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S-ADENOSYL-L-METHIONINE:(S)-COCLAURINE-N-METHYLTRANSFERASE FROM *TINOSPORA CORDIFOLIA**

SUSANNE LOEFFLER, BRIGITTE DEUS-NEUMANN and MEINHART H. ZENK†

Lehrstuhl für Pharmazeutische Biologie, Universität München, Karlstrasse 29, D-80333 München, Germany

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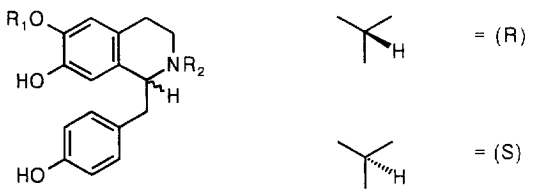
Key Word Index—*Tinospora cordifolia*; Menispermaceae; cell cultures; (*S*)-coclaurine-*N*-methyltransferase; *N*-methyltransferase survey in cell cultures.

Abstract—A survey of eight plant cell cultures belonging to four isoquinoline alkaloid producing plant families revealed that norcoclaurine is, in the presence of *S*-adenosyl-L-methionine (SAM), transformed into *N*-methylnorcoclaurine, coclaurine and *N*-methylcoclaurine. In *Tinospora cordifolia* (*S*)-norcoclaurine was exclusively *O*- and *N*-methylated and not its (*R*)-enantiomer. The *N*-methylating enzyme activity was purified and shown to catalyse stereoselectively only the methyl transfer from SAM to (*S*)-configured norcoclaurine and coclaurine. Of a total of 15 benzylisoquinoline alkaloids only (*S*)-coclaurine and (*S*)-norcoclaurine were accepted as substrates. The pH optimum of this enzyme is 8.6 and (*S*)-coclaurine as substrate yields a K_m of 36 μM, while the K_m for SAM is 44 μM. The enzyme is a single polypeptide with M_r 85 $\pm$ 2 $\times$ 10^3. This stereoselective enzyme is apparently only present in members of the Menispermaceae and in *Dicentra spectabilis* (Fumariaceae).

INTRODUCTION

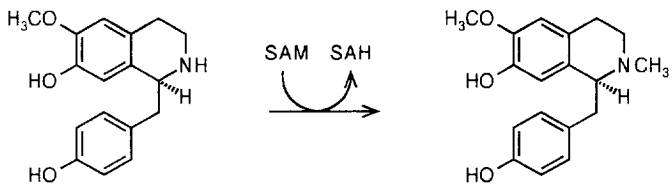
(*S*)-Reticuline is one of the most versatile metabolites in secondary metabolism, since its substitution pattern allows several very distinct intra- and intermolecular phenol oxidative coupling reactions, which by further modification can lead to a large number of different classes of isoquinoline alkaloids [1, 2]. The originally proposed pathway leading from the tetraoxygenated norlaudanosoline [3, 4] to reticuline had to be revised in light of the fact that the trihydroxylated alkaloid norcoclaurine was shown to be the true precursor of reticuline after performing *in vivo* and *in vitro* feeding experiments [2, 5–9]. The first step in norcoclaurine biosynthesis encompasses a condensation of dopamine with *p*-hydroxyphenylacetaldehyde. Transformation of norcoclaurine to reticuline involves two *O*-methylation, one hydroxylation and one *N*-methylation step. Methylation at the C-6 hydroxyl group yields coclaurine, which is subsequently *N*-methylated to give *N*-methylcoclaurine [8]. To date, several SAM-dependent *N*-methyltransferases that modify tetrahydroisoquinoline alkaloids have been described from various isoquinoline producing cell cultures [10, 11]. These enzymes, termed *S*-adenosyl-L-methionine:(*R*,*S*)-tetrahydrobenzylisoquinoline-*N*-methyltransferases [11] proved to be non-stereoselective, accepting both the (*R*)- and the (*S*)-configured substrates. These enzymes have proven to be very useful for the enzyme catalysed synthesis of (*R*)- and (*S*)-reticuline and other precursors labelled in the *N*-methyl group with radioactive or stable isotopes [10, 12]. In the course of our studies on the formation of the distant precursors of the isoquinoline pathway, we surveyed a considerable number of plant cell cultures for their ability to methylate early precursors of reticuline, and discovered in the family Menispermaceae members of the genus *Tinospora* which show a surprising substrate specificity. The *Tinospora N*-methyltransferase is highly substrate specific and methylates, besides (*S*)-norcoclaurine, exclusively (*S*)-coclaurine to form (*S*)-*N*-methylcoclaurine, which is an important branch point intermediate in benzylisoquinoline alkaloid biosynthesis (Fig. 1). This *N*-methylated alkaloid can either be channelled directly into the bisbenzylisoquinoline alkaloid biosynthetic route to yield structures of the berbamunine type [13, 14], or it can be metabolized via hydroxylation and additional *O*-methylation to afford (*S*)-reticuline. This paper deals with a



Norcoclaurine: $R_1 = R_2 = H$
Coclaurine: $R_1 = CH_3$; $R_2 = H$
N-Methylnorcoclaurine: $R_1 = H$; $R_2 = CH_3$
N-Methylcoclaurine: $R_1 = R_2 = CH_3$

*Dedicated to Professor Heinz Floss on the occasion of his 60th birthday.

