Phytochemistry, Vol. 29, No. 11, pp. 3491–3497, 1990
Printed in Great Britain.

0031-9422/90 $3.00 + 0.00

PURIFICATION AND CHARACTERIZATION OF THREE ISOFORMS OF *S*-ADENOSYL-L-METHIONINE: (*R,S*)-TETRAHYDROBENZYLISOQUINOLINE-*N*-METHYLTRANSFERASE FROM *BERBERIS KOETINEANA* CELL CULTURES

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(*Received* 20 *April* 1990)

Key Word Index—*Berberis koetineana*; Berberidaceae; cell cultures; tetrahydrobenzylisoquinoline-*N*-methyltransferase; isoforms; alkaloid biosynthesis.

Abstract—Three distinctly different isoforms of *S*-adenosyl-L-methionine-(*R,S*)-tetrahydrobenzylisoquinoline-*N*-methyltransferases could be isolated from cell suspension cultures of *Berberis koetineana*. These isoforms were designated NMT-I, -II and -III. The three enzymes have different molecular weights ($60–78 \times 10^3$), pH optima (6.8/7.4), kinetic properties and substrate specificities. NMT-I showed maximal activity with (*R*)-tetrahydropapaverine as substrate, NMT-II and -III were most active against (*R*)-coclaurine. The mixture of all three NMT's was immobilized on CH-Sepharose or CPG-10 glass beads. The enzymes under these conditions retained their properties and represent a useful tool for the preparative synthesis of isotopically labelled *N*-methylated benzylisoquinoline alkaloids.

INTRODUCTION

N-Methylation reactions play an important role in the biosynthesis of benzylisoquinoline alkaloids. The formation of (*S*)-reticuline, the central biosynthetic intermediate in this large group of alkaloids [1], involves two *O*- and one *N*-methylation steps. Recent investigations [2, 3] have refuted the intermediacy of the tetrahydroxylated alkaloid norlaudanosoline in the construction of the preeminent base reticuline. The now firmly established true physiological precursor of reticuline is the trioxygenated alkaloid norcoclaurine, formed by the condensation of dopamine with *p*-hydroxyphenylacetaldehyde. These discoveries necessitated a revision of the whole pathway leading from L-tyrosine to (*S*)-reticuline [3]. The hypothesis that *O*-methylation preceeds *N*-methylation in the formation of reticuline via *N*-methylation of norreticuline [4, 5], and facile enzymatic *N*-methylation of norreticuline *in vitro*, led to the designation *S*-adenosyl-L-methionine:norreticuline-*N*-methyltransferase for this enzyme [6]. In view of the now revised pathway [3], this enzyme has to be renamed because the physiological substrate is not norreticuline, which obviously does not exist in nature, but rather coclaurine. Due to its broad substrate specificity the enzyme should now be designated: *S*-adenosyl-L-methionine: (*R, S*)-tetrahydrobenzylisoquinoline-*N*-methyltransferase (NMT). One focal point of this work was the determination of the substrate specificity of the *N*-methyltransferase. *Berberis koetineana* cell cultures were chosen as starting material as these contained the highest amounts of NMT amongst the several plant species tested. It could also be demonstrated that this enzyme exists in multiple forms in this plant and it was therefore likely that the isoforms might show different substrate specificities.

RESULTS AND DISCUSSION

Occurrence of NMT

Wat *et al.* [7] have found NMT activity in seven plant cell suspension cultures of the genus *Berberis*. In order to extend our knowledge of the occurrence and taxonomic distribution of the NMT and to find the most suitable plant material yielding the highest total activity of NMT per volume of medium, we screened 37 cell suspension cultures for NMT activity employing standard assay conditions. The enzyme was present in a considerable number of the cell cultures investigated (Table 1). Most of the isoquinoline alkaloid containing cell cultures also revealed the presence of this enzyme pointing to a central role for NMT. No NMT activity directed against tetrahydrobenzylisoquinoline alkaloids was found in tissue cultures of *Nicotiana tabacum* and *Catharanthus roseus*, which both lack benzylisoquinoline alkaloids. Of all the cell suspension cultures tested, *Berberis koetineana* contained both the highest specific and the highest total NMT activity per volume of medium. Additionally, it is worth mentioning that some cell cultures such as *B. heteropoda*, *Eschscholtzia marginata* and *Fumaria officinalis* did not show any NMT activity in the crude cell-free extract, although isoquinoline alkaloids are present in these species. This finding can, most likely, be explained by the presence of low molecular enzyme inhibitors in these extracts, which cannot be removed by passage through XAD or dextran-coated charcoal. The high yield of enzyme, ease of cultivation and reproducibility made *B. koetineana* an excellent starting material for our studies.

