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This report is the 84th paper on ergot alkaloids. For the previous report in this series, see E. Schreier, *Helv. Chim. Acta*, **59**, 585 (1976).

Biosynthesis of Morphine Alkaloids in *Papaver bracteatum* Lindl.

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Abstract □ Administration of 1-^{3}H-*N*-methyl-^{14}C-(±)-reticuline to *Papaver bracteatum* gave good incorporation of carbon-14 into thebaine and a decrease in the tritium to carbon-14 ratio indicative of racemization. The incorporation of carbon-14 and the extent of tritium loss were the same whether reticuline was administered to the intact plant or to isolated leaves. Carrier dilution with cold codeine, codeinone, and morphine showed only insignificant incorporation of radioactivity into codeine and none at all into codeinone and morphine. When codeinone was administered to the living plant, it was converted to codeine rapidly and efficiently, but no *O*-demethylation to morphine could be detected. The experimental data indicate that the biosynthesis of thebaine in *P. bracteatum* proceeds by the same pathway as in the opium poppy. The limiting step in the sequence is the demethylation of the enol ether group of thebaine to neopinone.

Keyphrases □ Morphine alkaloids—biosynthesis in *Papaver bracteatum*, radiochemical study □ Alkaloids, morphine—biosynthesis in *Papaver bracteatum*, radiochemical study □ Biosynthesis—morphine alkaloids in *Papaver bracteatum*, radiochemical study □ *Papaver bracteatum*—biosynthesis of morphine alkaloids, radiochemical study □ Radiochemistry—study of biosynthesis of morphine alkaloids in *Papaver bracteatum*

In 1963, Neubauer and Mothes (1) reported on a strain of *Papaver bracteatum* Lindl. that produced thebaine in high yield but apparently contained neither codeine nor morphine. *P. bracteatum* is closely related to *P. orientale* and *P. pseudo-orientale*, which do not synthesize significant amounts of thebaine. They can be differentiated from *P. bracteatum* by cytological examination (2) and chemotaxonomic tests (3). In contrast to the opium poppy (*P. somniferum*), which produces a large number of alkaloids, several in appreciable concentrations, *P. bracteatum* contains mainly thebaine, which may account for 98% of the alkaloid content[1] (1, 4). Isolation and purification of thebaine from *P. bracteatum* are, therefore, relatively simple.