†Author to whom correspondence should be addressed.



(S)-Coclaurine (S)-N-Methylcoclaurine

Fig. 1. Reaction catalysed by action of *S*-adenosyl-L-methionine:(*S*)-coclaurine-*N*-methyltransferase in the presence of SAM.

survey of *O*- and *N*-methylation activities of early precursors of reticuline and the purification and characterization of a novel stereoselective enzyme which we designate *S*-adenosyl-L-methionine:(*S*)-coclaurine-*N*-methyltransferase.

RESULTS AND DISCUSSION

General survey of cell cultures for N-*methyltransferase activity*

The transformation of (*S*)-norcoclaurine, the first true alkaloidal intermediate in the biosynthesis of reticuline-derived alkaloids [5–7], involves *O*-methylation at C-6 followed by an *N*-methylating step to afford *N*-methylcoclaurine [2]. To distinguish between *O*- and *N*-methyltransferase activities in our cell cultures, the screening assay described in ref. [8] was employed. A survey for *O*- and *N*-methylating activities was performed using a variety of suspension cultures established from plants that are known to produce benzylisoquinoline alkaloids, employing (*R*)- and (*S*)-norcoclaurine as substrates. The results of this investigation are presented in Table 1. In order to differentiate between *O*- and *N*-methylated products, samples were incubated under standard assay conditions using a tetrahydroisoquinoline alkaloid as acceptor and [$^{14}CH_3$]SAM as methyl group donor, and subsequently subjected to TLC as described in the Experimental. Under the conditions of the standard enzyme assay, all cell extracts exhibited measurable norcoclaurine-6-*O*-methyltransferase activity. In most cases, this enzyme activity was accompanied by non-stereoselective *N*-methylating activities, which yielded (*R*)- and (*S*)-*N*-methylnorcoclaurine and -*N*-methylcoclaurine, respectively. However, only suspension cultures of *T. cordifolia* and *D. spectabilis*, showed stereoselective *N*-methyltransferase activity, taking exclusively (*S*)-enantiomers as substrates (Table 1). This is the first example of an absolutely stereoselective *N*-methyltransferase enzyme in isoquinoline alkaloid biosynthesis. In order to determine the optimal time for harvesting the cells, a kinetic analysis of cell growth (dry wt) and enzyme activity was conducted. Enzyme activity increased during the log phase and reached a maximum on day 5 of cultivation just before the stationary growth phase (Fig. 2). During this phase, the relative enzyme activity distinctly decreased to a minimum at the end of the growth period. For optimal enzyme yield, cells were harvested on day 5 of cultivation and used for enzyme purification.

Purification of N-*methyltransferase activity from* T. cordifolia

Purification of the enzyme was carried out using 375 g deep frozen 5-day-old cells of *T. cordifolia*. The enzyme was pre-purified by $(NH_4)_2SO_4$ precipitation (40%) and the supernatant applied to a Phenyl Sepharose column. The column was eluted with 10 mM KPO_4^{2-} buffer, pH 7.5, containing 20 mM MeSH. The active fractions were pooled and loaded onto a TSK Fractogel column (Fig. 3A). Elution was achieved as described above. Fractions containing the enzyme (100% yield) were combined and subjected to ion exchange chromatography employing a Mono Q matrix (Fig. 3B). The enzyme activity was eluted with a linear KCl gradient (0–1 M) in standard buffer. The active fractions were pooled and subsequently desalted by Sepharose G 25 chromatography. Elution was done with 10 mM KPO_4^{2-} buffer, pH 6.5, without addition of MeSH. The next purification step consisted of affinity chromatography employing coclaurine-bound epoxy-activated Sepharose 6B (Fig. 3C). Elution was performed with a KCl-step-gradient in 10 mM KPO_4^{2-} buffer with enzyme activity in the effluent, which was immediately brought to pH 9.0 with Na_2CO_3–$NaHCO_3$ and subsequently desalted by Sephadex G 25 chromatography. Finally, the enzyme preparation was subjected to disc electrophoresis. The active enzyme recovered after this step was used to characterize the *S*-adenosyl-L-methionine:(*S*)-coclaurine-*N*-methyltransferase. The final purification step yielded a 115-fold purified enzyme (Table 2). Of the initial protein, 99.9% was separated while 9% of enzyme activity could be recovered in the last purification step.