The first step was to investigate the growth parameters of the suspension culture and ascertain the time course of

Table 1. A survey of *N*-methyltransferase activity from isoquinoline alkaloid producing plant cell cultures grown in standard medium

Cell culture	Age (days)	NMT nkat l^{-1} medium	NMT pkat mg^{-1} protein
Berberidaceae			
Berberis koetineana	5	44.5	42.5
B. henryana	5	22.2	23.3
B. angulosa	4	8.0	8.2
B. beaniana	6	7.1	15.2
B. taliensis	4	6.3	13.1
B. stolonifera V 29	5	5.3	6.9
B. julia	4	4.9	5.5
B. brettschneideri	4	3.2	20.8
B. wilsoniae	6	3.0	7.2
Mahonia nervosa	5	2.8	3.8
B. dictophylla	4	2.7	4.6
B. gagnepainii	5	2.4	6.6
B. laxifolia	4	1.8	12.1
B. crataegina	4	1.6	7.4
Podophyllum hexandrum	7	1.5	2.5
B. carminea	4	1.4	3.6
B. atropurpurea	4	1.3	7.7
B. turcomanica	4	1.1	1.6
Podophyllum peltatum	7	1.1	2.2
B. stenophylla	4	0.8	1.8
B. vulgaris	4	0.6	4.5
B. heteropoda	4	0	0
Ranunculaceae			
Thalictrum dipterocarpum	5	3.0	3.3
Coptis japonica	5	0.5	0.7
Papaveraceae			
Glaucium corniculatum	7	1.1	2.9
Glaucium flavum	7	1.0	3.2
Eschscholtzia californica	7	1.0	1.3
Bacconia cordata	5	0.3	1.4
Eschsholtzia glauca	6	0.3	0.7
Glaucium elegans	7	0.2	0.5
Argemone platyceras	5	0.1	3.9
Eschscholtzia marginata	7	0	0
Fumariaceae			
Corydalis vaginans	6	0.5	0.7
Fumaria officinalis	6	0	0
Menispermaceae			
Tinospora caffra	5	7.7	21.5
Stephania delavagyi	5	1.0	2.4

enzyme formation. The enzyme was present in the inoculum (day 0) in a substantial amount (Fig. 1). The activity increased during the log phase and peaked at day 13 of cultivation, in parallel with the highest dry weight yield. A 2.5-fold increase in the enzyme activity and a four-fold increase in dry cell mass was determined. During the late stationary phase enzyme activity decreases. For the purification of the enzyme, cells harvested on days 11–13 were used.

Purification of NMT

Preliminary experiments had shown that chromatography of crude enzyme extracts of *B. koetineana* on DEAE-Sephacel yielded three distinct activity peaks. It was not the aim of this study to purify all three activities to homogeneity but rather to cleanly separate all three isoforms from each other in order to study their catalytic behaviour. The crude extract from frozen cells of *B. koetineana* in 30 mM standard buffer was passed through an XAD column to remove all coloured alkaloids which are considered to be potential inhibitors of the NMT. The eluate was then loaded onto a DEAE-Sephacel column where an initial separation of the three isoforms was achieved by a linear gradient of KCl (Fig. 2). Fractions containing the three isoforms were combined separately and used for further purification. The three individual fractions were adjusted to 15% saturation with