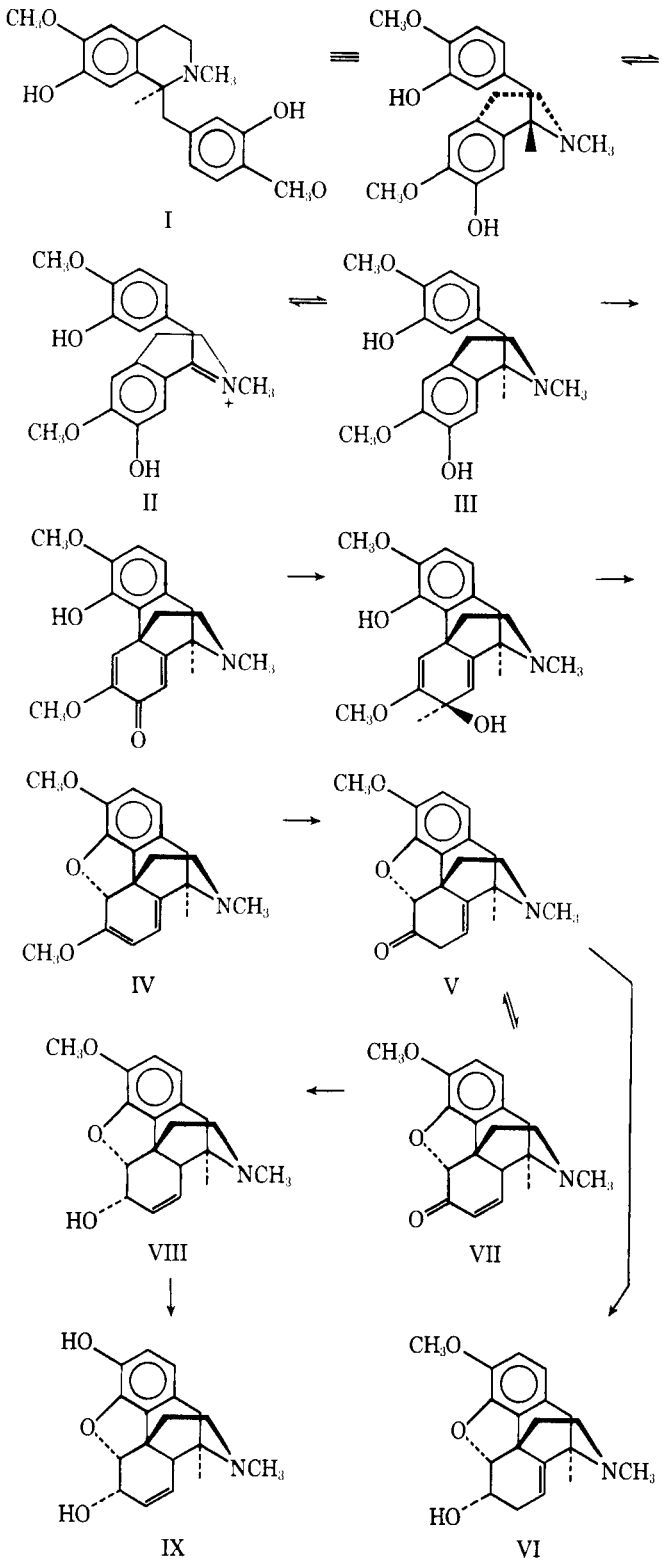
In recent years, considerable interest has developed in this plant as a source of thebaine; in the laboratory, thebaine can be converted to codeine and other narcotic analgesics and antitussives (6–8). Thebaine is also the raw material for naloxone and related narcotic antagonists and for the interesting *endo*-ethenotetrahydrothebaines (9). In 1972, a collaborative research project on *P. bracteatum* was initiated by the United Nations Narcotics Laboratory, which has been coordinating investigations carried out in many countries (10).

The purposes of the present investigation were to study the biosynthetic pathways of hydrophenanthrene alkaloids in *P. bracteatum* and to explore possible sites of biosynthesis.

BACKGROUND

The biosynthesis of morphine alkaloids in the opium poppy has been

[1] Several varieties, or chemical races, of *P. bracteatum* have been found, differing somewhat in alkaloid composition. The Arya II variety from western Iran has a particularly high content of thebaine (5).



Scheme I

described in detail (11–17). The last few steps of the pathway in *P. somniferum* are illustrated in Scheme I. Reticuline produced from L-tyrosine has the (*S*)-configuration and is dextrorotatory (I). Battersby *et al.* (13) demonstrated that reticuline undergoes racemization in the plant, a reaction essential for the formation of the morphine alkaloids derived from (−)-(*R*)-reticuline (III). This racemization is enzymatic and substrate specific (18) and proceeds by a reversible oxidation–reduction mechanism *via* the 1,2-dehydro derivative (II) (13). While *O*-demethylation of the enol ether group of thebaine (IV) is an enzymatic reaction, the rearrangement of neopinone (V) to codeinone (VII) does not require an enzyme. In aqueous solution, an equilibrium that favors codeinone is established (19).

Table I—Tracer Experiments on *P. bracteatum* with 1-^{3}H-*N*-Methyl-^{14}C-(±)-reticuline

Administration	Incorporation of ^{14}C, %, into Thebaine	Codeine[a]	Codeinone[a]	Morphine[a]
Intact plant by wick	12.03[b]	0.002	0.000	0.000
Isolated shoots	12.08[c]	0.000	—	0.000
Root of whole plant:				
Root	0.30[d]	—	—	—
Shoot	0.06[e]	—	—	—
Root slices	1.60[f]	—	—	—

[a] Isolated by dilution with cold carrier. [b] 4696 dpm/mg (^{14}C); 53.6% ^{3}H-loss. [c] 41,888 dpm/mg (^{14}C); 54.2% ^{3}H-loss. [d] 1071 dpm/mg (^{14}C); 55.3% ^{3}H-loss. [e] 267 dpm/mg (^{14}C); 58.9% ^{3}H-loss. [f] 2762 dpm/mg (^{14}C); 58.0% ^{3}H-loss.

