Molecular Characterization of the S-Adenosyl-L-methionine:3’-Hydroxy-N-methylcoclaurine 4’-O-Methyltransferase Involved in Isoquinoline Alkaloid Biosynthesis in Coptis japonica*  

Received for publication, March 23, 2000, and in revised form, April 27, 2000  
Published, JBC Papers in Press, May 12, 2000, DOI 10.1074/jbc.M002439200

Takashi Morishige‡, Tetsuya Tsujita§§, Yasuyuki Yamada¶¶, and Fumihiko Sato††‡‡**  
From the ‡Division of Applied Life Science, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan and the ¶¶Division of Integrated Life Sciences, Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan

S-Adenosyl-l-methionine:3’-hydroxy-N-methylcoclaurine 4’-O-methyltransferase (4’-OMT) catalyzes the conversion of 3’-hydroxy-N-methylcoclaurine to reticuline, an important intermediate in synthesizing isoquinoline alkaloids. In an earlier step in the biosynthetic pathway to reticuline, another O-methyltransferase, S-adenosyl-l-methionine:norcoclaurine 6-O-methyltransferase (6-OMT), catalyzes methylation of the 6-hydroxy group of norcoclaurine. We isolated two kinds of cDNA clones that correspond to the internal amino acid sequences of a 6-OMT/4’-OMT preparation from cultured Coptis japonica cells. Heterologously expressed proteins had 6-OMT or 4’-OMT activities, indicative that each cDNA encodes a different enzyme. 4’-OMT was purified using recombinant protein, and its enzymological properties were characterized. It had enzymological characteristics similar to those of 6-OMT; the active enzyme was the dimer of the subunit, no divalent cations were required for activity, and there was inhibition by Fe**+, Cu**+, Co**, Zn**, or Ni**, but none by the SH reagent. 4’-OMT clearly had different substrate specificity. It methylated (R,S)-6-O-methylnorlaudanosoline, as well as (R,S)-laudanosoline and (R,S)-norlaudanosoline. Laudanosoline, an N-methylated substrate, was a much better substrate for 4’-OMT than norlaudanosoline. 6-OMT methylated norlaudanosoline and laudanosoline equally. Further characterization of the substrate saturation and product inhibition kinetics indicated that 4’-OMT follows an ordered Bi Bi mechanism, whereas 6-OMT follows a Ping-Pong Bi Bi mechanism. The molecular evolution of these two related O-methyltransferases is discussed.

**This work was supported in part by Grant-in-aid B (08456172) from the Ministry of Education, Science, Sports and Culture, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) D29811 (for 6-OMT) and D29812 (for 4’-OMT).

§ Present address: Pharmaceutical Research Institute, Kyowa Hakko Kogyo, 1188 Shimotogari, Nagazumi-cho, Suntou-gun, Shizuoka 411-8731, Japan.

¶¶ Present address: Nara Institute of Science and Technology, Nara 630-0101, Japan.

** To whom all correspondence should be addressed. Tel.: 81-75-753-6380; Fax: 81-75-753-6398; E-mail: fumihiko@kais.kyoto-u.ac.jp.

1 The abbreviations used are: 6-OMT, S-adenosyl-l-methionine:norcoclaurine 6-O-methyltransferase; 4’-OMT, S-adenosyl-l-methionine:3’-hydroxy-N-methylcoclaurine 4’-O-methyltransferase; NMT, S-adenosyl-l-methionine:cochlaurine N-methyltransferase; AdoMet, S-adenosyl-l-methionine; SMT, S-adenosyl-l-methionine:scoulerine 9-O-methyltransferase; AdoHcy, S-adenosyl-l-homocysteine; OMT, O-methyltransferase; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography–mass spectroscopy; 6-OME-NLS, 6-O-methylnorlaudanosoline; NLS, norlaudanosoline; PAGE, polyacrylamide gel electrophoresis; CHES, 2-cyclohexylaminoethanesulfonic acid.

2 This paper is available online at http://www.jbc.org

This paper is available online at http://www.jbc.org
using recombinant proteins heterologously expressed in *Escherichia coli*. The recombinant protein expressed from the cDNA of the 41-kDa polypeptide had 4′-OMT activity and that of the 40-kDa one 6-OMT activity. Enzymological properties of 4′-OMT purified without 6-OMT contamination were determined from its recombinant protein.

**EXPERIMENTAL PROCEDURES**

**Cultured Cells**—The original cultured cells were induced from rootlets of *C. japonica* Makino var. dissecta (Yatabe) Nakai. A cell line (156-1) that produces large amounts of alkaloids was subcultured as described elsewhere (12). Fourteen-day-old cultured cells were harvested and used for the extraction of mRNA.