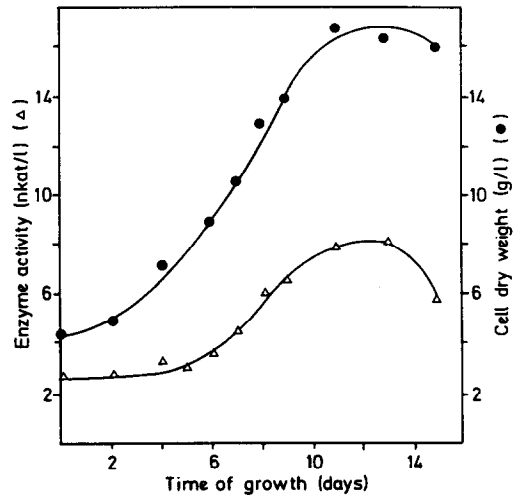

Fig. 1. Time course of *N*-methyltransferase activity (△) and cell dry weight (●) in a suspension culture of *B. koetineana* (1 l flask containing 250 ml medium).

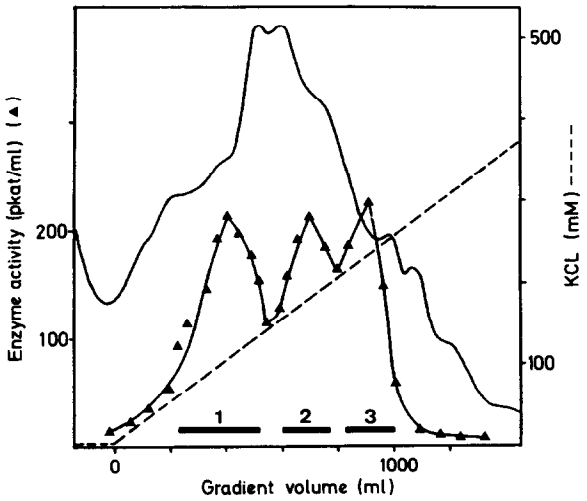

Fig. 2. Elution profile of protein and *N*-methyltransferase activity of a DEAE-Sephacel column. The slope of the gradient is 25 mM KCl per 100 ml in standard buffer. The bars indicate the combined fractions containing the isoforms for further purification.

$(NH_4)_2SO_4$, centrifuged and filtered (0.8 μm). These solutions were applied to a phenyl-Superose column which was equilibrated with standard buffer containing 15% $(NH_4)_2SO_4$. The column was eluted with a linear gradient up to 30% ethyleneglycol in standard buffer. The first isoform, NMT-I, eluted at 45% elution buffer, NMT-II at 70% and NMT-III at 90%. Overlapping fractions were rechromatographed. The three separated NMTs were dialysed against standard buffer and loaded onto a Mono Q FPLC column and eluted with a linear gradient from 0 to 250 mM KCl in standard buffer. NMT-I was free of contaminating isoforms at this point of purification (Fig. 3A). The separation of NMT-II and -III, that were not separated completely during the previous steps, is shown in Fig. 3B. Rechromatography of the individual forms did not lead to the re-appearance of the other two forms, which proves that these isoforms are not artifacts. By this procedure the isoforms were purified only *ca* 20–30-fold at a 1–5% yield, care being taken to discard overlapping fractions which lowered the overall yield drastically. However, these fractions were free from any interfering activities especially from those transforming the benzylisoquinoline substrate or methylating the hydroxyl groups.

Properties of the isoforms

To determine the product of the methylation reaction, all three isoforms were incubated with (*S*)-norreticuline under standard conditions in the presence of [$^{14}CH_3$]-SAM. The reaction product was isolated by TLC. The radiolabelled product of the reaction was further converted quantitatively in the presence of the berberine bridge enzyme [7]. The product of this reaction was identified unequivocally to be (*S*)-scoulerine by additional transformation with (*S*)-tetrahydroprotoberberine oxidase [8] to afford dehydroscoulerine. Employment of (*S*)-coclaurine as a substrate yielded *N*-methylcoclaurine as the enzymic product, identified by HPLC analysis. There was absolutely no indication of the occurrence of any *O*-methyltransferase reaction when norlaudanosoline or laudanosoline were used as substrates.