Product identification

The alkaloidal product formed in a large-scale incubation (100-fold) using the 115-fold purified enzyme was

Table 1. *N*- and *O*-methylating enzyme activities in crude extracts of different plant cell suspension cultures using [$^{14}CH_3$]SAM and the indicated alkaloids as substrates

Cell culture	Substrate	Enzyme activity: pkat mg^{-1} protein	Enzyme activity: nkat l^{-1} medium	Labelled products formed (%): *N*-CH_3-Norcoclaurine	Coclaurine	*N*-CH_3-Coclaurine
Fumariaceae						
Dicentra spectabilis	(*R*)-Norcoclaurine	0	0	0	0	0
	(*S*)-Norcoclaurine	1.3	0.5	0	64	36
Fumaria capreolata	(*R*)-Norcoclaurine	1.4	0.4	20	45	35
	(*S*)-Norcoclaurine	2.9	0.8	25	38	37
Menispermaceae						
Chondodendron tomentosum	(*R*)-Norcoclaurine	1.0	0.2	29	45	26
	(*S*)-Norcoclaurine	1.8	0.4	21	52	27
Tinospora cordifolia	(*R*)-Norcoclaurine	0	0	0	0	0
	(*S*)-Norcoclaurine	14.9	1.6	0	(0)*	100
Papaveraceae						
Argemone platyceras	(*R*)-Norcoclaurine	9.4	0.1	48	52	0
	(*S*)-Norcoclaurine	11.5	0.2	49	36	15
Papaver somniferum	(*R*)-Norcoclaurine	12.0	4.0	21	65	14
	(*S*)-Norcoclaurine	18.4	6.2	42	47	11
Berberidaceae						
Berberis stolonifera	(*R*)-Norcoclaurine	22.5	3.3	9	70	21
	(*S*)-Norcoclaurine	33.1	4.8	11	8	81
Berberis juliana	(*R*)-Norcoclaurine	2.0	0.6	0	78	22
	(*S*)-Norcoclaurine	9.4	2.8	36	5	59

*Product immediately converted to *N*-methylcoclaurine.

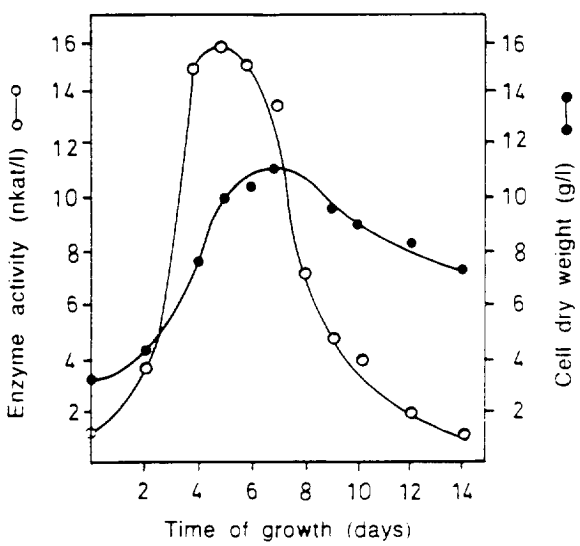


Fig. 2. Time course of *S*-adenosyl-L-methionine:(*S*)-coclaurine-*N*-methyltransferase activity (○—○) and cell dry weight (●—●) in a suspension culture of *Tinospora cordifolia*.

identified as *N*-methylcoclaurine by mass spectral analysis (CI, 180 eV): *m/z* (% relative intensity) 300 [M + H]$^+$ (100), 192 (7), 107 (4). Clearly only one methyl group is transferred from one molecule of SAM to (*S*)-coclaurine. The fragmentation pattern shows that methylation occurs at the nitrogen atom of coclaurine and does not involve one of the phenolic hydroxy groups.

*Properties of coclaurine-*N*-methyltransferase*

The 115-fold purified enzyme was used to characterize its catalytic properties. The M_r of the enzyme was determined by gel permeation HPLC to be 83 000 and by gel filtration to be 87 000, using in both cases appropriate marker proteins. The pH optimum of the methyltransferase was at 8.6 (Fig. 4). The temperature optimum of the enzyme was determined using triethanolamine buffer, pH 9.0, and a 23-fold purified enzyme preparation, and was in the range 34°–37° (Fig. 5).

Substrate specificity

The substrate specificity of the enzyme was analysed by incubating 20 nmol of various stereoisomers or racemates of benzylisoquinolines with 3 pkat of the 115-fold purified enzyme in 50 μmol triethanolamine buffer, pH 9.0, under standard assay conditions with subsequent TLC analysis of the reaction products. The results of this investigation are shown in Table 3. Of all the compounds tested only (*S*)-coclaurine and, to a minor extent, (*S*)-norcoclaurine (8%) were converted to their respective *N*-methylated derivatives. Minor activity was found with (*S*)-norreticuline (0.6%) and (*R,S*)-4′-*O*-methylnorlaudanosoline (0.7%). However, traces of impurities present in these synthetic compounds which could serve as

