Phenol Oxidative Coupling of Benzylisoquinoline Alkaloids is catalysed by Regio- and Stereo-selective Cytochrome P-450 linked Plant Enzymes: Salutaridine and Berbamunine

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The alkaloids salutaridine and berbamunine are formed by intramolecular C–C and intermolecular C–O coupling, respectively, catalysed by cytochrome P-450 linked NADPH and O_2 dependent microsomal bound plant specific enzymes.

The proposal of Barton and Cohen[1] correlated the structure of specific plant alkaloids in terms of the reaction mechanisms. The oxidation of phenols by one-electron transfer affords phenolic radicals which, by radical pairing, form new C–C or C–O bonds either intra- or inter-molecularily. The biocatalysts involved in these transformations have until now remained obscure. The hypothesis that the ubiquitous phenoloxidase, laccase, tyrosinase, and peroxidase enzymes are of importance could be excluded due to the lack of stereo- and substrate-specificity displayed by these enzymes.[2]

We present now two examples which unequivocally demonstrate that the formation of C–C and C–O bonds in the benzylisoquinoline alkaloid metabolism is catalysed by specific cytochrome P-450 linked microsomal bound plant enzymes.

Barton and Cohen[1] have proposed that the crucial C-12–C-13 bond of morphine alkaloids can be envisaged as being formed by intramolecular phenolic coupling of (*R*)-reticuline (**1**). Indeed, experimental proof has been obtained, by employing *in vivo* experiments, that (**1**) is transformed to salutaridine (**2**) by regioselective *para–ortho* oxidative coupling.[3] Attempts to synthesize the morphinandienone structure biomimetically have up to now resulted in rather mediocre yields.[4] An efficient chemical or enzymatic method for the phenolic oxidation of (**1**) to (**2**) has been the subject of intensive research for many years. The latter product can be converted chemically to thebaine (**3**), codeine (**4**), and morphine (**5**), thus allowing a chemo-enzymatic synthesis of the morphine alkaloids if the respective enzymes would be available. Claims[5] for the discovery of a cell-free system from

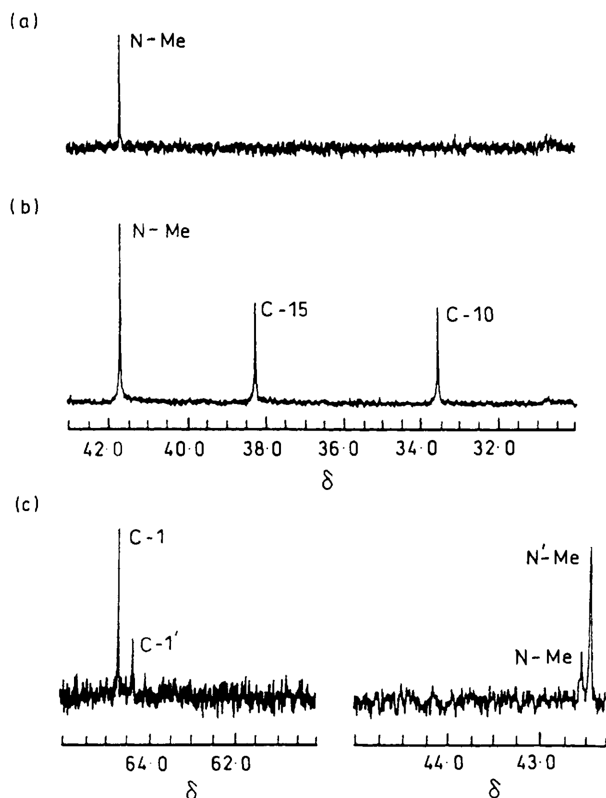
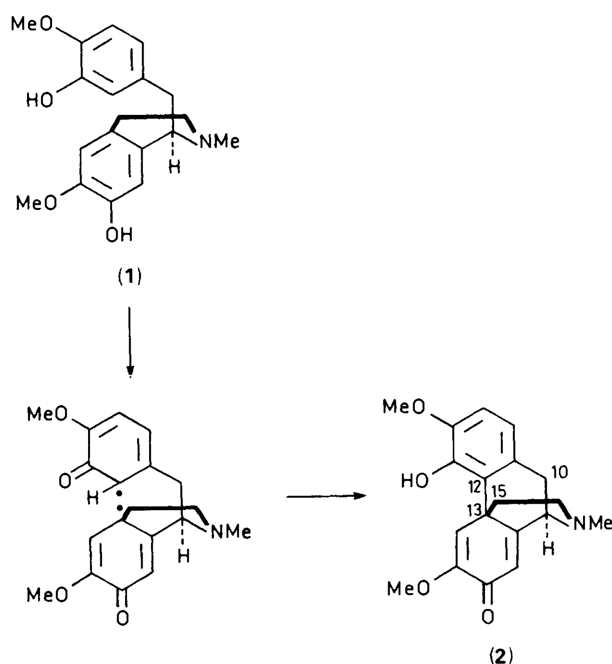
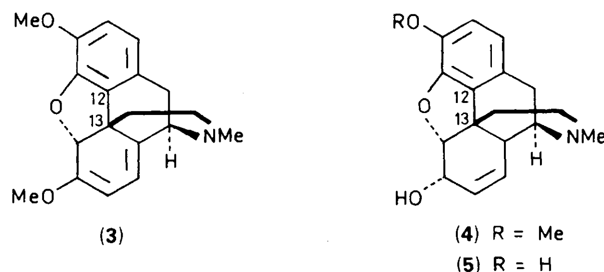
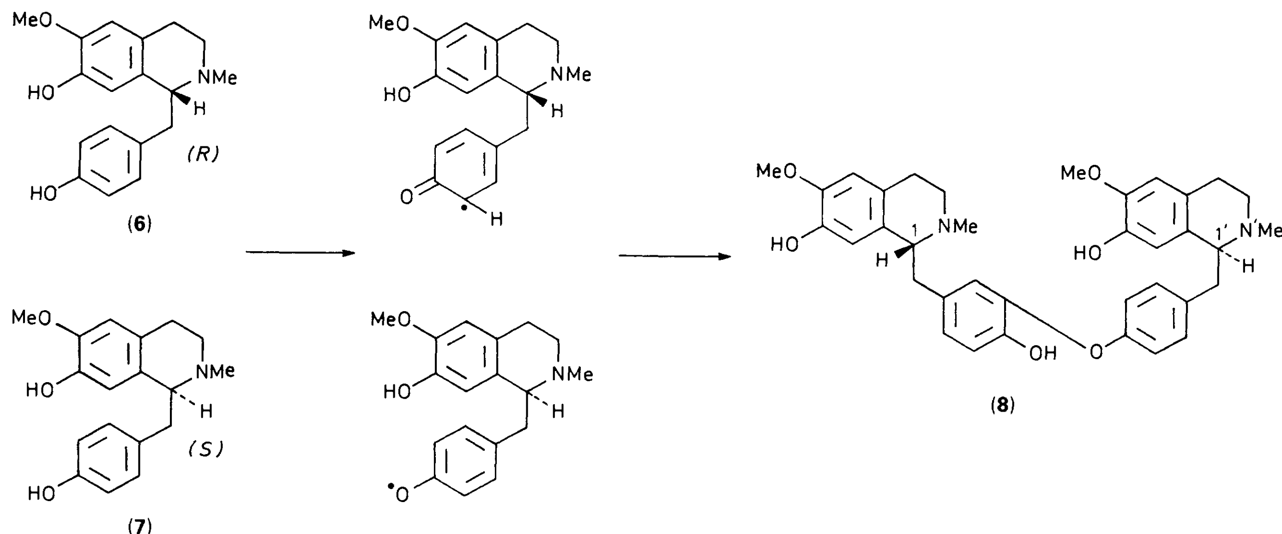


