Partial Purification and Characterization of S-Adenosyl-L-Methionine: Norreticuline N-Methyltransferases from *Berberis* Cell Suspension Cultures*

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Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

Isoquinoline Alkaloids, Norreticuline, Reticuline, Biosynthesis, Plant Cell Culture

Two new N-methyltransferases (NMT-I and NMT-II) were found to occur in *Berberis vulgaris* cell suspension cultures. One of these enzymes (NMT-I) was partially purified (100-fold) and characterized. This enzyme is specific for tetrahydrobenzylisoquinoline alkaloids and S-adenosyl-L-methionine serves as the methyl donor. The apparent molecular weight of the enzyme is 68,000. The pH optimum of the enzyme is 7.6, the temperature optimum 35 °C. Apparent K_M values for (*R*)-tetrahydropapaverin as substrate were 0.2 mM and for SAM 0.04 mM. The preparation of the same type of enzyme from *B. wilsoniae* var. *subcaulialata* was utilized as an efficient enzymatic system for the synthesis of stereochemically pure (*R*)- as well as (*S*)-reticuline labelled with tritium or ^{14}C at the $N-CH_3$ group. Enzymes catalyzing this type of reactions are named S-adenosyl-L-methionine: norreticuline N-methyltransferases.

Introduction

Reticuline, a tetrahydrobenzylisoquinoline alkaloid, is firmly established as the key intermediate in the biosynthesis of a vast number of isoquinoline alkaloids [1]. Among this group of alkaloids are compounds with such diverse types of structures as pavines, dibenzopyrrocolines, morphinans, aporphines, protoberberines, benzophenanthridines and phthalide isoquinolines. Investigating the early steps in the reticuline pathway, we succeeded in the isolation and partial purification of two new enzymes: (*S*)-norlaudanosoline synthase, the first enzyme in the benzylisoquinoline pathway which stereospecifically condenses dopamine with 3,4-dihydroxyphenylacetaldehyde and a specific O-methyltransferase which methylates norlaudanosoline predominantly at the 6-position yielding 6-O-methyl-norlaudanosoline [2, 3]. Two further methylation steps, one O-methylation at 4′ position and one N-methylation at atom 2, will transform this product into reticuline. It is generally assumed that O-methylation precedes N-methylation [4, 5] and our recent experimental evidence using cell-free extracts verifies this assumption. In the biosynthesis of orientaline, for instance, it has been clearly shown that 6-O-methylnorlaudanosoline is exclusively monomethylated at position 5′ by a highly specific enzyme to yield nororientaline [6]. While another enzyme specific for the methylation of 6-O-methylnorlaudanosoline at the 4′ position to give norreticuline is presently under investigation in our group, we report here the isolation of N-methyltransferases which show specificity towards certain benzylisoquinoline alkaloids and which are absolutely inactive towards simple biogenic amines. This N-methylation is the final reaction involved in the early steps of the tetrahydroisoquinoline pathway, that is, the formation of reticuline. The presence of N-methyltransferases had to be postulated, since it was shown previously that the latex of *Papaver somniferum*, when incubated with norlaudanosoline and ^{14}C-SAM, yielded labelled reticuline as well as morphinan alkaloids [7], and the same type of experiment with a cell-free extract from agar-grown callus of *Berberis aggregata* yielded labelled protoberberine alkaloids [8]. Furthermore, in this latter study [8], it was demonstrated that tetrahydropapaverine was N-methylated. We describe here the detection, partial purification and characterization of a norreticuline N-methyltransferase which is of considerable importance in the biosyn-

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Abbreviations: BSA, bovine serum albumin; NLS, norlaudanosoline; NMT, N-methyltransferase; NRK, norreticuline; OMT, O-methyltransferase; SAM, S-adenosyl-L-methionine; THP, tetrahydropapaverine.

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thesis of the numerous isoquinoline alkaloids and which can conveniently be used to introduce radioactive or stable isotopes into this important precursor.

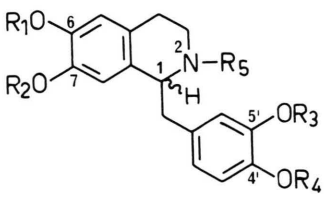
Materials and Methods

Plant material

Berberis vulgaris and *B. wilsoniae* var. *subcaulialata* as well as other *Berberis* cell cultures used were initiated and maintained since 1975 on Linsmaier and Skoog (LS) medium [9]. Batch cultures in 1-litre Erlenmeyer flasks containing 250 ml medium were agitated on a gyratory shaker (100 rpm) in diffuse light (750 lux) at 24 °C and were subcultured at weekly intervals using about 10% inoculum (v/v). The cells were harvested immediately after reaching stationary phase, frozen in liquid nitrogen and stored at −20 °C. Cell fresh weight was determined after collecting the cells on a fritted glass funnel with suction. All other cell cultures were from our culture collection and were grown under identical conditions as given above.

