Biotransformation of 1-Benzyl-1,2,3,4-tetrahydro-2-methylisoquinolines into Tetrahydroprotoberberines with Rat Liver Enzymes

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Abstract: The biotransformation of reticuline (**1**) into coreximine (**3**), scoulerine (**5**), and norreticuline (**7**) with a whole rat or a rat liver preparation in the presence of NADPH was demonstrated by tracer experiments using radioactive (±)-reticuline. Tritium-labeled laudanosine (**11**) was also incorporated into xylopinine (**4**), tetrahydropalmatine (**6**), and norlaudanosine (**8**) with the same rat liver preparation.

In connection with our interest in the biotransformation of isoquinoline alkaloids with animal tissue, we have previously reported in a communication that (+)-reticuline, an important precursor in the biogenesis of opium alkaloids such as berberine, morphine, benzophenanthridine, and phthalideisoquinoline alkaloids,[1,2] was transformed by rats into coreximine, which was identified by mass spectrometry and gas chromatographic behavior.[3] In further studies using radioactive substrates we have proved that enzymes in rat liver were responsible for transformation of 1-benzyl-1,2,3,4-tetrahydro-2-methylisoquinolines into tetrahydroprotoberberines and the corresponding *N*-nortetrahydroisoquinoline and we now wish to describe our findings.

(±)-Reticuline was prepared by methylation of 7-benzyloxy-1-(3-benzyloxy-4-methoxybenzyl)-3,4-dihydro-6-methoxyisoquinoline with methyl iodide, followed by the reduction of the methiodide with sodium borohydride and then the debenzylation of the resulting (±)-*O,O*-dibenzylreticuline with a mixture of concentrated hydrochloric acid and benzene according to reported methods.[4,5] Tritiation of (±)-reticuline (**1**) (Scheme I) with tritiated water in the presence of potassium *tert*-butoxide according to Kirby's method[6] gave (±)-[2′,6′,8-$^3H_3$]reticuline (**9**). A parallel experiment using deuterated water instead of tritiated water gave **10**, the NMR spectrum of which verified that three hydrogens at the ortho and para positions of the phenolic hydroxyl groups were equally substituted with deuterium. Methylation of (±)-[2′,6′,8-$^3H_3$]reticuline (**9**) with diazomethane afforded (±)-[2′,6′,8-$^3H_3$]laudanosine (**11**), which was free from radioactive reticuline (**9**) or laudanine (**12**) on the basis of radio thin-layer chromatographic analysis (Chart I).

Scheme I

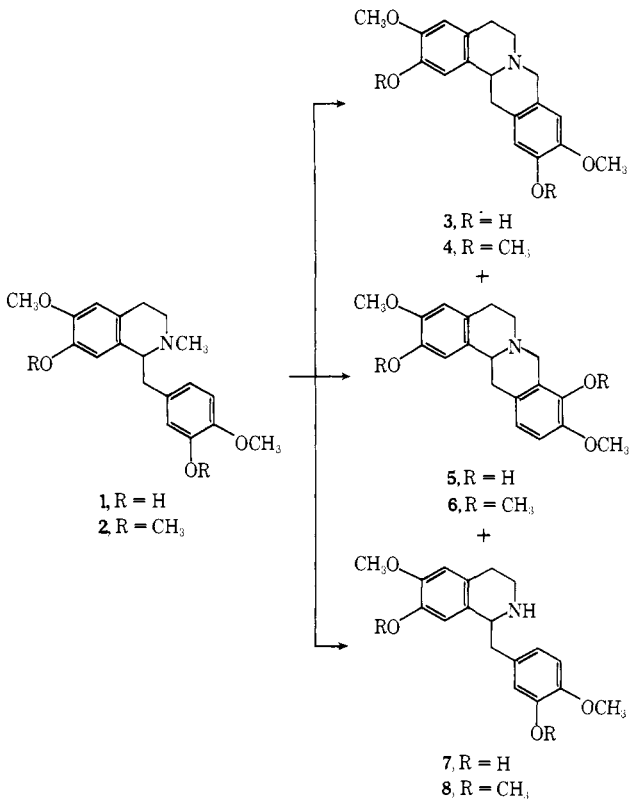
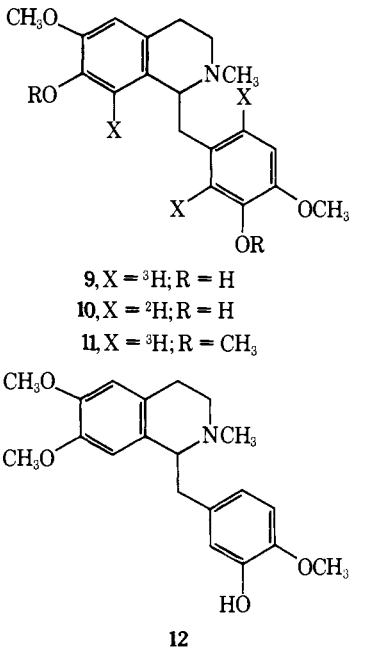