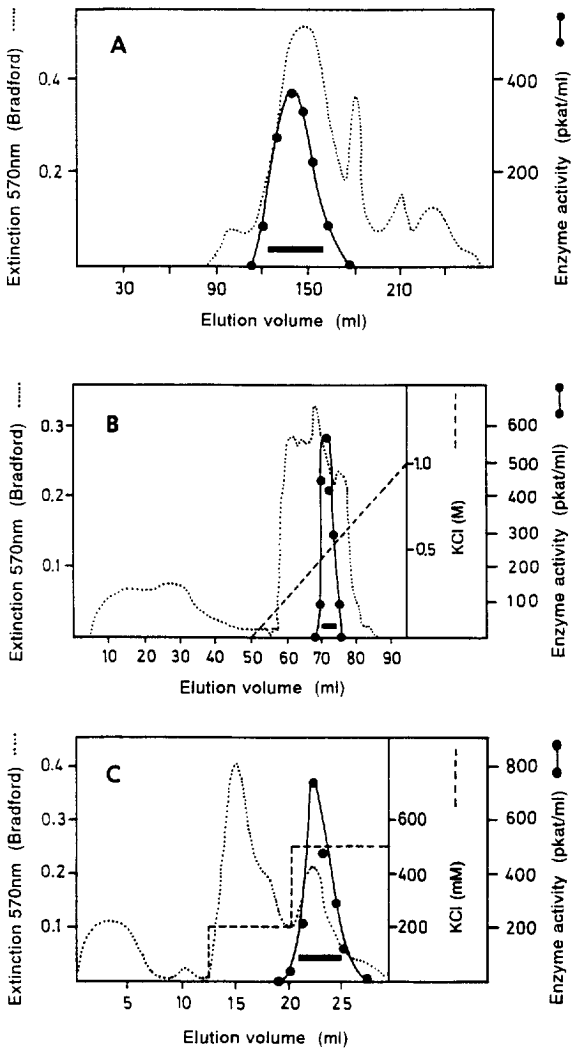


Fig. 3. (A) TSK Fractogel treatment of a pre-purified *S*-adenosyl-L-methionine:(*S*)-coclaurine-*N*-methyltransferase preparation obtained after Phenylsepharose chromatography. (B) Mono Q ion exchange chromatography of pooled active fractions from step (A). (C) Affinity chromatography using coclaurine-bound epoxy-activated Sepharose of the combined active fractions from (B). The bars indicate the combined fractions, which were used for subsequent enzyme purification.

substrates cannot be excluded. Methylation of coclaurine at the 7-*O*-position or changes in the substitution pattern of ring C impeded the enzymic reaction. Therefore, the coclaurine-*N*-methyltransferase was found to be highly substrate specific and exhibits strict stereoselectivity by converting only the (*S*)- but not the (*R*)-enantiomers of coclaurine and norcoclaurine. Most interestingly, however, (*S*)-norlaudanosoline, the previously postulated universal precursor for most benzylisoquinolines [3, 4] was not accepted as substrate by this highly specific enzyme. This fact again strongly supports the finding that the trihydroxylated norcoclaurine and not the tetrahydroxylated norlaudanosoline is the true precursor for benzylisoquinoline alkaloid biosynthesis in the plant kingdom [2, 8].

Michaelis–Menten kinetics displayed a sigmoidal plot of turnover rate vs substrate concentration (Fig. 6A). The resulting exponential trace obtained after Lineweaver–Burk transformation suggests a positive cooperative effect, i.e. an allosteric enzyme (Fig. 6B). To determine the K_m value, the Lineweaver–Burk plot was linearized by substituting $1/[S]$ with $1/[S]^n$. The Hill coefficient, n, was determined from the slope of the Hill plot as 2.6 (Fig. 6C). Linear Lineweaver–Burk plots gave apparent K_m values of 36 μM for (*S*)-coclaurine (Fig. 6D) and 52 μM for (*S*)-norcoclaurine with SAM (200 μM) as the second substrate. For SAM an apparent K_m value of 44 μM was determined with (*S*)-coclaurine at a concentration of 1 mM. The concentration of *S*-adenosyl-L-homocysteine (SAH) required for 50% inhibition (K_i) of the coclaurine-*N*-methyltransferase reaction with (*S*)-coclaurine as substrate was found to be 62 μM.

*Occurrence of coclaurine-*N*-methyltransferase*

In order to extend our knowledge of the occurrence and taxonomic distribution of the coclaurine-*N*-methyltransferase, 42 different 5-day-old cell suspension cultures representing seven plant families were screened for enzyme activity employing (*R*)- as well as (*S*)-coclaurine as substrates under standard assay conditions. The assay mixtures were tested for methyltransferase activities by 2D TLC [(1) $CHCl_3$–MeOH–NH_4OH, 90:9:1; (2)

Table 2. Summary of an average purification of *S*-adenosyl-L-methionine:(*S*)-coclaurine-*N*-methyltransferase from 375 g (fr. wt), 5-day-old cells of *T. cordifolia*

Purification step	Volume (ml)	Protein (mg)	Total activity (nkat)	Specific activity (pkat mg^{-1})	Yield (%)	Purification (-fold)
Crude extract	830	382	22	58	100	1
$(NH_4)_2SO_4$ precipitation (40%)	920	230	19	83	86	1
Phenylsepharose	17	104	16	154	73	3
TSK Fractogel	33	69	16	232	73	4
Mono Q	6	4.4	6	1364	27	24
Affinity gel	10	0.7	3	4286	14	74
Disc electrophoresis	17	0.3	2	6667	9	115