Chemicals

(*R,S*)-Reticuline, (*R,S*)-laudanine, (*R,S*)-orientaline perchlorate, (*R,S*)-nororientaline·HCl, (*R,S*)-noriso-orientaline·HCl, and (*R,S*)-norprotosinomenine·HCl were samples from Prof. E. Brochmann-Hanssen, San Francisco; norglaucine, norboldine, and caaverine from Prof. Cavé, Chatenay-Malabry; (*R*)- and (*S*)-reticuline and (*R,S*)-norreticuline from Prof. N. Nagakura, Kobe; (*R,S*)-laudanidine from Merck, Darmstadt, and laudanosine from Aldrich Chem. Co. (*R,S*)-Tetrahydropapaverine·HCl was prepared by the method described in [10] and resolved into each enantiomer as described in [11]. (*R,S*)-Norlaudanosoline was synthesized by a Pictet-Spengler condensation of dopamine and 3,4-dihydroxyphenylacetaldehyde [2] in water at pH 4.



	R_1	R_2	R_3	R_4	R_5
Norlaudanosoline	H	H	H	H	H
Laudanosoline	H	H	H	H	CH_3
Norreticuline	CH_3	H	H	CH_3	H
Reticuline	CH_3	H	H	CH_3	CH_3
Nororientaline	CH_3	H	CH_3	H	H
Orientaline	CH_3	H	CH_3	H	CH_3
Norprotosinomenine	H	CH_3	H	CH_3	H
Protosinomenine	H	CH_3	H	CH_3	CH_3
Norisoorientaline	H	CH_3	CH_3	H	H
Isoorientaline	H	CH_3	CH_3	H	CH_3
Tetrahydropapaverine	CH_3	CH_3	CH_3	CH_3	H
Laudanosine	CH_3	CH_3	CH_3	CH_3	CH_3

1H = α (*S*); 1H = β = (*R*).

Assay methods

Three substrates were used for assaying the enzymes. THP and NRK were used to test for the N-methyltransferases and caffeic acid was used for unspecific O-methyltransferases. The standard incubation mixture consisted of 10 µl substrate (THP, 100 nmol, other substrates 50 nmol in DMSO), 20 µl $^{14}CH_3$-S-adenosylmethionine (0.1 µCi/102 nmol, Amersham), appropriate amount of enzyme and potassium phosphate buffer (0.1 to 0.125 M, pH 7.5) to give a final volume of 100 µl. For monitoring the activity of enzyme from column fractions, SAM at a concentration of 26 nmol/20 µl per assay (0.05 µCi) was used. Incubation was carried out for 40 min at 30 °C. The reaction was stopped by addition of 400 µl 1 M KCl in 1 M phosphate buffer (pH 8) at 4 °C and 500 µl ethyl acetate. The mixture was rotated for 15 min at room temperature and centrifuged (Eppendorf system). An aliquot (300 µl) of the ethyl acetate was removed and the radioactivity determined in 5 ml Bray's solution. For caffeic acid, the pH of the incubation mixture was 6.8 and the reaction stopped by the addition of 400 µl 2 N HCl. An alternative assay, for NMT activity only, consisted of 15 µmol potassium phosphate buffer (pH 7.5), 38 nmol (*R,S*)-norreticuline·HCl in DMSO (final conc. 5% v/v), 15 µmol Na ascorbate, 10.4 nmol $^{14}CH_3$-SAM (2.4 µCi/µmol) and up to 100 µg enzyme in a total volume of 150 µl. Incubation was for 60 min at 30 °C. The incubation was terminated by the addition of 200 µl 0.2 M Na_2CO_3/$NaHCO_3$, pH 10, and 500 µl isoamyl alcohol. The mixture was rotated for 20 min at room temperature. After centrifugation, 300 µl of the organic phase was taken and the radioactivity determined.

Efficiency of extraction of laudanosine

Two methods were used to determine the efficiency of EtOAc extraction of laudanosine, the N-methylated product of THP, from the reaction mixture as assayed above. The first method involved a large scale incubation consisting of $^{14}CH_3$-SAM, the enzyme and THP. After purifying the labelled product by TLC with solvent systems I and II (see below) it was dissolved in DMSO. An aliquot (10 µl) of this labelled compound was added to vials containing varied amounts of unlabelled laudanosine (0–200 nmol) in the extraction buffer and the solution was mixed with EtOAc as described above. The efficiency of the extraction was found to be over 95% when the concentration of laudanosine present was up to 25 nmol. In the second method, 20 µl of assay mixture was applied on a Sil G/UV_{254} Polygram plate (Macherey and Nagel, Düren) and developed in solvent system II. The chromatogram was then scanned (Berthold, Wildbad). The ratio of activity of the residual SAM which remained at the origin to that of [^{14}C]laudanosine was calculated by cutting out and weighing the area under the peaks obtained from the scan. The amount of activity in laudanosine obtained by this TLC method was found to correspond within ± 20% to that determined by the EtOAc extraction method.

