BIOSYNTHESIS OF OPIUM ALKALOIDS: THE EFFECTS OF STRUCTURAL MODIFICATION OF RETICULINE ON RACEMIZATION AND BIOTRANSFORMATION

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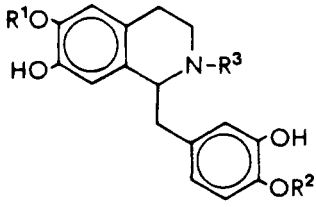
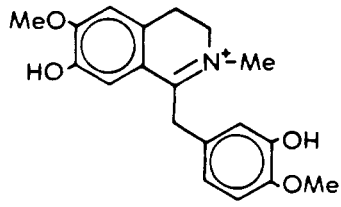
ABSTRACT.—The enzymatic racemization of reticuline, so essential to the biosynthesis of opium alkaloids, is very substrate specific and is completely blocked by minor structural modifications. The *N*-ethyl, 6-ethoxy and 4'-ethoxy analogues of reticuline are completely resistant to racemization, while they permit biotransformation of the R(−)-enantiomers to hydrophenanthrene alkaloids.

Biosynthetic reactions of alkaloids exhibit varying degrees of substrate specificity and stereoselectivity. The racemization of reticuline (**1**), which is an essential step in the biosynthesis of hydrophenanthrene alkaloids in *Papaver somniferum*, shows little or no stereoselectivity (1,2) while being amazingly substrate specific (3). Earlier work has indicated that an *N*-substituent and the 7 and 3' hydroxyl groups are essential features for racemization, but it is not known which roles the 6 and 4' methoxyl groups and the *N*-methyl group play or to what extent they may be modified without interfering with racemization and further biotransformation.





1: $R^1 = R^2 = R^3 = Me$

2: $R^1 = Et$, $R^2 = R^3 = Me$

3: $R^2 = Et$, $R^1 = R^3 = Me$

4: $R^3 = Et$, $R^1 = R^2 = Me$

5

In an effort to gain greater insight into this important reaction and, at the same time, obtain information on the specificity of the enzymes catalyzing the biotransformation of reticuline, feeding experiments were carried out with three close structural analogues of reticuline. The structural modifications consisted of replacing the *N*-methyl group by an ethyl group and the methoxy groups by ethoxy groups. These unnatural substrates represented by structures **2**, **3**, and **4** were labeled by ^{14}C at position 3 and by ^{3}H at the asymmetric center (C–1) and administered to separate batches of *Papaver somniferum* as the racemic mixtures. Since racemization proceeds *via* the 1,2-dehydroreticulinium ion (**5**) (1,4) loss of ^{3}H, as observed by a decrease of the $^{3}H:{}^{14}C$ ratio, would be indicative of racemization. Dilution with cold carriers was used for isolation of residual precursors and their unnatural metabolites. A control experiment carried out at the same time with 3–^{14}C, 1–^{3}H–(±)–reticuline ($^{3}H:{}^{14}C = 16.80:1$) gave radioactive morphine with a ^{3}H to ^{14}C ratio of 6.61:1, corresponding to a ^{3}H loss of 60.7%.

Table 1. Results of feeding experiments with *Papaver somniferum*.

Expt. No.	Compound fed	Amt. fed ^{14}C μCi	^{3}H: ^{14}C	^{14}C Incorporation (%) and/or ^{3}H retention (%) in								
				Residual Precursor[a]	Morphine-3-ethyl ether[a]		Morphine		N-Ethylnor-codeine[a]		N-Ethylnor-morphine[a]	
				^{3}H ret. (±1%)	^{14}C inc.	^{3}H ret.	^{14}C inc.	^{3}H ret	^{14}C inc.	^{3}H ret.	^{14}C inc.	^{3}H ret.
1	(±)-6-Ethyl-4'-methyl-laudanosoline[b] (**2**)	6.60	29.26	100	—	—	3.28	100	—	—	—	—
2	(±)-4'-Ethyl-6-methyl-laudanosoline[b] (**3**)	16.40	12.40	100	0.44	100	0.56	100	—	—	—	—
3	(±)-N-Ethylnor-reticuline[b] (**4**)	1.15	6.70	100	—	—	—	—	—	—	0.71	100
4	(±)-N-Ethylnor-reticuline[c] (**4**)	74.0	—	—	—	—	—	—	0.65	—	0.95	—

[a]Isolated by carrier dilution. [b][3-^{14}C, 1-^{3}H]. [c][3-^{14}C].