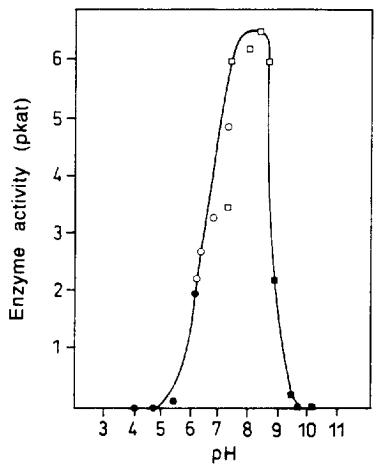


Fig. 4. pH Profile of the catalytic activity of *S*-adenosyl-L-methionine:(*S*)-coclaurine-*N*-methyltransferase. Buffers used (200 mM) ●—● citrate/phosphate, ○—○ KPO_4^{2-}, □—□ triethanolamine, ■—■ Na_2CO_3–$NaHCO_3$.

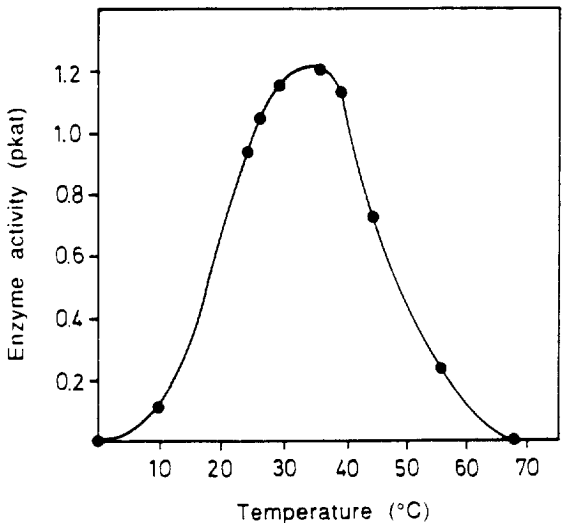
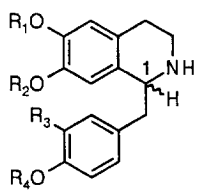


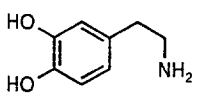
Fig. 5. The effect of temperature on the catalytic activity of *S*-adenosyl-L-methionine:(*S*)-coclaurine-*N*-methyltransferase. Buffer used: 200 mM triethanolamine, pH 9.0.

Table 3. Substrate specificity of 115-fold purified *S*-adenosyl-L-methionine:(*S*)-coclaurine-*N*-methyltransferase



1H = α = (S)

1H = β = (R)



Dopamine

Substrate*	Conversion rate (%)	R_1	R_2	R_3	R_4
(*S*)-Coclaurine	100	CH_3	H	H	H
(*R*)-Coclaurine	0	CH_3	H	H	H
(*S*)-Norcoclaurine	8.3	H	H	H	H
(*R*)-Norcoclaurine	0	H	H	H	H
(*R,S*)-Isococlaurine	0	H	CH_3	H	H
(*R,S*)-Norarmepavine	0	CH_3	CH_3	H	H
(*S*)-Norlaudanosoline	0	H	H	OH	H
(*R*)-Norlaudanosoline	0	H	H	OH	H
(*S*)-6-*O*-Methylnorlaudanosoline	0	CH_3	H	OH	H
(*R*)-6-*O*-Methylnorlaudanosoline	0	CH_3	H	OH	H
(*R,S*)-7-*O*-Methylnorlaudanosoline	0	H	CH_3	OH	H
(*R,S*)-4′-*O*-Methylnorlaudanosoline	0.7	H	H	OH	CH_3
(*S*)-Norreticuline	0.6	CH_3	H	OH	CH_3
(*R*)-Norreticuline	0	CH_3	H	OH	CH_3
(*R,S*)-Norprotosinomenine	0	H	CH_3	OH	CH_3
(*R,S*)-Nororientaline	0	CH_3	H	OCH_3	H
(*R,S*)-Norisoorientaline	0	H	CH_3	OCH_3	H
(*S*)-Tetrahydropapaverine	0	CH_3	CH_3	OCH_3	CH_3
(*R*)-Tetrahydropapaverine	0	CH_3	CH_3	OCH_3	CH_3
Dopamine	0				

*Substrate (20 nmol) was incubated together with 50 μmol triethanolamine buffer, pH 9.0, and 3 pkat 115-fold purified enzyme under standard assay conditions.