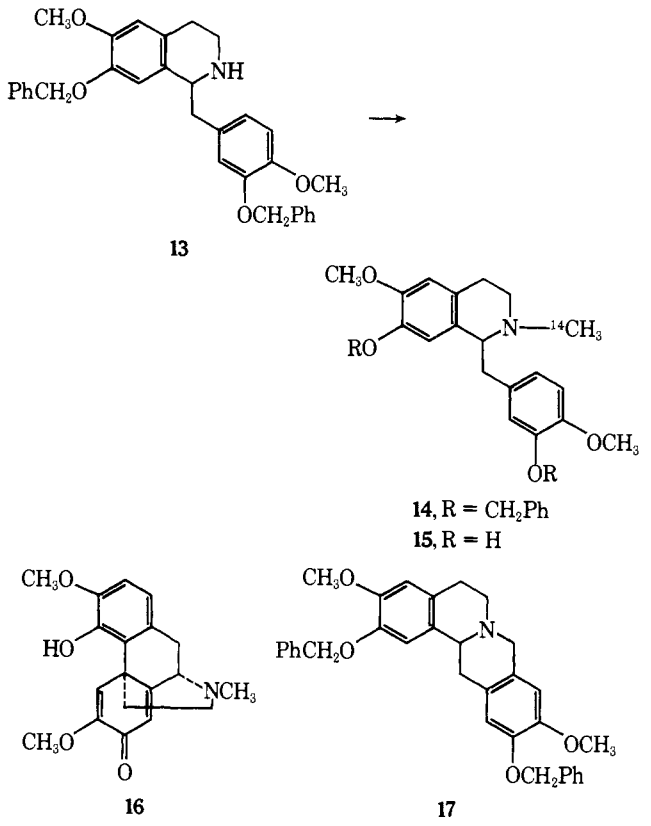
Chart I



(±)-[N-$^{14}CH_3$]Reticuline (**15**) was prepared by the reductive methylation of (±)-*O,O*-dibenzylnorreticuline (**13**)[7] with [^{14}C]formalin and sodium borohydride in methanol, followed by debenzylation of (±)-[N-$^{14}CH_3$]-*O,O*-dibenzylreticuline (**14**) (Scheme II).

A solution of (±)-[N-$^{14}CH_3$]reticuline (**15**) was administered to a female rat of Wistar strain. After treatment of the collected urine and dilution with nonradioactive (±)-coreximine (**3**), radioactive (±)-coreximine was isolated in a pure state in 0.03% yield.

Scheme II



The biotransformation was also demonstrated by incubating a 20% homogenate of rat liver, prepared in phosphate buffer at pH 7.4 with (±)-[2′,6′,8-$^3H_3$]- (**9**) and (±)-[N-$^{14}CH_3$]reticuline (**15**). After incubation at 37° for 2 h, to each reaction mixture carrier alkaloid was added and then purified by preparative TLC, followed by recrystallization to constant activity. Scoulerine (**5**)[8,9] was recrystallized as the salt of the hydrochloride. Sinoacutine (**16**)[10] was purified via the picrate and then converted into the free base, which was repeatedly recrystallized.

The results of the above tracer experiments are shown in Table I. In experiment 4, yields were calculated as 1.5 times greater than that of the total activities determined in pure product, since one tritium was lost in the cyclization. Formation of coreximine and scoulerine from (±)-reticuline with the homogenized rat liver was proved by both experiments with (±)-reticuline labeled with carbon-14 or tritium. In the experiments, in which ring-tritiated precursors were utilized, percent incorporation observed may possibly be lower than actual values, because some tritium may be expected to exchange with hydrogen. No radioactivity was found in pure sinoacutine (**16**). In the experiments using a whole rat and a large amount of nonradioactive (+)-reticuline, we observed no formation of morphinandienone-type alkaloids and aporphine alkaloids on the basis of the inspection by TLC or gas chromatography of crude products. It seems that there is no enzyme in rat liver which is responsible for the phenolic oxidative coupling of reticuline.