RESULTS AND DISCUSSION

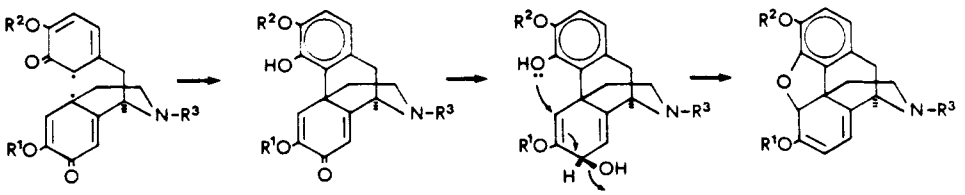
The results of the feeding experiments are illustrated in table 1. In the experiments in which double labeling was used, the extracted unmetabolized substrates and their natural and unnatural metabolites had the same ^{3}H: ^{14}C activity as the substrates before administration. Therefore, no racemization had taken place with any of the structural modifications of reticuline, thus confirming the exceptional substrate specificity of this reaction. However, the structural modifications did not prevent the reticuline analogous from being metabolized along the same pathway as reticuline itself, although the rate of biotransformation appeared to be somewhat reduced. Morphine and *N*-ethylnormorphine, which were isolated from the feeding experiments, had the ^{14}C label in the appropriate position (C–16) proving that the biotransformations had taken place without scrambling of the label which might have resulted from decomposition and reassembly of smaller fragments (table 2). Our earlier work has shown that both

TABLE 2. Controlled degradation of radioactive alkaloids.

Compound fed	Alkaloid degraded	Rel. ^{14}C activity at C–16 (%)
(±)-6-Ethyl-4'-methyl-landanosoline[a]	Morphine	98.9
(±)-4'-Ethyl-6-methyl-landanosoline[a]	Morphine	100.0
(±)-N-Ethylnor-reticuline[a]	*N*-Ethylnormorphine	98.1
(±)-N-Ethylnor-reticuline[b]	*N*-Ethylnormorphine	99.2

[a][3-^{14}C,1-^{3}H]. [b][3-^{14}C].

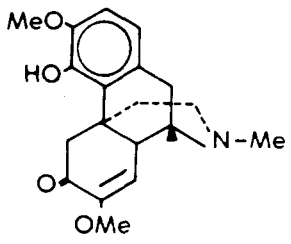
the 3-ethyl and 6-ethyl analogues of thebaine are efficiently converted to morphine in the opium poppy (5,6). The present study indicates that the same is true for the *N*-ethyl analogue. Moreover, it also shows that the biradical coupling to the salutaridine analogues followed by stereoselective reduction to the corresponding dienols and rearrangement to the thebaine analogues are not seriously affected by minor structural modifications of reticuline (scheme 1). On the



SCHEME 1.

other hand, enantiomeric modification due to a chiral center changes the geometry of the molecule in a profound way which is more important than minor changes in structure. It is, therefore, reasonable to conclude that only the R(−)-enantiomers of the reticuline analogues participated in the biosynthesis of the morphine alkaloids. Oxidative phenol coupling of S(+)-reticuline to sinoacutine, the enantiomer of salutaridine, has been reported in *Sinominium acutum* (7). Sinoacutine is further transformed to sinomenine (**6**) (8). There is no evidence that these reactions operate in the opium poppy (9). Consequently, the actual incorporations of the R-enantiomers in the biosyntheses are twice as great as the values reported in table 1, in which incorporations have been calculated on the basis of the racemic mixtures which were administered.

