Tetrahedron Letters,Vol.30,No.39,pp 5257-5260,1989 0040-4039/89 $3.00 + .00
Printed in Great Britain Pergamon Press plc

FORMATION OF BOTH METHYLENEDIOXY GROUPS IN THE ALKALOID (S)-STYLOPINE IS CATALYZED BY CYTOCHROME P-450 ENZYMES

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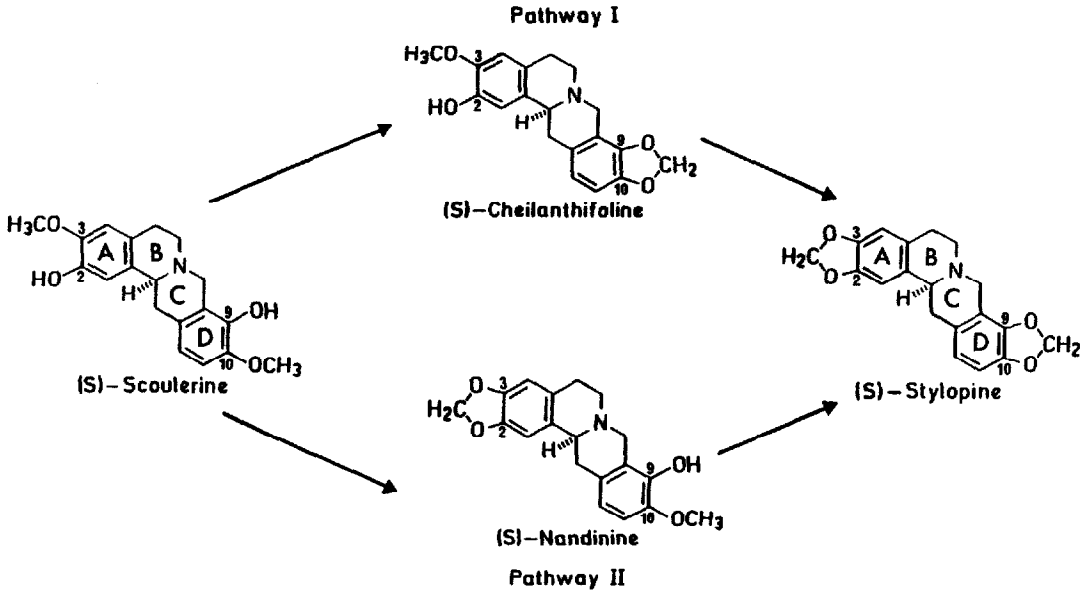
Abstract: Two highly stereospecific microsomal cytochrome P-450 NADPH dependent enzymes have been discovered and characterized which are responsible for the methylenedioxy group formation from (S)-scoulerine via (S)-cheilanthifoline to (S)-stylopine.

(S)-Stylopine, a tetrahydrobenzylisoquinoline alkaloid containing two methylenedioxy groups in rings A and D, is an important branch point intermediate. It can for instance undergo oxidation of ring C to yield the protoberberine coptisine, or alternatively N-methylation opens the pathway to the benzophenanthridine alkaloids.[1] In vivo experiments employing ^{14}C-labelled precursors demonstrated that the methylenedioxy groups found in benzylisoquinoline alkaloids are derived by cyclization of an o-methoxyphenol to furnish the methylenedioxy ring.[2-4] Elaborate experiments involving multiply labelled (S)-scoulerine (derived from (S)-reticuline) demonstrated that this precursor is transformed into (S)-stylopine in the intact plant.[5] Furthermore, evidence has been obtained that during the transition of (S)-scoulerine to (S)-stylopine the methylenedioxy group in ring D (9,10-position) is formed prior to the $-OCH_2O-$bridge in ring A of scoulerine.[6] Subsequently (S)-cheilanthifoline was unequivocally proven to be the ultimate precursor of (S)-stylopine.[7] On the other hand, (S)-nandinine, containing the methylenedioxy group in ring A at the 2,3-position also exists in plants.[8] This intermediate could potentially also serve as a precursor for stylopine. The determination of which pathway (Scheme 1) is operative in the plant and also which type of reaction leads to the formation of the methylenedioxy groups in stylopine prompted us to investigate this phenomenon on the enzyme level.

