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PARTIAL PURIFICATION AND PROPERTIES OF *S*-ADENOSYL-L-METHIONINE: (*S*)-TETRAHYDROPROTOBERBERINE-*CIS*-*N*-METHYLTRANSFERASE FROM SUSPENSION-CULTURED CELLS OF *ESCHSCHOLTZIA* AND *CORYDALIS*

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Key Word Index—*Corydalis vaginans*; Fumariaceae; *Eschscholtzia californica*; Papaveraceae; SAM: (*S*)-tetrahydroprotoberberine-*cis*-*N*-methyltransferase.

Abstract—An enzyme has been found in different species of isoquinoline alkaloid-producing plant cell cultures which specifically *N*-methylates certain (*S*)-tetrahydroprotoberberine alkaloids such as (*S*)-canadine and (*S*)-stylopine at the expense of *S*-adenosyl-L-methionine (SAM). It was partially purified (90-fold) from *Eschscholtzia californica* cell suspension cultures and characterized. The enzyme has a pH optimum of 8.9, a temperature optimum at 40° and a M_r of about 78 000 ± 10%. The K_m for (*S*)-canadine was determined to be 6.4 μM, for (*S*)-stylopine 3.1 μM and for SAM 12 μM. The enzyme is inhibited by *S*-adenosyl-L-homocysteine (SAH) with a K_i of 24 μM.

INTRODUCTION

(*S*)-Tetrahydroprotoberberine alkaloids can either be transformed into protoberberine molecules by enzymatic oxidation of ring C [1], or they can be *N*-methylated and then serve as precursors for large groups of isoquinoline alkaloids such as the benzophenanthridines, protopines, tetrahydrobenzazepines (rhoeadine type), spirobenzylisoquinolines (ochotensane type), etc. [2] (Fig. 1). Thus, *N*-methylation of the tetrahydroprotoberberine alkaloids serves as an important branch point in benzylisoquinoline metabolism in plants. Takao *et al.* [3] demonstrated by *in vivo* application experiments that only *cis*-*N*-methyl derivatives of tetrahydroprotoberberines can be stereospecifically metabolized to the benzophenanthridine and protopine type skeleton. In our attempt to investigate the individual and complete steps of reaction sequences leading from the primary metabolite L-tyrosine to the benzophenanthridine alkaloids sanguinarine and macarpine, we searched for the enzyme which catalyses the *N*-methylation of tetrahydroprotoberberines.

The *N*-methylation of (*S*)-7,8,13,14-tetrahydroberberine (canadine) or (*S*)-stylopine, which implies transfer of the *S*-adenosyl-L-methionine (SAM) methyl group onto the chiral alkaloidal skeleton in a stereospecific (*cis*) mode, is catalysed by a highly specific *N*-methyltransferase [4]. We now report on the partial purification and some properties of this *N*-methyltransferase, which occupies a key position in isoquinoline alkaloid biosynthesis, from suspension cells of *Eschscholtzia californica* and *Corydalis vaginans*.

RESULTS

Detection and assay of the transferase

Incubation of (*R*,*S*)-tetrahydroberberine (=canadine) with [Me-^{14}C]SAM in the presence of crude extracts of *C. vaginans* cells at pH 8.0 resulted in the formation of a product which upon TLC was indistinguishable from the reference compound *N*-methyl canadine. The same compound was formed when (*R*,*S*)-[8,14-^{3}H]canadine was incubated with unlabelled SAM. On prolonged incubation exactly 50% of the tetrahydroprotoberberine was metabolized indicating methylation of only one enantiomer. Separate incubations with either (*S*)- or (*R*)-canadine displayed turnover of only the (*S*)-enantiomer. This indicates that the enzyme under investigation shows high stereospecificity with respect to the substrate. The reaction product was unequivocally identified as *N*-methyltetrahydroprotoberberine by large scale incubation (standard incubation × 500) containing a total of 80 mg of the protein of the crude extract. The reaction product was purified by TLC, isolated and subsequently analysed by mass spectroscopy [*m*/*z* 353 $[M]^+$ (5.6), 339 (19.4), 176 (27.8), 174 (30.6), 164 (100), 149 (83.5)]. The mass spectrum was in every respect identical to *N*-methylcanadine [5]. In order to determine the stereochemistry of the *N*-methyl group, a further scale up of the incubation was performed in order to obtain sufficient material for NMR studies. Part of the ^{1}H NMR spectrum of the enzymically synthesized compound is shown in Fig. 2a, in comparison to *trans*-*N*-methyl-canadine (Fig. 2b). The most salient features of the *cis*-*N*-methylcanadine spectrum include: (i) a prominent downfield shift (δ3.27) of the deshielded *N*-methyl group in comparison to the *trans* counterpart (δ2.94), (ii) overlap of the two aromatic protons H-4 and H-1 at δ6.80. These data are in agreement with Takao [3]. The NMR spectrum unequivocally proves that the enzymatically formed product is the *cis*-derivative, which upon feeding experiments using synthetic material was previously shown to be the metabolic precursor of the benzophenanthridines [3]. Therefore, this experiment confirms the previously obtained data [3, 4].