Identification of methylated products

For the identification of benzylisoquinolines, MN-Polygram Sil G/UV_{254} was used with the following solvent systems: (I) $CHCl_3$: methanol 20:1, (II) toluene : EtOAc : diethylamine 7:2:1, (III) $CHCl_3$: acetone : diethylamine 5:4:1, (IV) cyclohexane : diethylamine 9:1, (V) xylene : methylethyl ketone : methanol : diethylamine 20:20:3:1, (VI) acetone : methanol : HCOOH : H_2O 5:3:1:1, (VII) EtOAc : methylethyl ketone : HCOOH : methanol 12:12:3:2, (VIII) EtOAc : methylethyl ketone : HCOOH : H_2O 6:2:1:1. The N-$^{14}CH_3$ product of THP (laudanosine) was further identified by co-crystallization with laudanosine. The labelled product obtained by incubation with $^{14}CH_3$-SAM was first purified by TLC with solvent systems II and I. Laudanosine (52 mg) was added, and the mixture recrystallized from EtOH (919 dpm/µmol), hexane (823 dpm/µmol) and petrol (830 dpm/µmol). This labelled laudanosine was further converted to laudanosine methiodide according to the procedure described in [12]. After evaporation of the solvent and excess Me I, the residue was recrystallized first from methanol-Et_2O (873 dpm/µmol) and then from methanol (836 dpm/µmol), m.p. 215–216 °C, UV_{max}^{EtOH} = 280 nm (4.75). MS (80 eV, direct inlet) *m/e* (%): 372(M^+,2), 207(15), 206(100), 191(12), 190(18), 151(8), 142(22), 127(20), 58(72), corresponding to the N-methyl laudanosine ion of laudanosine methiodide.

Enzyme purification

All buffers used were at pH 7.5 containing 20 mM β-mercaptoethanol unless otherwise stated. Typically, 300 g of frozen cells were stirred for 30 min in 450 ml 0.1 M potassium phosphate buffer containing 60 mM β-mercaptoethanol. After filtering through cheesecloth, the filtrate was centrifuged at 27,000 × *g* for 10 min. The supernatant was fractionated with $(NH_4)_2SO_4$ and the protein which precipitated between 35–70% saturation was dissolved in 40 ml of the extraction buffer. The solution was stirred for 10 min with 7 g Dowex 1 × 4 (100–200 mesh, phosphate form) and filtered through sintered glass funnel. Dextran-coated charcoal (200 mg) was added to the filtrate, stirred for 10 min and centrifuged. The supernatant was dialyzed against 0.05 M phosphate buffer. The dialyzed enzyme was passed through a DEAE-cellulose column (2.5 × 15 cm, Whatman DE-52). After washing with starting buffer, a linear gradient (0–0.5 M KCl) in 0.05 M phosphate buffer was applied. Fractions of 5 ml were collected at a flow rate of 1 ml/min. NMT and OMT activities in the fractions were monitored by assays with (*R,S*)-THP, (*R,S*)-NRK, and caffeic acid. The fractions containing NMT activity which were eluted with the starting buffer were pooled (NMT-I) and dialyzed against 0.02 M phosphate buffer.

The active fractions of NMT-I were subsequently passed through a column of CM-cellulose (1 × 8 cm) equilibrated with dialysis buffer.

NMT-II was eluted from the DEAE-column at about 0.12 M KCl; details for this enzyme will be published later.

Molecular weight determination

The NMT-I enzyme, after it was eluted from its respective cellulose columns, was dialyzed against 0.1 M phosphate buffer. The enzyme (1.8 ml) together with ferritin (0.5 mg, 450 kD), cytochrome *c*

(2 mg, 12.5 kD), and glycerol (0.2 ml) was applied to an Ultrogel AcA-44 column (2.3 × 88 cm) equilibrated with the dialysis buffer. Fractions of 3 ml were collected at a flow rate of 24 ml/h. The column was previously calibrated with blue dextran, catalase (240 kD), aldolase (158 kD), bovine serum albumin (68 kD) or albumin (43 kD), chymotrypsinogen A (25 kD) and cytochrome *c* (12.5 kD) (Boehringer, Mannheim). The markers were measured either by their light absorption, catalytic activity, or both.

Affinity chromatography

The enzyme preparation (NMT-I) used in this chromatographic step had been prepurified by two subsequent DEAE-cellulose columns and was dialyzed against 0.02 M phosphate buffer, the starting buffer for all columns used below. The protein concentration of this preparation was 0.6 mg/ml. (*R*)-THP was used as the substrate in the assays.

SAH-AH-Sepharose 4B: The ligand SAH was coupled to AH-Sepharose 4B by the procedure described in [13] and the residual amino groups on the gel were blocked by a further carbodiimide reaction with acetic acid. After applying the enzyme solution (3.2 ml), the column (0.9 × 5 cm) was first washed with 16 ml 0.02 M KCl in starting buffer containing varied amounts of SAM followed by 16 ml 0.5 M KCl in starting buffer. Fractions of 1.5 ml were collected at a flow rate of 0.3 ml/min.

SAH-CH-Sepharose 4B. The affinity gel was prepared as described in [14] and equilibrated in the starting buffer. Column dimensions and enzyme application/elution were as described above.