The most interesting and potentially promising results were obtained with the



6

N-ethyl analogue of reticuline which gave good incorporation into *N*-ethylnormorphine (Experiment 3). This experiment was repeated with a precursor of greater specific activity and a time of 10 to 14 days between feeding and harvesting, compared to 3 to 5 days in experiment 3. Single ^{14}C labeling was used in experiment 4. Radioactive *N*-ethylnorcodeine and *N*-ethylnormorphine were isolated with good incorporation of ^{14}C. The bioconversion of *N*-ethylnorreticuline to *N*-ethylnormorphine suggests the interesting possibility of biosynthetic production of narcotic antagonists. Experiments along this line are in progress.

Morphine isolated from opium poppies which had been fed 3-^{14}C, 1-^{3}H-*N*-ethylnor-reticuline (Experiment 3) contained a level of radioactivity which was barely detectable and a ^{3}H: ^{14}C ratio of 2.3:1, indicating loss of ^{3}H. The incorporation of ^{14}C into morphine amounted to 0.03%. Papaverine isolated from the same batch of plants was also slightly radioactive (incorporation 0.02%). It is most logical to explain these observations by assuming that the precursor contained a trace of nor-reticuline. In the plant, nor-reticuline is *O*-methylated to tetrahydropapaverine which is the immediate precursor of papaverine (3) and *N*-methylated to reticuline which racemizes, thereby losing ^{3}H, before being converted to morphine.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The Noordster variety of *Papaver somniferum* was used in these txperiments. The methods for cultivation of the plants and administration of the labeled substrates have been described in previous communications (10,11). All melting points were determined with the Thomas-Hoover melting point apparatus and were uncorrected. The nmr spectra were determined in deuteriochloroform with tetramethylsilane as internal standard.

SYNTHESIS OF LABELED SUBSTRATES.—Several practice runs were always made with nonradioactive materials prior to the synthesis of the labeled compounds. The nonradioactive intermediates and final products were characterized by spectroscopic and chromatographic methods and by melting points. The radioactive compounds were identified by comparison with the nonradioactive compounds by tlc and glc.[1] Double labeled compounds were made by mixing single labeled compounds in a known proportion. The calculated ^{3}H: ^{14}C ratio was compared with that determined by actual double label counting in a liquid scintillation counter, the latter being used for calculation of ^{14}C incorporation and ^{3}H retention in the isolated alkaloids.

3-^{14}C,1-^{3}H-(±)-N-Ethylnor-reticuline (4).—1-(3-Benzyloxy-4-methoxybenzyl)-7-benzyloxy-6-methoxy-3,4-dihydroisoquinoline hydrochloride (12,13) (200 mg) was reduced with 5 mg (0.9 mCi) of sodium ^{3}H-borohydride in anhydrous 2-propanol under a head of nitrogen. After 4 hr at room temperature, sodium borohydride was added in small portions to complete the reduction. The product was isolated and purified by crystallization from ethanol to give 135 mg of pure 1-^{3}H-(±)-*O,O*-dibenzylnor-reticuline, specific activity, 1.35 mCi/mmole. 3-^{14}C(±)-*O,O*-Dibenzylnor-reticuline was prepared as described previously (11), specific activity, 0.116 mCi/mmole. 3-^{14}C, 1-^{3}H-(±)-*O,O*-Dibenzylnor-reticuline was obtained by mixing the ^{14}C-labeled compound with enough ^{3}H-labeled compound to give a ^{3}H: ^{14}C ratio of about 7:1. The mixture was dissolved in 0.5 ml of ethanol and 0.5 ml of concentrated hydrochloric acid, refluxed for 1 hr and evaporated to dryness under reduced pressure. The residue was crystal-

[1]The Varian 2100 gas chromatograph was used. The columns were 6 ft long and 2 mm I.D. and packed with OV-1 (3%) and with OV-17 (3%) on high performance Chromosorb W, 100-120 mesh.

