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Research Articles

Partial Purification and Properties of S-Adenosylmethionine: (R), (S)-Norlaudanosoline-6-O-Methyltransferase from Argemone platyceras Cell Cultures

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Argemone platyceras; Papaveraceae; Cell Suspension Cultures; Alkaloid Biosynthesis; S-Adenosylmethionine: (R), (S)-Norlaudanosoline-6-O-Methyltransferase.

Abstract

A new enzyme, S-adenosylmethionine: (R), (S)-norlaudanosoline-6-O-methyltransferase, was isolated from the soluble protein extract of *A. platyceras* cell cultures and purified approximately 80-fold. This enzyme catalyses the formation of 6-O-methylnorlaudanosoline, and, to a minor extent, 7-O-methylnorlaudanosoline from SAM and (S), as well as (R), norlaudanosoline. The apparent molcular weight of the enzyme is 47000 Dalton. The pH-optimum of the enzyme is 7.5, the temperature optimum, 35° C. Apparent K_M values for (S) and (R)-norlaudanosoline were 0.2 mM, and for SAM, 0.05 mM. The transferase shows high substrate specificity for tetrahydrobenzylisoquinoline alkaloids. Simple orthophenols, like phenylpropane derivatives, coumarins or flavonoids, are not accepted as substrates. The enzyme is widely distributed in benzylisoquinoline-containing plant cell cultures and is present in differentiated plants like *Papaver somniferum*.

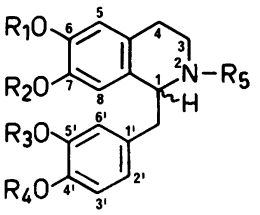
Introduction

(S)-NLS is now firmly established as the first intermediate in benzylisoquinoline alkaloid biosynthesis [1]. A recently discovered enzyme [2] catalyses its formation from dopamine and 3,4-dihydroxyphenylacetaldehyde.

Further down the pathway, reticuline is proven to be a branch point intermediate in the biosynthesis of a vast array of structure types of benzylisoquinoline alkaloids, as for instance: morphinanes, protoberberines, proaporphines, cularines, dibenzopyrrocolines etc. (e.g. 3]. This means that NLS must be transformed to reticuline by three methylation reactions, two O-methylations at positions 6 and 4′ and one N-methylation (at atom 2). Since nor-reticuline has been amply demonstrated by feeding experiments to be a precursor of reticuline *in vivo* [4, 5], one has to assume that O-methylation of NLS precedes N-methylation for instance in opium alkaloids. O-Methylation of NLS is therefore an important reaction in the early steps of the biosynthesis of reticuline, the universal branch point intermediate. O-Methyltransferases have been reported to mediate the transfer of methyl groups from SAM to phenylpropanoid and flavonoid compounds mainly at the *meta* position of the aromatic system, though *para*-O-methylation is not uncommon [6 and literature cited therein]. Specific-O-methyltransferases derived from plants and acting on tetrahydrobenzylisoquinolines have so far not been reported. Yet incubation of poppy latex with (R,S)-norlaudanosoline and ^{14}C-SAM has led to the formation of labelled opium alkaloids, thus demonstrating indirectly the presence of methyltransferase enzymes [7]. There are, however, several reports on NLS-O-methylation, by animal enzymes [e.g. 8].

In our attempt to elucidate the enzymatic steps involved in isoquinoline biosynthesis in plants, we have investigated the O-methylation of NLS. In this report, we present the partial purification and properties of a new enzyme named norlaudanosoline-6-O-methyltransferase from *A. platyceras* cell suspension cultures. This enzyme catalyses the predominant transfer of the S-methyl group of SAM to the phenolic OH-group at position 6 of NLS, and to a lesser extent, to the 7-O-position.

Abbreviations: NLS = Norlaudanosoline; SAM = S-Adenosyl-L-methionine; SAH = S-Adenosylhomocysteine, NLS-OMT = S-Adenosylmethionine: (R), (S)-Norlaudanosoline-6-O-Methyltransferase.