**Chemicals**—Berberine was purchased from Wako Pure Chemicals (Osaka, Japan). (R,S)-Norlaudanosoline and (R,S)-6-O-methyl-norlaudanosoline (squared) were used as the substrates for the routine assay of 6- and 4′-OMT, respectively.

**Amino Acid Sequence Analysis**—6-OMT was purified from cultured cells, after which two 41- and 40-kDa polypeptides were isolated in vivo by reverse phase HPLC as described previously (4). The 41-kDa polypeptide was digested with trypsin, the 40-kDa one with the lysyl endopeptidase from *Achromobacter* (model 477A/120A; Applied Biosystems). The amino acid sequences of the peptides were determined with a protein sequencer (TaKaRa). The amino acid sequence in motif A of the AdoMet-dependent methyltransferase (13). The 41-kDa probe 1, 5′-TCCACCGACGTCAACAAGTGAGTC-3′, and the 40 kDa probe 2, 5′-CACTTGACGTCGGTGGAGG-3′, corresponded to GTNIGuyoMA and the 41-kDa probe 1, 5′-ATHYTICAYGAYTGGAAYGA-3′, to FNEAMAND (in which probes I = inosine, H = A or C or T, Y = C or T, R = A or G). The first screening with the 41-kDa probe 1 yielded 18 positive signals, and the third screening yielded 9 independent phages. Similarly, 70 positive signals were obtained by the first screening with 40-kDa probe 1 and 18 independent phages by the third screening. The cDNA inserts of the plasmid vector (pBluescript II SK-) were excised with a ZAP-cDNA synthesis kit and used for transformation to *E. coli* XL1-Blue. The cDNA clones (designated pBS41 for the 41-kDa polypeptide and pBS40 for the 40-kDa polypeptide) were analyzed by Southern blot hybridization using both probes and by digestion with the restriction enzyme.

**Construction of Expression Vectors for the 41- and 40-kDa Polypeptides**—Expression vectors for these polypeptides were constructed with a ZAP-cDNA synthesis kit (Stratagene) as described elsewhere (11). The oligonucleotide probes used to screen the cDNA library were based on the internal amino acid sequences of the 41- and 40-kDa polypeptides. The 41-kDa probe 1, 5′-ATGGTCTCCATGATHYTIGGIAT-GACGTTGCCAAR-3′, corresponded to MVPMIKGTMQK and the 41-kDa probe 2, 5′-ATHYTICAYGAYTGGAAYGA-3′, to ILHDWND. The 40-kDa probe 1, 5′-GGACIAATATTGGGTATATTGCGC-3′, corresponded to GTNIGuyoMA and the 40-kDa probe 2, 5′-TCCTTTGAA-3′, to GTNIGuyoMA and the 41-kDa probe 1, 5′-ATHYTICAYGAYTGGAAYGA-3′, to FNEAMAND (in which probes I = inosine, H = A or C or T, Y = C or T, R = A or G). The first screening with the 41-kDa probe 1 yielded 18 positive signals, and the third screening yielded 9 independent phages. Similarly, 70 positive signals were obtained by the first screening with 40-kDa probe 1 and 18 independent phages by the third screening. The cDNA inserts of the plasmid vector (pBluescript II SK-) were excised with a ZAP-cDNA synthesis kit and used for transformation to *E. coli* XL1-Blue. The cDNA clones (designated pBS41 for the 41-kDa polypeptide and pBS40 for the 40-kDa polypeptide) were analyzed by Southern blot hybridization using both probes and by digestion with the restriction enzyme.

**Screening of cDNAs and Nucleotide Sequencing**—A cDNA library of cultured *Coptis* cells was prepared with a ZAP-cDNA synthesis kit (Stratagene) as described elsewhere (11). The oligonucleotide probes used to screen the cDNA library were based on the internal amino acid sequences of the 41- and 40-kDa polypeptides. The 41-kDa probe 1, 5′-ATGGTCTCCATGATHYTIGGIAT-GACGTTGCCAAR-3′, corresponded to MVPMIKGTMQK and the 41-kDa probe 2, 5′-ATHYTICAYGAYTGGAAYGA-3′, to ILHDWND. The 40-kDa probe 1, 5′-GGACIAATATTGGGTATATTGCGC-3′, corresponded to GTNIGuyoMA and the 40-kDa probe 2, 5′-TCCTTTGAA-3′, to GTNIGuyoMA and the 41-kDa probe 1, 5′-ATHYTICAYGAYTGGAAYGA-3′, to FNEAMAND (in which probes I = inosine, H = A or C or T, Y = C or T, R = A or G). The first screening with the 41-kDa probe 1 yielded 18 positive signals, and the third screening yielded 9 independent phages. Similarly, 70 positive signals were obtained by the first screening with 40-kDa probe 1 and 18 independent phages by the third screening. The cDNA inserts of the plasmid vector (pBluescript II SK-) were excised with a ZAP-cDNA synthesis kit and used for transformation to *E. coli* XL1-Blue. The cDNA clones (designated pBS41 for the 41-kDa polypeptide and pBS40 for the 40-kDa polypeptide) were analyzed by Southern blot hybridization using both probes and by digestion with the restriction enzyme.