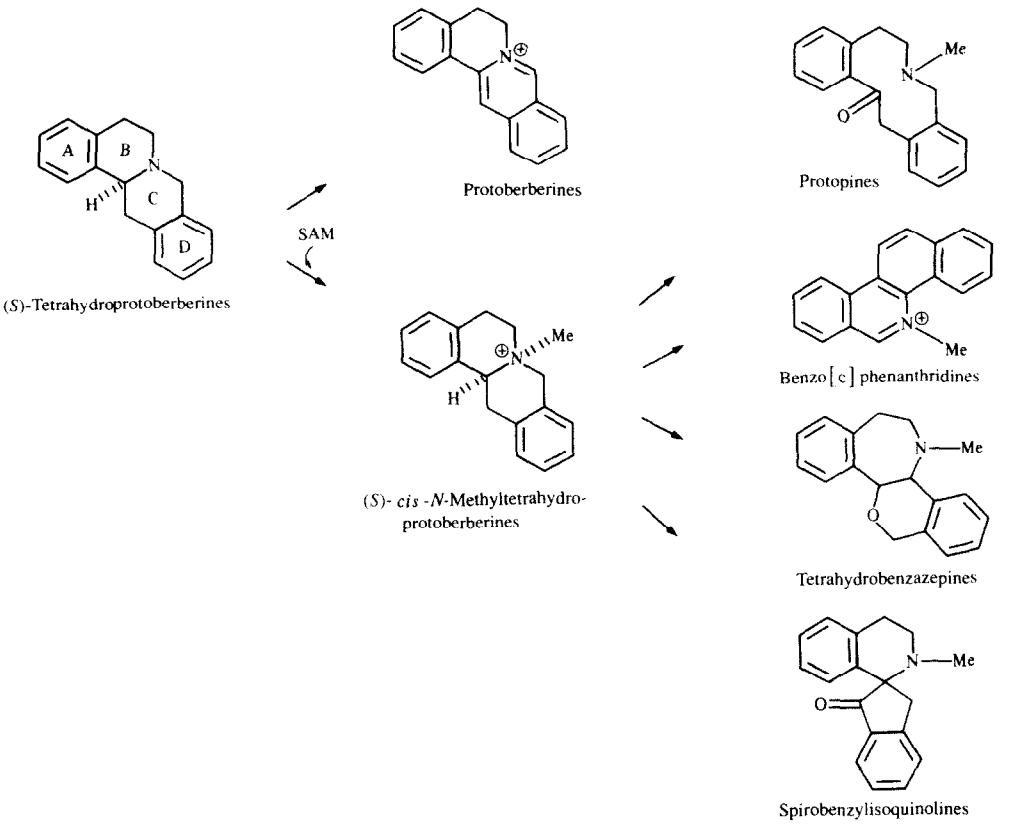


Fig. 1. The branched pathway leading from (*S*)-tetrahydroprotoberberine to protoberberine or to *cis*-*N*-methyltetrahydroprotoberberine which in turn is transformed into various alkaloidal skeletons.

Corydalis vaginans and *E. californica* were chosen as the best starting material for purification of the transferase, they showed the highest enzyme activities (*C. vaginans*, 6-8 nkat l^{-1}; *E. californica*, 25.2 nkat l^{-1}) and were also reliable with regard to both biomass and alkaloid production. A time course study was undertaken in order to determine the right day for harvesting the cells as judged by the highest total enzyme activity. Extracts were made from *E. californica* cells without or with elicitor. The latter dramatically increases the yield of benzophenanthridine alkaloids in this culture [6] and, therefore, could also influence enzyme yields in this culture.