	R_1	R_2	R_3	R_4	R_5
Norlaudanosoline	H	H	H	H	H
6-O-methylnorlaudanosoline	CH_3	H	H	H	H
7-O-methylnorlaudanosoline	H	CH_3	H	H	H
5'-O-methylnorlaudanosoline	H	H	CH_3	H	H
4'-O-methylnorlaudanosoline	H	H	H	CH_3	H
Laudanosoline	H	H	H	H	CH_3
Norreticuline	CH_3	H	H	CH_3	H
Nororientaline	CH_3	H	CH_3	H	H
Laudanidine	CH_3	CH_3	H	CH_3	CH_3

1H = α = (S); 1H = β = (R)

Materials and Methods

Plant Material

A. platyceras cell culture was initiated and maintained since 1975 on Linsmeyer 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. The cells were harvested after 8 days, frozen in liquid nitrogen and stored at −20° C. Cell fresh weight was determined after filtering the cells through a fritted glass funnel using suction. Aliquots were used for dry-weight determination. All other cell cultures were from our culture collections and were grown under identical conditions as given above.

Chemicals

The following compounds were obtained from the indicated sources: S-adenosyl-L-methionine hydrogen sulphate, Combithek (calibration proteins), S-adenosylhomocysteine, all from Boehringer, Mannheim; S-adenosyl-L-methyl-^{3}H-methionine was prepared enzymatically from methionine -S-methyl-^{3}H, (Radiochemical Centre Amersham) using standard methods. All benzylisoquinoline alkaloids were synthesized according to standard procedures. ACA 34 was purchased from LKB, DEAE-cellulose-microgranular form from Whatman, and hydroxyapatite from Bio-Rad. All other materials were of reagent grade. Liquid scintillation counting was performed in a toluene mixture (Rotiszint 22, Roth).

O-Methyltransferase Assay and Product Identification

During enzyme purification, OMT activity was assayed against R/S-norlaudanosoline, except that with crude enzyme preparations, laudanosoline was substituted for its nor-derivative. The assay mixture consisted of KPO_4^{2-}-buffer, pH 7.5 (130 mM), ascorbate (130 mM) R/S-norlaudanosoline (0.3 mM), ^{3}H-SAM (0.1 mM, 10000 cpm), and varying amounts of enzyme in a total volume of 150 µl.

The mixture was incubated for 45 minutes at 35° C and the reaction was terminated by addition of 200 µl Na_2CO_3-buffer (1 M, pH 9.5). The methylated products were extracted by adding 400 µl isoamylalcohol and shaking for 45 min. The turbid mixture was cleared by centrifugation in an Eppendorf centrifuge for 5 min. 200 µl of the organic phase were transferred to scintillation vials and counted for radioactivity. Recovery of the methylated products was 95 % under these conditions. Blank mixtures containing either no enzyme or no substrate (NLS) yielded a blank value of about 8 % radioactivity, a value which was subtracted from all incubation mixtures.

For product identification, the products were separated by HPLC using a Nucleosil-SA-column (25 mm × 3.2 mm i.d.) and 0.5 ammonium phosphate : methanol (80:20) as a solvent system. Retention times of the potential products were: 5'-O-methyl-NLS, 6.45 min.; 7-O-methyl-NLS, 7.35 min.; 4'-O-methyl-NLS, 8.31 min.; 6-O-methyl-NLS, 9.18 min. Preparative isolations were done by using the above incubation mixture × 100. The reaction product was extracted by ethylacetate and subjected to TLC (Si-gel; solvent system: Chloroform : methanol : acetic acid : water = 18:6:3:0.3). The zone containing radioactivity was scraped off, purified for a second time in the solvent system: Chloroform : n-propanol : methanol : water = 45:15:60:40 ($CHCl_3$-phase), and its mass spectrum was measured in a Finnigan MAT 44S instrument.

Enzyme purification

Step 1: 100 g frozen tissue was allowed to thaw in 0.1 M KPO_4^{2-}-buffer, pH 7.5, containing 20 mM β-mercaptoethanol, stirred for 20 minutes, pressed through cheese cloth, and centrifuged at 48000 xg for 10 min. Ammoniumsulphate precipitation was done from 0–70 % saturation, centrifuged again for 10 min. at 48000 xg. The pellet was taken up in 0.1 M KPO_4^{2-}-buffer, pH 7.5, containing 20 mM β-mercaptoethanol.