Very few biosynthetic studies have been performed with *P. bracteatum*. Neubauer (20) showed that short-term (6 hr) exposure of leaves to 2-^{14}C-DL-tyrosine gave incorporation of radioactivity into thebaine with a proper labeling pattern. Nordal *et al.* (21) obtained radioactive thebaine from uniformly labeled phenylalanine, tyrosine, glycine, and urea when the radioactive substances were applied to the surface of leaves and stems in a surfactant solution.

To obtain more detailed information about the biosynthetic pathway, reticuline was chosen as the precursor because—unlike the amino acids—it is not a primary metabolite and its racemization plays a crucial part in the biosynthesis of morphine in the opium poppy. Racemization was studied by means of double-labeled (±)-reticuline containing a ^{14}C-*N*-methyl, a ^{3}H-label at the asymmetric center, and a known ratio of specific activities. Previously (12, 13), it was demonstrated that no significant *N*-demethylation takes place in the biosynthesis of thebaine from reticuline in the opium poppy.

If the isotope ratio of the biosynthetic product remains unchanged, no racemization has taken place and only the *levo*-enantiomer has been utilized in the reaction. A ^{3}H-loss of 50% or less means that racemization has occurred, but it may only have affected the dextrorotatory isomer. If the ^{3}H-loss is greater than 50%, even the levorotatory isomer of required stereochemistry must have been subjected to some racemization and, consequently, the reaction is reversible. The labeling pattern of isolated thebaine was established by rearrangement to thebenine (11), followed by exhaustive methylation and Hoffman degradation. Trimethylamine carrying the ^{14}C-label was isolated as benzyltrimethylammonium bromide.

To determine the limiting step in the biosynthesis of hydrophenanthrene alkaloids in *P. bracteatum*, feeding experiments also were performed with codeinone. This alkaloid was administered as the nonradioactive substance. The alkaloids produced by the plant were isolated and identified by GLC and IR spectroscopy.

In young plants of *P. bracteatum*, the thebaine content increases most rapidly in the roots and tends to stabilize there after a few weeks, whereupon the thebaine concentration increases in the shoots (22). It was suggested, therefore, that thebaine is biosynthesized in the root and translocated to the shoots. As the plant reaches maturity during its 2nd year of growth, the highest concentration of thebaine is found in the capsule, reaching a peak 4–6 weeks after flowering (8, 23). If the plant is disbudded and thereby prevented from flowering and forming capsules, most thebaine remains in the roots, which increase in size and weight (7).

RESULTS AND DISCUSSION

The results of the feeding experiments indicate that the biosynthesis of thebaine in *P. bracteatum* occurs by the same route as in the opium poppy. Double-labeled (±)-reticuline showed good incorporation into thebaine by several methods of administration (Table I). The plant is capable of racemization of reticuline, and the reaction is reversible as indicated by a ^{3}H-loss greater than 50%. Chemical degradation of thebaine showed that all ^{14}C-activity was located in the *N*-methyl group (Table II). Rearrangement to thebenine took place without loss of tritium. Immature plants fed (±)-reticuline through their leaves gave thebaine with the highest specific ^{14}C-activity (~42,000 dpm/mg), but the percent incorporation was the same as that obtained by wick feeding because of the much larger amount of thebaine present in the whole plants.

The negligible incorporation of radioactivity into codeine (VIII), codeinone (VII), and morphine (IX), isolated by dilution with cold carriers, indicates that these alkaloids are not present in the plant. However,

Table II—Controlled Degradation of Radioactive Thebaine

Fragment	Relative ^{14}C-Activity in Isolated Fragment, %	^{3}H-Loss
Methebenine methosulfate	99.6	0.00
Benzyltrimethylammonium bromide	99.5	100.0

Küppers *et al.* (24) recently reported the isolation of trace amounts (0.003–0.004%) of neopine (VI) and codeine from the Arya I variety of *P. bracteatum*. Unequivocal detection of such small amounts by the isotope dilution method requires a precursor of much greater specific activity than was used in these experiments. Nevertheless, the small incorporation of reticuline into codeine is consistent with the results of Küppers *et al.* (24).