The results obtained (Fig. 3) show that an increase in enzyme activity was paralleled for about five days by an increase in the dry mass of the cells. A sharp increase in enzyme activity was obtained on day 6 of cultivation (late log phase of the culture) but then declined to a minimal value on day 8. The addition of elicitor just about doubled (1.8-fold) the total enzyme activity and was without effect on the growth rate. Day 6 was therefore chosen as the optimal period for harvesting the cells for enzyme purification from either induced or non-induced cultures.

Enzyme purification

The data obtained in the purification procedure are shown in Table 1. The *N*-methyltransferase in the extract from suspended cells was purified 90-fold with an overall yield of 8%. Following the same general procedure, the *N*-methyltransferase from *C. vaginans* was purified 40-fold with an overall yield of 8%. The specific activities obtained were 1.2 nkat mg^{-1} and 0.16 nkat mg^{-1} for the *E. californica* and *C. vaginans* transferases, respectively. In a final purification step the specific (*S*)-tetrahydroprotoberberine-*N*-methyltransferase of *E. californica* could be separated from other contaminating methyltransferases by standard slab gel electrophoresis. The enzyme was found to be nearly homogeneous upon SDS-PAGE electrophoresis. However, the total yield had dropped to only 0.38%. The substrate specificities were conducted with the essentially pure enzyme.

Properties of the enzyme

The M_r of the *N*-methyltransferase from *E. californica* was determined to be 78000 (±10%) and from *C. vaginans* 72000 (±10%) on a calibrated Sephadex G-100 column. The pH optimum of the enzyme was 8.0, showing a half maximal activity at pH 6.6 and 9.1. The optimal temperature for the catalytic activity using the 90-fold purified enzyme was determined to be 37–40°. As determined by isoelectric focusing, the enzyme showed a IEP of 4.7. The enzyme is clearly not compartmentalized, unlike other enzymes of the protoberberine pathway such as the C ring-aromatizing (*S*)-tetrahydroprotoberberine oxidase [1]. The enzyme, if kept in the presence of 0.05% NaN_3 to assure sterile conditions, showed a half life of 5 days at 20°, 10 days at 4°, and 5 days at −20° with 20% glycerol.