Step 2: The crude extract from step 1 (15 ml) was put on an Ultrogel ACA 34 column (l = 90 cm, ø = 2.5 cm), equilibrated with 10 mM KPO_4^{2-}-buffer, pH 7.5, 20 mM β-mercaptoethanol. Fractions of 4 ml were collected at a flow rate of 8 ml/h. The fractions (45–61) containing the enzyme activity were pooled and subjected to the next step.

Step 3: The fractions containing the enzyme (59 ml), were subjected to ionexchange chromatography on DEAE-cellulose (l = 10 cm, ø = 1.5 cm). The column was equilibrated with 10 mM KPO_4^{2-}-buffer, pH 7.5, containing 20 mM β-mercaptoethanol, and a gradient was applied from 0–300 mM KCl (8 hrs). 4 ml Fractions were collected at a flow rate of 40 ml/h. The enzyme was found in fractions 56–64. The fractions containing the enzyme were pooled and applied to the next step.

Step 4: The protein solution was added to a hydroxyapatite column (l = 10 cm, ø = 1 cm), equilibrated with 10 mM KPO_4^{2-}-buffer, pH 7.5, 20 mM β-mercaptoethanol, and a gradient of 10–200 mM KPO_4^{2-}, pH 7.5, was applied (8 hrs). 2 ml Fractions were collected at a flow rate of 30 ml/h. Enzyme activity was found in frac-

tions 43–54 with NLS as a substrate. Soluble protein was determined as described previously [10] or in more highly purified samples, with an optical method [11].

Molecular Weight Determination

The molecular weight determination of the purified O-methyltransferase was carried out by gel filtration on a calibrated G-100-superfine column. Although only the Stokes radii of the proteins can be determined by this method, it is often used for the determination of the molecular weight assuming globular shape for the proteins. The column, 168 ml (l = 95 cm, ø = 1.5 cm), equilibrated with 10 mM KPO_4^{2-}-buffer, pH 7.5, 20 mM β-mercaptoethanol, was eluted at a flow rate of 15 ml/h in 100 fractions of 2 ml. The column was calibrated with the proteins of the Combithek. Ferritin (MW: 450000) was used for the determination of the void volume of the column. The standards were monitored by the absorbance at 280 nm. The results are given as Stokes radii.

Results

The presence of O-methyltransferases in crude enzyme extracts of plant cell cultures of different taxonomic origin was investigated using (R, S)-laudanosoline and C^3H_3-SAM as substrates. Laudanosoline was chosen as an initial methyl group acceptor to avoid interference with N-methyltransferases potentially present in the assay. Using these substrates in the standard assay, O-methyltransferases were detected in all cell cultures containing isoquinoline alkaloids thus far tested (Table I). Cell cultures of four different plant families tested for the presence of O-methyltransferase enzymes showed positive results. By far the highest absolute amount of O-methyltransferase per unit culture fluid was found in *Argemone platyceras* (Papaveraceae), a plant species known to contain isoquinoline alkaloids of the pavine, protoberberine, and aporphine types [12]. It was therefore decided to use cells of this species to purify and characterize the O-methyltransferase. (R, S)-norlaudanosoline was used as a substrate in the purification of the enzyme since it was possible to demonstrate that using this plant tissue, there was no interference with N-methyltransferases under the conditons chosen for the assay.

All of the NLS-methylating emzyme activity was detected in the 100000 xg supernatant of the homogenate of the *Argemone* cells. It could not be found in the culture filtrate. The enzyme was isolated and partly purified by ammonium sulphate precipitation, ACA 34, DEAE-cellulose, and hydroxyapatite chromatography as described under "Materials and Methods". This procedure yielded a purification of approximately 80-fold with a recovery of 13 %. The data for a typical purification procedure are summarized in Table II. The protein solution at the stage of highest purification did not contain any other enzymes of the isoquinoline biosynthetic pathway thus far tested. A typical elution profile of a hydroxyapatite column is shown in Fig. 1.

