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ONE STEP ENZYMATIC SYNTHESIS OF DIHYDROSANGUINARINE FROM PROTOPINE

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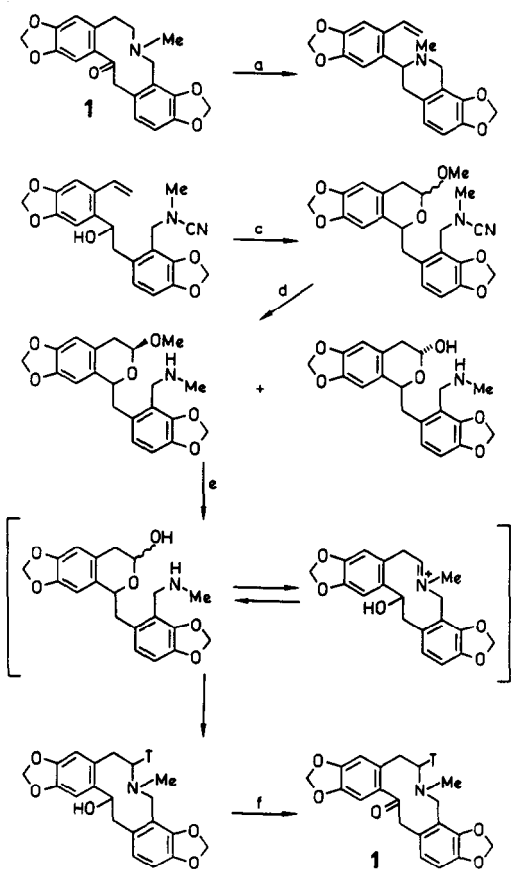
Abstract: A microsomal cytochrome P-450 NADPH dependent enzyme which hydroxylates carbon-6 of protopine has been discovered and characterized; the monooxygenase reaction leads to 6-hydroxyprotopine which spontaneously rearranges to form dihydrosanguinarine.

Sanguinarine is the prototype of benzophenanthridine alkaloids which occur frequently in plants of the family Papaveraceae.[1] It has anticancer activity[2] and has recently gained commercial interest because of its antibacterial properties.[3] The biosynthesis of this compound has been investigated by precursor feeding experiments to intact plants. It could be demonstrated that benzophenanthridine alkaloids are derived from (S)-reticuline and the tetrahydroprotoberberine, stylopine.[4] Furthermore, (S)-cis-N-methylstylopine and protopine (**1**) were unequivocally shown to be metabolites in this pathway.[5] Fission of the C(6)-N(7)-bond of the tetrahydroprotoberberine with subsequent bond formation of C-6 to C-13 should ultimately yield the benzophenanthridine carbon skeleton. No intermediates for this and subsequent stages have until now been detected. Recently, however, it was possible to discover, partially purify and characterize an enzyme from cell suspension cultures of Eschscholtzia californica which catalyzes the oxidation of dihydrobenzophenanthridines to the quaternary alkaloids in the presence of oxygen.[6] This enzyme is assumed to catalyze the terminal step in the formation of benzophenanthridine alkaloids which in turn means that the dihydrobenzophenanthridines should by complex reactions be derived from protopine (**1**) or derivatives of this compound.

The C(6)-N ring fission at the protopine stage involves most likely a hydroxylation at C-6 leading to a hypothetical aldehyde-enamine intermediate. Since it had been previously shown that the hydroxylation of (S)-N-methyltetrahydroprotoberberines to the corresponding protopine molecules involves a NADPH driven microsomal bound monooxygenase,[7] it was obvious to test microsomal preparations for potential 6-hydroxylase activity. To monitor this reaction, use was made of the fact that in this case a proton at C-6 of protopine (**1**) would be replaced by a hydroxyl group. A tritium at C-6 would yield a convenient assay in that hydroxylation of this position would release half of the label into the aqueous medium while the other half should stay in the metabolite formed, thus allowing the identification of the intermediate. [6-^{3}H]-Protopine (**1**) was synthesized as shown in