The pH optima of the three isoforms were determined using 0.5 pkat of catalytic activity each in the standard assay with (*R,S*)-coclaurine or (*R,S*)-norreticuline as substrates. The incubation time was 15 min at 30°. The enzyme activity was assayed in 0.2 pH steps in the range of pH 5–9. NMT-I and -II displayed an absolutely identical pH profile with a pH optimum at 7.4 (Fig. 4). NMT-III, however, was distinctly different and showed optimal activity at pH 6.8. The temperature optimum for all three enzymes was between 35 and 40°. The M_r of the NMTs was determined by gel filtration chromatography. NMT-I and -III showed a distribution coefficient that corresponds to a M_r of 60×10^3, NMT-II of 78×10^3, assuming a globular shape of the enzyme molecules. The heterogeneity of the three NMT isoforms is clearly shown by the pH optima and the clearly distinct M_rs, which are also different from the NMT enzyme from *Berberis vulgaris* which was determined to be 68×10^3. The substrate specificity of the three enzyme forms was of particular interest to further corroborate the different catalytic properties of these proteins. Under standard assay conditions tetrahydrobenzylisoquinoline alkaloids differing in their peripheral substitution patterns were analysed as acceptors for the catalytic transfer of methyl groups from radiolabelled SAM. The relative activities towards different substrates are listed in Table 2. The activities are given in per cent of the best substrate for each isoform tested. Again there are significant differences among the individual NMTs. In the case of NMT-I, the highest activity was observed with (*R*)-tetrahydropapaverine. NMT-II and -III showed definitely higher turnover rate of the alkaloid (*R*)-coclaurine. In all three cases, the (*R*)-configurated compounds were slightly better substrates. As has been observed previously [6], 6-*O*-methylation of the benzylisoquinolines rendered these more favourable substrates for the *N*-methyltransferases as compared with the 6-hydroxylated bases. The only exception to this rule was norcoclaurine which was methylated by all three

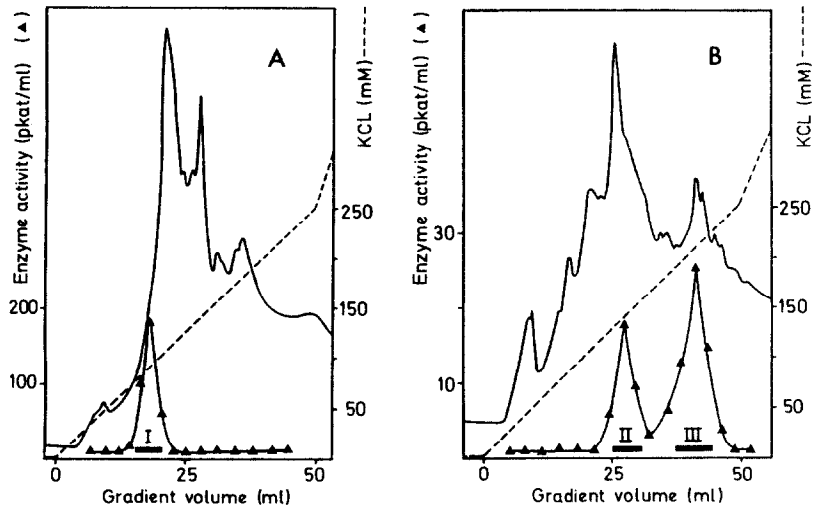

Fig. 3. Separation of the NMT isoforms prepurified on DEAE and phenyl superose on a Mono Q column (0.5 × 5 cm). Elution was achieved by a linear gradient with a slope of 5 mM KCl ml^{-1} in standard buffer up to 250 mM KCl. The bars indicate the combined fractions, which were used for subsequent characterization of the NMT isoforms.