The amount of the alkaloids which were formed by the above enzymic reaction was too small to measure the optical rotation. It is sometimes possible to isolate an optically active compound from the racemate and vice versa by crystallization. There are many reports which provide the concurrent isolation of an optically active isomer and the racemate from the same plant.[11,12] It was expected that if a small amount of the radioactive product, which is optically active, was diluted with a large amount of the enantiomer,

Table I. Biotransformation of Radioactive Reticuline Using a Whole Rat or a Homogenized Rat Liver

Expt no.	Substrate	Total act. fed, dpm	Carrier alkaloid	Yield, %
1[a]	(±)-[N-$^{14}CH_3$]Reticuline	3.27×10^6	(±)-Coreximine (**3**)	0.030
2[b]	(±)-[N-$^{14}CH_3$]Reticuline	3.79×10^6	(±)-Coreximine (**3**)	0.083
3[b]	(±)-[N-$^{14}CH_3$]Reticuline	5.52×10^6	(−)-Scoulerine (**5**) Sinoacutine (**16**)	0.042 0
4[b]	(±)-[2′,6′,8-$^3H_3$]Reticuline	1.57×10^7	(±)-Coreximine (**3**) (±)-Scoulerine (**5**)	0.110 0.034
5[b]	(±)-[N-$^{14}CH_3$]Reticuline	1.06×10^7	(±)-Coreximine (+)-Coreximine (−)-Coreximine	0.090 0.044 0.050

[a] A whole rat experiment. [b] A homogenized rat liver experiment.

the repeated recrystallizations eventually showed no radioactivity.

(+)- and (−)-coreximine were synthesized by the optical resolution of (±)-*O,O*-dibenzylcoreximine (**17**) with (+)-di-*p*-toluoyltartaric acid. In previous experiments,[13] two optical isomers of di-*p*-toluoyltartaric acid were used to obtain both enantiomers. We have now found that one isomer of the acid is enough to accomplish the complete optical resolution. Recrystallization of the coreximine obtained from the mother liquor during resolution gave the pure enantiomer. This fact suggested that optically active coreximine is separable from the racemate by repeated recrystallizations.

Thus, after incubation, the resulting homogenate was equally separated into three fractions, to which (−)-, (+)- and (±)-coreximine were added. The radiochemically pure bases were obtained by rigorous preparative TLC, followed by repeated recrystallizations. The yields, which were calculated on the basis of the amount of "cold" carrier added, are shown in the last experiment of Table I.

The radiochemical yield of the product from dilution with (±)-coreximine was nearly twice of that obtained from dilution with (−)- or (+)-coreximine, respectively, the result of which suggests that the coreximine formed was racemate. It is interesting that the above enzymic transformation of reticuline into coreximine was not stereospecific while in a similar case Battersby and his co-workers have found that reticuline undergoes rapid racemization in plants.[14]