(*R,S*)-THP-CH-Sepharose 4B. The preparation of this matrix was essentially the same as described in [14] with (*R,S*)-THP substituting for SAH. The UV spectrum of the buffer was scanned between 200–350 nm at the beginning and at the end of the reaction time. There was a 40% decrease in the absorbance of the peak at 230 nm. This was taken as an indication that (*R,S*)-THP was removed from the solution and bound to the gel. The column (0.9 × 8 cm) was equilibrated with the starting buffer containing 50 μM SAM. The enzyme solution (5 ml), to which SAM was added to give a final concentration of 50 μM, was then applied. The column was successively washed with 30 ml starting buffer containing 50 μM SAM, 30 ml starting buffer, 10 ml starting buffer containing 40 μM SAM and 10 μM (*R,S*)-THP, and finally, with 30 ml starting buffer containing 50 μM SAM, 10 μM (*R,S*)-THP and 0.1 M KCl. Fractions of 3 ml were collected at a flow rate of 0.3 ml/min.

NMT for preparative purpose

Frozen tissue of *B. wilsoniae* var. *subcaulialata* (100 g fresh weight) was suspended in 250 ml of 0.05 M phosphate buffer, pH 7.4, containing 20 mM β-mercaptoethanol and stirred at room temperature to homogeneity. The mixture was pressed through cheesecloth and the filtrate centrifuged for 10 min at 18,000 × *g*. $(NH_4)_2SO_4$ was added to the extract until 70% saturation was reached. After centrifugation the pellet was resuspended in 14 ml starting buffer and desalted on a Sephadex G-25 column (2.3 × 18 cm) equilibrated with 0.01 M phosphate buffer, pH 7.4. The protein-containing fraction was subjected to ion exchange chromatography (DEAE-Sephacel, Pharmacia; 1.6 × 12.5 cm) and eluted with a KCl gradient (0–400 mM) in 0.01 M phosphate buffer. Fractions between 0.14 and 0.18 M KCl were pooled and used as enzyme source for the preparative synthesis of labelled N-methylated products; this preparation was devoid of any O-methyltransferase activity using caffeic acid or laudanosoline as substrate. In this case, (*R,S*)-NRK was used as the substrate and the alternative enzyme assay given above was employed. For protein determinations the modified Folin method [15] was used with bovine serum albumin as reference.

Results

General survey of cell cultures for NMT

Using (*R*)- and (*S*)-THP as substrates, a survey for N-methylating activity was made on more than 50 cell suspension cultures propagated from plants that produce isoquinoline alkaloids. Under the conditions used in the assay, the preparations from the genus *Berberis* showed the highest activity towards these two substrates, and consistently with higher activity toward the (*R*)-isomer (Table I). Cultures from other genera such as *Corydalis, Eschscholtzia, Fumaria,* and *Thalictrum* gave preparations with less activity. As controls, preparations from tobacco and carrot cell suspension cultures were used and neither one possessed NMT activity towards THP.

Table I. A survey of (*R*)- and (*S*)-tetrahydropapaverine specific N-methyltransferase activity from *Berberis* cell cultures, grown in Linsmaier and Skoog medium [9].

Cell cultures	Days of growth	Specific activity (pkat/mg protein) (*R*) THP	(*S*) THP
B. Coryi	6	1.7	0.5
B. henryana	16	5.0	2.4
B. heteropoda	16	7.3	2.1
B. stolonifera	7	12.0	7.7
B. vernae	11	3.8	2.8
B. vulgaris	11	5.1	2.2
B. wilsoniae var. *sub.*	8	29.3	13.0
controls:			
Nicotiana tabacum	13	0	0
Daucus carota	7	0	0

Purification of NMT from B. vulgaris

The enzyme was first prepurified by $(NH_4)_2SO_4$ precipitation, Dowex-A and dextran-coated charcoal treatment. The enzyme preparation thus obtained showed methylation activities towards (*R*)- and (*S*)-THP (N-methylation), (*R,S*)-norlaudanosoline, (*R,S*)-norreticuline (O- and N-methylation), (*R,S*)-laudanosoline and caffeic acid (O-methylation). Assays were carried out with the EtOAc extraction method. Though a 84–99% recovery of laudanosine in the concentration range from 0–200 nmol was obtained, however, the extraction of only partially methylated products with hydroxyl groups still present was not complete by this procedure.

By DEAE-cellulose chromatography with 0.5 M phosphate as starting buffer, two pools of enzymes with N-methylation activities were obtained (Fig. 1). The first one was eluted with the starting buffer. On pooling the most active fractions from this peak, this enzyme (NMT-I) showed activity towards (*R*)- and (*S*)-THP, (*R,S*)-NRK, and (*R,S*)-NLS only. The second peak was eluted from the column with a KCl gradient at about 0.12 M KCl. This protein solution containing NMT-II possessed activities for N- and O-methylation. In this paper only the purification and characterization of NMT-I will be given.