Figure 1. Proton-decoupled ^{13}C NMR spectra (90 MHz, Bruker AM360) of; (a) salutaridine (**2**) enzymically synthesized from (*R*)-[N-$^{13}CH_3$]reticuline (**1**); (b) unlabelled salutaridine added to biosynthesized (**2**) from experiment (a); (c) berbamunine (**8**) enzymically synthesized from (*R*)-[1-^{13}C]*N*-methylcoclaurine (**6**) and (*S*)-[N-$^{13}CH_3$]*N*-methylcoclaurine (**7**).



Scheme 1. Intramolecular *para–ortho* coupling of the (*R*)-reticuline (**1**) derived biradical intermediate generated by a microsomal cytochrome P-450 enzyme to salutaridine (**2**).





Scheme 2. Biosynthesis of the dimer berbamunine (**8**) by intermolecular coupling of the monomeric radicals catalysed by a microsomal P-450 enzyme.

Papaver somniferum converting (*R*,*S*)-reticuline to (**2**) have been refuted.[6]

Cytochrome P-450 enzymes can function in alkaloid biosynthesis both as oxygenases in O_2 and NADPH dependent substrate hydroxylations,[7,8] and in the synthesis of C–O bonds exemplified by the formation of methylenedioxy groups from *O*-methoxy phenols.[9] This prompted us to investigate the microsomal fractions of the flowering stages of *Papaver somniferum* capsules as to their ability to form (**2**). Microsomes were prepared in the conventional manner[10] and an aliquot (0.15 mg protein) incubated aerobically in the presence of 200 mM KPO_4^{-2} buffer at pH 7.5, 1 mM NADPH and 200 μM (**1**) in a total reaction volume of 100 μl for 60 min at 25 °C. Production of (**2**) was detected and quantified by a salutaridine-specific radioimmunoassay[11] and HPLC. The rate of salutaridine (**2**) formation was determined as 0.2 pkat/standard sample assay. Absolutely no transformation of (**1**) was observed if either NADPH, oxygen, or substrate were omitted from the incubation mixture or if heat denatured microsomes were added. The product was further rigorously identified as (**2**) by large scale incubation with (*R*)-[N-$^{13}CH_3$]reticuline (**1**) and subsequent ^{13}C NMR spectral analysis (solvent CD_3OD) of the purified product, depicted in Figure 1 (a) before and (b) after addition of unlabelled (**2**). The N–Me signal appeared at δ 40.95 for (**1**) and 41.65 for (**2**). Similar incubations with (*S*)-reticuline as a substrate absolutely failed to afford (**2**), substantiating the stereoselectivity of this enzyme catalysed reaction. The optimum pH for the enzyme was 7.5 and the reaction proceeded linearly with time for 60 min. The K_M values for (**1**) and NADPH were determined as 12 and 140 μM, respectively. The addition of catalase and superoxide dismutase did not impede the reaction excluding the participation of H_2O_2 or $O_2^{\cdot-}$. Typical cytochrome P-450 inhibitors were also employed and the concentrations necessary for 50% inhibition of the reaction determined: prochloraz (15 μM), ancymidole (30 μM), ketoconazole (34 μM), and cytochrome c (1.6 μM). Furthermore, the enzyme was inhibited (90%) by a mixture of $CO:O_2 = 9:1$. This inhibition was alleviated by illumination with blue light but not with red light. The enzyme is absent in microsomal particles isolated from poppy latex. Also, no activity could be registered in microsomes originating from cell cultures or plants that do not elaborate the morphinane skeleton such as *Eschscholtzia californica, Fumaria capreolata, Corydalis cava*, and *Berberis stolonifera*. The enzyme is, however, present in microsomes obtained from specific cell culture strains of *Papaver somniferum* that produce substantial amounts of (**3**). Therefore, it can be deduced that the formation of this crucial C-12–C-13 bond in morphine type compounds is catalysed by a highly regio- and stereo-selective cytochrome P-450 linked microsomal *Papaver* enzyme (Scheme 1).

The second example given here involves a cytochrome P-450 catalysed intermolecular coupling of, most likely, phenolate radicals to form C–O bonds. The example demonstrated is the formation of the bisbenzylisoquinoline alkaloid berbamunine (**8**), which is formed from coclaurine. According to biogenetic theory the phenyl ether bond is formed by oxidative phenolic coupling.[1] It has been previously shown that the *N*-methyl derivatives of both enantiomeric coclaurines (**6**, **7**) act as precursors of (**8**) and that their incorporation is highly stereoselective in cell cultures of *Berberis stolonifera* plants.[12] Using this plant source microsomal fractions were prepared as described above[10] but instead of $MgCl_2$ precipitation a high spin fraction (48 000 g) was used. The pellet was suspended in 100 mM tricine–NaOH buffer (pH 8.5) and an aliquote (0.03 mg) incubated aerobically in the presence of 500 μM NADPH, 100 μM (*R*)-*N*-methylcoclaurine (**6**), and 100 μM (*S*)-*N*-methylcoclaurine (**7**) in a total volume of 100 μl for 40 min at 30 °C. The rate of formation of (**8**) was measured by HPLC analysis. A reaction rate of 0.5 pkat was monitored per standard incubation mixture. Absolutely no activity could be detected if NADPH was omitted or under anaerobic conditions. Also, incubations containing only one of the enantiomers, *i.e.* (**6**) or (**7**), did not afford the dimeric product. The optimum pH for the reaction was at 8.5 and the reaction proceeded linearly for up to 40 min. The K_M values for (**6**), (**7**), and NADPH were determined as 16, 12, and 160 μM, respectively. The presence of catalase and superoxide dismutase did not show any detrimental effect on the turnover rate. Inhibition (50%) of enzyme activity was achieved with the following cytochrome P-450 inhibitors: prochloraz (8 μM), tetcyclacis (100 μM), ketoconazole (110 μM), and cytochrome c

(2.5 μM). The reaction was inhibited (95%) by a mixture (9 : 1) of CO : O_2, which was reversed by illumination with blue light but not with red light. Further structural confirmation of the dimerization product was obtained by a large scale standard incubation with (*R*)-[1-^{13}C]*N*-methylcoclaurine (**6**) and (*S*)-[N-$^{13}CH_3$]*N*-methylcoclaurine (**7**) and subsequent NMR spectral analysis of the purified product. Figure 1(c) depicts an excerpt of the ^{13}C NMR spectrum of (**8**) (solvent $CDCl_3$) clearly illustrating the enriched resonances (δ 64.61 for C-1 and δ 42.44 for N′–Me). The natural abundance carbon signals C-1′ and N–Me are visible after addition of unlabelled (**8**). The enzyme was also present in microsomes from other *Berberis* species known to produce these biscoclaurines, but was absent in *Papaver somniferum* and other species that do not elaborate dimeric alkaloids.

The data presented here leads to the inference that intermolecular C–O bond formation is undoubtedly catalysed by a highly stereo- and regio-selective cytochrome P-450 complex, in which the enzyme surface certainly exerts a major influence on the way the radicals combine (Scheme 2).

Microsomal preparations harbouring cytochrome P-450 enzymes evidently play a crucial role in the biosynthesis of alkaloids and other important natural products. Furthermore, this coupling mechanism involving the respective phenoxy radicals proceeds in a highly specific manner, as has been predicted previously.[1]

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