lized from ethanol. 3-^{14}C,1-^{3}H-(±)-Nor-reticuline (42 mg), obtained from its hydrochloride salt, was dissolved in 3 ml of acetic anhydride. Pyridine (0.3 ml) was added and the mixture stirred at 120° under nitrogen for 3 hr. Water was added to decompose excess acetic anhydride and the solution was extracted with chloroform. The chloroform extract was washed several times with 0.5 *N* hydrochloric acid, then with dilute ammonia solution (2%) and, finally, with water. The chloroform solution was dried and evaporated to dryness. The residue of triacetylnor-reticuline was dissolved in 4 ml of anhydrous tetrahydrofuran (THF) and the solution added dropwise to a stirred suspension of lithium aluminum hydride (25 mg) in 3 ml of anhydrous THF. The mixture was stirred overnight at room temperature. The reaction was quenched with a saturated solution of potassium sodium tartrate in water. The precipitate was removed by filtration and washed with chloroform. The combined filtrate and chloroform washings were evaporated to dryness. The residue was dissolved in 0.1 *N* hydrochloric acid, the solution washed with chloroform and adjusted to pH 13-14 with aqueous potassium hydroxide solution. The alkaline solution was washed with chloroform, adjusted to pH 8.5-9.0 with solid ammonium chloride and extracted several times with ether. The combined ether extracts were dried over anhydrous sodium sulfate and evaporated to dryness. The residue (19.2 mg) was identical with a nonradioactive sample of *N*-ethylnor-reticuline prepared for carrier dilution, specific activity: ^{14}C, 0.0205 mCi/mmole; ^{3}H, 0.1396 mCi/mmole; ^{3}H: ^{14}C=6.80:1.

Another batch of 3-^{14}C-(±)-*N*-ethylnor-reticuline was prepared in the same way as described above; specific activity: ^{14}C, 0.566 mCi/mmole.

3-^{14}C,1-^{3}H-(±)-6-Ethyl-4'-methyl-laudanosoline (**2**).—The synthesis of this double labeled 6-ethoxy analogue of reticuline was carried out by standard methods (2,11,12), 3-ethoxy-4-hydroxybenzaldehyde being used instead of vanillin as the starting material for ring A. The compound was identical with the nonradioactive compound prepared as a carrier; specific activity: ^{14}C, 0.126 mCi/mmole; ^{3}H, 3.687 mCi/mmole; ^{3}H: ^{14}C=29.26:1.

3-^{14}C,1-^{3}H-(±)-4'-Ethyl-6-methyl-laudanosoline (**3**).—This doubly labeled 4'-ethoxy analogue of reticuline was prepared in the same way as reticuline (2,11,12), 4-ethoxy-3-hydroxybenzaldehyde being used instead of isovanillin as the starting material for ring C. The compound was identical with the nonradioactive compound prepared for carrier dilution, specific activity: ^{14}C, 0.303 mCi/mmole; ^{3}H, 3.757 mCi/mmole; ^{3}H: ^{14}C=12.40:1.

SYNTHESIS OF UNNATURAL ALKALOIDS FOR REVERSE ISOTOPE DILUTION.—(±)-*N*-Ethylnor-reticuline was synthesized as described for the labeled substrate with the exception that the acetylation and reduction were performed before debenzylation. (±)-*O,O*-Dibenzylnor-reticuline (192 mg) was acetylated with acetic anhydride and pyridine. The resulting amide was crystallized from absolute ethanol, yield 162 mg, mp 128-129.5°, pure by tlc. Reduction with lithium aluminum hydride gave a crude product which was chromatographed on a column of silica gel with benzene-chloroform (9:1); ^{1}H nmr, δ 1.13 (t, N-CH$_2$*CH$_3$*, *J*=7.0 Hz), 2.70 (q, N-*CH$_2$*-CH$_3$, *J*=7.0 Hz), 3.83 (s, OCH$_3$), 4.57 (s, 7-O*CH$_2$*C$_6$H$_5$), 5.07 (s, 3'-O*CH$_2$*C$_6$H$_5$), 5.94-7.45 (m, arom. H's); ms (ei) *m/e* 523 M$^+$, C$_{34}$H$_{37}$O$_4$N). *O,O*-Dibenzyl-*N*-ethylnor-reticuline (173 mg) was dissolved in 10 ml of concentrated hydrochloric acid, 25 ml of benzene was added, and the mixture was stirred vigorously under nitrogen at room temperature (14). After 10 hr, 20 ml of water was added and the two layers separated. The acid solution, when cooled in ice water and neutralized with concentrated aqueous ammonia to pH 8.5-9 and extracted several times with chloroform, gave 132 mg of a crude product, which was purified by column chromatography on silica gel with chloroform containing increasing concentrations of methanol ranging from 1 to 4%. Finally, the product was crystallized from benzene-hexane; mp 85-88°; ^{1}H nmr, δ 1.12 (t, N-CH$_2$-*CH$_3$*), 2.73 (q, N-*CH$_2$*-CH$_3$), 3.84 and 3.86 (2s, OCH$_3$), 6.26-6.75 (m, arom. H's); ms (ei), *m/e* 341 (M-2)$^+$.

