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# Cytochrome P-450-dependent formation of Isoandrocymbine from Autumnaline in Colchicine Biosynthesis

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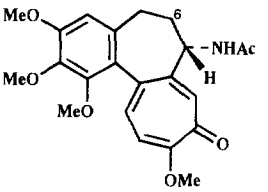
*Abstract:* A highly substrate-specific microsomal-bound cytochrome P-450 NADPH and $O_2$-dependent enzyme system has been discovered in immature seeds of *Colchicum autumnale* L. that is responsible for the intramolecular phenol-oxidative coupling of autumnaline to isoandrocymbine during colchicine biosynthesis. Copyright © 1996 Elsevier Science Ltd

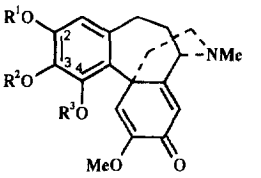
The oxidation of plant phenols by one-electron transfer affords phenolic radicals which by radical pairing form new C-C or C-O bonds both intra- and intermolecularly. This reaction was proposed as one of the principal biosynthetic mechanisms for the formation of plant alkaloids by Barton and Cohen.[1] This proposal was verified when it could be shown that highly regio- and stereoselective membrane-bound cytochrome P-450 enzymes[2] are catalyzing both intramolecular phenol coupling intermediates such as salutaridine[3] in morphine biosynthesis[4] and intermolecular coupling of monomeric benzylisoquinoline alkaloids of the *N*-methylcoclaurine type to form the dimeric alkaloid berbamunine.[5,6] Early on, this mechanism was also extended to the biosynthesis of the tropolone ring-containing alkaloid colchicine (**1**), for which a precursor dienone alkaloid (*O*-methylandrocymbine (**2**)) was demonstrated to be an intermediate.[7] In order to investigate this specific phenol coupling reaction in colchicine biosynthesis, we now investigated the enzymic transformation of autumnaline[7] (**3**) to the suspected dienone intermediate. Autumnaline (**3**) and congeners were synthesized by standard techniques[7] containing label either in the 6-*O*-$CH_3$ group with $^{14}C$ or in positions 3 and 9 with $^{13}C$.

Since extremely long application times of labeled precursors to *Colchicum* capsules have previously been used,[8] we first optimized the physiological parameters for feeding experiments. Immature *Colchicum autumnale* seeds having an average weight of 12-15 mg per seed were used throughout this study. Eight seeds were incubated in 1 ml of water containing the labeled precursor in $3 \cdot 10^{-4}$ M concentration under agitation for 48 hrs. Under these conditions, an incorporation of [3,9-$^{13}C$](*R,S*)-autumnaline of 18% into colchicine was observed and specific incorporation was verified by $^{13}CNMR$ spectroscopy. In contrast, [3-$^{13}C$](*R,S*)-*N*-norautumnaline (**4**) was incorporated to only 1.6%, demonstrating the high specificity of the enzyme system involved in that biosynthetic transformation. Furthermore, the incorporation study using [3,9-$^{13}C$](*R,S*)-autumnaline (**3**) showed that the label in position 3 was entirely lost during the transition into colchicine, while that at position 9 was strongly

observed in carbon 6 (36.2 ppm) of the tropolonic alkaloid (**1**). This verifies the previously published results,[9] which were attained by incorporation of [3,9-$^{14}$C]autumnaline (**3**) into colchicine (**1**). Modification of the C-ring in the (*R,S*)-phenethylamine precursor, for instance, to a trimethoxy derivative (**5**) or a 3,5-dimethoxy-4-hydroxy derivative (**6**) yielded incorporation of only 10% or less than that of (*R,S*)-autumnaline (**3**). All of these experiments taken together with previous results[7] indicate that (*S*)-autumnaline is the immediate precursor of the phenol coupling reaction, yielding eventually an androcymbine derivative as precursor for colchicine.

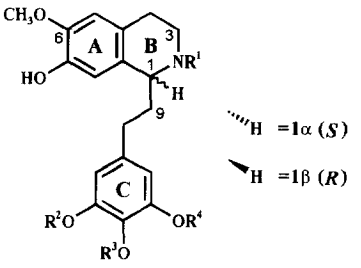



(1)



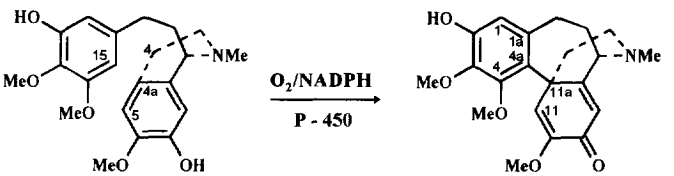

| | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| (2) | $OCH_3$ | $OCH_3$ | $OCH_3$ |
| (7) | $OCH_3$ | OH | $OCH_3$ |
| (8) | OH | $OCH_3$ | $OCH_3$ |

