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ENZYMATIC STUDY OF THE LATE STAGES OF PROTOBERBERINE ALKALOID BIOSYNTHESIS

C.W.W. Beecher and W.J. Kelleher*

University of Connecticut, School of Pharmacy
Storrs, CT 06268, U.S.A.

Abstract: A specific methyltransferase which converts tetrahydrocolumbamine to tetrahydropalmatine, and oxidase activity which converts tetrahydroberberine, tetrahydrocolumbamine and tetrahydropalmatine, but not tetrahydrojatrorrhizine, to their quaternary counterparts are demonstrated.

The benzyltetrahydroisoquinoline, (S)-reticuline (1), has repeatedly been shown to be the preferred precursor to the protoberberine skeleton.[1,2] Oxidative cyclization of 1, provides the tetrahydroprotoberberine, (S)-scoulerine (2). Two O-methylations and oxidation to the quaternary structure are needed to convert 2 to palmatine (3). Some question remains regarding the order in which these late reactions occur. The possibilities are as follows: (i) O-methylation may occur at either or both oxidation states, viz. the tetrahydro- and quaternary states; (ii) the order of methylation (i.e., at the 2-hydroxyl and the 9-hydroxyl) is directed by specific methyltransferases or is more or less random; and (iii) in light of the demonstration by us[3] (confirmed by Rueffer et al.[4]) that jatrorrhizine (4), a monophenol, is derived from berberine (5), methylation of 4 or its tetrahydro derivative at the 3-hydroxyl is the route to palmatine (3). Examination of these possibilities can be best achieved by the use of enzyme extracts of a protoberberine alkaloid-producing callus culture.

We wish to report the demonstration of a specific methyltransferase which transfers a methyl group from S-adenosylmethionine (SAM) to tetrahydrocolumbamine, thereby producing tetrahydropalmatine. The enzyme extract was prepared as previously described from an alkaloid-producing suspension culture of Berberis aggregata.[5] A typical incubation mixture contained the following in a final volume of 100 µl: (RS)-tetrahydrocolumbamine, 31 nmoles; [$^{14}CH_3$]-SAM, 1 x 10^5 dpm, 0.87 µmoles (dissolved in 10 µl ethanol); dithiothreitol, 0.45 µmoles; $MgCl_2$, 0.27 µmoles; tris chloride buffer, pH 8.0, 9 µmoles; and enzyme solution, 90 µl (1.4-2.4 mg/ml protein). Incubation was conducted aerobically at 30° for two hours. After incubation, the mixture was treated with an equal volume of saturated aqueous sodium chloride and then extracted three times, each with one ml of chloroform. The chloroform extracts were combined and aliquots were taken for radioactivity determination and tlc.[3] The palmatine band from eight incubations was eluted, and the eluate was treated with 100 mg of authentic palmatine iodide. Crystallization of palmatine iodide was repeated until the product reached constant specific activity (1674 dpm/mg). A similar incubation (200 µl) in the presence of 0.33 µM morin[6] (an oxidase inhibitor) gave only labeled tetrahydropalmatine, which after addition of 62 mg of carrier and crystallization to constant specific activity gave a product with 618 dpm/mg. Tetrahydropalmatine was also the major product obtained when the incubation was conducted under an atmosphere of nitrogen. The same enzyme extract showed no methyltransferase activity