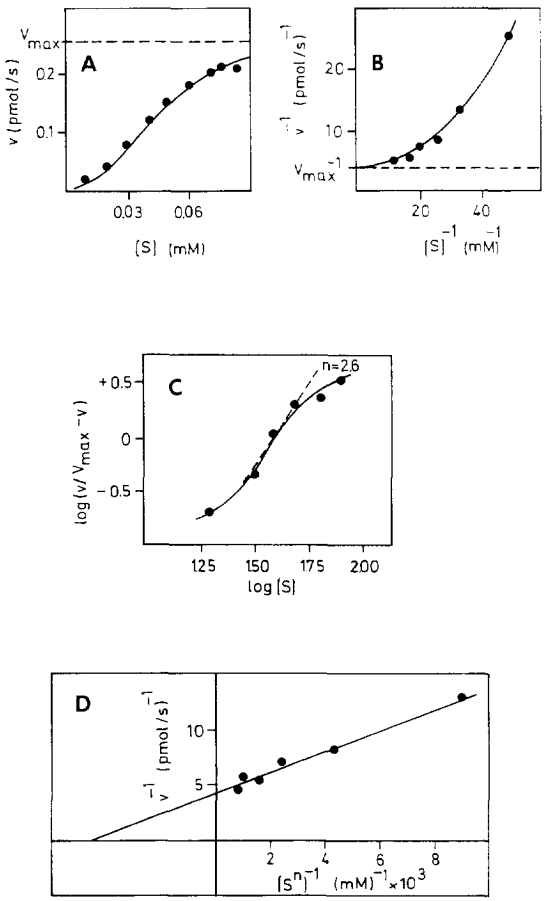


Fig. 6. The effect of (*S*)-coclaurine concentration on the activity of *S*-adenosyl-L-methionine:(*S*)-coclaurine-*N*-methyltransferase. Standard incubation mixture with 200 μM SAM, 1.3 pkat enzyme, pH 8.6, 30°. (A) Michaelis–Menten plot. (B) Lineweaver–Burk plot; V_{max} determined by extrapolation. (C) Hill plot with determination of the Hill coefficient ($n = 2.6$). (D) Linear Lineweaver–Burk plot, K_m 36 μM.

EtOAc–MeCOEt–HCO_2H–H_2O, 5:3:1:1] with subsequent autoradiographic detection of the reaction products. The results of this investigation are partly shown in Table 4. Coclaurine-*N*-methyltransferase activity was present in all isoquinoline alkaloid producing cell cultures tested. The highest enzyme activities (as nkat l^{-1} medium) were found in enzyme extracts of the families Menispermaceae and Berberidaceae. Cell cultures of *T. cordifolia* showed the highest enzyme activity (13 nkat l^{-1} medium). Exclusively the (*S*)-enantiomer of coclaurine was converted, thus demonstrating the (*S*)-stereospecificity of the coclaurine-*N*-methyltransferase. This exclusiveness for the (*S*)-configured substrate was only shown by some members of the Menispermaceae and one member of the Fumariaceae, *Dicentra spectabilis*. In contrast, cell cultures belonging to the Berberidaceae, Fumariaceae, Papaveraceae and Ranunculaceae converted both enantiomers to their respective methylated derivatives, however, at a different rate. In most cases the enzyme showed higher activity with the (*S*)-enantiomer, while enzyme extracts of *Berberis koetineana* and *Fumaria officinalis* were the only ones which converted both enantiomers at the same rate. Cell cultures which are known to be devoid of isoquinoline alkaloids (e.g. *Catharanthus roseus* and *Rauvolfia mannii*) served as controls and did not show any coclaurine-*N*-methyltransferase activity.

*Immobilization of coclaurine-*N*-methyltransferase*

This new enzyme facilitates the preparation of enantiomerically pure labelled (*S*)-*N*-methylcoclaurine for biosynthetic purposes using isotopically labelled substrates allowing precursor synthesis with extremely high specific activities (Bjorklund, Floss *et al.*, in press). Now it should be even possible to separate labelled racemic coclaurine mixtures into (*R*)-coclaurine and (*S*)-*N*-methylcoclaurine by applying this new enzyme. A continuous process using immobilization of the coclaurine-*N*-methyltransferase would be desirable for these purposes. The enzyme was partly purified by $(NH_4)_2SO_4$ precipitation (70%) and was subsequently desalted by Sephadex G 25 chromatography. The pre-purified enzyme was used for immobilization with Controlled pore glass as described in ref. [15]. With this method, an immobilization yield of 22% was achieved with regard to the catalytic activity of coclaurine-*N*-methyltransferase. The stability of the immobilized coclaurine-*N*-methyltransferase was tested at different temperatures (Fig. 7) and compared with that of non-immobilized enzyme. However, there was no significant difference in stability between the immobilized and the soluble enzyme at room temperature or 4°. The immobilized enzyme showed higher stability than the soluble form at $-20°$ up to 100 days. Longer incubation times of the immobilized enzyme at $-20°$ caused a significant drop in activity over 300 days. Immobilization of coclaurine-*N*-methyltransferase may, therefore, be advantageous when used within a period of 100 days.

