] The present reaction opens the possibility for the chemoenzymatic preparation of aphelandrine (**1**)-type alkaloids. Barley seeds offer big advantages compared with *Aphelandra* plants as they are easily available, the material can be used 8 days after planting and the reaction does not require co-factors as NADPH. The whole cell-free extract from barley can be used for this transformation without preliminary separation. Further investigations in this direction are in progress.

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