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Dihydroxyverbacine is the terminal precursor in the biosynthesis of aphelandrine and orantine

Lenka Nezbedová, Manfred Hesse,* Konstantin Drandarov and Christa Werner

Organisch-chemisches Institut der Universität Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland

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Abstract

A microsomal-bond cytochrome P-450 enzyme system has been discovered in the roots of *Aphelandra squarrosa* that is responsible for the stereospecific intramolecular phenol coupling of (*S*)-dihydroxyverbacine to aphelandrine. The biogenesis of the closely related alkaloids orantine (ephedradine A) and ephedradines B, C and D should follow a similar biogenetic pattern. © 2000 Elsevier Science Ltd. All rights reserved.

A number of phenol coupled natural products are known in higher plants. Their formation follows a radical mechanism postulated earlier by Barton and Cohen.[1] It has been shown that highly specific cytochrome P-450 enzymes are in several cases the biocatalysts responsible for this phenol coupling.[2–4] The bicyclic spermine alkaloids aphelandrine (**1**) and its diastereoisomer orantine (**2**), found in *Aphelandra* plants (Acanthaceae),[5–7] are members of a group of similarly constituted compounds containing 13- and 17-membered rings derived from spermine and two coumaric acid units.[8,9] Obviously, their biosynthesis is accomplished by an oxidative phenol coupling. The initial biosynthetic experiments, conducted by feeding potential precursors such as ^{3}H and ^{14}C labeled putrescine, spermidine and coumaric acid to intact *Aphelandra* plants, confirmed the biogenetic structural units of aphelandrine (**1**), but whether mono- (**6b**) or dicoumaroylspermine are its precursors remained uncertain.[10]

Recently, using HPLC-MS and HPLC-MS/MS techniques, the 17-membered phenolic macrolactam **4**, named prelandrine, was detected in the roots of *A. squarrosa*. Prelandrine (**4**) is presumably biosynthesized from a *N*(1)-cinnamoylspermine (**6a**) or *N*(1)-*p*-coumaroylspermine (**6b**) by intramolecular Michael addition. If *N*(1)-cinnamoylspermine (**6a**) is cyclized by Michael addition a hydroxylation step from **5** to **4** would be expected.[11] An additional acylation of prelandrine (**4**) at the *N*(1) atom with *p*-coumaroyl-CoA thioester is necessary to obtain the expected terminal precursor (*S*)-dihydroxyverbacine (**3**). Then the last, crucial step in the

* Corresponding author. Fax: +1 635 68 12; e-mail: cwerner@oci.unizh.ch

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biosynthesis of aphelandrine (**1**) is the phenol oxidative coupling of (*S*)-dihydroxyverbacine (**3**) (Fig. 1), which is presented in this paper.