**Construction of Expression Vectors for the 41- and 40-kDa Polypeptides**—Expression vectors for these polypeptides were constructed without the fused peptides derived from vector sequence in a pET-21d vector (Novagen). The 5′-fragment of the 41-kDa polypeptide cDNA (pBS41) was amplified by PCR by use of the following primers: the forward primer 5′-CATGTTAGTGAAGAAGAAGG-3′ and the reverse primer 5′-GACICARAA-3′, corresponded to MVPMILMGTVK and the 41-kDa probe 1, 5′-ATHYTICAYGAYTGGAAYGA-3′, to ILHDWND. The 40-kDa probe 1, 5′-GGACIAATATTGGGTATATTGCGC-3′, corresponded to GTNIGuyoMA and the 40-kDa probe 2, 5′-TCCTTTGAA-3′, to GTNIGuyoMA and the 41-kDa probe 1, 5′-ATHYTICAYGAYTGGAAYGA-3′, to FNEAMAND (in which probes I = inosine, H = A or C or T, Y = C or T, R = A or G). The first screening with the 41-kDa probe 1 yielded 18 positive signals, and the third screening yielded 9 independent phages. Similarly, 70 positive signals were obtained by the first screening with 40-kDa probe 1 and 18 independent phages by the third screening. The cDNA inserts of the plasmid vector (pBluescript II SK-) were excised with a ZAP-cDNA synthesis kit and used for transformation to *E. coli* XL1-Blue. The cDNA clones (designated pBS41 for the 41-kDa polypeptide and pBS40 for the 40-kDa polypeptide) were analyzed by Southern blot hybridization using both probes and by digestion with the restriction enzyme.

**Screening of cDNAs and Nucleotide Sequencing**—A cDNA library of cultured *Coptis* cells was prepared with a ZAP-cDNA synthesis kit (Stratagene) as described elsewhere (11). The oligonucleotide probes used to screen the cDNA library were based on the internal amino acid sequences of the 41- and 40-kDa polypeptides. The 41-kDa probe 1, 5′-ATGGTCTCCATGATHYTIGGIAT-GACGTTGCCAAR-3′, corresponded to MVPMIKGTMQK and the 41-kDa probe 2, 5′-ATHYTICAYGAYTGGAAYGA-3′, to ILHDWND. The 40-kDa probe 1, 5′-GGACIAATATTGGGTATATTGCGC-3′, corresponded to GTNIGuyoMA and the 40-kDa probe 2, 5′-TCCTTTGAA-3′, to GTNIGuyoMA and the 41-kDa probe 1, 5′-ATHYTICAYGAYTGGAAYGA-3′, to FNEAMAND (in which probes I = inosine, H = A or C or T, Y = C or T, R = A or G). The first screening with the 41-kDa probe 1 yielded 18 positive signals, and the third screening yielded 9 independent phages. Similarly, 70 positive signals were obtained by the first screening with 40-kDa probe 1 and 18 independent phages by the third screening. The cDNA inserts of the plasmid vector (pBluescript II SK-) were excised with a ZAP-cDNA synthesis kit and used for transformation to *E. coli* XL1-Blue. The cDNA clones (designated pBS41 for the 41-kDa polypeptide and pBS40 for the 40-kDa polypeptide) were analyzed by Southern blot hybridization using both probes and by digestion with the restriction enzyme.