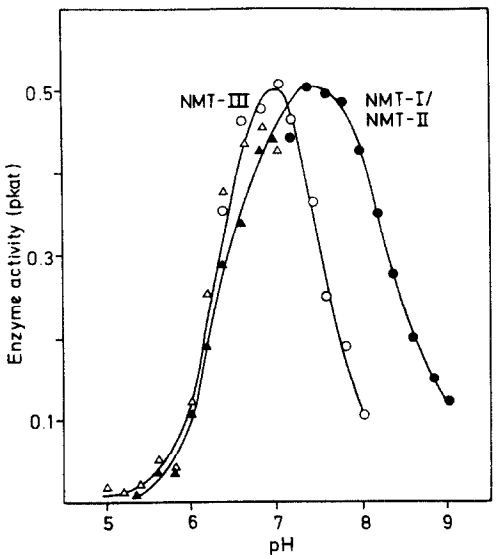

Fig. 4. pH Profile of the catalytic activity of the three NMT isoforms. Buffers used (67 mM); △—△ ▲—▲, citrate–NaOH, ○—○, $NaKPO_4$; ●—●, Tris–HCl. NMT-I and -II have an identical profile.

enzymes to a considerable extent. Methoxyl groups in all other than the 6-position did not influence the compounds' ability to serve as a substrate. *N*-Methylated bases were excluding the formation of quaternary *N*-dimethyl derivatives. While NMT-II preferred the (*R*)-configurated substrates in all cases, there seems to be no clear cut stereoselective preference observed in the case of the two other NMTs. None of the NMTs tested here displayed any activity towards L-tyrosine, dopamine, tyramine, and 3-hydroxytyramine. According to the recently revised pathway leading to (*S*)-reticuline [3], and the pathway leading to the dimeric (*R*,*S*)-*N*-methylcoclaurines of the berbamunine type [9], (*R*)-coclaurine also serves as an early acceptor of the SAM methyl group.

From a biosynthetic point of view (*R*)- or (*S*)-norreticuline and (*R*)- and (*S*)-6-*O*-methylnorlaudanosoline are likely natural substrates. To find out whether the three isoforms express any preference towards these potential substrates the corresponding K_m values were determined. The concentration range chosen to determine the apparent K_m values as well as the constants measured for all three isoforms in the presence of 50 μM SAM are listed in Table 3. Again there are distinct differences in the apparent K_m values of the three enzymes. In almost all cases the (*S*)-enantiomers are preferred substrates as indicated by their lower K_m values.

Immobilization of NMT

To facilitate the preparation of substantial amounts of *N*-methylcoclaurine or reticuline (radiolabelled at the *N*-methyl group), the immobilization of the NMT was attempted. In this case no separation of the respective NMT isoforms was conducted. The NMTs were partly purified to ensure separation of *O*-methylating activity (Table 4). The 12-fold purified enzyme was subsequently used for immobilization. Fractions of the NMT (90 pkat, 450 μg protein each) were bound to 100 mg (dry wt) of various activated immobilization matrices. The residual activated groups were blocked by using Tris, unbound protein being removed by repeated washing. The results of the immobilization procedure with different matrices is shown in Table 5. The best immobilization yield of active enzyme was found employing activated CH-Sepharose and controlled pore glass (CPG). Because of the better flow rate through columns filled with glass beads, this matrix was used for further experiments. The pH optimum for the immobilized NMT isoform mixture

Table 2. Relative rates of conversion of tetrahydrobenzylisoquinoline alkaloids to their *N*-methylated analogues by the three respective *N*-methyltransferase isoforms