N-Ethylnormorphine.—Morphine was *N*-demethylated as described previously (5,15), and normorphine, when acetylated with acetic anhydride and pyridine, yielded *N,O,O*-triacetylnormorphine, which was reduced with lithium aluminum hydride in anhydrous THF to give *N*-ethylnormorphine. It was purified by crystallization from 50% aqueous ethanol, mp 220-223° (dec.); ^{1}H nmr, δ 1.18 (t, N-CH$_2$*CH$_3$*, *J*=7.2 Hz), 1.75-3.10 (m. incl. N-*CH$_2$*CH$_3$), 4.86 and 4.93 (2d, H-5 and H-6, *J*=1.0 Hz), 5.25 and 5.54 (2dd, H-7 and H-8), 6.60 (2d, H-1 and H-2, *J*=8 Hz); ms (ei) *m/e* 299 (M$^+$). *N*-Ethylnorcodeine was prepared from *N*-ethylnormorphine by methylation with trimethylanilinium hydroxide (16) and crystallized from benzene, mp 97-100°; ^{1}H nmr, δ 1.12 (t, N-CH$_2$*CH$_3$*, *J*=7.2 Hz), 2.47 (q, N-*CH$_2$*CH$_3$, *J*=7.2 Hz), 3.82 (s, OCH$_3$), 5.25 and 5.67 (2dd, H-7 and H-8), 6.58 (2d, H-1 and H-2, *J*=8 Hz); ms (ei) *m/e* 313 (M$^+$). (±)-6-Ethyl-4'-methyl-laudanosoline was prepared as described for the labeled substrate. It was purified by column chromatography on silica gel with chloroform containing increasing concentrations of methanol ranging from 2-10% and, finally, crystallized from ether-hexane, mp 87-89°; ^{1}H nmr, δ 1.40 (t, 6-OCH$_2$*CH$_3$*, *J*=7.2 Hz), 2.43 (s, N-CH$_3$), 2.61-3.25 (m, 6H), 3.65 (t, 1H, *J*=6.0 Hz), 3.82 (s, 4'-OCH$_3$), 4.05 (q, 6-O*CH$_2$*CH$_3$, *J*=7.2 Hz), 6.40-6.77 (m, arom. H's); high resolution ms (ei), *m/e* 341.1609 ((M-2)$^+$, C$_{20}$H$_{23}$O$_4$N, 10%), 206.1176 (C$_{12}$H$_{16}$O$_2$N, 100%). It was found that M$^+$ peaks of reticuline analogues and of an authentic sample of reticuline could not be observed under ei conditions. Invariably, (M-2)$^+$ peaks were the highest *m/e*'s observed. However, the *O,O*-dibenzyl derivatives gave the correct M$^+$ peaks. The base peaks are due to the isoquinoline fragment resulting from cleavage of the benzylic portion of the molecules. 4'-Ethyl-6-methyl-laudanosoline was prepared as described for the labeled substrate. It was purified by column chromatography on silica gel with chloroform-methanol and crystallized from ether-hexane, mp 58-61°; ^{1}H nmr, δ 1.32 (t, 4'-OCH$_2$*CH$_3$*, *J*=7.0 Hz), 2.36 (s, N-CH$_3$), 3.60 (t, H-1, *J*=7.2 Hz), 3.74 (s, 6-OCH$_3$), 3.93 (q, 4'-O*CH$_2$*CH$_3$, *J*=7.0 Hz), 6.29-6.70 (m, arom. H's); high resolution ms (ei), *m/e* 341.1623 ((M-2)$^+$, C$_{20}$H$_{23}$O$_4$N, 1.53%), 192.1018 (C$_{11}$H$_{14}$O$_2$N, 100%).