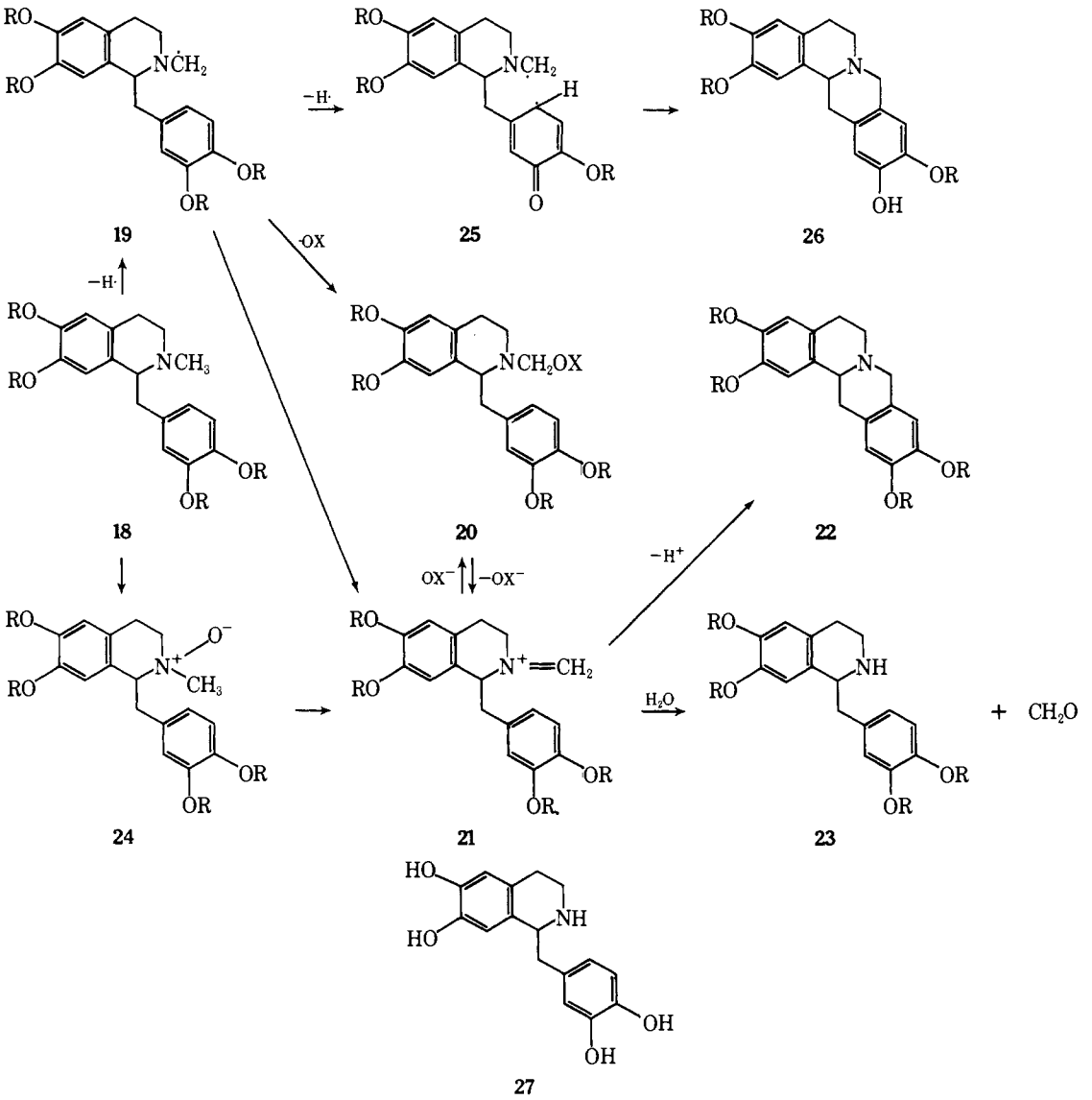
The transformation of (±)-reticuline was further studied using a 9000*g* supernatant of rat liver homogenates (Table II). Without cofactor, the radioactivity of (±)-[2′,6′,8-$^3H_3$]reticuline (**9**) was incorporated into (±)-coreximine (**3**) in 0.160% and (±)-norreticuline (**7**) in 0.410% yield (experiment 6). An addition of NADPH into the supernatant increased the formation of (±)-coreximine and (±)-norreticuline to 3.31 and 1.35%, respectively (experiment 7). Furthermore, the yield of coreximine was enhanced by an addition of magnesium chloride. Thus, in the presence of NADPH and magnesium chloride, the incorporation of (±)-reticuline into (±)-coreximine was 11.68% (experiment 8). Using the supernatant together with glucose 6-phosphate, NADP, nicotinamide, and magnesium chloride, (±)-reticuline was converted into (±)-coreximine in 5.99% yield (experiment 9).

In the case of nonphenolic tetrahydroisoquinolines, the radioactivity of (±)-[2′,6′,8-$^3H_3$]laudanosine (**11**) was incorporated into (±)-norlaudanosine (**8**, 14.13%) in the pres-

Table II. Biotransformation of Tritium-Labeled Reticuline and Laudanosine Using a Rat Liver 9000*g* Supernatant

Expt no.	Radioactive substrate	Cofactors	Total act. fed, dpm	Carrier alkaloid	Yield, %
6	(±)-Reticuline (**9**) (15.3 μmol)	Nil	1.16×10^7	(±)-Coreximine (**3**)	0.160
				(±)-Norreticuline (**7**)	0.410
7	(±)-Reticuline (**9**) (16.5 μmol)	NADPH (16.5 μmol)	1.26×10^7	(±)-Coreximine (**3**)	3.31
				(±)-Norreticuline (**7**)	1.35
8	(±)-Reticuline (**9**) (8.7 μmol)	NADPH (8.7 μmol), $MgCl_2$ (1 mmol)	6.65×10^6	(±)-Coreximine (**3**)	11.68
9	(±)-Reticuline (**9**) (8.0 μmol)	NADP (8.0 μmol), $MgCl_2$ (1 mmol), Nicotinamide (1 mmol), G-6-P (30 μmol)	6.05×10^6	(±)-Coreximine (**3**)	5.99
10	(±)-Laudanosine (**11**) (46.5 μmol)	NADPH (55.2 μmol)	3.54×10^7	(±)-Xylopinine (**4**)	0.019
				(−)-Tetrahydropalmatine (**6**)	0.007
				(±)-Norlaudanosine (**8**)	14.13