Substrate	Relative activity (%) NMT-I	NMT-II	NMT-III	N	Position of methyl groups 6	7	3′	4′
(*R*)-Norcoclaurine	28	31	4	H	OH	OH	H	OH
(*S*)-Norcoclaurine	14	31	24	H	OH	OH	H	OH
(*R*)-Coclaurine	56	100	100	H	OMe	OH	H	OH
(*S*)-Coclaurine	75	91	62	H	OMe	OH	H	OH
(*R,S*)-Isococlaurine	34	—	5	H	OH	OMe	H	OH
(*R,S*)-Norarmepavine	49	—	73	H	OMe	OMe	H	OH
(*R*)-Norlaudanosoline	0	0	0	H	OH	OH	OH	OH
(*S*)-Norlaudanosoline	0	0	0	H	OH	OH	OH	OH
(*R,S*)-Laudanosoline	0	0	0	Me	OH	OH	OH	OH
(*R*)-6-*O*-Methyllaudanosoline	0	0	0	Me	OMe	OH	OH	OH
(*S*)-6-*O*-Methyllaudanosoline	0	0	0	Me	OMe	OH	OH	OH
(*R*)-6-*O*-Methylnorlaudanosoline	67	72	37	H	OMe	OH	OH	OH
(*S*)-6-*O*-Methylnorlaudanosoline	44	49	30	H	OMe	OH	OH	OH
(*R,S*)-7-*O*-Methylnorlaudanosoline	12	0	0	H	OH	OMe	OH	OH
(*R,S*)-4′-*O*-Methylnorlaudanosoline	3	0	0	H	OH	OH	OH	OMe
(*R*)-Norreticuline	70	76	63	H	OMe	OH	OH	OMe
(*S*)-Norreticuline	65	71	72	H	OMe	OH	OH	OMe
(*R*)-Norprotosinomenine	6	0	0	H	OH	OMe	OH	OMe
(*R,S*)-Nororientaline	77	65	55	H	OMe	OH	OMe	OH
(*R,S*)-Norisoorientaline	7	—	0	H	OH	OME	OMe	OH
(*R,S*)-Norcodamine	78	—	—	H	OMe	OH	OMe	OMe
(*R*)-Tetrahydropapaverine	100	83	13	H	OMe	OMe	OMe	OMe
(*S*)-Tetrahydropapaverine	54	45	12	H	OMe	OMe	OMe	OMe

Table 3. Summary of the apparent K_m values for the three NMT isoforms, determined under standard assay conditions with (*R*)- and (*S*)-norreticuline and 6-*O*-methylnorlaudanosoline as substrates (the chosen concentration ranges for the substrates are indicated

Substrate	NMT-I Conc. (μM)	K_m (μM)	NMT-II Conc. (μM)	K_m (μM)	NMT-III Conc. (μM)	K_m (μM)
(*R*)-Norreticuline	1–70	18	1–70	15	1–100	12
(*S*)-Norreticuline	1–100	17	1–70	9	1–100	5
(*R*)-6-*O*-Methylnorlaudanosoline	1–25	5	0.5–40	4	1–100	50
(*S*)-6-*O*-Methylnorlaudanosoline	1–30	12	0.5–30	3	0.5–50	1.5

SAM was 50 μM.

Table 4. Purification of *N*-methyltransferases from 480 g, 12-day-old cells of *B. koetineana*

Purification step	Total volume (ml)	Total protein (mg)	Specific activity (nkat mg^{-1})	Yield (%)	Purification (-fold)
Crude extract via XAD	1180	2630	27	100	1
DEAE-Sephacel	670	1130	52	83	2
Phenyl-Scpharose	160	210	240	72	9
TSK-Fraktogel	43	135	330	63	12

The standard assay with (*R,S*)-norreticuline as substrate was used to determine the enzyme activity.

Table 5. Immobilization of NMT from *B. koetineana* onto different matrices

Matrix	Protein (%) bound	Activity (%)
Activated CH-Sepharose	95	45
CNBr-Sepharose	51	16
Tresyl-Sepharose	35	7
Glutardialdehyde-coupled AH-Sepharose	98	40
CPG-10 beads	98	42

The amount of bound enzyme is given as percentage of the initial amount of soluble enzyme added to the activated matrix.

Table 6. Substrate specificity of CPG-10 immobilized mixture of NMT isoforms from *B. koetineana* using the standard assay and the most important alkaloids as substrates