Subsequently, an attempt was made to further purify NMT-I by affinity chromatography. Three affinity matrices were prepared: SAH-AH-Sepharose 4B, SAH-CH-Sepharose 4B and THP-CH-Sepharose 4B. With SAH-CH-Sepharose, the enzyme was not retained. With THP-CH-Sepharose all the protein was retained but the enzyme could not be

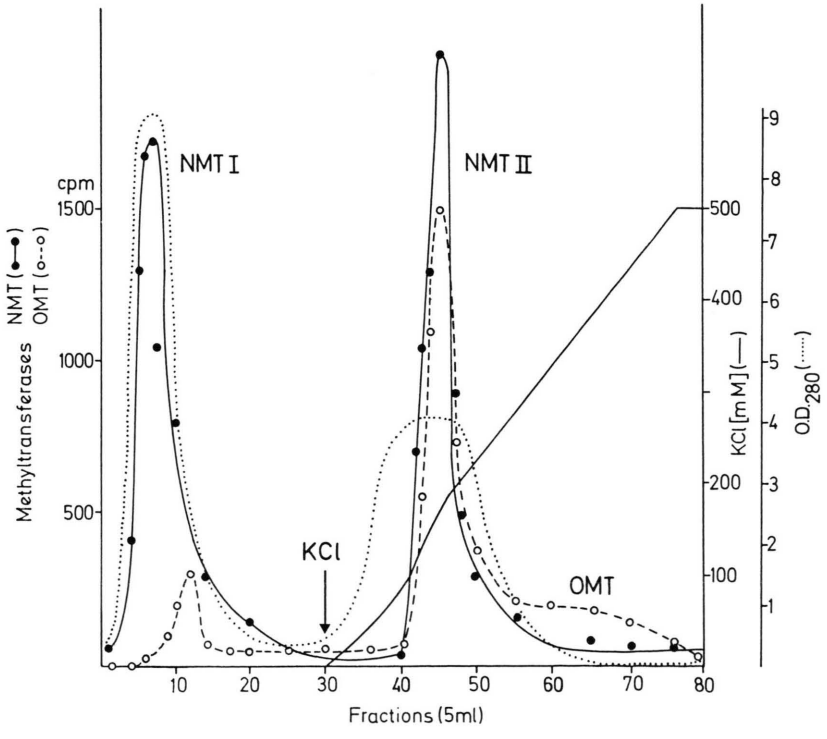


Fig. 1. Elution profile of NMT-I and NMT-II as well as unspecific O-methyltransferase activities from *B. vulgaris* cell cultures on DEAE-cellulose columns with (*R,S*)-tetrahydropapaverine as substrate (standard assay conditions).

Table II. Purification procedure for N-methyltransferase-I from *B. vulgaris* cell suspension cultures [(*R*)-THP as substrate].

Purification step	Total activity [pkat]	Total protein [mg]	Specific activity [pkat/mg]	Recovery [%]	Purification - fold
Crude dialysate	114	572	0.2	100	1
Ammoniumsulfate-precipitation (35–70%)	140	127.4	1.1	123	5.5
DEAE-Cellulose chromatography	43	27.0	1.6	38	8
CM-Cellulose chromatography	38	10.6	3.6	33	18
SAH-AH-Sepharose chromatography	5.5	0.27	20.4	5	102

eluted, neither when SAM was omitted from the buffer nor when SAM (50 μM), (*R,S*)-THP (10 μM) and KCl (1.0 M) were present. With SAH-AH-Sepharose, the NMT was retained on the column but could not be eluted by the inclusion of SAM in the buffer (up to 0.1 mM). It was, however, released by a KCl gradient or by first washing with 0.02 M KCl followed by 0.5 M KCl in starting buffer. The recovery of the activity was only 5% with about 5-fold purification compared with the previous step and 102-fold overall. A typical purification procedure is summarized in Table II. NMT-I was stable for at least two weeks when kept at 4 °C; on freezing the catalytic activity was, however, totally lost. Attempts to stabilize the enzyme with varying amounts of sucrose, glycerol or DMSO at −20 °C failed.

Properties of NMT-I

The ca. 100-fold purified sample of the NMT-I enzyme was used to determine its catalytic properties as shown in Table III. The enzyme was found by gel filtration to have a molecular weight of about 68,000. Under standard assay conditions the time course of the reaction of the enzyme was linear at least up to 1 hr. For the NMT-I enzyme there is a sharp pH optimum at 7.65 (Fig. 2). The purified enzyme shows a temperature optimum at 35 °C. K_M for (*R*)-THP as substrate was in the range of 0.2 mM, while the K_M for SAM was 0.04 mM. The concentrations of SAH

Table III. Some properties determined for purified NMT-I from *B. vulgaris* cell suspension cultures.

Molecular weight	68 kD
pH-optimum	7.6
Temperature optimum	35 °
K_M for (*R*)-THP*	180 μM
K_M for (*S*)-THP*	30 μM
K_M for SAM**	40 μM
SAH concentration for 50% inhibition	80 μM

* at 1 mM SAM.
** at 1 mM of (*R*)-THP.