Table I

Survey of distribution of (R, S)-laudanosoline-methyltransferase activity in species of different isoquinoline alkaloid containing families (laudanosoline served as substrate)

Plant material	Family	Enzyme activity pkat/l medium	pkat/mg protein
Cell cultures:			
Argemone platyceras	Papaveraceae	6350	13.8
Corydalis sempervirens	„	4620	12.2
Glaucium flavum	„	2855	27.7
Fumaria officinalis	„	1950	11.1
Corydalis pallida	„	1480	8.2
Argemone intermedia	„	1360	33.7
Adlumia fungosa	„	1070	12.5
Papaver somniferum	„	1020	9.3
Chelidonium majus	„	915	6.8
Eschscholtzia tenuifolia	„	590	4.2
Berberis henryana	Berberidaceae	2050	38.7
Berberis wilsonae	„	1650	13.9
Berberis stolonifera	„	1260	18.9
Thalictrum tuberosum	Ranunculaceae	2060	26.6
Thalictrum sparsiflorum	„	570	2.4
Cissampelos mucronata	Menispermaceae	940	3.7
Differentiated plant:		pkat/gdwt	pkat/mg
Papaver somniferum		218	1.6

Properties of the O-Methyltransferase

The 80-fold purified enzyme was used to determine the catalytic properties. The activity of the

Table II

Purification procedure for O-methyltransferase from *Argemone platyceras* (R, S-norlaudanosoline as substrate)

Purification step	Total activity (pkat)	Total protein (mg)	Specific activity pkat/mg	Recovery (%)	Purification -fold
Crude extract	3626	259.6	14	100	1
Ammoniumsulfate-precipitation (0–70 %)	3409	178.3	19	94	1.4
Gel filtration (ACA = 34)	2290	15.2	150	63.2	10
DEAE-cellulose chromatography	1124	2.5	459	31.0	32
Chromatography on hydroxyapatite	477	0.4	1173	13.2	83

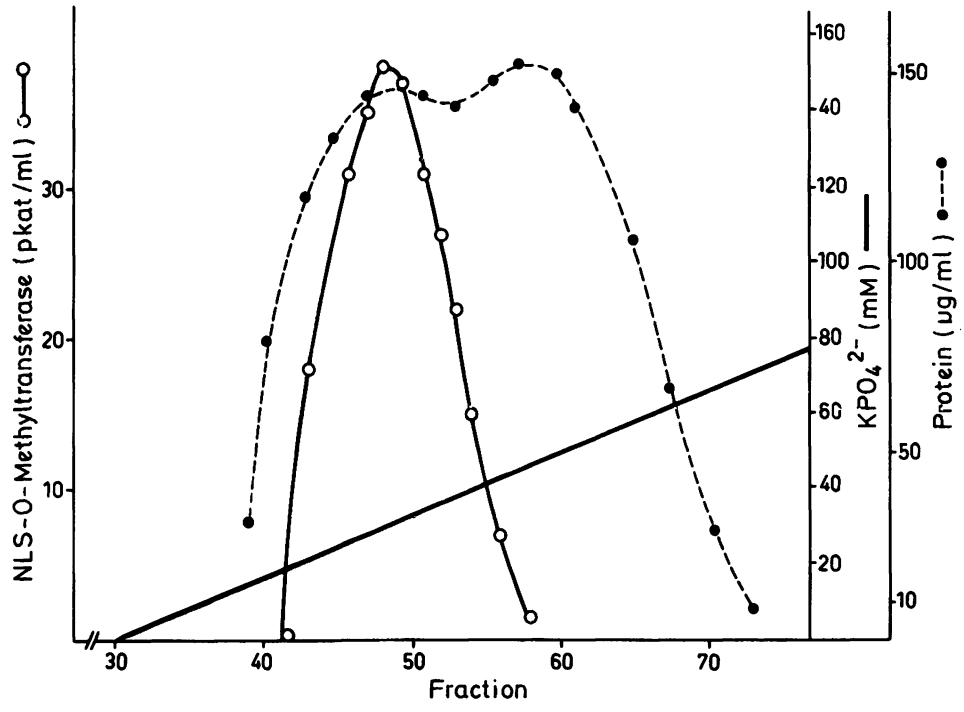


Fig. 1. Elution profile of NLS-OMT from a hydroxyapatite column.