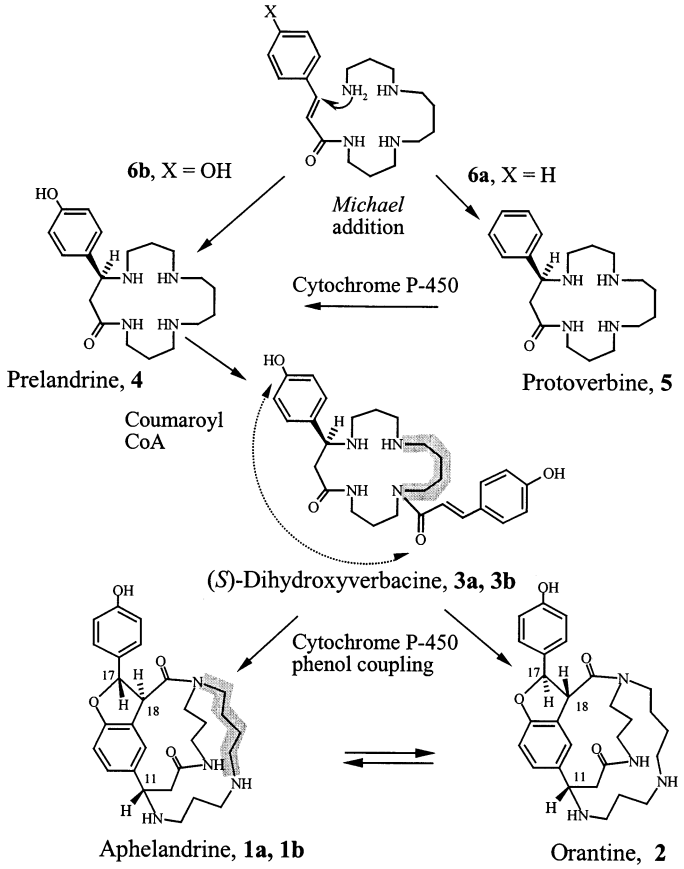

Figure 1. The biosynthetic formation of aphelandrine from prelandrine (**4**) via phenol oxidative coupling of (*S*)-dihydroxyverbacine. ═ C_4H_8 in the native aphelandrine (**1a**) and in (*S*)-dihydroxyverbacine (**3a**), C_4D_8 in (*S*)-D_8-dihydroxyverbacine (**3b**) and in enzymatically formed D_8-aphelandrine (**1b**)

To avoid possible interference with the endogenous aphelandrine (**1a**) present in the plant material, the labeled **3b** was synthetically prepared.[12] Phenol oxidative coupling of the intermediate **3b** was studied in the microsomal fraction from the 12-week-old roots of *A. squarrosa*. In this order, washed roots (125 g fr. wt) were frozen for 1 h at –20°C then cut into small pieces and homogenized in 400 ml of 0.1 M K/Na phosphate buffer (pH 7.4), containing 0.6 M mannitol, 10 mM mercaptoethanol, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The homogenate was filtered through four layers of cheesecloth, and centrifuged for 20 min at 20 41g. The supernatant was centrifuged for 90 min at 200 7862g. The microsomal pellet was resuspended twice in 0.1 M K/Na phosphate buffer (pH 7.4), containing 1 mM EDTA and recentrifuged (200 000g, 30 min). Finally the microsomal pellet was resuspended in 4 ml of phosphate buffer (pH 7.4), containing 1 mM EDTA and 30% (v/v) glycerol and stored frozen at –80°C until required. The incubation mixture for the attempted phenol coupling reaction contained 0.99 mM **3b**, 5 mM NADPH, and 400 µl microsomal suspension (60–100 µl protein) in a total volume of 500 µl. The mixture was incubated for 1 h at 25°C with gentle shaking. The

reaction was stopped by adding 100 μl AcOH in 20 ml MeOH and centrifuged for 10 min at 341*g*. The supernatant was evaporated to dryness (30°C) than dissolved in 0.1 N aq. HCl and extracted ×3 with EtOAc. The water layer was adjusted to pH 9 (K_2CO_3) and extracted ×5 with $CHCl_3$. The organic phase was dried (Na_2SO_4) and evaporated. The residues were taken up in 100 μl MeOH and analyzed by on-line coupled HPLC-UV(DAD)-ESI (electrospray ionization) 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 (A:B) within 70 min; R_t: **3b** = 1.7 min, **2** = 2.3 min, **1b** = 11.3 min). The ESI-MS detector (quadrupole ion trap instrument), interfaced directly to the output of the UV detector.

In the ESI-MS a $[M+H]^+$ quasi molecular peak at m/z 501, corresponding to **1b** was detected with exactly the same retention time (11.3 min) as the natural aphelandrine (**1a**). The MS/MS showed the fragmentation pattern corresponding to aphelandrine (**1b**), fully deuterated in its putrescine C_4H_8 chain. The fragmentation ions of **1a** (m/z 493) and labeled enzymatically formed **1b** (m/z 501) were identical or differed by 8 amu. Thus, it was shown that the labeled precursor (*S*)-(D_8)-dihydroxyverbacine (**3b**) was enzymatically transformed by the microsomal fraction into (D_8)-labeled aphelandrine (**1b**). This enzymatic reaction was strictly dependent on the presence of microsomal protein. The heat denatured enzyme did not give the product. The reaction catalyzed by the microsomal fraction was strictly dependent on O_2 as well as NADPH. When NADPH was substituted with NADH less then 10% of the activity was detected. When the reaction mixture was bubbled with N_2 gas to displace O_2 only 5% of enzyme activity was detected. An absolute requirement for oxygen was next demonstrated by removing O_2 from the assay through addition of the glucose/glucose oxidase/catalase system.[13] In this case, the enzyme was completely inactivated. No reduction in the enzyme activity was observed when the preincubation was performed with heat-inactivated glucose oxidase. The enzyme activity was strongly inhibited by $CO:O_2$ (9:1) in the dark.