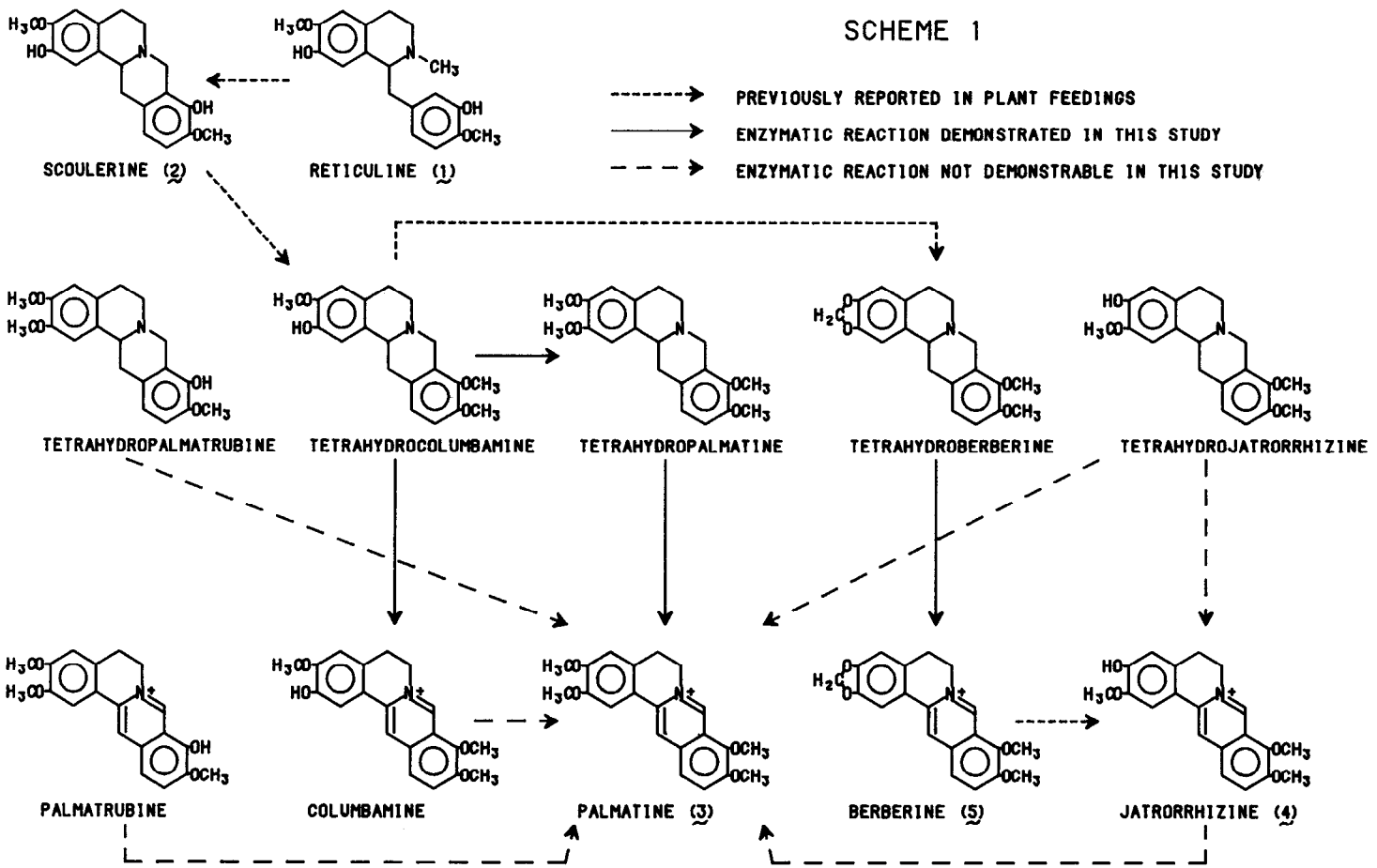
when tetrahydrojatrorrhizine, tetrahydropalmatrubine, as well as columbamine, jatrorrhizine and palmatrubine were used as methyl group acceptors.

We also report the presence of oxidase activity in our cell-free extract which catalyzes the oxidation of tetrahydroberberine, tetrahydrocolumbamine, and tetrahydropalmatine to their quaternary counterparts. A typical mixture contained the following in a final volume of 100 µl: 3-O-[$^{14}CH_3$]-(RS)-tetrahydrocolumbamine, 1 x 10^5 dpm, 0.87 umoles; dithiothreitol, 0.45 µmoles; tris chloride buffer, pH 8.0, 9 µmoles; and enzyme solution, 90 µl (1.4-2.4 mg/ml protein). The incubation was conducted aerobically at 30° for two hours. Work-up was the same as that described for the methyltransferase incubations. Incubations with heat-inactivated enzyme served as controls for autooxidation. Radioactivity in the quaternary alkaloid fraction from the control incubations (about 2 to 10% of that from the enzyme-catalyzed oxidation) was subtracted from the corresponding value obtained form the complete incubation mixture. The same cell-free extract also showed strong oxidase activity with 9-O-[$^{14}CH_3$]-(RS)-tetrahydroberberine and 3-O-[$^{14}CH_3$]-(RS)-tetrahydropalmatine as substrates. 2-O-[$^{14}CH_3$]-(RS)-tetrahydrojatrorrhizine was not oxidized in these incubations.