The enzyme described here, is the first strictly stereoselective methyltransferase in benzylisoquinoline biosynthesis. Up till now, only *N*-methyltransferases have been isolated which *N*-methylate both of the (*S*)- and (*R*)-enantiomers at approximately the same rate [10, 11]. It is of interest to note that during evolution both types of *N*-methyltransferases, stereoselective and non-selective, have evolved. Future research will be directed at comparing the molecular structures of these enzymes.

EXPERIMENTAL

Plant material. Cell cultures of *Tinospora cordifolia* were kindly provided by A. Nattermann & Cie. GmbH (Cologne, Germany) and cultivated in 250 ml B5C2 medium [16] in 1 l flasks at 23° under constant illumination (650 lux) at 100 rpm. Tissue was harvested by suction

Table 4. Occurrence of *S*-adenosyl-L-methionine:coclaurine-*N*-methyltransferase activity in plant cell cultures representing six plant families

Cell culture	(*S*)-Coclaurine enzyme activity		(*R*)-Coclaurine enzyme activity	
	pkat mg^{-1} protein	nkat l^{-1} medium	pkat mg^{-1} protein	nkat l^{-1} medium
Berberidaceae				
Berberis canadensis	34.0	2.6	34.0	3.4
B. koetineana	35.0	5.5	34.0	5.4
B. stolonifera	13.4	4.9	9.0	3.3
Fumariaceae				
Corydalis vaginans	11.9	2.9	2.2	0.5
Dicentra spectabilis	1.6	0.5	0	0
Fumaria officinalis	1.6	0.4	1.1	0.3
Menispermaceae				
Cocculus trilobus	12.8	0.8	0	0
Stephania delavayi	17.8	1.2	0	0
Tinospora cordifolia	59.8	13.0	0	0
T. fragosa	51.6	7.4	0.8	0.1
Papaveraceae				
Bocconia cordata	12.5	2.9	1.9	0.4
Eschscholtzia californica	20.8	2.7	13.9	1.7
Ranunculaceae				
Thalictrum dasycarpum	11.1	2.7	2.3	0.6
T. flavum	5.0	0.3	0	0
T. glaucum	10.9	1.8	1.7	0.3
Apocynaceae				
Catharanthus roseus	0	0	0	0
Rauvolfia mannii	0	0	0	0

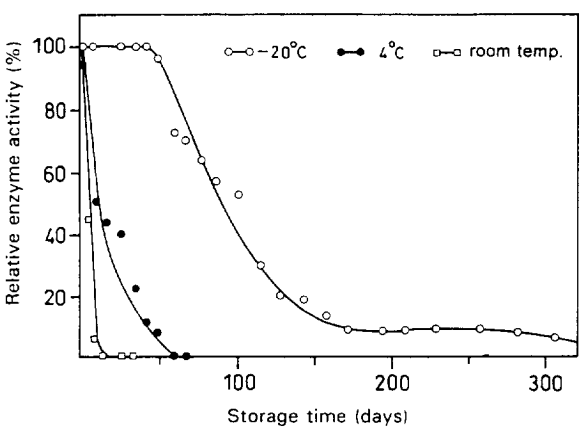


Fig. 7. Thermal stability of *S*-adenosyl-L-methionine:(*S*)-coclaurine-*N*-methyltransferase immobilized on CPG-10 at different temperatures. Enzyme activity was measured under standard assay conditions.

filtration after a growth period of 7 days, shock frozen in liquid N_2 and stored at − 20°. Aliquots of the cells were used for dry weight determination. All other cell cultures were from the departmental cell culture collection.

Chemicals. Optically pure (*S*)-coclaurine was kindly provided by Dr R. Stadler. All other alkaloids used were from the departmental alkaloid collection. [C^3H_3]SAM and [$^{14}CH_3$]SAM were obtained from Dr T. Frenzel, Munich [11]. Materials for chromatography were purchased from Pharmacia LKB, Uppsala (Phenyl-Sepharose, Mono Q, epoxy activated Sepharose 6B, Sephadex G 25) and Merck, Darmstadt (TSK Fractogel).

Enzyme purification. Deep frozen 5-day-old cells of *T. cordifolia* (375 g) were suspended in 750 ml 100 mM KPO_4^{2-} buffer, pH 7.5, containing 20 mM MeSH. The cells were macerated until a homogeneous brei was obtained which was pressed through 4-layered cheesecloth and subsequently centrifuged at 10000 *g*. The clear supernatant was supplemented with $(NH_4)_2SO_4$ to a final concentration of 40% within 10 min under stirring. The suspension was centrifuged for 10 min at 10000 *g* and the resulting supernatant applied to a Phenyl–Sepharose column (2 × 15 cm), pre-equilibrated with 10 mM KPO_4^{2-} buffer, pH 7.5, with 20 mM MeSH (standard buffer) and containing 40% $(NH_4)_2SO_4$. Elution was performed with 10 mM KPO_4^{2-} buffer, pH 7.5, containing 20 mM MeSH. The proteinaceous solution was complemented with sucrose and then applied to a TSK Fractogel column (3 × 47 cm), pre-equilibrated with standard buffer. Elution was performed with the same buffer at a flow rate of 15 ml hr^{-1}. The enzyme eluted