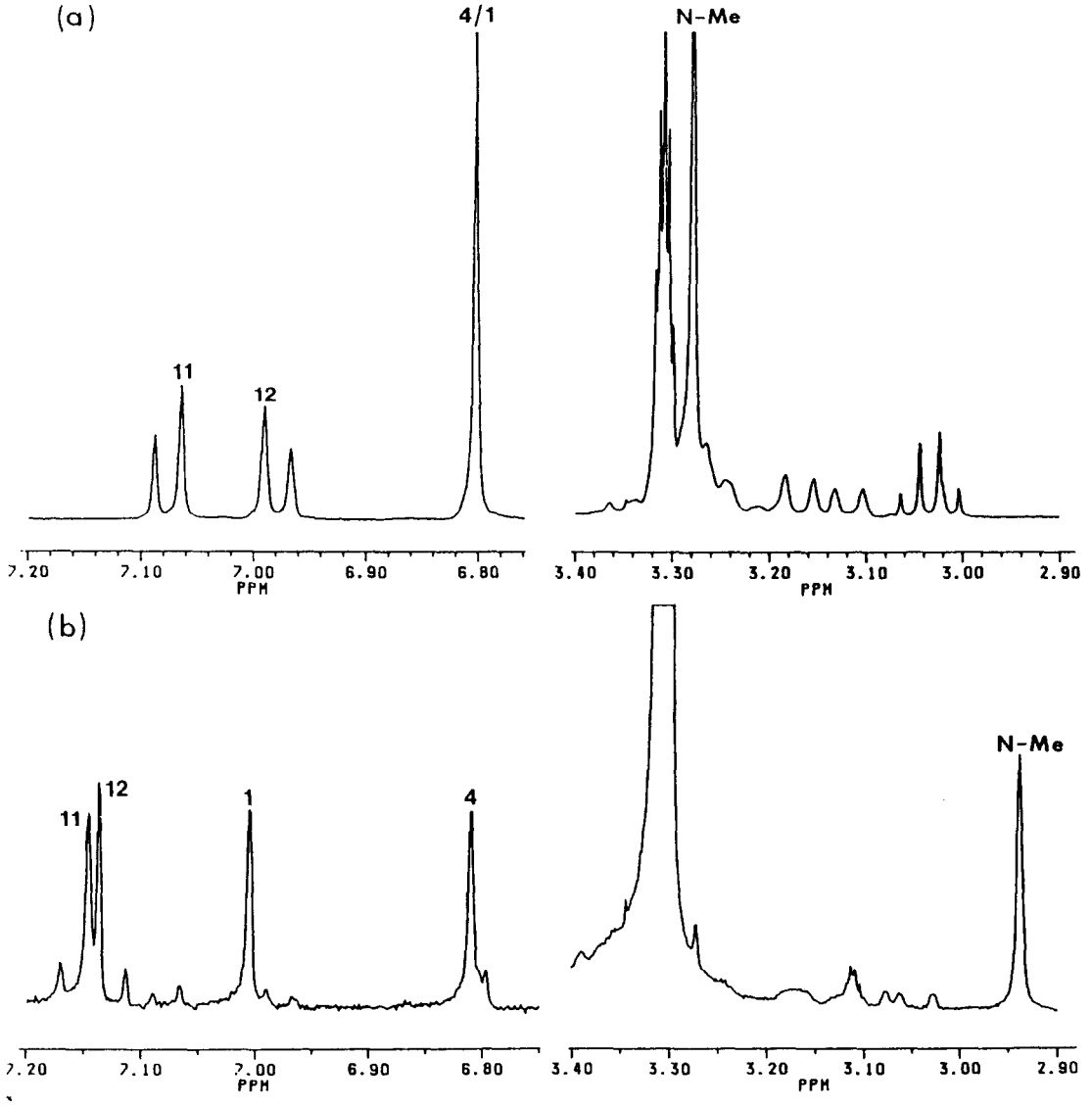


Fig. 2. Partial ^{1}H NMR spectra of (a) enzymatically formed product of the SAM: (*S*)-tetrahydroprotoberberine-*cis*-*N*-methyltransferase reaction with (*R*,*S*)-canadine as substrate, (b) *trans*-*N*-methylcanadine reference material.

Finally, the substrate specificities of both enzymes were determined using the standard assay with 4 pkat activity by measurement of the transfer of the [Me-^{14}C]-group of [Me-^{14}C]SAM onto the nitrogen group of differently substituted benzylisoquinoline molecules. The structural formulae and relative rates of conversion of potential benzylisoquinoline substrates are given in Table 2. Absolutely no reaction could be measured with the following substrates: (*R*,*S*)-cheilanthifoline, (*R*,*S*)-coclaurine, (*R*,*S*)-coreximine, (*R*,*S*)-corydaline, (*R*,*S*)-corydalmine, (*R*,*S*)-corygovanine, (*R*,*S*)-2,3-dihydroxy-9,10-dimethoxytetrahydroprotoberberine, (*R*,*S*)-methoxycorytenchirine, (*R*,*S*)-2,3-methylenedioxy-10-methoxy-11-hydroxytetrahydroprotoberberine, (*R*,*S*)-*nor*-reticuline, (*R*,*S*)-pseudostylopine, (*R*,*S*)-sinactine, (*R*,*S*)-tetrahydrocolumbamine, (*R*,*S*)-tetrahydrocorysamine, (*R*,*S*)-tetrahydrojatrorrhizine, (*R*,*S*)-tetrahydropalmatine, (*R*,*S*)-tetrahydrothalifaurine, (*R*,*S*)-tetrahydrothalifendine and (*R*,*S*)-2,3,9,10-terahydroxytetrahydroprotoberberine.

Lineweaver–Burk plots gave a K_m value of 7.0 μM for (*S*)-canadine, for (*S*)-stylopine 4.0 μM and a K_m for SAM of 1.7 μM, while the K_i for SAH was 24 μM in the case of the *C. vaginans* enzyme. For the enzyme from *E. californica* a K_m value of 12 μM for SAM, 6.4 μM for (*S*)-canadine, 3.1 μM for (*S*)-stylopine and a K_i value of 50 μM for SAH was determined.