When codeinone was administered to the intact plant and to isolated leaves, rapid and efficient reduction to codeine took place, as demonstrated by actual isolation of codeine (Table III). Little or no codeinone could be recovered unchanged. The difference between the amount of codeinone administered and the amount of codeine isolated was due primarily to losses during the extensive purification of codeine. No morphine could be detected. Therefore, the limiting step in the biosynthetic sequence apparently is the *O*-6-demethylation of thebaine to neopinone. In view of the large amount of thebaine present in *P. bracteatum* and the facile reduction of the ketones, as demonstrated for codeinone, the minute amounts of codeine and neopine reported by Küppers *et al.* (24) perhaps may be best explained by nonenzymatic hydrolysis of the enol ether group of thebaine because of the acidic conditions prevailing in the plant. Neopinone and codeinone produced in this way would be rapidly reduced to the corresponding alcohols in the living plant.

The feeding experiments with young, isolated leaves prior to stem formation make it clear that alkaloid biosynthesis occurs at a fast rate in the aerial parts (Table I). When labeled reticuline was fed through the roots of young plants placed in vermiculite and the roots and shoots were analyzed separately, significant incorporation of radioactivity into thebaine could be observed in the roots. A much smaller incorporation was found in the aerial parts, perhaps because the time of the experiment was too short (2 days) for an appreciable translocation from the roots.

High incorporation of radioactivity into thebaine was achieved when the roots were cut into small sections and allowed to remain in contact with radioactive reticuline solution. This result was undoubtedly due to the larger surface area and the direct contact of the precursor with the enzymes exposed by the cross-sections of the roots. Therefore, alkaloid biosynthesis in *P. bracteatum* is not limited to any one part of the plant. As in the opium poppy, alkaloid biosynthesis probably takes place in the laticiferous vessels that form an anastomotic network throughout the plant (25, 26). This finding is consistent with the fact that the latex that accumulates in the capsule after petal drop is the single richest source of thebaine, containing as much as 20% on a dry weight basis (27).

EXPERIMENTAL[2]

Synthesis of Precursors—*1-^{3}H-N-Methyl-^{14}C-(±)-reticuline*—*N*-Methyl-^{14}C-(±)-*O,O*-dibenzylreticuline was prepared as described previously (28); the specific activity was 1.65 mCi/mmole.

1-(3-Benzyloxy-4-methoxybenzyl)-7-benzyloxy-6-methoxy-3,4-dihydroisoquinoline methiodide (200 mg) (28) was dissolved in 25 ml of anhydrous dimethyl sulfoxide and reduced with 8 mCi (1.6 mg) of sodium ^{3}H-borohydride overnight. Cold sodium borohydride was added to complete the reduction, and the product was isolated and purified by crystallization to give 166 mg of pure 1-^{3}H-(±)-*O,O*-dibenzylreticuline, specific activity 14.00 mCi/mmole.

N-Methyl-^{14}C-(±)-*O,O*-dibenzylreticuline (667 mg) and 1-^{3}H-(±)-*O,O*-dibenzylreticuline (150 mg) were dissolved in 50 ml of hydrochloric acid (38%) in a 250-ml round-bottom flask. Then 50 ml of benzene was added, and the mixture was stirred vigorously under nitrogen for about 14 hr at room temperature (29). The acid layer was separated, cooled in ice water, and made alkaline to pH 13 with 6 *N* NaOH. After extraction with ether, the aqueous phase was adjusted to pH 9 with ammonium chloride and extracted several times with ether. The ether extracts obtained at pH 9 were dried over anhydrous sodium sulfate and evaporated to dryness. The residue (428 mg) was purified by repeated precipitation from ether–petroleum ether until pure by TLC on silica gel and GLC[3] of the base and its bis(trimethylsilyl) derivative on OV-1 (3%); specific activities were: ^{14}C, 1.35 mCi/mmole; ^{3}H, 2.57 mCi/mmole; and ^{3}H to ^{14}C ratio, 1.90.