**Construction of Expression Vectors for the 41- and 40-kDa Polypeptides**—Expression vectors for these polypeptides were constructed without the fused peptides derived from vector sequence in a pET-21d vector (Novagen). The 5′-fragment of the 41-kDa polypeptide cDNA (pBS41) was amplified by PCR by use of the following primers: the forward primer 5′-GAGATTTAACATGTGTCCTTCCATGGG-3′ was designed to introduce an AfIIIII site (AcPGaPyGT) at the ATG start codon and the reverse primer 5′-CTCCACCGACGTCAACAAGTGAGTC-3′ with no modification of the conserved amino acid sequence in motif A of the AdoMet-dependent methyltransferase (13). The 41- and 40-kDa polypeptides were constructed with a ZAP-cDNA synthesis kit (Stratagene) and an ABI 373A DNA sequencer (Applied Biosystems) with fluorescein isothiocyanate-labeled primers.
FIG. 3. Phylogenetic tree of plant S-adenosyl-L-methionine-dependent O-methyltransferase sequences. OMT protein sequences obtained from GenBank™ were used for tree building. Totally, 19 sequences were aligned by the multisequence alignment program in GeneWorks 2.5.1 (IntelliGenetics, Inc.) using the UPGMA (unweighted pair group maximum average) method.

Results

Isolation of cDNAs of the 41- and 40-kDa Polypeptides—A cDNA library prepared from *C. japonica* cells was screened with oligonucleotide probes designed for the internal amino acid sequences of the purified 41- and 40-kDa polypeptides. After approximately 200,000 plaque-forming units had been screened, 9 positive plaques were identified for the 41-kDa polypeptide and 18 for the 40-kDa one. These constructs were completely sequenced to confirm that no changes were introduced by the subcloning process.

Heterologous Expression of the 41- and 40-kDa Polypeptides in *E. coli*—The expression vector for the 41- or 40-kDa polypeptide was introduced into *E. coli* BL21 (DE3). Cells were induced with 1 mM isopropylthiogalactoside, incubated at 16 °C for 24 h, then harvested and sonicated in extraction buffer (0.1 M Tris-HCl, pH 7.5, containing 10 mM ascorbate and 20 mM 2-mercaptoethanol). The supernatant was desalted through an NAP-5 column (Amersham Pharmacia Biotech) and used to assay both 4'-OMT and 6-OMT activities.

Assay of Enzymatic Activity—4'-OMT and 6-OMT activities were detected by HPLC and liquid chromatography-mass spectroscopy (LC-MS). Whereas (S)-3'-hydroxy-N-methylcoclaurine and (S)-norcoaclarine are the true intermediates in reticuline biosynthesis (3), (R)-S)-6-O-methylnorlaudanosoline (6-OMe-NLS) and (R)-S-norlaudanosoline (NLS) were used as the substrates for the routine assays of 4'-OMT and 6-OMT, respectively, due to the availability of these compounds and similar product formation in the reaction (Fig. 1) (4, 5). 4'-OMT reaction mixture consisted of 0.3 M potassium phosphate (pH 7.4), 25 mM sodium ascorbate, 1 mM NLS, 1 mM AdoMet, and the enzyme preparation. The standard 6-OMT reaction mixture consisted of 0.3 M CHES-NaOH (pH 9.0), 25 mM sodium ascorbate, 1 mM NLS, 1 mM AdoMet, and the enzyme preparation. The assay mixture was incubated at 30 °C for 1 h, after which the reaction was terminated by the addition of methanol. After protein precipitation, the reaction product was detected by reverse phase HPLC (mobile phase, 22% acetonitrile/H2O for the 4'-OMT assay and 18% for the 6-OMT assay containing 1% acetic acid; column, LiChrsopher 100 RP-18(e) (4 × 250 mm; Cica-Merck); flow rate, 0.8 ml/min; detection, absorbance measurement at 280 nm). Mass spectra were obtained with an API165 (Perkin-Elmer).

To quantify the enzymatic activity of 4'-OMT, the transfer of the 3H-labeled methyl group of S-adenosyl-L-[methyl-3H]methionine (NEN Life Science Products) to the product was measured. Reaction conditions have been described elsewhere (4).

Purification of 4'-OMT from *E. coli* Lysate—Large scale production of 4'-OMT was obtained with 600-ml cultures of *E. coli*. Purification was done at 4 °C. All the buffers used contained 4 mM 2-mercaptoethanol and 10% glycerol. The crude bacterial lysate was applied to a Q-Sepharose Fast Flow column (2.5 × 10 cm; Amersham Pharmacia Biotech) equilibrated with 20 mM Tris-HCl (pH 7.5). Proteins were eluted with a linear NaCl gradient of 0–1 M in 20 mM Tris-HCl (pH 7.5) (total volume: 100 ml). Active fractions were applied to a Bio-Gel HTP column (2.5 × 10 cm; Bio-Rad) equilibrated with 10 mM potassium phosphate (pH 7.0). Proteins were eluted with a linear potassium phosphate gradient of 10–500 mM (total volume: 160 ml). Protein was determined according to Bradford (14) with bovine serum albumin as the standard.