Occurrence of the enzyme

In our preliminary note [4] we observed the occurrence of the *N*-methyltransferase in five species of isoquinoline-containing plants. This study was extended here (Table 3) and the enzyme was found in a considerable number of cell culture species known to contain isoquinoline alkaloids derived from *N*-methyltetrahydroprotopines. Only low activity of the target enzyme was detected in members of the Berberidaceae which contain mainly protoberberine alkaloids. Fumariaceae and

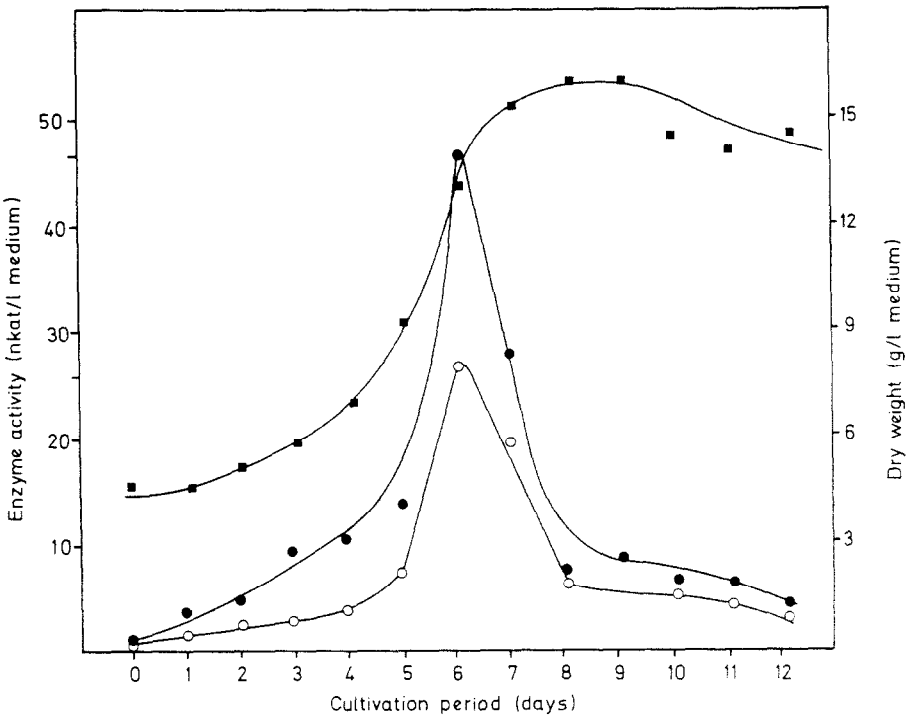


Fig. 3. Time course of SAM: (*S*)-tetrahydroprotoberberine-*cis*-*N*-methyltransferase activity and cell dry mass (■) in cell suspension cultures of *E. californica* without (○) and with (●) addition of yeast elicitor (10 mg to 1 l Erlenmeyer flasks containing 250 ml medium).

Table 1. Purification of the SAM from *E. californica* cell suspension cultures

Purification step	Total activity (nkat)	Total protein (mg)	Specific activity (pkat mg^{-1})	Yield (%)	Purification (*x*-fold)
Crude extract	24.5	1850	27	100	1
DEAE eluate	41.0	924	44	167	3
Phenylsepharose eluate	26.0	172	150	106	11
Hydroxyapatite eluate	15.0	32	480	61	35
SAH-AH-Sepharose eluate	2.0	1.5	1214	8	90

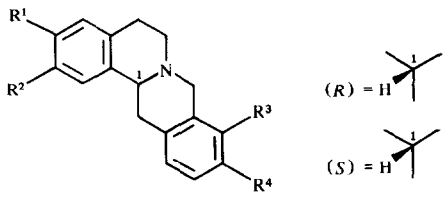
Activities were measured by the assay using [^{3}H]SAM as described in 'Experimental'.

Papaveraceae show considerable enzyme activity towards tetrahydroprotoberberine substrates, stylopine being the preferred substrate in most cases but also the tetramethoxylated alkaloid tetrahydropalmatine serves as a methyl group acceptor in most species investigated. This is at variance with the purified enzyme and it can be expected that there is a second *N*-methyltransferase activity present in crude extracts. The control species were *Rosa canina* and *Rauvolfia serpentina*. None of the above cultures showed any enzyme activity. This demonstrates the specificity of the enzyme under investigation and its exclusive role in isoquinoline alkaloid metabolism. This enzyme is not involved in primary metabolism.