Scheme III



ence of NADPH. The incorporations into (±)-xylopinine (**4**) and (−)-tetrahydropalmatine (**6**) were very small but not zero (experiment 10).

It is reasonable on the basis of the above results that 1-benzyl-1,2,3,4-tetrahydro-2-methylisoquinolines (**18**) would give rise to immonium cations (**21**), which afford tetrahydroprotoberberines by cyclization or an N-demethylated product by hydrolysis. All possible mechanisms for the formation of the immonium cation **21** are outlined in Scheme III. The responsible rat liver enzyme would require NADPH and molecular oxygen as in the known enzymic N-demethylation.[15,16] The formation of xylopinine (**4**) and tetrahydropalmatine (**6**) from laudanosine (**11**) would exclude the possibility that the radical **19** is enzymatically oxidized to the diradical **25**, which affords a protoberberine by a radical coupling. It is difficult to determine whether the cyclization step of immonium cation **21** is enzymic or nonenzymic.

Recently Virginia Davis and co-workers found the in vivo and in vitro conversion of tetrahydropapaveroline (**27**) to

coreximine and related berberines by mammalian systems in the presence of *S*-adenosylmethionine.[17,18] We believe the cyclization occurred after N-methylation.

Experimental Section

Optical rotations were measured with a JASCO-PIP-SL automatic polarimeter. The radioactivities were determined by liquid scintillation counting with a Packard (Model 3380) Tri-Carb liquid scintillation spectrometer equipped with an absolute activity analyzer (Model 544). A mixture of 4 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)benzene, 700 ml of toluene, and 300 ml of methanol was used as a scintillator. About 1 mg of the sample was weighed accurately and dissolved in 0.1 ml of methanol and then 10 ml of the scintillator was added. The efficiencies for tritium and carbon-14 ranged from 49 to 53%. The radio thin-layer chromatography was detected by an Aloka thin-layer chromatogram scanner (Model JTC-203).

Resolution of (±)-*O,O*-Dibenzylcoreximine (17). A solution of 700 mg of *O,O*-dibenzylcoreximine (**17**) in 70 ml of methanol was treated with 505 mg of (+)-di-*p*-toluoyltartaric acid. The precipitate was repeatedly recrystallized from methanol to give 420 mg of the pure salt, mp 190° dec. The salt liberated 200 mg of (−)-*O,O*-dibenzylcoreximine, which was then refluxed for 3 h with a mixture of 8 ml of ethanol and 10 ml of concentrated hydrochloric acid. After evaporation of the solvent, an excess of 10% ammonium hydroxide was added to the residue and the free base was extracted with chloroform. The extract was washed with water, dried over Na_2SO_4, and evaporated to give a powder, recrystallization of which from methanol gave 105 mg of (−)-coreximine: mp 261–262°; $[\alpha]^{20}D$ −350° (*c* 0.021, MeOH).

The former filtrate during the above resolution was worked up to give the crude (+)-*O,O*-dibenzylcoreximine, which was directly debenzylated as above. After repeated recrystallization, 60 mg of (+)-coreximine had mp 261–262° and $[\alpha]^{20}D$ +340° (*c* 0.022, MeOH).

(±)-[N-^{14}CH$_3$]Reticuline (15). A solution of 50 mg of (±)-*O,O*-dibenzylnorreticuline (**13**) and 0.15 mg of [^{14}C]formalin (50 μCi) in 1 ml of methanol was stirred at room temperature. After 10 min, 1 ml of formaldehyde solution consisting of 37% formalin and methanol (1:99 v/v) was added to the above mixture, which was further stirred for 20 min. To this solution 50 mg of sodium borohydride was added and the resulting mixture was stirred for 1 h at room temperature. After addition of 10 ml of water the mixture was extracted with chloroform three times. The extract was washed with water, dried over Na_2SO_4, and evaporated to give a syrup, to which 50 mg of nonlabeled (±)-*O,O*-dibenzylreticuline was added. Recrystallization from ethanol to constant molecular activity gave 80 mg of (±)-[N-^{14}CH$_3$]-*O,O*-dibenzylreticuline (**14**, 150 μCi/mmol).