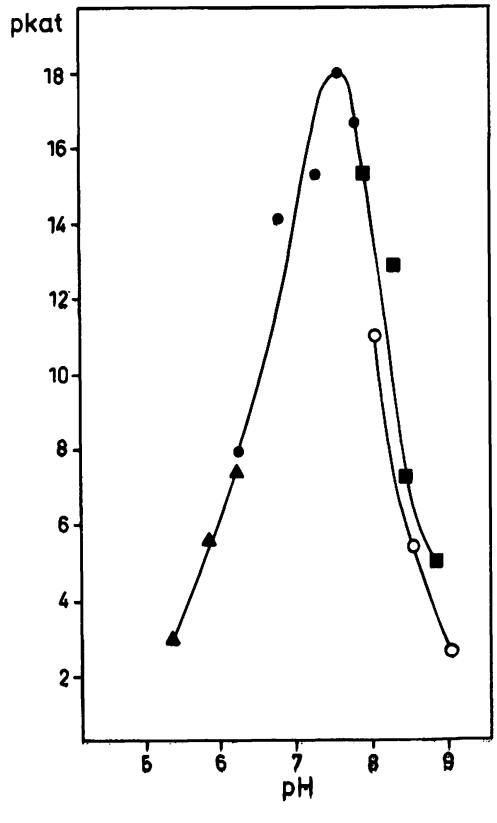


Fig. 2. pH profile of the catalytic activity of purified NLS-OMT from *A. platyceras* cell cultures. Buffers used: ▲ – ▲ Na-maleate/NaOH; ● – ● K_2HPO_4/KH_2PO_4; ■ – ■ Tris/HCl; ● – ● Na-borate.

enzyme was measured at a range of pH 5–9 with different buffers as shown in Fig. 2.

The O-methyltransferase from *Argemone* shows a clear pH-optimum at pH 7.5. The enzyme exhibits a temperature optimum at 35° C. The molecular weight of the O-methyltransferase determined by gel filtration on Sephadex G 100 was 47000 daltons. The enzyme was inhibited by preincubation for 15 min. at 35° C with the following metal ions added as sulphates to a final concentration of 5 mM, and the reaction started by the addition of the labelled substrate: Cu^{++} (100 % inhibition), Mn^{++} (15 %), Zn^{++} (100 %), Mg^{++} (0 %), Co^{++} (81 %), Ni^{++} (100 %), Sn^{++} (55 %), Hg^{++} (100 %), Fe^{++} (90 %). No inhibition was found after preincubation with 25 mM EDTA. Of the organic enzyme inhibitors tested only p-chloromercuribenzoate (25 mM, 70 %) and iodobenzoic acid (10 mM, 73 %) resulted in substantial inhibition of the enzyme. This indicates that there is an SH-group requirement for full enzyme activity. A competitive inhibition was observed using S-adenosyl-homocysteine in the presence of ^{3}H-SAM and NLS, as substrates. A Ki of 10 µm was determined for SAH. The enzyme shows a half life of 8 hrs at 30° C. At 4° C after a 4 week storage period, 50 % of the initial activity of the enzyme was lost. All enzyme activity was lost when the enzyme was frozen in 30 % glycerol solution.

As shown in Table III, the purified enzyme is absolutely specific for the tetrahydrobenzylisoquinoline

Table III

Substrate specificity of partially purified NLS-OMT from *A. platyceras* cell cultures

Substrate	Enzyme pkat/mg enzyme	activity %*	Reaction product
(S)-Norlaudanosoline	313	100	6-O-methylnorlaudanosoline: 7-O-methylnorlaudanosoline = 8:2
(R)-Norlaudanosoline	288	92	same as above
(R, S) 4'-O-methylnorlaudanosoline	107	34	Norprotosinomenine
(R, S) 5'-O-methylnorlaudanosoline	255	81	Nororientaline
(R, S)-Laudanosoline	247	79	6-O-methyllaudanosoline
(R, S)-Norlaudanosoline-1-carboxylic acid	15	5	nd
(R, S)-Norreticuline	3	1	nd
(R, S)-Nororientaline	0	0	–
(R, S)-Laudanidine	0	0	–
(S)-Scoulerine	3	1	Tetrahydrocolumbamine
2,3-Dihydroxy-9,10-dimethoxy protoberberine	22	7	Jatrorrhizine
(R, S)-2,3-Dihydroxy-9,10-dimethoxy tetrahydroprotoberberine	15	5	Tetrahydrojatrorrhizine
Adrenaline	0	0	–
Aesculetine	0	0	–
Caffeic acid	0	0	–
Catechol	0	0	–
Dopamine	0	0	–
Quercetin	0	0	–