DISCUSSION

The *S*-adenosyl-L-methionine: (*S*)-tetrahydroprotoberberine-*cis*-*N*-methyltransferase has been partially purified and characterized. In the presence of SAM and a specifically substituted (*S*)-tetrahydroprotoberberine the transferase catalysed the *cis*-*N*-methylation of the alkaloid (Fig. 4). This reaction opens a branch pathway. The *N*-methylated tetrahydroprotoberberines cannot be aromatized in ring C by the specific oxidase [1] and will be transformed by oxidation at position 14 to the protopines [7], that either accumulate in the plant or in turn can be further metabolized by 6-hydroxylation to dihydrobenzophenanthridines [8]. Other possibilities of transformation of the *N*-substituted alkaloids involve the formation of the tetrahydrobenzazepines and spirobenzylisoquinolines (Fig. 1). A comparison of both enzymes, catalysing one and the same reaction but isolated from two different plant cell cultures, is shown in Table 4. It is noteworthy that the *N*-methyltransferase is absolutely inactive towards the (*S*)-tetrahydrobenzylisoquinoline alkaloids scoulerine and tetrahydrocolumbamine, which

Table 2. Relative rates of conversion of tetrahydroprotoberberines to *N*-methyltetrahydroprotoberberines catalysed by SAM



Substrate	R^1	R^2	R^3	R^4	Rate of transformation (%) *C. vaginans*	*E. californica*
(*S*)-Canadine	O–CH_2–O		OMe	OMe	100	100
(*R*)-Canadine	O–CH_2–O		OMe	OMe	0	0
(*S*)-Stylopine	O–CH_2–O		O–CH_2–O		104	109
(*R*)-Stylopine	O–CH_2–O		O–CH_2–O		0	0
(*R,S*)-Nandinine	O–CH_2–O		OH	OMe	90	94
(*R,S*)-Tetrahydrogroenlandicine	OH	OMe	O–CH_2–O		12	17
(*S*)-Scoulerine	OMe	OH	OH	OMe	0	0

are respectively both the first and common intermediates starting from the central intermediate (*S*)-reticuline, leading either to the protoberberines or the series of *N*-substituted tetrahydroprotoberberine derived alkaloids (Fig. 1). (*S*)-Scoulerine is formed through action of the berberine bridge enzyme contained in a special vesicle with a density of $\rho = 1.14\ g\ ml^{-1}$ [9]. (*S*)-Scoulerine is released from the vesicle and methylation to tetrahydrocolumbamine occurs by a cytoplasmic *O*-methyltransferase [10]. Alternatively formation of both methylenedioxy groups on the way to (*S*)-stylopine occurs by microsomal bound enzymes [11]. Only after substitution is completed to afford (*S*)-tetrahydroberberine (canadine) or (*S*)-stylopine, can the specific *N*-methyltransferase, described here, act on these molecules. An unusually high degree of substrate specificity is observed in this enzyme, where clearly the substitution pattern of the alkaloidal substrate determines whether a metabolic branch is opened or not. This enzyme specificity in hand with compartmentalization *en route* to the different metabolites, determines which of the end products finally accumulates in the plant or cell culture.

EXPERIMENTAL

Plant material. Plant cell suspension cultures were provided by the cell culture laboratory of our department. Cells were routinely grown in 1 l conical flasks containing 250 ml Linsmaier–Skoog medium [12] over a period of 7–14 days at 23° on a gyratory shaker (100 rpm) in diffuse light (750 lux). After filtration by suction the tissue was immediately frozen with liquid nitrogen, stored at −20° and used as an enzyme source. In some cases *E. californica* cultures were elicited as described previously [6] by addition of 10 mg yeast elicitor per 250 ml medium.

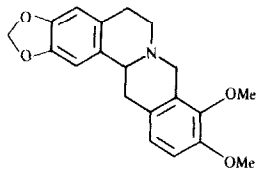
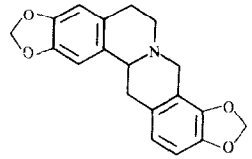
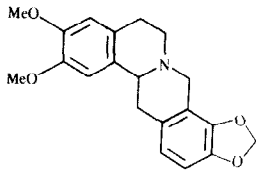
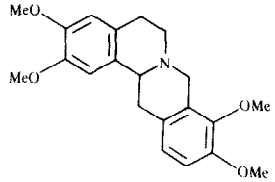
Chemicals. SAM und SAH were purchased from Boehringer (Mannheim). Stylopine and (*R*)- and (*S*)-canadine were kind gifts of Prof. Nagakura (Kobe) and (*S*)-*trans*-*N*-methylcanadine was provided by Dr Tanahashi (Kobe). All other alkaloids were synthesized in our laboratory according to standard procedures. Buffer salts were purchased from Merck (Darmstadt).

Radiochemicals. ^{3}H-SAM and ^{14}C-SAM were synthesized with 95% yield from [^{3}H]methionine (spec. act.: 3.03 TBq mmol^{-1} = 82 mCi μmol^{-1}) and [^{14}C]methionine (spec. act.: 2.07 GBq mmol^{-1} = 56 μCi μmol^{-1}) (both Amersham-Buchler) with a partially purified SAM-synthetase according to ref. [13].