Additionally, the microsomal fraction prepared from the roots of *A. squarrosa* (as described above) catalyzed the aromatic hydroxylation of the synthetically prepared (*S*)-protoverbine[14] (**5**) to prelandrine (**4**) (Fig. 1). Also in this case the product formation was clearly dependent on NADPH and on O_2. With boiled enzyme, by bubbling N_2, or gassing the mixture with $CO:O_2$ (9:1) no transformation could be observed.

The data shown infer that the microsomal fraction from young roots of *A. squarrosa* contain the enzymatic system required to catalyze two steps in the biosynthesis of aphelandrine (**1**) in the presence of the necessary cofactors, the aromatic hydroxylation step of protoverbine (**5**) to prelandrine (**4**) and the phenol oxidative coupling of (*S*)-dihydroxyverbacine (**3**) to aphelandrine (**1**).

In *Aphelandra* sp. orantine is present as a minor alkaloid together with aphelandrine (**1**). The biogenesis of orantine (**2**) should follow the same reaction sequence as postulated herein for aphelandrine (**1**), but no orantine (**2**) was detected in the HPLC-MS analysis of the chloroformic extracts from incubates. This shows that (*S*)-dihydroxyverbacine (**3**) was diastereoselectively transformed to aphelandrine (**1**) by cytochrome P-450. The possibility of an enzymatic isomerization of aphelandrine to orantine in cells has to be proven.

In principle, the biogenesis of the aphelandrine and orantine (ephedradine A)[8] closely related alkaloids ephedradines B, C, and D[8] should follow a similar biogenetic pattern, as described above.

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References

1. Barton, D. H. R.; Cohen, T. *Festschrift Prof. Dr. A. Stoll*; Birkhäuser: Basel, 1957; pp. 117–143.
2. Gerardy, R.; Zenk, M. H. *Phytochemistry* **1993**, *32*, 79–86.
3. Stadler, R.; Zenk, M. H. *J. Biol. Chem.* **1993**, *268*, 823–831.
4. Peters, P.; Schmidt, W.; Beerhues, L. *Planta* **1998**, *204*, 64–69.
5. Bosshardt, H.; Guggisberg, A.; Johne, S.; Hesse, M. *Pharm. Acta Helv.* **1978**, *53*, 355–357.
6. Dätwyler, P.; Bosshardt, H.; Bernhard, H. O.; Hesse, M. *Helv. Chim. Acta* **1978**, *61*, 2646–2671.
7. Youhnovski, N.; Filipov, S.; Linden, A.; Werner, C.; Hesse, M. *Phytochemistry* **1999**, *52*, 1717–1723.
8. Hikino, H.; Ogata, M.; Konno, C. *Heterocycles* **1982**, *17*, 155–158.
9. Guggisberg, A.; Hesse, M. In *The Alkaloids*, *Chemistry and Biology*; Cordell, G. A., Ed; Academic Press: New York, 1998; Vol. 50, pp. 219–256.
10. Papazoglou, G.; Sierra, J.; Homberger, K.; Guggisberg, A.; Woggon, W. D.; Hesse, M. *Helv. Chim. Acta* **1991**, *74*, 565–571.
11. Nezbedová, L.; Hesse, M.; Drandarov, K.; Werner, C. *Helv. Chim. Acta* **2000**, in press.
12. Nezbedová, L.; Drandarov, K.; Werner, C.; Hesse, M. *Helv. Chim. Acta* **2000**, in press.
13. Kochs, G.; Grisebach, H. *Z. Naturforsch.* **1987**, *42c*, 343–348.
14. Guggisberg, A.; Drandarov, K.; Hesse, M. *Helv. Chim. Acta* **2000**, in press.