Other Methods—The subunit molecular mass of the enzyme was ascertained by SDS-PAGE (10% polyacrylamide), and the molecular mass of the native enzyme by gel filtration chromatography through a Superose 12 column (Amersham Pharmacia Biotech) in fast protein liquid chromatography. Protein was determined according to Bradford (14) with bovine serum albumin as the standard.

Nucleotide Sequence and Predicted Amino Acid Sequences of
pBS41 and pBS40—The nucleotide sequences of the cDNA inserts of pBS41 and pBS40, which encoded the longest polypeptide, and of other cDNA inserts were determined (GenBank™ accession numbers: D29812 for 41 kDa and D29811 for 40 kDa). Sequences respectively corresponding to the 41- and 40-kDa polypeptides carried 1,280 and 1,267 nucleotides, with open reading frames that encoded for 350 and 347 amino acids (Fig. 2). The calculated molecular masses of the pBS41 (38,731 Da) and pBS40 (38,655 Da) inserts were less than the observed molecular masses of the 41- and 40-kDa polypeptides from Coptis cells, but the deduced amino acid sequences had almost all the internal amino acid sequences determined from the purified polypeptides.

Both polypeptide sequences had conserved putative AdoMet binding domains at the C-terminal end (Fig. 2, motif A-C) (13), and both had 52 amino acids between motifs A and B and 30 amino acids between motifs B and C. This spatial relationship is identical to that of the caffeic acid 3-O-methyltransferases. The 41- and 40-kDa polypeptides therefore belong to the Pl (plant)-OMT II group (13).

Their deduced amino acid sequences have a high degree of similarity (52% identity); whereas SMT, which also functions

![Image](image-url)

**Fig. 4.** LC-MS analysis of the 41-kDa reaction product (A), authentic norreticuline (B), 40-kDa reaction product (C), and authentic 6-O-methylnorlaudanosoline (D).

| Purification step | Total protein (mg) | Total activity (nkat) | Specific activity (pkat/mg) | Purification fold | Recovery (%) |
|-------------------|-------------------|-----------------------|-----------------------------|------------------|-------------|
| Crude extract     | 163               | 7.5                   | 46.1                        | 1.0              | 100         |
| Q-Sepharose       | 65.8              | 3.9                   | 58.7                        | 1.3              | 51.3        |
| Bio-Gel HTP       | 6.0               | 2.7                   | 453                         | 9.8              | 35.9        |
in berberine biosynthesis, showed much lower homology when its amino acid sequence was compared with that of the 41- or 40-kDa polypeptide (about 30% identity, see Fig. 2). Both polypeptides had a high degree of similarity (about 40% identity) to the iso flavone 7-O-methyltransferase of alfalfa (15), 6a-hydroxyxmaackiain 3-O-methyltransferase of pea (16), and hydroxycinnamic acids/hydroxycinnamoyl CoA esters O-methyltransferase of lobolly pine (17). SMT had greater similarity to the caffeic acid 3-O-methyltransferases and catechol O-methyltransferases of several plant species. Both the 41- and 40-kDa polypeptides had a somewhat low identity (24 and 35%, respectively) to catechol OMTs from *Thalictrum tuberosum* (Thatu 2) and 2, respectively, GenBank accession numbers AF064693 and AF064694), even though these catechol OMTs from cultured *T. tuberosum* cells could methylate the 6-hydroxy group of norcoclaurine (18). Phylogenetic analysis clearly indicated that both the polypeptides belong to a different branch than the catechol OMT from *Thalictrum* (Thatu 2) (Fig. 3).

Expression of the 41- and 40-kDa Polypeptides in *E. coli*—Expression vectors (pE41 and pE40, respectively) to produce recombinant proteins in *E. coli* were constructed to identify the enzymological activities of the 41- and 40-kDa polypeptides. We modified the first methionine codons of both CDNAs to fit the Neol-XhoI sites of the *E. coli* expression vector pET-21d to obtain expression of nontagged polypeptides. These constructs then were introduced into *E. coli* cells, and recombinant protein production was induced. The crude *E. coli* lysate was used to identify the enzymatic activities of both 4′- and 6-OMT. HPLC analysis clearly showed that the cell lysate that expresses pE41 had methylation activity for 3′-hydroxy-N-methylecoclaurine, but not for norcoclaurine, whereas the lysate containing pE40 had methylation activity for norcoclaurine but not for 3′-hydroxy-N-methylecoclaurine (data not shown). The crude *E. coli* lysate carrying the pET-21d vector showed no enzymatic activity. LC-MS analysis confirmed that the respective reaction products of 41 kDa with 6-OMe-NLS and of 40 kDa with NLS were norreticuline and 6-OMe-NLS (Fig. 4), indicative that the 41-kDa polypeptide corresponds to 4′-OMT and the 40-kDa one to 6-OMT.