* relative to (S)-NLS; nd = not determined

nucleus. None of the phenylpropanoids, phenolics, biogenic amines, coumarins or flavonoids were methylated by action of this enzyme. The best substrates were the ones with four free phenolic-hydroxy-functions, followed by mono-methyl-derivatives. Di-O-methylated alkaloids are very poor substrates. K_M values for this enzyme were determined using the following substrates: K_M (S)-norlaudanosoline 0.2 mM; (R)-norlaudanosoline 0.2 mM; (R,S)-4'-O-methylnorlaudanosoline 1.1 mM; (R, S)-laudanosoline 0.3 mM. SAM 0.05 mM (with (S)-NLS as substrate).

The 80-fold purified OMT is absolutely free of any N-methylating activity as demonstrated by its lack of activity on norreticuline, nororintaline or tetrahydropapaverine.

The best enzyme substrate of the compounds tested so far proved to be (S)-norlaudanosoline. The product of the reaction catalysed by the transferase using (S)-NLS and ^{14}C-SAM as substrates was subsequently investigated. HPLC-chromatography under the conditions given, showed that only two radioactive products were formed, one compound with a retention time of 7.2 min. (20 yield) and the major compound with a retention time of 9.2 min. (80 % yield). The retention times and co-chromatography with all four possible authentic monomethylated norlaudanosolines indicated the minor compound to be 7-O-methyl-norlaudanosoline and the major compound to be 6-O-methyl-norlaudanosoline. Preparative isolation of the major compound from a 15 ml incubation mixture containing (R, S)-laudanosoline as substrate, mass spectroscopy of the purified major unknown product showed a clear fragment (3,4-dihydro-6-methoxy-7-hydroxy-N-methyl-isochinolinium cation) with m/z 192 (100 %) containing one N and one O-methyl-group which further fragmented to m/z 177 (192-CH_3, 30 %), and m/z 162 (177-CH_3, 4 %). The spectrum clearly indicated methylation at the A-ring and was identical in every respect with the mass spectrum of authentic 6-O-methyllaudanosoline. Thus the major product of the NLS-OMT reaction was proven to be 6-O-methylnorlaudanosoline.

The time course of the enzyme formation in suspension cultures of *Argemone* cells is shown in Fig. 3. The enzyme is present in the inoculum only in low amounts. The activity peaks at day 8 of cultivation exactly at the point when the culture is leaving the logarithmic growth phase. A 30-fold increase in activity can be seen as compared with only 10-fold increase in dry cell matter. During stationary phase there is a drastic decrease of total activity of the enzyme.

Discussion

Isoquinoline alkaloids comprise the largest group of alkaloids in plant kingdom. Relatively little is known about their biosynthesis at the cell-free level. In an attempt to elucidate the enzymatic steps involved in benzylisoquinoline synthesis, it is our primary