Table III—Biosynthetic Conversion of Codeinone to Codeine in *P. bracteatum*

Administration	Codeinone, mg Administered	Codeinone, mg Recovered	Codeine Isolated, mg
Intact plant by wick	6	0	2
Isolated shoots	30	Trace	21

Codeinone—This compound was prepared by oxidation of codeine with manganese dioxide as described by Highet and Wildman (30). It was purified by chromatography on alumina[4] with benzene containing 20% chloroform and by crystallization from benzene–hexane until free of codeine as demonstrated by GLC on OV-1 (3%), mp 185°.

Cultivation of Plants and Administration of Labeled Reticuline—Seeds[5] of *P. bracteatum*, Arya II variety, were sown on free land in December 1974 and in flowerpots in a greenhouse in January 1976. The precursor solutions were prepared by dissolving the compound in an equivalent amount of 0.1 *N* H_2SO_4 and diluting with water to a concentration of 1 mg/ml.

Wick Feeding—A cotton thread was inserted through the main stem near its base of 12 plants, 16–18 months old, as they were just beginning to blossom. Both ends of the thread were twisted together and placed in a vial containing the precursor solution. The amount of precursor per plant ranged from 1 to 3 mg, depending on plant size. The plants were harvested after 10 days and placed in a freezer until they could be extracted.

Feeding of Isolated Shoots—Sixty leaves from 30 immature plants, about 15 months old, were cut off near the roots and immediately placed in a beaker of water. About 1 cm of the end of the leaf stem was cut off under water and the leaf was transferred, with a drop of water clinging to the end, to a small test tube, 15 mm i.d. × 10 cm long. The test tube contained 0.25 mg of labeled reticuline in 1 ml of water. Two leaves were placed in each test tube. Water was added as needed during the experiment, and the leaves were harvested when they showed signs of wilting (36–48 hr).

Root Feeding of Whole Plants—Four immature plants, about 15 months old, were carefully removed from the soil, rinsed with water, and placed in moist vermiculite in test tubes. A solution of the precursor containing 3 mg of labeled reticuline was added to each test tube around the root system. The plants were harvested after 2 days when they began to wilt. About 1 cm of the intersection between the root and the shoot was removed, and the roots and the leaves were extracted separately.

Tracer Experiments with Root Slices—Six immature plants, about 15 months old, were removed carefully from the soil and rinsed with water. The shoots were removed, including about 1 cm of the uppermost part of the root. The rest of the root was cut into 1-cm slices, which were placed in a dark-brown glass bottle and moistened with a solution containing 10 mg of labeled reticuline. The bottle was rotated several times a day for 4 days to allow the root pieces to contact the radioactive precursor solution.

Extraction, Separation, and Purification of Alkaloids—The fresh or frozen plants were extracted with methanol in a high-speed blender[6], and the cold alkaloid carriers were added. The suspension was poured into a glass percolator and extracted with methanol until the extract gave negative tests for alkaloids. The extract was concentrated in a rotary vacuum evaporator to remove the methanol, transferred to a separator, and shaken with several small portions of ethyl acetate. The combined ethyl acetate extracts were washed three times with 0.5 *N* HCl, and the acid washings were combined with the original aqueous solution (total alkaloids).

The total alkaloid solution was cooled in ice water, 6 *N* NaOH was added to pH 13, and the solution was extracted several times with chloroform. Chloroform evaporation gave the nonphenolic alkaloids. The aqueous solution containing the phenolic alkaloids was adjusted to pH 9 with ammonium chloride and extracted with 50, 30, and 20 ml of chlo-

[2] All melting points are uncorrected.

[3] Varian Aerograph model 2100 gas chromatograph with glass column, 1.82 m (6 ft) long, 2 mm i.d.

[4] Neutral aluminum oxide, Woelm, activity III.

[5] Obtained from the United Nations Narcotic Laboratory, courtesy of Dr. O. J. Braenden.

[6] Waring Products Co.

roform–isopropyl alcohol (3:1). The organic extract was shaken, first with 10 ml and then with 5 ml of 0.1 *N* NaOH.