Enzyme assay. (*R,S*)-Canadine (40 nM), ^{3}H-SAM (8 nM; 17 000 cpm) and the enzyme preparation (15 μg–1 mg protein depending on the state of purification) were incubated in a final vol. of 250 μl with 200 mM glycine buffer (pH 8.0) for 20 min at 40°. The incubation was stopped by addition of 400 μl isoamylalcohol, the mixture shaken mechanically for 20 min, and subjected to Eppendorf centrifugation for 3 min. The organic phase (200 μl) was transferred to a scintillation vial with 5 ml scintillator (Emulsifier Safe, Packard) and counted (Betaszint BF 8000). Assays with boiled enzyme solns served as controls. To verify the nature of the *N*-methylated tetrahydroprotoberberines, the incubation mixtures were subjected to TLC (Polygram SIL G/UV$_{254}$, Macherey–Nagel) using $CHCl_3$–MeOH–NH_3 (350:100:3) as solvent system (SAM R_f 0.0, *N*-methylcanadine R_f 0.5 *N*-methylstylopine R_f 0.45, stylopine R_f 1.0).

Enzyme preparation and purification. All extraction and purification procedures were performed at 0–4°. Unless otherwise stated, all chromatography columns were equilibrated with the standard buffer 10 mM K-Pi, pH 7.5 (20 mM 2-mercaptoethanol). For extraction of the cells, 1 kg of deep frozen cells were thawed with stirring in standard buffer, filtrated through cheesecloth and the filtrate centrifuged for 15 min at 13 000 *g*. The clear supernatant was pumped onto a DEAE-column (Amicon matrex AH-cellufine, 5 cm $\phi \times 20$ cm) operating at a flow rate of 180 ml hr^{-1}. Protein not absorbed was washed off with 700 ml standard buffer and thereafter the enzyme eluted with a 0–1 M KCl gradient in standard buffer 1.44 l, 8 hr). The enzyme was eluted in 220 ml at *ca* 0.7 M KCl. The fractions containing the enzyme were combined and the KCl concn in the eluate raised to 1 M. This soln was applied to a phenyl Sepharose column (Pharmacia, 5 cm $\phi \times 20$ cm) under hydrostatic pressure, equilibrated with 1 M KCl in standard buffer. The enzyme was eluted with standard buffer (172 ml) under hydrostatic pressure and applied to a hydroxyapatite column (Biorad, 1.5 $\phi \times 10$ cm) at a flow rate of 20 ml hr^{-1}. After washing the column with 2 vols

Table 3. Occurrence of SAM in different plant cell cultures originating from plants containing benzylisoquinoline alkaloids

Cell culture	Substrate: (*R,S*)-Canadine	(*R,S*)-Stylopine	(*R,S*)-Sinactine	(*R,S*)-Tetrahydropalmatine
				
	(pkat g^{-1} dry weight)			
Berberidaceae				
Berberis beaniana	0.9	0.9	1.7	0
B. notabilis	0	0	0	0
B. wilsoniae	0	0.9	2	1
Fumariaceae				
Corydalis vaginans	52	80	29	31
Dicentra spectabilis	28	40	12	50
Fumaria officinalis	44	42	n.d.	52
Menispermaceae				
Chondodendron tomentosum	2	2	n.d.	4
Tinospora cordifolia	0	0	0	0
Papaveraceae				
Argemone platyceras	41	41	57	49
Bocconia cordata	20	18	12	20
Eschscholtzia caespitosa	77	107	30	74
E. californica	55	55	2	39
Ranunculaceae				
Thalictrum glaucum	0.4	0.4	0.8	1.1
T. bautrinii	0	4	0	0

Cell cultures from *Rosa canina* (Rosaceae) and *Rauvolfia serpentina* (Apocynaceae) served as control cultures containing no benzylisoquinolines and no tetrahydroprotoberberine-*N*-methyltransferase activity.