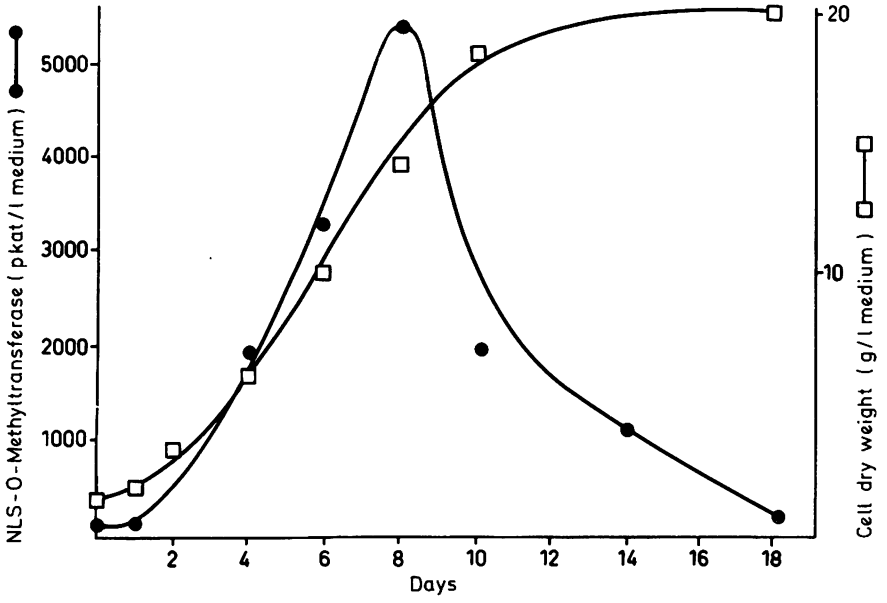


Fig. 3. Time course of NLS-O-methyltransferase activity and cell dry weight in a suspension culture of *A. platyceras*.

aim to isolate and characterize those enzymes which are involved in reticuline biosynthesis, the central and branch-point intermediate in benzylisoquinoline metabolism in plants [3]. In previous studies, we were able to demonstrate that the initial reaction in formation of the benzylisoquinoline skeleton is the stereospecific condensation of dopamine and 3,4-dihydroxyphenylacetaldehyde to yield (S)-norlaudanosoline [1, 2]. The aldehyde, rather than the 3,4-dihydroxyphenylpyruvate, is the substrate for this condensation reaction. The previously postulated intermediate norlaudanosoline-1-carboxylic acid [13–15] is most probably an artefact. Between the now recognized first metabolite in the pathway norlaudanosoline and reticuline, three methylation steps are involved, two O-methylations at carbon atom 6 and 4′ and one N-methylation at atom 2. Since nor-reticuline has been amply demonstrated by *in vivo* experiments to be the immediate precursor of reticuline [e.g. 4, 5], the question arises, which of the phenolic groups of the norlaudanosoline molecule is methylated first on its way to nor-reticuline, the one in 6 or the one in 4′ position. A survey of plant cell cultures using (R,S)-laudanosoline and ^{3}-H-SAM as substrates (in order to prevent N-methylation) demonstrated that all isoquinoline containing species contained good methylating activity. No methyltransferase activity was observed using (R,S)-NLS and ^{3}H-SAM as substrate with for instance *Catharanthus roseus* (Apocynaceae), a species which is known *not* to contain any benzylisoquinoline alkaloids. The highest amount of methyltransferase per volume of medium was observed in *A. platyceras* cell cultures, and it was decided to use this species for the isolation and characterization of the NLS-O-methyltransferase enzyme. The test used for the analysis of the methylated NLS formed involved differential extraction of the labelled product at pH 9.5 with isoamylalcohol leaving residual ^{3}H-SAM in the aqueous solution.

By using this assay the methylating enzyme could be purified about 80-fold and the major (80 %) product of reaction could unequivocally be identified as 6-O-methylnorlaudanosoline by HPLC and mass spectroscopy and comparison with the other four authentic mono-methyl-NLS species. The minor reaction product (20 %) was identified as 7-O-methyl-NLS. It is noteworthy that using the plant enzyme there was not much difference in the methylation rate using the stereoisomers (S)- or (R)-NLS. Furthermore there was no difference in methylation pattern observed using the pure optical isomers as substrates. In both cases 80 % of the product formed was 6-O-methyl-NLS, formed regardless of whether (R)- or (S)-NLS was used as substrate. This demonstration is