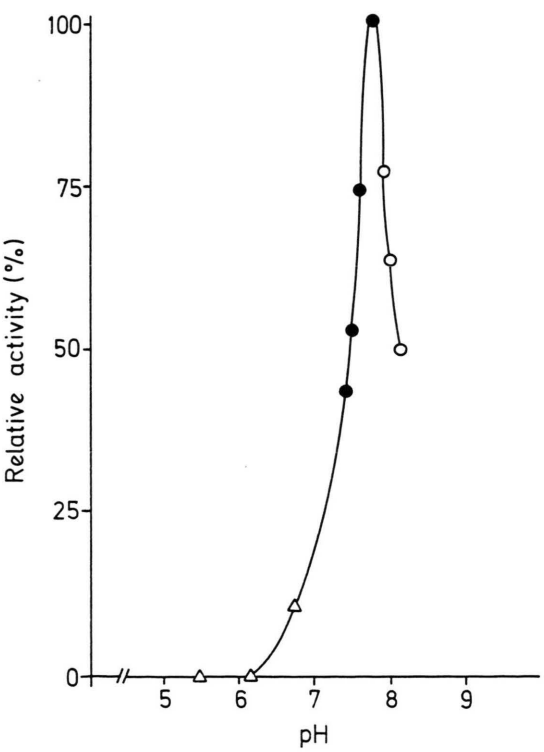


Fig. 2. pH profile of the catalytic activity of purified N-methyltransferase I from *B. vulgaris* cell cultures; △ – △ citric acid/Na-acetate; ● – ● KH_2PO_4/K_2HPO_4; ○ – ○ Tris/HCl at 0.05 M. Standard assay conditions with (*R*)-THP as substrate.

Table IV. Substrate specificity of 100 fold purified NMT-I from *B. vulgaris* cell cultures.

Substrate	Relative activity* [%]	Reaction product
(*R,S*)-Norlaudanosoline	16	laudanosoline
(*R,S*)-Norreticuline	190	reticuline
(*R,S*)-Nororientaline	190	orientaline
(*R,S*)-Noriso-orientaline	3	nd
(*R,S*)-Norprotosinomenine	0	–
(*R*)-Tetrahydropapaverine	100	laudanosine
(*S*)-Tetrahydropapaverine	20	laudanosine
Norboldine	0	–
Norglaucine	0	–
Caaverine	0	–
Tyramine	0	–
Dopamine	0	–
3-Methoxy-4-hydroxytyramine	0	–
3,4-Dimethoxytyramine	0	–
Caffeic acid	0	–

* Relative to (*R*)-THP; nd = not determined.

required to give 50% inhibition of the reaction with (*R*)-THP as substrate was 80 μM for NMT-I. Substrate specificity for NMt-I is depicted in Table IV. The best substrates so far tested were norreticuline and nororientaline followed by the standard substrate used here, (*R*)-THP. (*S*)-THP was methylated at about the same rate as norlaudanosoline. Rigorous proof of the reaction product was made for laudanosoline formed by enzymatic methylation of (*R*)-THP. As given in the data of the methods section, the product was purified to a constant specific activity and converted to a crystalline derivative (methiodide) which carried the same specific radioactivity as the reaction product. The identity of the other reaction products were determined by cochromatography using several TLC systems.

Several isoquinoline alkaloids regarded as intermediates or end products were tested with respect to their influence on the methylation reaction catalyzed by NMT-I with (*R*)-THP as substrate. At 1 mM concentration, only (*R,S*)-laudanine and berberine (a metabolite produced by the *Berberis* cells) inhibited the reaction significantly (by ca. 60%). Neither dopamine, 3,4-dihydroxyphenylacetaldehyde, (R,S),-norlaudanosoline, (*R,S*)-laudanosoline, (*R,S*)-reticuline, (*R,S*)-orientaline, glaucine nor boldine showed any drastic inhibition. Furthermore, the reaction was not significantly inhibited by usual inhibitors of enzyme reactions (at 1 mM conc.), such as: iodoacetamide, iodoacetic acid, N-ethylmaleimide, 5,5′-dithiobis (2-nitrobenzoic acid), *o*-iodosobenzoic acid, *p*-hydroxymercuribenzoate, KCN, NaN_3, sodium diethyldithiocarbamate, α,α,-dipyridyl and EDTA.

NMT for preparative purposes from B. wilsoniae

The richest source for NMT was found to be a subspecies of *B. wilsoniae* (Table I). From this mate-