A mixture of 76 mg of the above radioactive base, 3.8 ml of benzene, and 3.8 ml of concentrated hydrochloric acid was stirred vigorously for 24 h under a current of nitrogen at room temperature. The benzene layer was then separated and extracted with diluted hydrochloric acid. The combined aqueous layer was basified with 10% ammonium hydroxide and extracted with chloroform. The extract was washed with water, dried over Na_2SO_4, and evaporated to give 43 mg of (±)-[N-^{14}CH$_3$]reticuline (**15**, 150 μCi/mmol).

(±)-[2′,6′,8-^{3}H$_3$]Reticuline (9). A mixture of 100 mg of (±)-reticuline (**1**), 150 mg of potassium *tert*-butoxide, and 1.0 ml of tritiated water (25 mCi) was sealed under a current of nitrogen and heated at 100° for 7 days. The contents of the tube were washed out with water, and ammonium chloride was added to neutralize the resulting solution. Extraction with chloroform three times, followed by washing with water, drying over Na_2SO_4, and evaporation of the solvent, gave a syrup, whose perchlorate was recrystallized together with 50 mg of cold (±)-reticuline perchlorate from methanol–ether to constant activity to afford 120.5 mg of (±)-[2′,6′,8-^{3}H$_3$]reticuline (**9**) perchlorate (346 μCi/mmol).

(±)-[2′,6′,8-^{3}H$_3$]Laudanosine (11). To a solution of 15.3 mg of the tritium-labeled reticuline (**9**), liberated from the above perchlorate, in 4 ml of methanol was added a solution of an excess of diazomethane in ether, prepared from *p*-toluenesulfonylmethylnitrosamide and the mixture was allowed to stand for 16 h at room temperature. Evaporation of the excess reagent and solvent left a caramel-like residue, which was used in the next incubation without further purification. It was confirmed by a thin-layer chromatogram scanner that the product was free from radioactive reticuline (**9**) or laudanine (**12**).[19] R_f values of laudanosine (**2**), laudanine (**12**), and reticuline (**1**) on thin-layer chromatography using silica gel (Merck F_{254}) developing with chloroform–methanol (9:1 v/v) were 0.71, 0.49, and 0.28, respectively.

Administration of (±)-[N-^{14}CH$_3$]Reticuline into Whole Rat. A solution of 3.2 mg of (±)-[N-^{14}CH$_3$]reticuline (**15**) in propylene glycol was injected intraperioneally into a 160-g rat, and the urine was collected in the bottle containing a few drops of toluene for 4 days after injection. The pooled urine was adjusted to pH 5 with diluted sulfuric acid and then to pH 4.5 with 0.1 M acetate buffer and incubated with β-glucuronidase at 37° for 2 days. The hydrolyzate was adjusted to pH 1 with diluted hydrochloric acid, and the resulting solution was washed with ether, then made basic with diluted ammonia, and extracted with ethyl acetate after an addition of 14.0 mg of nonradioactive (±)-coreximine. The basic fraction was purified by preparative TLC on silica gel developing in chloroform–methanol (10:1 v/v). The fraction of (±)-coreximine was recrystallized from methanol to constant activity (2.19×10^4 dpm/mmol).

Incubation of the Labeled (±)-Reticuline with Homogenized Rat Liver. Rats were killed by decapitation and the livers were homogenized in phosphate buffer (5 vol) at pH 7.4 with a Potter-Elvehjem homogenizer, which was cooled with ice. To this homogenate radioactive (±)-reticuline (ca. 1 mg/10 ml) was added and the mixture was shaken for 2 h at 37°. After addition of 10–15 mg of an unlabeled alkaloid, the homogenate was extracted several times with ethyl acetate. The combined extracts were washed with water and dried over Na_2SO_4. All emulsions formed during the above extractions and washings were removed by centrifugation. After evaporation of the solvent, the crude extract was purified by a preparative TLC on silica gel developing in chloroform–methanol (10:1 v/v). The alkaloidal fraction was scratched from the plate and eluted with 15% methanolic chloroform. In the case of coreximine, the product was recrystallized from methanol to constant activity. Scoulerine was recrystallized as the hydrochloride from methanol. Sinoacutine was purified firstly as picrate and then converted into the free base, which was recrystallized from methanol–ether.

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