These results are summarized in Scheme 1 and they make it possible for us to rule out a number of alternate pathways to palmatine and jatrorrhizine. The entry to the protoberberine skeleton is scoulerine which possesses the reticuline pattern of O-methylation. Methylation at the 9-hydroxyl gives tetrahydrocolumbamine which is now shown to reside at a triple branch-point: (i) oxidative cyclization to form the methylenedioxy ring as shown in feeding experiments by others;[7] (ii) enzymatic oxidation to columbamine as shown in this study; and (iii) enzymatic methylation at the 2-hydroxyl as described herein. In the case of berberine, columbamine and palmatine, the substitution pattern (hydroxyl, methoxyl or methylenedioxy) is established at the tetrahydro state of oxidation since none of the monophenolic quaternary alkaloids served as a substrate for the methyltransferase. Jatrorrhizine, the fourth and major protoberberine produced by our culture, is unique in that the entry point into its formation is at the quaternary state of oxidation as shown in earlier studies.[3,4] When this is taken into account, our failure to find oxidase activity acting on tetrahydrojatrorrhizine is not surprising.

In the present study, we did not examine scoulerine as a substrate for the methyltransferase or the oxidase. Scheme 1 depicts scoulerine as being methylated first at the 9-hydroxyl. The other possibility, methylation first at the 2-hydroxyl, would lead to tetrahydropalmatrubine. We have shown that neither this compound, nor palmatrubine is a substrate for methylation. In earlier work,[5] we found that palmatine and columbamine, but not palmatrubine, were formed when reticuline and SAM were incubated with an enzyme extract. On the basis of these observations, and also taking into account that neither tetrahydropalmatrubine nor palmatrubine are produced by the callus culture, we feel justified in proposing the order of methylation shown in Scheme 1.

Amann et al.[8] recently reported the presence of an oxidase in cell cultures of several Berberis species and in Papaver somniferum plants which catalyzes the oxidation of some 20 different (S)-tetrahydroprotoberberines. Only two of these substrates were named, tetrahydrojatrorrhizine and tetrahydroberberine (= canadine), and the respective K_m values were given as


SCHEME 1
PREVIOUSLY REPORTED IN PLANT FEEDINGS
ENZYMATIC REACTION DEMONSTRATED IN THIS STUDY
ENZYMATIC REACTION NOT DEMONSTRABLE IN THIS STUDY
SCOULERINE (2)
RETICULINE (1)
TETRAHYDROPALMATRUBINE
TETRAHYDROCOLUMBAMINE
TETRAHYDROPALMATINE
TETRAHYDROBERBERINE
TETRAHYDROJATRORRHIZINE
PALMATRUBINE
COLUMBAMINE
PALMATINE (3)
BERBERINE (5)
JATRORRHIZINE (4)

1.3 μM and 26.7 μM, for the enzyme from Berberis wilsoniae var. subcaulialata. The significant discrepancy in the activity towards tetrahydrojatrorrhizine caused us to repeat the incubations several times, each with a freshly prepared enzyme extract that did show activity towards tetrahydroberberine and tetrahydrocolumbamine. We considered the possibility that the chloroform was extracting unchanged tetrahydrojatrorrhizine and leaving its oxidation product in the aqueous phase. n-Butanol was found to be a better solvent for extracting jatrorrhizine, and when substituted for chloroform in the extraction procedure, extracted greater than 95% of the radioactivity present in the incubation mixture. Radioscanning of thin-layer chromatograms showed that virtually all of the radioactivity was associated with the tetrahydrojatrorrhizine band.

The Berberis cultures used in Zenk's laboratory produce essentially the same pattern and distribution of protoberberines as does ours.[3,9] Since the report of Amann et al. provided no experimental descriptions, it is not possible to speculate on causes for the contradictory results.

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