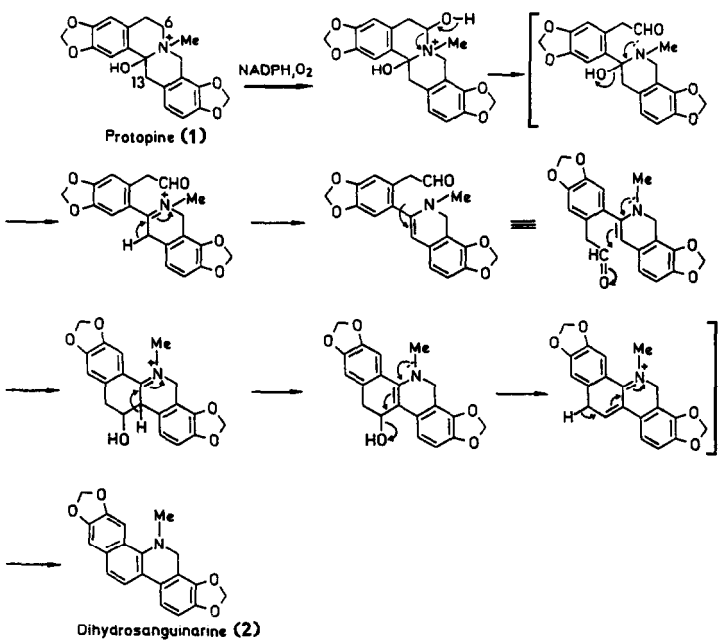


Scheme 1. Chemical synthesis of [6-^{3}H]-protopine (1). Reagents: (a) i. $POCl_3$, CH_3CN, reflux; ii. 25% KOH-MeOH, reflux; iii. $NaBH_3CN$, MeOH, pH 5-6, rt. (b) BrCN, MgO, THF-H_2O, rt. (c) TTN, MeOH, -10°C to 0°C. (d) $LiAlH_4$, THF, 0°C. (e) i. 10% HCl, THF, rt., then $NaHCO_3$, pH 6.5 - 7.0; ii. $NaBT_3CN$, MeOH-THF-H_2O, rt. (f) PCC, NaOAc, CH_2Cl_2, rt.

Scheme 1 with a 13% radiochemical yield. Microsomes from five day old cultured suspension cells of elicited E. californica, which produce substantial quantities of quaternary alkaloids,[8] were prepared by standard methods.[9] The $MgCl_2$ pelleted microsomal fraction was resuspended in 0.1 M tricine buffer pH 7.5, containing 50 mM $MgCl_2$ and 5 mM thioglycolic acid. The microsomes (10 - 100 µg protein) were incubated in the presence of 200 mM tricine buffer pH 7.5, 200 µM NADPH and 5 µM [6-^{3}H]-protopine (1) (60 000 dpm), in a total volume of 250 µl for 20 min at 30°C. Under these conditions tritium removal was observed (6 - 80% of theoretical). Comparable microsomal preparations from Berberis stolonifera or Catharanthus roseus, which do not produce benzophenanthridine alkaloids, did not show any tritium release. Fractionation of the incubation mixture by XAD column chromatography followed by TLC (cyclohexane : diethylamine = 9 : 1; n-hexane : Et_2O = 6 : 1) and radio scanning identified mainly two labelled products, one with an Rf-value (0.70; 0.43) identical to that of dihydrosanguinarine (2) (ca. 10% of label). Incubation of microsomes with labelled protopine (1) in the presence of dihydrosanguinarine (2) (54 µM) led to an accumulation of radioactivity in the zone with the Rf of dihydrosanguinarine (2). This labelled unknown product when oxidized (silica/O_2) yielded a compound chromatographically indistinguishable from sanguinarine. The radioactive metabolite was diluted with sangui-

narine (2.08 x 10^4 cpm.μM^{-1}) and a known cyano adduct[10] was prepared. The product was recrystallized eight times to a spec. act. of 1.90 x 10^4 cpm.μM^{-1}. The oxidized compound upon radio HPLC (Nucleosil C_{18}, $CH_3CN/H_2O/H_3PO_4$) eluted as one radioactive peak with the retention time of the authentic material. We conclude therefore that the product formed by the microsomal oxidation is dihydrosanguinarine (**2**). The second metabolite mentioned above is a derivative originating from dihydrosanguinarine (**2**).

Characterization of the 6-hydroxylation reaction yielded optima at pH 7.5 and 30°C with a K_M of 2 µM for protopine (**1**). Omission of oxygen from the reaction mixture rendered the enzyme complex completely inactive. Replacement of NADPH with NADH resulted in only 6% of the original activity while NAD and NADP were both ineffective. Typical inhibitors for cytochrome P-450 enzymes were found to be active (50% inhibition was found at 1.4 µM prochloraz; 1.5 µM cytochrome c; 11 µM ketoconazole; 85 µM metyrapone). Neither CN^- nor EDTA inhibited the enzyme activity at concentrations of 10^{-3} M. Obviously a cytochrome P-450, this enzyme complex acts in the oxidation of protopine (**1**) as a monooxygenase: R-H + NADPH + O_2 + $2H^+$ → R-OH + $NADP^+$ + H_2O. The enzyme activity responsible for the hydroxylation of protopine should be termed: protopine-6-hydroxylase. As it had been previously predicted on purely chemical reasoning,[11] the hydroxylation of the C-6 position of protopine (**1**) most likely leads to an enamine-aldehyde intermediate which spontaneously undergoes intramolecular condensation between C-6 and C-13 as depicted in Scheme 2.



Scheme 2. Reaction catalyzed by protopine-6-hydroxylase and the subsequent spontaneous rearrangement of 6-hydroxyprotopine to dihydrosanguinarine (**2**).

It was surprising to see that a single, specific enzymic hydroxylation at C-6 of protopine (**1**) leads to the spontaneous rearrangement of this molecule to yield a dihydrobenzophenanthridine structure. This reaction is the key to benzophenanthridines. Subsequent oxidation of the dihydrobenzophenanthridine by the known enzyme, dihydrobenzophenanthridine oxidase,[6] yields the fully aromatized quaternary benzo[c]phenanthridine, sanguinarine. After berberine,[12] sanguinarine is now the second alkaloid whose biosynthesis is completely understood at the enzyme level. Tyrosine was established in 1963 as the precursor of the benzophenanthridine skeleton.[13] We now know that in the conversion of this amino acid into sanguinarine, 17 enzymes are involved, all of which have been identified and characterized.

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