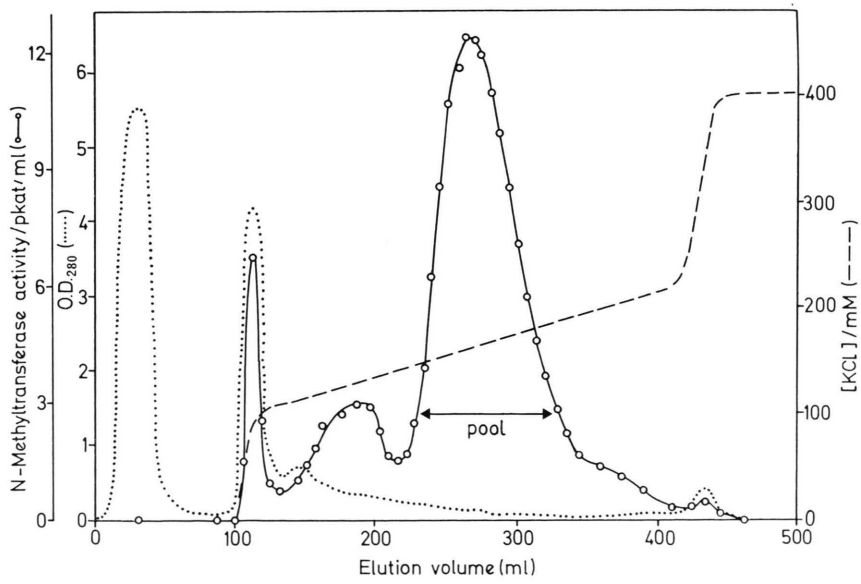


Fig. 3. Elution profile of N-methyltransferase from *B. wilsoniae* var. *subcaulialata* on DEAE Sephacel [(*R,S*)-NRK as substrate] (standard assay conditions).

rial the enzyme was isolated in 37% yield and with a six-fold purification.

Ammonium sulfate precipitation (0–70%), desalting with Sephadex G-25 and DEAE ion exchange chromatography yielded the pool fraction (Fig. 3) with 16.6 mg protein and a specific activity of 173 pkat/mg protein. At this stage the enzyme was already free of any O-methyltransferase. The ion exchange chromatography step (Fig. 3) was found to be absolutely necessary. Prior to this step, the enzyme is strongly inhibited by impurities in the protein preparation and yields of N-methylated product do not exceed 20%. Using this enzyme preparation (550 pkat), quantitative conversion of 125 μCi (2.1 μmol) [$^{14}CH_3$]-SAM, and in a separate experiment, of 5 mCi (57 nmol) [C^3H_3]-SAM in the presence of at least a 10-fold excess of unlabelled (*R*)- or (*S*)-norreticuline as a cosubstrate in a total volume of 28 ml containing each 5 mM ascorbate and phosphate buffer pH 7.4 was observed. Reticuline was recovered from the incubation mixture by ethylacetate extraction (at pH 8.5) and was the sole reaction product as judged by TLC and derivative (acetate) formation. The final specific activities of the products observed were determined by the "self-displacement" method using a radioimmunoassay specific for (*R*)- and (*S*)-reticuline (U. Wieczorek and M. H. Zenk, unpublished results) to be about 5 Ci/mmol for [N-C^3H_3]-reticuline and 45 mCi/mmol for [N-$^{14}CH_3$]-reticuline. Substrate specificity and other characteristica (pH, and temperature optimum, molecular weight etc.) of this enzyme is almost identical to NMT-I from *B. vulgaris*.

Discussion

Isoquinoline alkaloids comprise the largest group of alkaloids in the plant kingdom [1]. Reticuline, the central precursor of most isoquinoline alkaloids, is formed by condensation of dopamine with 3,4-dihydroxyphenylacetaldehyde [2] with subsequent O- and N-methylations. *In vivo* experiments have clearly shown that both types of methyl groups are derived from L-methionine [1]. S-Adenosyl-L-methionine is presumed to be the immediate methyl donor in these transmethylation reactions. This concept has been strengthened by the recent finding that (*R,S*)-norlaudanosoline in the presence of $^{14}CH_3$-SAM and cell-free extracts derived from *Berberis aggregata* callus lead to a compound similar to palmatine in its fluorescent properties and chromatographic mobility [8]. A similar incubation with the non-penolic (*R,S*)-THP provided labelled laudanosine [8]. These experiments indicated that under cell-free conditions O- and N-methylation reactions occur at the expense of SAM. From our previous work [3, 6] it is now clear that several distinct O-methyltransferases in benzylisoquinoline synthesis exist in plant tissues. Studies of their characteristics have shown that these enzymes possess a much narrower substrate specificity than previously imagined. In order to understand fully the enzymatic formation of reticuline, we have isolated and characterized the norreticuline-N-methyltransferase from *B. vulgaris*. THP and NRK was used as substrate for the assay of this enzyme. THP was used for a general survey of different plant cell cultures in order to exclusively detect only N-methyltransfer reactions and to exclude any interference by O-methylation reactions. Later on with the *B. wilsoniae* tissue, however, no O-methyltransferase activity with NRK as substrate was detected and thus NRK was used as substrate for the N-methyltransfer reaction in an alternative assay. A survey for the enzyme led to the detection of N-methyltransferase activity in several cell cultures of the genera *Corydalis, Eschscholtzia, Fumaria, Papaver,* and *Thalictrum* (to be published), as well as in *Berberis,* where the highest enzyme activity was detected. The enzyme involved was purified 100-fold from *B. vulgaris* and named systematically: S-Adenosyl-L-methionine: (*R,S*)-norreticuline N-methyltransferase. Two distinct forms of the enzyme were found and named NMT-I and NMT-II. Only NMT-I was further characterized in this paper.