Purification of 4′-OMT and Its Characterization—Because preparation of 4′-OMT without 6-OMT contamination is now possible, we tried to prepare highly purified 4′-OMT from *E. coli*. The final purification was 9.8-fold and the yield 35.9% (Table I and Fig. 5). SDS-PAGE analysis showed clearly that the purified fraction was almost homogeneous. The molecular mass of active 4′-OMT was estimated to be about 80 kDa by gel filtration chromatography, evidence that active 4′-OMT is a dimer of the 41-kDa subunit. Enzyme assays at various pH values indicated that the optimum pH for the methylation of 6-OMe-NLS was about 8.0. Half-maximal activity was found at pH 6.6 or 8.6.

4′-OMT, like *Coptis* 6-OMT, did not require divalent cations for activity. The addition of Fe²⁺, Cu²⁺, Co²⁺, Zn²⁺, or Ni²⁺ at 5 mM inhibited 4′-OMT activity, respectively, by 87%, 100%, 86%, 48%, or 96%. These cations also inhibited 6-OMT activity (4). Other cations (Ca²⁺, Mg²⁺, Mn²⁺) had no effect on 4′-OMT activity. The enzymatic activity of 4′-OMT, like that of 6-OMT, was negligibly inhibited by SH reagents (p-chloromercuribenzenesulfonate and iodoacetamide) or EDTA at 1 mM. When, however, berberine (the end product of isoquinoline alkaloid biosynthesis in *Coptis* cells) was added to the assay mixture at 2.5 mM, 4′-OMT activity was not inhibited, whereas 1 mM berberine inhibited 6-OMT activity by 30% (4).

Purified 4′-OMT was used as the antigen to prepare anti-4′-OMT polyclonal antibody. In a Western blot analysis with this antibody, both the crude extract of *E. coli*-expressing 4′-OMT and the *Coptis* proteins had a major immunoreactive band of the same molecular mass (data not shown). This suggests that 4′-OMT is produced in mature form in *E. coli*, whereas the SMT expressed in *E. coli* apparently is longer than that purified from *Coptis* cells (10). Hydrophobicity analysis of full-length 4′-OMT, 6-OMT, and SMT showed a difference in the hydrophobicity of their N termini. There was no evidence that 4′- or 6-OMT is located in specific vesicles, whereas SMT had a hydrophobic signal sequence of 10 amino acids at its N terminus (10) (data not shown).

Substrate Specificity of 4′-OMT—The incorporation of radioactivity from S-adenosyl-L-[methyl-³H]methionine to the products was used as a marker of substrate specificity (Fig. 6). When (R,S)-6-OMe-NLS was the control substrate (i.e., relative incorporation 100%), the respective relative activities with (R,S)-laudanosoline and (R,S)-norlaudanosoline were 767 and 118%, whereas no significant methylation was found for the other substrates. The preferential methylation of laudanosoline rather than norlaudanosoline suggests that N-methylation proceeds before 4′-O-methylation, as suggested by Stadler and Zenk (1).

Dependence of Initial Velocity on Substrate Concentrations and Product Inhibition Kinetics— Whereas (S)-3′-hydroxy-N-methylecoclaurine is the true intermediate in reticuline biosynthesis (3), 6-OMe-NLS was used as the substrate for the kinetic assay of 4′-OMT due to the availability of this compound and similar product formation in the reaction (5). Substrate-saturation kinetics of the purified 4′-OMT for 6-OMe-NLS and AdoMet were the typical Michaelis-Menten type. Kinetic parameters therefore were estimated from double-reciprocal plots of the initial velocity versus the substrate concentration. By varying the concentration of 6-OMe-NLS and [³H]AdoMet in the range of 8–125 μM, a set of apparent *Kₘ* and *Vₘₐₓ* values could be calculated and replotted to determine the real *Kₘ* and *Vₘₐₓ*. The respective *Kₘ* values of 4′-OMT for (R,S)-6-OMe-NLS and AdoMet were 42 and 68 μM, with *Vₘₐₓ* of 1.8 nkat/mg protein. The pattern of primary reciprocal plots was representative of a sequential substrate binding mechanism
Product inhibition analysis was used to determine the reaction mechanism. 4'-O-methylated 6-OMe-NLS (norreticuline) was used for inhibition by the methylated alkaloid. Double-reciprocal plots of the initial velocity versus the concentrations of 6-OMe-NLS, with respect to different fixed concentrations of S-adenosyl-L-homocysteine (AdoHcy), gave a series of lines intersecting to the left of the y-axis (Fig. 7A). AdoHcy inhibition therefore was noncompetitive with respect to variations in 6-OMe-NLS as the substrate, and the $K_i$ value was 43 μM. The inhibition constants were obtained as described elsewhere (19).