Codeine 3-ethyl ether for carrier dilution was obtained from Merck & Co.

EXTRACTION, SEPARATION AND PURIFICATION OF ALKALOIDS.—The fresh or frozen plants were cut with pruning shears and homogenized with methanol in a high-speed blender. The cold carriers were added to the slurry which was poured into a glass percolator and extracted with methanol until the extract gave negative tests for alkaloids. The extract was concentrated under reduced pressure, transferred to a separatory funnel and shaken with several small portions of ethyl acetate to remove chlorophyll. The combined ethyl acetate extracts were shaken with 3 x 50 ml of 0.5 *N* hydrochloric acid and the acid extracts combined with the original aqueous solution (total alkaloids). The alkaloids were separated into a weakly basic fraction by extraction of the acidic solution with chloroform, a moderately basic nonphenolic fraction by extraction with chloroform at pH 13–14, a fraction containing mainly reticuline and reticuline analogues by extraction with ether at pH 8.5–9 and morphine and *N*-ethylnormorphine by extraction with chloroform-2-propanol (3:1) at pH 9.0. *N*-Ethylnormorphine (experiments 3 and 4) is considerably more soluble in ether than morphine and was found partly in the reticuline fraction and partly in the morphine fraction. The morphine fraction was dissolved in 25 ml of 0.5 *N* sodium hydroxide solution in a 250 ml glass-stoppered flask, 100 ml of ether was added and the mixture shaken vigorously. After adjustment of pH to 9.0 with solid ammonium chloride, the mixture was shaken again for 5 min and placed in a refrigerator overnight. The ether layer was decanted off, 100 ml of ether was added again, shaken and decanted off. The remainder was filtered to collect the crude morphine crystals, which were crystallized from methanol to constant radioactivity. The ether extracts were dried, evaporated to dryness and the residue of *N*-ethylnormorphine purified by preparative tlc, first on 2 mm plates of silica gel, then on 0.5 mm plates with chloroform-methanol (8:2) and crystallized from 50% ethanol to constant radioactivity. Codeine 3-ethyl ether (Experiment 2) and *N*-ethylnorcodeine (Experiment 4) were isolated from the moderately basic nonphenolic fraction and purified, first by column chromatography on alumina with benzene-chloroform (4:1), then by preparative tlc on 0.5 mm silica gel with chloroform-methanol (9:1) and crystallized from benzene-hexane to constant radioactivity. The reticuline analogues (Experiments 1,2,3) were purified by preparative tlc on 2 mm and 0.5 mm silica gel with chloroform-methanol (8:2) until pure by analytical tlc and glc and of constant radioactivity. Papaverine (Experiment 3) was isolated from the weakly basic alkaloid fraction and purified by preparative tlc on silica gel, first with chloroform-methanol (9:1), then with ethyl acetate-methanol (96:4) and crystallized from ether-methanol to constant radioactivity.

CONTROLLED DEGRADATION OF MORPHINE AND *N*-ETHYLNORMORPHINE FOR ISOLATION OF THE CARBON ATOM AT POSITION 16.—The radioactive alkaloid was subjected to exhaustive methylation and Hofmann degradation according to Pschorr and Dickhäuser (17). The resulting α-dimethylmorphimethine was reduced catalytically with platinum oxide catalyst to give the tetrahydroderivative which was methylated with methyl iodide, converted to the free base with silver oxide and subjected to a second Hofmann degradation (18). This gave trimethylamine and 6-methoxy-13-vinyloctahydromethylmorphenol. Ozonolysis[2] of the latter compound according to Battersby *et al.* (12) liberated the original C–16 of morphine as formaldehyde which was distilled into a solution of dimedone in 50% ethanol. The formaldehydedimedone adduct separated as white needles, which were crystallized from aqueous ethanol to constant radioactivity.

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[2]Welsbach ozonator.