Methylenedioxy group formation from an o-methoxyphenol should involve the loss of one hydrogen atom from the methyl group. Introduction of a tritium labelled methoxy group either in position 3 or 10 of the putative precursors should provide a convenient assay system. If the methylenedioxy group is formed by an enzymatic reaction, theoretically one third of the tritium activity residing in the methyl group should be released into the aqueous medium. The remaining two thirds of the label should be located in the methylenedioxy group, thus enabling the identification of the product. The compounds (S)-(10-O-C^3H_3)-, (S)-(3-O-C^3H_3)-scoulerine, (S)-(10-O-C^3H_3)-nandinine and (S)-(3-O-C^3H_3)-chei-



Scheme 1. Two possible pathways leading from (S)-scoulerine to (S)-stylopine

lanthifoline were synthesized enzymatically by standard techniques employing catechol-O-methyl transferase (Sigma), 4'-O-methyl transferase,[9] berberine bridge enzyme,[10] using the appropriate benzylisoquinoline alkaloids and C^3H_3-SAM (85 Ci/mmol) as substrates. The compounds were stereochemically pure except for cheilanthifoline which contained 13% of the (R)-enantiomer. Analogously, the ^{14}C-labelled compounds were prepared using $^{14}CH_3$-SAM (59 mCi/mmol). The corresponding (R)-enantiomers were synthesized by subjecting the (S)-enantiomers to (S)-tetrahydroprotoberberine oxidase action. The resulting dehydro-derivatives were reduced with BH_4^- and the remaining (S)-analogues of the racemates selectively reoxidized repeatedly in the same manner.[11]

Eschscholtzia californica cell suspension cultures were used as an enzyme source, since these cells proved also to be a rich source of benzophenanthridine alkaloids, especially after elicitation with yeast cell wall fractions[12] and therefore must involve the stylopine pathway.[7] Enzyme preparations of E. californica (cytosol and subcellular particles) subjected to the highly sensitive ABTS-test[13] revealed absolutely no peroxidase activity. Therefore, peroxidase dependent demethylation of the radio-labelled methoxy-groups, as observed in Berberis cultures,[14] did not impede the tritium assay. Since the incubation of cytosolic enzyme preparations of E. californica with the labelled substrates did not effect any tritium release into the aqueous medium, microsomal fractions were tried as enzyme source. Microsomes from five day old elicited suspension cells were prepared by standard methods.[15] 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 microsomal fraction (10 - 100 µg protein) was incubated in the presence of 200 mM tricine

buffer pH 8.0, 200 µM NADPH and the $-OC^3H_3$ labelled substrates (2 µM; 70 000 dpm) in a total volume of 250 µl for 20 min at 30°C. Under these conditions tritium release of up to 90% of the theoretical value was observed using (S)-(10-O-C^3H_3)-scoulerine and (S)-(3-O-C^3H_3)-cheilanthifoline as substrates. Tritium release from (S)-(3-O-C^3H_3)-scoulerine was only half as high as the above mentioned value and (S)-(10-O-C^3H_3)-nandinine showed no tritium release. All (R)-enantiomers displayed no reaction.

To identify the reaction products, ^{14}C-labelled (S)-scoulerine was incubated under standard conditions. The incubation mixture was subjected to TLC analysis (toluene : ethylacetate : diethylamine = 7 : 2 : 1; cyclohexane : diethylamine = 9 : 1; cyclohexane : chloroform : acetic acid = 45 : 45 : 10).