Substrate	Relative activity (%)	N	Position of methyl groups 6	7	3′	4′
(*R*)-Coclaurine	92	H	OMe	OH	H	OH
(*S*)-Coclaurine	100	H	OMe	OH	H	OH
(*R*)-Norlaudanosoline	0	H	OH	OH	OH	OH
(*S*)-Norlaudanosoline	0	H	OH	OH	OH	OH
(*R*)-Norreticuline	83	H	OMe	OH	OH	OMe
(*S*)-Norreticuline	45	H	OMe	OH	OH	OMe
(*R*)-Tetrahydropapaverine	100	H	OMe	OMe	OMe	OMe
(*S*)-Tetrahydropapaverine	34	H	OMe	OMe	OMe	OMe

showed a biphasic curve with maximal activity at pH 6.9 and 7.5, corresponding exactly to the pH optima of the soluble isoforms. The most important substrates were once again tested with the immobilized NMT. The immobilized enzymes showed maximal turnover of (*S*)-coclaurine and (*R*)-tetrahydropapaverine (Table 6). (*R*)-Norreticuline was methylated at a higher rate than its (*S*)-counterpart. Norlaudanosoline did not serve as a substrate. There were minor differences compared to the substrate specificity of the soluble individual NMT isoforms, (Table 2), this is probably due to an uneven ratio of the isoforms present in the mixture. The half life of the immobilized enzymes was determined to be 7 days at $+20°$, 20 days at $+6°$, and 40 days at $-20°$ (in the presence of 20% glycerol). These values do not differ significantly from the soluble enzyme. The NMT immobilized to CH-Sepharose showed almost identical values. The advantage of the immobilized enzyme, however, lies in the fact that the enzymic catalyst can be used to generate larger quantities of product without contamination with protein, thus facilitating the isolation procedure. This allowed the synthesis of labelled and unlabelled alkaloids in the 100 μmol range. Three NMT enzymes could be isolated and partly characterized. The enzymes showed a low order of stereoselectivity in that they *N*-methylated both the (*R*)- and (*S*)-enantiomers, as previously predicated by precursor feeding experiments [5]. On the basis of the now revised pathway leading from L-tyrosine to reticuline [3], the natural substrate for these enzymes is the alkaloid (*S*)-coclaurine which is biotransformed to (*S*)-*N*-methylcoclaurine. Subsequent 3′-hydroxylation and 4′-*O*-methylation yield reticuline. The hitherto proposed base norreticuline [4] is not a natural substrate. All three enzyme isoforms differ with respect to their M_r, pH optima and substrate specificity. Basically, however, they catalyse the same reaction by transferring the methyl group of SAM onto the free nitrogen atom of various tetrahydrobenzylisoquinoline molecules, regardless of their stereochemistry at the chiral centre (C-1). (*R*)- and (*S*)-configurated alkaloids are thereby *N*-methylated, thus enabling the synthesis of a variety of important precursor molecules with a site specific label of very high isotopic specific activity. Why nature has developed three isoforms with subtle but distinct differences for one and the same reaction cannot presently be explained.

EXPERIMENTAL

Materials. Cell cultures were provided by the cell culture collection of this department. *Berberis koetineana* was grown under conditions as described [10]. The cells were harvested by suction filtration, frozen in liquid N_2 and stored at $-20°$. Alkaloids not commercially available were either synthesized according to standard techniques [11] or supplied by Prof. N. Nagakura (Kobe). ($^{14}CH_3$–; C^3H_3–)-Methionine (50 mCi mM^{-1}; 15 Ci mM^{-1}) was purchased from Amersham (U.K.) and bioconverted to radiolabelled SAM by action of SAM synthase, isolated from *E. coli* EWH 47 according to ref. [12].

Enzyme activities. The enzyme assays for *N*-methyltransferase were conducted as follows; HEPES buffer 67 mM pH 7.4 or 6.8, ascorbate 16 mM, alkaloidal substrate (usually racemic norreticuline) 167 μM, (C^3H_3–)-SAM 33 μM/15000 cpm, and

enzyme in a total volume of 150 μl were incubated at 37° for 30–60 min, so that not more than 10–15% of the methyl group SAM was transferred. The reaction was terminated by the addition of 200 μl cold 0.2 M $NaHCO_3$ solution and 400 μl isoamylalcohol. The samples were agitated for 5 min, centrifuged (all Eppendorf systems) and 300 μl of the organic phase removed and counted in a liquid scintillation counter [13]. The recovery rate for reticuline was 85–90% within a range of 0.5–100 nmol per assay. Berberine bridge enzyme and (*S*)-tetrahydroprotoberberine oxidase were assayed according to refs [7, 8].