Comparison with other types of N-methyltransferase from other plant sources [18] shows that the enzyme described here has a somewhat lower pH optimum (7.65) than the majority of the enzymes reported up to now. The activity was not affected by Mg^{2+} or EDTA and was not inhibited by SH-interfering reagents (at 1 mM). Only tetrahydrobenzylisoquinoline alkaloids served as substrates and none of the precursor molecules such as dopamine or tyramine was methylated, thus clearly showing a complete difference to the previously described tyramine N-methyltransferases [19]. The fact that nor-aporphine alkaloids were not N-methylated by the enzymes demonstrates their unique involvement in N-methyl-benzylisoquinoline alkaloid biosynthesis. These enzymes are most active with norreticuline

and nororientaline as substrates. Both stereoisomers of THP as well as NRK are methylated. This remarkable lack of substrate stereospecificity of these enzymes also demonstrates that stereochemical control during the biosynthesis is neither exerted at the first O-methylation [18] nor at the N-methylation level. The much lower activity (ca. 10%) shown towards (*R,S*)-norlaudanosoline indicates that some level of O-methylation might be necessary before N-methylation could take place *in vivo*. Both observations are in absolute agreement with results obtained by Brochmann-Hanssen *et al.* [5]. By *in vivo* feeding experiments using opium poppy plants, they demonstrated a low order of stereoselectivity and that O-methylation precedes N-methylation. The lack of N-methylating activity for norprotosinomenine and norisoorientaline by this enzyme further demonstrates that a 7-O-methyl substitution pattern renders the benzylisoquinoline molecule almost inactive for N-methylation. Methylation at the 4′ OH group (NRK) or at the 5′ OH group (nororientaline) has absolutely no effect on this SAM-dependent transmethylase.

For biosynthetic studies there is a definite need to have available benzylisoquinoline precursors carrying a label in the N-methyl group such as reticuline or related compounds [16]. There are several methods for the chemical N-methylation of norbenzylisoquinolines [17]. However, these are cumbersome if material highly labelled with isotopes of carbon and hydrogen is wanted. This enzyme, NRK N-methyltransferase, just described from *B. wilsoniae*, can be obtained easily in satisfactory yield (Fig. 3) and it has no absolute preference for either the (*R*)- or (*S*)-norcompound. Starting with (*S*)- or (*R*)-NRK, the N-methyl group of stereochemically pure reticuline could be labelled with ^{2}H,^{3}H,^{13}C or ^{14}C.

The degree of isotope labelling obtained would be of the same order as that of commercially available methyl-labelled-SAM or that obtained enzymatically from labelled L-methionine. This procedure will prove to be superior for obtaining labelled reticulines for biosynthetic purposes to chemial methods [17] used hitherto to resolve enantiomers of this important intermediate.

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[1] M. Shamma, The Isoquinoline Alkaloids, Academic Press, New York and London (1972).
M. Shamma and J. L. Moniot, Isoquinoline Alkaloids Research 1972–1977. Plenum Press, New York.
[2] H. M. Schumacher, M. Rueffer, N. Nagakura, and M. H. Zenk, Planta med. **48,** 212 (1983).
[3] M. Rueffer, N. Nagakura, and M. H. Zenk, Planta med. **49,** 131 (1983).
[4] A. R. Battersby, R. Binks, R. J. Francis, D. J. McCaldin, and H. Ramuz, J. Chem. Soc. **1964,** 3600.
[5] E. Brochmann-Hanssen, C. Chen, R. C. Chen, H. Chiang, A. Y. Leung, and K. McMurtrey, J. Chem. Soc. Perkin I, **1975,** 1531.
[6] M. Rueffer, N. Nagakura, and M. H. Zenk, Planta med. **49,** 196 (1983).
[7] M. D. Antoun and M. F. Roberts, Planta med. **28,** 6 (1975).
[8] W. J. Kelleher, A. Rother, E. Wellmann, and H. Grisebach, Planta med. **40,** 127 (1980).
[9] E. Linsmaier and F. Skoog, Physiol. Plant. **18,** 100 (1965).
[10] E. Späth and A. Burger, Ber. deutsch. chem. Ges. **60,** 704 (1927).
[11] H. Corrodi and E. Hardegger, Helv. Chim. Acta **39,** 889 (1956).
[12] A. R. Battersby and B. J. T. Harper, J. Chem. Soc. **1962,** 3526.
[13] S. K. Sharma and S. A. Brown, J. Chromatogr. **157,** 427 (1978).
[14] P. Izzo and R. Gantt, Biochemistry **16,** 3576 (1977).
[15] A. Bensadoun and D. Weinstein, Anal. Biochem. **70,** 241 (1976).
[16] M. Rueffer, O. Ekundayo, N. Nagakura, and M. H. Zenk, Tetrahedron letters **24,** 2643 (1983).
[17] A. R. Battersby, D. M. Foulkes, and R. Binks, J. Chem. Soc., **1965,** 3323.
[18] J. E. Poulton, in: P. K. Stumpf, E. E. Conn (eds.): The Biochemistry of Plants, **Vol. 7,** Academic Press, 668–723.
[19] E. Meyer, Plant Cell Reports **1,** 236 (1982).