The combined alkaline extracts were placed in a 50-ml glass-stoppered flask and 20 ml of ether, 500 mg of ammonium chloride, and 1 ml of pH 9.0 ammonium chloride–ammonium hydroxide buffer were added. The flask was stoppered, shaken vigorously for about 2 min, and placed in a refrigerator for crystallization of morphine. The crystals were collected and purified by crystallization from methanol to constant radioactivity (31).

The fraction containing the nonphenolic alkaloids was separated by chromatography on alumina[4] with benzene containing 10% chloroform. The elution was monitored by micro-TLC. After thebaine had been collected, the concentration of chloroform in the eluting solvent was increased gradually to 35% for elution of codeinone and codeine. Thebaine was purified by fractional crystallization from benzene–hexane to constant radioactivity. Codeinone and codeine were first purified by preparative TLC on alumina with 8% ethanol in benzene. Codeinone was then crystallized from benzene–hexane to constant radioactivity. Codeine was converted to 6-acetylcodeine by means of acetic anhydride and pyridine and crystallized from hexane to constant radioactivity.

Feeding Experiments with Codeinone—In one experiment, 6 mg of codeinone was administered as the sulfate to a mature flowering plant by cotton wick. The plant was harvested after 7 days and extracted as described. GC of the total alkaloid extract (116 mg) revealed thebaine as the major component with smaller amounts of several other alkaloids. One minor alkaloid had the same retention time as codeine. The gas chromatogram showed no detectable amount of codeinone.

The major portion of thebaine was removed by crystallization from ether, and the minor alkaloids were separated by preparative TLC on silica gel with chloroform–methanol (9:1) into three fractions, one of which contained codeine as its major component. This fraction was further purified by TLC on silica gel with benzene–ethanol (8:2) (double development) to afford pure codeine (2 mg), identified by GLC of the base and of its trimethylsilyl derivative on two stationary phases of different polarity (OV-1 and OV-17). There was no evidence of morphine.

In another experiment, 30 mg of codeinone was administered to 66 large leaves cut from 35 5-month-old plants as described for reticuline. The alkaloids were extracted and separated into phenolic and nonphenolic fractions. The nonphenolic fraction (172 mg), consisting mainly of thebaine, displayed a distinct peak on the gas chromatogram corresponding to codeine but showed only a trace of codeinone. It was separated by preparative TLC on alumina with benzene–ethanol (92:8) into six fractions, one of which was almost pure codeine (21 mg), identified by GLC and IR spectroscopy. The phenolic fraction (91 mg) was purified by preparative TLC on silica gel with chloroform–methanol (9:1). No morphine could be detected by GLC of the fractions obtained.

The same extraction and separation procedures were applied to 45 leaves of *P. bracteatum* that had not received codeinone. No codeine could be detected by GLC of the total nonphenolic fraction or of the fractions obtained by preparative TLC on silica gel with chloroform–methanol (9:1).

Degradation of Thebaine—Thebaine (200 mg) was dissolved in a mixture of 2 ml of 15% hydrochloric acid and 0.4 ml of methanol and refluxed under nitrogen for 2 min. The solution was cooled, and the liquid was decanted from the gummy precipitate of thebenine. Thebenine was methylated at room temperature in a nitrogen stream with dimethyl sulfate. Sodium hydroxide solution (11 *N*) was added dropwise to maintain the pH of about 10. The reaction mixture was cooled in the refrigerator overnight to produce green crystals of methebenine methosulfate (97 mg), mp 270–273° [lit. (11) mp 273–275°]. It was recrystallized from methanol–ether to constant radioactivity.

Methebenine methosulfate (88 mg) was placed in a two-necked flask fitted with a reflux condenser and a nitrogen inlet tube. The top of the condenser was connected to a flask containing 1 ml of benzyl bromide in 10 ml of methylene chloride. A slow nitrogen stream was passed into the reaction flask, bubbling through the solution of benzyl bromide. Five milliliters of 6 *N* NaOH was added to the reaction flask, and the solution was refluxed overnight. The methylene chloride solution was concentrated, and benzyltrimethylammonium bromide was precipitated with ether. The precipitate (26 mg) was recrystallized from ether–chloroform to constant radioactivity, mp 232–234° [lit. (32) mp 235°].

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