For the screening of different plant species for NMT activity, 5 g (fr. wt) of deep frozen cells were stirred at room temp. in 10 ml standard buffer (100 mM Pi; pH 7.8; 20 mM mercaptoethanol) until a homogeneous suspension was obtained. The mixt. was pressed through cheesecloth, and the filtrate centrifuged for 10 min at 2×10^4 rpm. In order to remove alkaloids 100 μl of dextran coated charcoal (0.5 g dextran, 5 g charcoal in 100 ml H_2O) were added per ml of crude extract. After stirring for 5 min the mixt. was centrifuged. The supernatant was removed to determine the enzyme activity.

Enzyme purification. All operations were carried out at 0–4°. *Ca* 650 g deep frozen cells of *B. koetineana* harvested after a cultivation period of 12 days were extracted in the same way as given above but using 30 mM Pi buffer. The crude extract was passed through a XAD column (2.5 × 20 cm), equilibrated with the same buffer, and was then applied to a DEAE-Sephacel column (5 × 20 cm) at a flow rate of 4 ml min^{-1}. Elution was performed initially with a linear gradient up to 400 mM KCl in standard buffer in a vol. of 1.6 l at a flow rate of 4 ml min^{-1}. The fractions containing NMT-I: 110 mM, NMT-II: 180 mM, NMT-III: 230 mM KCl were collected separately. They were adjusted to 15% $(NH_4)_2SO_4$, centrifuged, filtered through a 0.8 μm membrane and applied to a phenyl-Superose column (1 × 10 cm; 8 ml gel) pre-equilibrated with 15% $(NH_4)_2SO_4$. Elution followed with a linear gradient up to 30% ethylene glycol in standard buffer in a vol. of 50 ml and at a flow rate of 0.5 ml/min. NMT-I eluted at 45% elution buffer, NMT-II at 70% and NMT-III at 90%. The active fractions were combined separately and dialysed against standard buffer. Fractions of 5 mg protein of each NMT were applied to a Mono Q column equilibrated in standard buffer and eluted with a linear KCl gradient (0–250 mM) in a vol. of 50 ml at 1 ml min^{-1}. NMT-I eluated at 90 mM, NMT-II at 140 mM and NMT-III at 200 mM KCl. If necessary, the individual fractions had to be re-chromatographed to obtain pure isoforms free of contamination. Only the peak fractions were combined and kept for further studies.

Analytical procedures. Benzylisoquinolines and tetrahydroprotoberberines were separated by TLC (Polygram Sil G/UV_{254}, Macherey Nagel) developed with $CHCl_3$–Me_2CO–$NHEt_2$ (5:4:1); CH_2Cl_2–MeOH–NH_3 (90:9:1); EtOAc–MeCOEt–HCO_2H–H_2O (5:3:1:1) or by HPLC on nucleosil SA (cation exchanger, 4 mm × 25 cm) using 0.4 M $(NH_4)H_2PO_4$ in 10% MeOH as solvent A and 0.4 M $(NH_4)H_2PO_4$ in 40% MeOH as solvent B. A gradient from 100% A to 100% B within 5 min after the injection of the sample was developed to separate most of the benzylisoquinoline alkaloids. Relative protein concentrations were determined according to ref. [14]. The M_rs of the enzymes were determined with HPLC-GPC on a TSK G-3000 SW column (7 × 60 mm). Pi buffer (100 mM, pH 6.5) with a flow rate of 0.25 ml min^{-1} was used as eluent. Fractions of 200 μl were collected and assayed for NMT activity. The elution vol. was compared to that of reference proteins of known M_r.

Immobilization of enzymes. Activated CH-Sepharose, CNBr-Sepharose and tresyl-Sepharose were used according to the instructions of the suppliers. For the immobilization on glutaraldehyde substituted CP G-10 glass, the procedure cited in ref. [15] was followed.

Acknowledgements—The linguistic help of Dr R. Stadler is gratefully acknowledged. This work was supported by SFB 145 of Deutsche Forschungsgemeinschaft, Bonn, and Fonds der Chemischen Industrie.

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