Norreticuline inhibition with respect to varying the 6-OMe-NLS and AdoMet concentrations gave a series of lines intersecting to the left of the y-axis (Fig. 7, C and D). Norreticuline inhibition with respect to 6-OMe-
Table II

| Enzyme (origin) | Molecular mass (kDa) | pH optimum | SDS-PAGE | Reaction mechanism |
|-----------------|----------------------|------------|----------|--------------------|
| 4'-OMT (Coptis) | 90                   | 8.0        | 41       | Ordered Bi Bi      |
| 4'-OMT (Berberis) | 90                  | 7.5        | 40       | Ordered Bi Bi      |
| 6-OMT (Coptis) | 120–140              | 9.0–9.5    | 41       | Ordered Bi Bi      |
| SMT (Coptis) | 120–140              | 9.0–9.5    | 41       | Ordered Bi Bi      |

| Enzyme (origin) | Molecular mass (kDa) | pH optimum | SDS-PAGE | Reaction mechanism |
|-----------------|----------------------|------------|----------|--------------------|
| 4'-OMT (Coptis) | 90                   | 8.0        | 41       | Ordered Bi Bi      |
| 4'-OMT (Berberis) | 90                  | 7.5        | 40       | Ordered Bi Bi      |
| 6-OMT (Coptis) | 120–140              | 9.0–9.5    | 41       | Ordered Bi Bi      |
| SMT (Coptis) | 120–140              | 9.0–9.5    | 41       | Ordered Bi Bi      |

NLS and AdoMet therefore was noncompetitive. The respective $K_v$ values for norreticuline versus 6-O-Me-NLS and AdoMet were 103 and 115 $\mu$M. These findings indicate that 4'-OMT followed an ordered Bi Bi mechanism, in which AdoMet binds to the enzyme before the alkaloid substrate binds to the enzyme, after which the methylated alkaloid and AdoHcy are released sequentially.

**DISCUSSION**

We isolated full-length cDNA clones that encode 3'-hydroxy-N-methylcoclaurine 4'-OMT and 6-OMT from cultured *C. japonica* cells. Clone identity was confirmed by the catalytic activities of the heterologously expressed polypeptides. Because these enzymes had very similar physical properties, it was not possible to separate them in the active form (4, 5). Our study provides the first evidence that 4'-OMT and 6-OMT are encoded by different polypeptides.

Characterization of the recombinant 4'-OMT indicated that previous data obtained for partially purified *Berberis* 4'-OMT was reliable. *Coptis* 4'-OMT has a substrate specificity similar to that of the *Berberis* enzyme; an adjacent 3'-hydroxyl group is essential for the 4'-O-methylation reaction, and N-methylation of the substrate enhances the reaction rate.

Previous reports suggested that 4'- and 6-OMT have similar physical properties (4, 5). Indeed, they have similar enzymological properties that are distinct from those of SMT: active 4'- and 6-OMT are dimers of the subunit, whereas SMT is a trimer. 4'- and 6-OMT activities are negligibly inhibited by SH reagents, whereas SMT activity is inhibited by the SH reagent, $p$-chloromercuribenzoate, evidence that the SH group(s) is involved in SMT activities. Effects of cations on the 4'- and 6-OMT activities were very similar, but each polypeptide has distinctive properties in terms of $K_m$ values, substrate specificity, and the proposed reaction mechanism. Interestingly, 4'-OMT activity was not sensitive to berberine, the end product of alkaloid biosynthesis in *Coptis*, whereas the inhibitory effect of alkaloids on *Berberis* 4'-OMT (5) and *Coptis* 6-OMT and SMT has been reported (4, 8).

OMT characteristics are summarized in Table II. The deduced amino acid sequences of 4'- and 6-OMT have 52% identity, and SMT has 30% identity to both 4'- and 6-OMT. The enzymological similarities and differences are interesting in terms of molecular evolution and sequence similarities and diversities, in particular the aspects of substrate recognition.