It has been shown by us that microsomal fractions of differentiated plants or cell cultures catalyze select phenol coupling reactions.[2,4,5] In order to test for phenol coupling capacity in microsomes of *C. autumnale*, immature seeds of a stage yielding maximal incorporation of autumnaline into colchicine were used as experimental material. 200 g seeds (fwt) were shock-frozen with liquid $N_2$ and ground with sea sand in 400 ml 0.1 M tricine buffer (pH 7.5) containing 20 mM mercaptoethanol and 20 g polyvinylpyrrolidone at 0 °C. The homogenate was centrifuged for 10 min at 3000 x g at 4 °C and the supernatant was passed through an XAD-2 column to remove adhering alkaloids. The crude extract was subsequently centrifuged at 4 °C for 30 min at 50 000 x g.




| | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| (3) | $CH_3$ | OH | $CH_3$ | $CH_3$ |
| (4) | H | OH | $CH_3$ | $CH_3$ |
| (5) | $CH_3$ | $CH_3$ | $CH_3$ | $CH_3$ |
| (6) | $CH_3$ | $CH_3$ | OH | $CH_3$ |

The sediment was taken up in 40 ml of the above buffer and the protein content determined to be 3.1 mg/ml. The incubation mixture for the attempted phenol coupling reaction contained 50 µmol glycine-buffer pH 8.5, 200 nmol NADPH, 100 nmol NADP, 500 nmol glucose-6-phosphate, 3 units glucose-6-phosphate dehydrogenase, 5 nmol (*R,S*)-[6-*O*-$^{14}CH_3$]autumnaline (1 µCi) and 300 µg of microsomal protein in a total volume of 200 µl. The mixture was incubated for 2 h at 30 °C. The mixture was then extracted with ethyl acetate and the organic phase separated by TLC ($CHCl_3$:$(CH_3)_2CO$:$(CH_3CH_2)_2NH$ = 7:2:1). Three labeled products with Rf values of 0.16 (5%), 0.23 (13%) and 0.78 (27%) were observed in addition to unchanged autumnaline (Rf 0.31). All three unknown compounds were isolated, purified and fed to cholchicum seeds. All three compounds were incorporated into colchicine and, therefore, seem to be precursors of this alkaloid. The most abundant product with the highest Rf value was isolated from a large scale incubation using non-radioactive substrate and was further purified (HPLC). This reaction product, when treated with diazomethane, was converted to *O*-methylandrocymbine (**2**). Incubation of a soluble protein extract from *C. autumnale* seeds prepurified by DEAE-column chromatography (elution at 0.3 M KCl) and the unknown product in the presence of [$^{14}CH_3$]*S*-adenosylmethionine, yielded a radioactive product indistinguishable from *O*-methylandrocymbine (**2**) while under the same conditions androcymbine (**7**) was not methylated. 3,5-Dimethoxy-4-hydroxyphenethylisoquinoline (**6**) and 3,4,5-trimethoxyphenethylisoquinoline (**5**) were completely inactive as substrates for the enzymic phenol coupling reaction, demonstrating the high substrate selectivity of the microsomal system used. The product is, therefore, identified as the expected dienone carrying a 2-OH-3,4-di$OCH_3$ substitution pattern and will be called isoandrocymbine (**8**). The transformation of **3** to **8** was strictly dependent on the presence of microsomal protein. Heat-denatured enzyme did not yield the dienone. The reaction catalyzed by the microsomal fraction was strictly dependent on $O_2$ as well as NADPH. The $K_m$ value for autumnaline is 37 µM and for NADPH 75 µM. Typical cytochrome P-450 inhibitors were also employed and cytochrome *c* and juglone showed complete inhibition of the phenol coupling reaction at 50 µM. At 500 µM, propiconazol showed 70%, metyrapone 42%, ketoconazol 34%, and prochloraz 29% inhibition. Furthermore, the enzyme was totally inhibited (100%) by a mixture of CO:$O_2$ of 9:1.




(3) (8)

Fig. 1 The formation of isoandrocymbine from autumnaline in colchicine biosynthesis is catalyzed by a microsomal cytochrome P-450 enzyme.

This inhibition was alleviated (47%) by illumination with light. No inhibition of the reaction was observed by a mixture of $N_2$:$O_2$ = 9:1 either in the dark or in light. In defining characteristics, the autumnaline oxidase is a cytochrome P-450 enzyme catalyzing the phenol coupling of **3** to **8**, as shown in Fig. 1. Again a phenol coupling reaction in plant alkaloid biosynthesis is catalyzed by a microsomal bound cytochrome P-450 enzyme, as has been demonstrated previously in the biosynthesis of morphine[2,4] and berbamunine.[5,6]

The data shown infer that intramolecular $C_{4a}$-$C_{15}$ bond formation involving (*S*)-autumnaline[9] (**3**) is undoubtedly catalyzed by a highly stereo- and regioselective cytochrome P-450 complex, yielding isoandrocymbine (**8**), the first phenol coupled intermediate in colchicine biosynthesis. This coupling mechanism involving the respective phenoxy radicals generated in (*S*)-autumnaline (**3**) proceeds in a highly specific manner, as has been predicted previously.[1]

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