Two radio-labelled bands were identified which corresponded to cheilanthifoline and stylopine, respectively. The labelled cheilanthifoline band was eluted, methylated (CH_2N_2) and the reaction product purified by TLC. The resulting compound (8.31×10^3 dpm.µmol^{-1}) was then diluted with cold (R,S)-sinactine and recrystallized six times to constant spec. act. (8.17×10^3 dpm.µmol^{-1}). We therefore conclude that one of the reaction products *formed by the microsomal oxidation of (S)-scoulerine is (S)-cheilanthifoline. The second* metabolite mentioned above was suspected to be (S)-stylopine. The TLC purified compound was diluted with carrier stylopine (1.49×10^4 dpm.µmol^{-1}) and was brought to a constant spec. act. after six recrystallizations (1.47×10^4 dpm.µmol^{-1}). In the case of the microsomal (3-O-$^{14}CH_3$)-cheilanthifoline incubation only one new peak was observed on radio-TLC analysis, which comigrated with stylopine. The radio-labelled compound was diluted with non-radioactive carrier (1.71×10^4 dpm.µmol^{-1}) and recrystallized six times to constant spec. act. (1.72×10^4 dpm.µmol^{-1}). These results prove that the labelled compound formed from (S)-cheilanthifoline is indeed (S)-stylopine. Microsomal incubations with labelled (S)-nandinine or the (R)-congeners of the aforementioned compounds as substrates did not furnish any labelled reaction products.

The addition of 25 nmol unlabelled (R,S)-nandinine to the incubation mixture in the presence of (S)-(8-^{14}C)-scoulerine did not result in accumulation of radioactivity in the nandinine band (TLC). There was also no interference in the transformation of the labelled precursor to cheilanthifoline (31%) and stylopine (46%). If, however, to the same incubation mixture containing ^{14}C-scoulerine as substrate, (R,S)-cheilanthifoline (25 nmol) was added, 42% of the total radioactivity besides starting material resided after TLC in the cheilanthifoline band. No labelled stylopine was detected. This points to the fact that two enzyme systems are present in the microsomal fraction catalyzing pathway I (Scheme 1): Enzyme I transforming (S)-scoulerine to (S)-cheilanthifoline and enzyme II transforming (S)-cheilanthifoline to (S)-stylopine.

Both enzyme systems are highly substrate specific. No reaction was observed after additon of the following ^3H- or ^{14}C-labelled substrates: (R,S)-tetrahydrogroenlandicine, (R,S)-corygovanine, (R,S)-tetrahydrothalifaurine, (R,S)-tetrahydrocolumbamine, dehydroscoulerine, dehydrocheilanthifoline, berberrubine, groenlandicine, dehydrocorygovanine, thalifaurine. Characterization of both methylenedioxy group forming enzymes showed optima for both enzymes at pH 8.0 and 30°C, with a K_M of 0.9 µM for scoulerine and 0.4 µM for cheilanthifoline as substrate. Omission of oxygen from the reaction mixture or replacement

of NADPH with other cofactors such as NADH, NAD or NADP rendered the enzyme complex completely inactive. Typical inhibitors for cytochrome P-450 enzymes were found to be active.

The following inhibitors showed a drastic difference in the concentration necessary for 50% inhibition of the two individual enzymes:

	Enzyme I	Enzyme II
Ancymidole	19 µM	4 µM
Metyrapone	1500 µM	75 µM
Prochloraz	23 µM	2 µM

No inhibition was observed at 2 mM EDTA or KCN.

Carbon monoxide (90% CO, 10% O_2) inhibited both enzymes to 10% of the original activity in the dark. Illumination of the samples containing the inhibitor restored activity to about 57%.

Both enzymes are present in a number of cell suspension cultures such as: Corydalis vaginans, Fumaria parviflora, Eschscholtzia glauca, E. lobbii, and Chelidonium majus. Enzymes I and II are absent in mouse-liver microsomes. Due to the fact that cytochrome P-450 dependent monooxygenases are involved in the above-mentioned reactions, we assume that methylenedioxy group formation is initiated via hydroxylation of the attached methoxy groups. This step would generate a hemiformal, possibly representing the crucial intermediate in the reaction mechanism.[4] The picture now emerging indicates that microsomal bound P-450 enzymes play a major role in the catalyzation of reactions in alkaloid biosynthesis which are difficult to mimic in organic chemistry.

Acknowledgement:

Our thanks are due to Mr. R. Stadler for linguistic help in the preparation of this manuscript. Supported by SFB 145 of Deutsche Forschungsgemeinschaft, Bonn, and Fonds der Chemischen Industrie.

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(Received in Germany 17 July 1989)