The berberine bridge enzyme (20–22) and (5)-N-methylcoclaurine 3'-hydroxylase (CYP80B1) (23) utilize benzylisoquinoline alkaloids as substrate. The multiple sequence alignments of *Coptis* OMTs and these enzymes did not, however, show any sequence homology (data not shown). As with many proteins, the three-dimensional structure, rather than the primary sequence, is more important for substrate recognition. The C-terminal ends of 4'-OMT and 6-OMT are highly conserved (58% identity) for the putative AdoMet binding, but the N-terminal ends are more divergent even among these related OMTs (47% identity) (Fig. 2). These findings suggest that the N-terminal end constitutes the alkaloid-binding pocket and that these sequence diversities reflect the substrate specificities of the enzymes. X-ray diffraction studies of *Coptis* OMTs, characterization of the chimeric enzymes among the *Coptis* OMTs, or both, should prove useful for understanding the importance of the N-terminal end of OMT in substrate recognition.

Frick and Kutchan (19) reported that catechol OMTs (Thatu 1 and 2) isolated from *T. tuberosum* cells have very broad substrate specificity and catalyze the 6-hydroxyl methylation of norcoclaurine. These enzymes catalyzed the same reaction for 6-OMT, but showed fairly low identity with 6-OMT (35% identity). They obtained another catechol OMT (Thatu 4), which...
could not catalyze the methylation of isoquinoline alkaloid. Thatu 1 and Thatu 4 differ by only one amino acid; Thatu 1 has tyrosine, and Thatu 4 has cysteine at position 21. As shown in Fig. 3, the alkaloid and phenylpropanoid OMTs may be related evolutionarily, whereas the 4’- and 6-OMT obviously belong to a different branch of the phylogenetic tree. OMTs provide us useful information on the molecular evolution of secondary plant metabolism.

Acknowledgments—We thank Dr. N. Nagakura and Mitsui Petrochemical Industries Ltd. for their generous gifts of the alkaloids, and we are grateful to Dr. A. Ishihara of Kyoto University for his technical assistance in the LC-MS analysis.

REFERENCES
1. Stadler, R., and Zenk, M. H. (1990) Liebigs Ann. Chem. 6, 555–562
2. Ruffer, M., Nagakura, N., and Zenk, M. H. (1983) Planta Med. 49, 131–137
3. Müller, M. J., and Zenk, M. H. (1992) Planta Med. 58, 524–527
4. Sato, F., Tsujiita, T., Katagiri, Y., Yoshida, S., and Yamada, Y. (1994) Eur. J. Biochem. 225, 125–131
5. Frenzel, T., and Zenk, M. H. (1990) Phytochemistry 29, 3505–3511
6. Wat, C.-K., Steffens, P., and Zenk, M. H. (1986) Z. Naturforsch. 41c, 126–134
7. Frenzel, T., and Zenk, M. H. (1990) Phytochemistry 29, 3491–3497
8. Poulton, J. E. (1981) The Biochemistry of Plants (Conn, E. E., ed) Vol. 7, pp. 667–723, Academic Press, New York
9. Muenmier, S., Ruffer, M., and Zenk, M. H. (1985) Plant Cell Rep. 4, 36–39
10. Sato, F., Takeshita, N., Fitchen, J. H., Fujiwara, H., and Yamada, Y. (1993) Phytochemistry 32, 659–664
11. Takeshita, N., Fujiwara, H., Mizura, H., Fitchen, J. H., Yamada, Y., and Sato, F. (1995) Plant Cell Physiol. 36, 29–36
12. Sato, F., and Yamada, Y. (1984) Phytochemistry 23, 281–285
13. Joshi, C. P., and Chiang, V. L. (1998) Plant Mol. Biol. 37, 663–674
14. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
15. He, X. Z., Reddy, J. T., and Dixon, R. A. (1998) Plant Mol. Biol. 36, 43–54
16. Wu, Q., Perisig, C. L., and Van Etten, H. D. (1997) Plant Mol. Biol. 35, 551–560
17. Li, L., Popko, J. L., Zhang, X. H., Osakabe, K., Tsai, C. J., Joshi, C. P., and Chiang, V. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5461–5467
18. Frick, S., and Kutchan, T. M. (1999) Plant J. 17, 329–339
19. Dixon, M., and Webb, E. C. (1979) Enzymes, 3rd Ed., Longman Group Ltd., London
20. Dittrich, H., and Kutchan, T. M. (1991) Proc. Natl. Acad. U. S. A. 88, 9969–9973
21. Facchin, P. J., Penzes, C., Johnson, A. G., and Bull, D. (1996) Plant Physiol. 112, 1669–1677
22. Haensch, K., Pauli, H. H., and Kutchan, T. M. (1998) Plant Mol. Biol. 36, 473–478
23. Pauli, H. H., and Kutchan, T. M. (1998) Plant J. 13, 793–801