Table IV

Comparison of Norlaudanosoline-O-methyltransferases of plant and animal origin

Characteristics	Enzyme source		
	A. platyceras	Rat [17]	Mouse*
(S)-NLS:K_M	0.2 mM	–	0.8 mM
(R)-NLS:K_M	0.2 mM	–	2.2 mM
(R, S)-NLS:K_M	–	1.3 mM	–
SAM:K_M	0.05 mM	6.2 mM	1.0 mM
pH-optimum	7.5	7.7–8.0	7.5
Position of methylation	6 and 7	6 and 7	6 and 7
Ratio for (S)-NLS	80:20	79:14	53:47
Ratio for (R)-NLS	80:20	26:68	47:53
Caffeic acid methylated	No	N.d.	Yes

* This investigation was carried out in our laboratory using a mouse liver preparation according to [17]

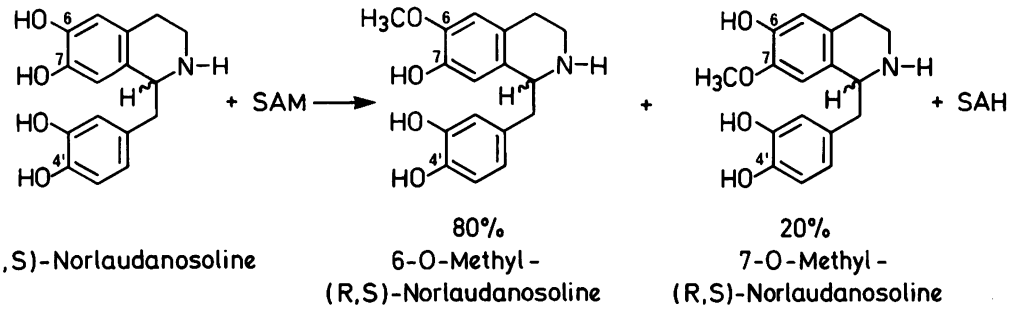


Fig. 4. Reaction sequence catalysed by S-Adenosyl-L-methionine: (R), (S)-Norlaudanosoline-6-O-methyltransferase.

at variance with observations using a purified O-methyltransferase from rat liver [8, 16, 17], where it could be clearly shown that methylation of (S)-NLS yielded predominantly (79 %) 6-O-methyl-NLS while the (R)-NLS isomer gave rise to 7-O-methyl-NLS (68 %). Thus in the animal system, the position of O-methylation (either at atom 6 or 7) is largely directed by the particular isomeric form of the substrate. A comparison of NLS-O-methyltransferases of plant and animal origin are given in Table IV.

There are distinct differences between the animal and plant enzyme. The plant enzyme shows a considerably higher affinity for the substrates than the animal enzymes, as demonstrated by the low K_M value for NLS and SAM. The animal systems which do not show, in contrast to the plant enzyme, a high substrate specificity clearly demonstrate that the stereoisomeric form of the alkaloid substrate dictates the site of enzymatic O-methylation. The plant enzyme prefers the 6 positions for methylation regardless of the steric configuration of the NLS substrate.

It is also noteworthy that the previously assumed intermediate, (R,S)-norlaudanosoline-1-carboxylic acid [13–15] shows only about 5 % of the activity of (S)-NLS. This fact would indicate that even if the carboxylic acid were a natural intermediate in benzylisoquinoline biosynthesis, decarboxylation would have to precede methylation of this molecule.

The observation presented above that the first methylation reaction of NLS occurs at the 6-O-position (see Fig. 4) is in perfect agreement with a finding by Brochmann-Hanssen et al. [5]. Their results, obtained from *in vivo* experiments, indicate that during the early steps in papaverine formation 6-O-methylation must precede 7-O-methylation. This is also consistent with the sequence of O-methylation of phenethylamine and tetrahydroisoquinolines in the biosynthesis of peyote alkaloids [18]. The observation that (R,S)-[1-^{3}H]-4′-O-methylnorlaudanosoline (0.18 %) is slightly better incorporated into reticuline than (R,S)-[1-^{3}H]-6-O-methylnorlaudanosoline (0.12 %) in feeding experiments to *Litsea glutinosa* [19] may be within experimental error, but demonstrates that the enzyme systems introducing the second O-methyl-group are probably rather unspecific.

The search for methylating enzymes in the pathway leading to reticuline must now concentrate on an O-methyltransferase methylating the 4′-position in 6-O-methyl-NLS, as well as on a norreticuline-N-methyltransferase.

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