between 120–156 ml with 100% yield of enzyme activity. The active fractions were collected and subsequently loaded onto a FPLC Mono Q column (flow rate: 1 ml min^{-1}), pre-equilibrated with standard buffer. The bound enzyme was eluted with a linear KCl gradient (0–1 M KCl) in standard buffer (1 ml frs). Fractions containing the enzyme (70–75 ml) were pooled and immediately applied to a Sephadex G 25 column (1.5 × 5 cm). Elution was performed with 10 mM KPO_4^{2-} buffer, pH 6.5, without addition of MeSH. The desalted protein solution was then applied in three portions (2 ml each) to a coclaurine-bound epoxy activated Sepharose 6B column (1.5 ml gel volume) with a flow rate of 4 ml hr^{-1}. The enzyme was eluted with a KCl-step-gradient (0–200 mM and 200–500 mM KCl, 7.5 ml each) in 10 mM KPO_4^{2-} buffer, pH 6.5. Fractions of 1 ml were collected and immediately combined with 50 μl 1 M Na_2CO_3–$NaHCO_3$, pH 9.0. The enzyme eluted with the 200–500 mM KCl step. The active fractions were combined, desalted by Sephadex G 25 chromatography as given above and then applied to disc gel electrophoresis (7.5% acrylamide) [17]. The gel was divided into zones and bands were removed and eluted with buffer. Those showing activity were pooled and used for the enzyme characterization.

Preparation of coclaurine-bound epoxy activated Sepharose. Epoxy activated Sepharose 6B (0.5 g) were presoaked in 10 ml H_2O and subsequently washed with 100 ml H_2O (bidest.). (*R,S*)-Coclaurine (15 mg; 47 μmol) was dissolved in a total volume of 0.5 ml DMSO–H_2O (2:3) and added to the gel. The gel suspension was adjusted to pH 9.0 by the addition of 10 μl 1 M Na_2CO_3–$NaHCO_3$, pH 9.5, purged with nitrogen gas, and then agitated for 18 hr at 35°. The gel was alternately washed with (a) 0.1 M citrate buffer, pH 3.0, (b) 0.1 M Na_2CO_3–$NaHCO_3$, pH 9.5, complemented with 40% DMSO, and H_2O. Thereafter, the gel was incubated with 1 M ethanolamine overnight at room temperature and washed again under the conditions described above. A 1.5 ml aliquot of the gel material was poured into a plugged Pasteur pipette and used for affinity chromatography.

Enzyme assay. The routine assay mixture contained in a total volume of 250 μl: 200 mM KPO_4^{2-} buffer, pH 7.5, 200 mM sodium ascorbate, 200 μM (*S*)-coclaurine, 80 μM SAM, 33 μM [C^3H_3]SAM (15000 cpm) and 120 μl enzyme solution. The reaction was terminated after 30 min incubation at 30° by addition of 200 μl Na_2CO_3–$NaHCO_3$, pH 9.5, and 400 μl isoamyl alcohol. Tubes were shaken for 30 min and the two phases were then separated by centrifugation (*ca* 10 sec; Eppendorf system). A 200 μl aliquot of the organic layer was removed for liquid scintillation counting.

Identification of enzyme product. For MS analysis of the enzyme product, 2 M ascorbate, 2 M KPO_4^{2-} buffer, pH 7.5, 120 μM SAM, 100 μM (*S*)-coclaurine, and 207 pkat (0.03 mg) 115-fold purified enzyme were incubated in a total volume of 25 ml for 3 hr at 30°. The pH of the assay mixture was then adjusted to pH 9.0 by the addition of 1 M Na_2CO_3–$NaHCO_3$ and extracted with EtOAc. The organic phase was removed and concd *in vacuo* at 35°. The enzymic product was isolated by TLC on Macherey & Nagel Polygram SIL G/UV_{254} sheets (0.25 mm), developed in Me_2CO–$CHCl_3$–Et_2NH (5:4:1), visualized by UV and the corresponding band eluted with MeOH. CI-MS: $[M + 1]^+$ 300 (100%); *m/z* 192 (7%), 107 (4%).

Analytical procedures. Relative protein values were determined according to ref. [18]. Molecular mass analysis of the enzyme was performed with HPLC-GPC on a TSK G-3000 SW column (2.15 × 60 cm) with 25 mM KPO_4^{2-} buffer, pH 6.0, as eluent (flow rate: 3 ml min^{-1}), as well as with gel filtration on a Sephadex G-100 column (2.5 × 109 cm), pre-equilibrated with 10 mM KPO_4^{2-} buffer, pH 7.5 containing 20 mM MeSH, at a flow rate of 12 ml hr^{-1}. The distribution of radiolabelled products on 2D TLC plates was determined with Industrex AX 18 × 24 films (Kodak).

Enzyme immobilization. For the immobilization on glutaraldehyde substituted Controlled pore glass (CPG-10; Serva, Heidelberg), the procedure cited under ref. [15] was followed.

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