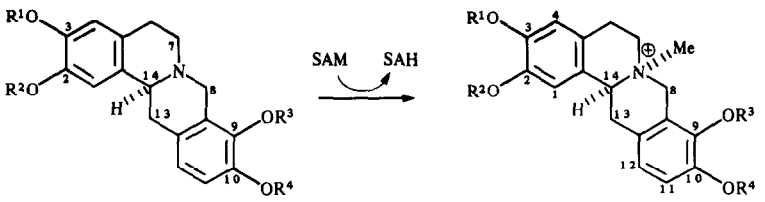


Fig. 4. Reaction catalysed by SAM: (*S*)-tetrahydroprotoberberine-*cis*-*N*-methyltransferase.
(*S*)-Canadine: R^1–R^2 = O–CH_2–O, $R^3 = R^4$ = OMe
(*S*)-Stylopine: R^1–R^2 = O–CH_2–O, R^3–R^4 = O–CH_2–O
(*S*)-Nandinine: R^1–R^2 = O–CH_2–O, R^3–OH, R^4 = OMe
(*S*)-Tetrahydrogroenlandicine: R^1 = OH, R^2 = OMe, R^3–R^4 = O–CH_2–O.

Table 4. Comparison of the most important characteristics of the SAM isolated from two different plant cell cultures

Properties	*C. vaginans*	*E. californica*
M_r	72 000 ± 10%	78 000 ± 10%
pH optimum	pH 8.0	pH 8.0
Temperature optimum	40°	40°
K_m (*S*)-canadine	7.0 μM	6.4 μM
K_m (*S*)-stylopine	4.0 μM	3.1 μM
K_m SAM	1.7 μM	12.0 μM
K_i SAM	24.0 μM	50.0 μM

of standard buffer, the elution was performed with a gradient of 10–500 mM K-Pi, pH 7.5 (20 mM 2-mercaptoethanol) (180 ml/8 hr). Enzyme activity was eluted in 32 ml at a concentration of 0.1 M K-Pi. The eluate was desalted and concd by ultrafiltration (Amicon flow cell, membrane: YM 10) and subjected to an affinity column (SAH-coupled by the carbodiimide method to AH-Sepharose 4B, Pharmacia, 1 cm $\phi \times 4$ cm) at a flow rate of 20 ml hr^{-1}. The enzyme was eluted with a gradient of 0–1 M KCl in standard buffer (100 ml per 4 hr). The fractions containing enzyme activity were combined (100 ml) and used for the characterization of the enzyme.

Protein determination. Protein concentrations were determined using Coomassie Brilliant Blue G-250 (Serva) with BSA as standard according to ref. [14].

M_r *determination.* The M_r of the enzyme was determined by gel filtration in standard buffer on a Sephadex G-100 column (Pharmacia, 2.5 cm $\phi \times 100$ cm) at a flow rate of 20 ml/hr. The markers used were aldolase (M_r 142 000), BSA (M_r 67 000), ovalbumin (M_r 45 000), myoglobin (M_r 17 800), and cytochrome *c* (M_r 12 300).

Electrophoresis. Native PAGE was carried out using 7.5% acrylamide (Serva) at pH 8.9 according to ref. [15]. SDS-electrophoresis was performed using 12.5% polyacrylamide and buffer system according to ref. [16].

Isoelectric focusing. Isoelectric focusing was performed in a granulated gel (Sephadex G-200, Pharmacia) according to ref. [17].

Product isolation. For product identification 26 μmol SAM and 132 μmol (*R,S*)-canadine were incubated in 0.5 M glycine buffer (pH 8.0) with 1 g protein (crude extract after Sephadex G-25 filtration) in a total vol. of 3 l at 30° (12 hr). The reaction product and unreacted substrate were adsorbed onto XAD-2, the column washed extensively with H_2O and the adhering constituents eluted with MeOH. After concn the residue was subjected to TLC (solvent system: $CHCl_3$–MeOH–NH_3, 140:40:1) and the compound with an R_f-value of 0.3 eluted with MeOH. After drying *in vacuo* the yield was 5.6 mg (65.9% calculated).

NMR analysis. ^{1}H spectra were recorded at 350 MHz in CD_3OD and with TMS as int. standard (300 scans). Analysis of the enzymatically formed product showed the following spectrum ^{1}H NMR: δ3.27 (3H, *s*, N-Me), 3.02–3.64 (6H, *m*, H-5, H-6, H-13), 3.86 (3H, *s*, O-Me), 3.88 (3H, *s*, O-Me), 4.73 (1H, *m*, H-14), 5.98 (2H, *dd*, O-CH_2-O), 6.80 (2H, *s*, H-4, H-1), 6.98 (1H, *d*, J = 8.5 Hz, H-12), 7.07 (1H, *d*, J = 8.5 Hz, H-11).

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