Phytochemistry, 1972, Vol. 11, pp. 2657 to 2662. Pergamon Press. Printed in England.

# *O*-METHYLTRANSFERASE ACTIVITY FROM JAPANESE BLACK PINE

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(*Received* 1 *April* 1972)

**Key Word Index**—*Pinus thunbergii*; Angiosperm; Gymnosperm; *O*-methyltransferase; lignin biosynthesis; methylation; softwood lignin; lignan; role of 5-hydroxyferulic acid.

**Abstract**—*O*-Methyltransferase catalyzing the methylation of caffeic acid to ferulic acid was extracted from growing pine seedlings. The partially purified enzyme required Mg ion for maximal formation of ferulic acid; divalent metals such as Co, Zn, Cd, and Ni ions potently inhibited the reaction. EDTA, PCMB and $ICH_2$-$CO_2H$ also inhibited activity. The enzyme, exhibiting the maximal activity at pH 7·5, was found to be *meta*-specific for various catecholic substrates. Caffeic acid served as the best substrate among 16 phenolics tested; protocatechuic aldehyde and 3,4-dihydroxyphenylacetic acid were methylated to a certain degree. On the other hand, 5-hydroxyferulic acid, an important precursor for syringly lignin, was a poor substrate. Thus, the pine enzyme differed markedly in substrate specificity from comparable enzymes from bamboo, poplar and callus tissues of angiosperms.

## INTRODUCTION

THE INCORPORATION of the methyl group of methionine into the methoxyl groups of lignin was first demonstrated *in vivo* by Byerrum *et al.*[1] It was previously reported[2,3] that the methyl group of the amino acid was transferred *in vitro* via *S*-adenosylmethionine (AME) to caffeic acid (CA) in the presence of ATP. Now that AME:catechol *O*-methyltransferase (OMT, E.C. 2.1.1.6), first extracted from plants by Finkle *et al.*,[4,5] has been investigated by several workers,[2–9] it has been established that plant OMT's catalyze in the presence of AME the methylation of CA to ferulic acid (FA),[2–9] 5-hydroxyferulic acid (5-HFA) to sinapic acid (SA),[2,7–9] and 3,4,5-trihydroxycinnamic acid (THC) to both 5-HFA and SA.[2,8,9] These findings support the cinnamate pathway for lignin biosynthesis, which has been proposed principally on the basis of tracer studies with $^{14}C$-labelled compounds.[10] In connection with the mechanism of the methoxylation of lignin precursors, interest has also been focused on the fact that angiosperm lignin consists of both guaiacyl and syringyl units,

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[2] T. HIGUCHI, M. SHIMADA and H. OHASHI, *Agric. Biol. Chem.* **31**, 1459 (1967).
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[8] M. SHIMADA, H. OHASHI and T. HIGUCHI, *Phytochem.* **9**, 2463 (1970).
[9] M. SHIMADA and T. HIGUCHI, *Wood Research* (*Bulletin of the Wood Research Institute of Kyoto University*), No. 50, 19 (1970).
[10] K. FREUDENBERG and A. C. NEISH, *Molecular Biology Biochemistry and Biophysics II* (*Constitution and Biosynthesis of Lignins*), Springer, Berlin (1968).

whereas gymnosperm lignin lacks syringyl units.[11–14] From a biochemical point of view, gymnospermous plants lack an enzyme or enzyme systems necessary for the formation of the syringyl units. The present authors demonstrated that FA-O$^{14}CH_3$ was efficiently incorporated via 5-HFA into the syringyl units of grass lignin,[15] showing that the hydroxylation of FA at the 5-position is a diveriging step in the biosynthesis of syringyl lignin. On this point, it is of importance to determine whether or not gymnosperm OMT acts on both CA and 5-HFA, since these substrates are methylated equally by OMT's from bamboo, poplar[8] and callus tissues of angiosperms.[16]

The first cell-free extraction of OMT from gymnosperms has already been reported,[17] showing that gymnosperm OMT should be regarded as a key enzyme for the biosynthesis of softwood lignin (guaiacyl lignin). The present paper described further studies on OMT from pine seedlings, and the role of this enzyme is discussed in relation to biochemical differences in methoxyl patterns between angiosperm and gymnosperm lignins.

## RESULTS

### *Enzymatic Formation of FA*

The reaction product formed by pine OMT in the presence of AME and CA was identified as FA by both radiochromatography and mass spectrometry with a GC–MS spectrometer, also confirming that no *para*-methylated product (*iso*-FA) was produced. The amount of FA formed increased with the incubation time and the methylation rate was dependent on the enzyme concentrations used. The enzymatic formation of FA is also demonstrated by the results given in Table 1, showing that no FA was produced in the reaction systems without either CA, AME, or the enzyme.

TABLE 1. ENZYMIC FORMATION OF FERULIC ACID

| System | cpm | FA formed (nmol/mg protein) |
|---|---|---|
| Complete | 26 440 | 13·8 |
| Minus CA | 1520 | 0·7 |
| Minus *S*-AMe | 60 | 0 |
| Minus enzyme | 560 | 0·3 |

The assay conditions are as described in the text.

### *Characteristics of Pine OMT*

Pine OMT was purified 4-fold over the crude extract by ammonium sulfate fractionation. The enzyme activity was retained without any loss for 3 days at 4° in the presence of mercaptoethanol (5 mM), which served as an enzyme stabilizer, although it was reported that

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'Nerine OMT' from a plant[18] of the Amaryllidaceae was potently inhibited by mercaptoethanol ($10^{-5}$ M).

TABLE 2. EFFECT OF METAL IONS ON METHYLTRANSFERASE ACTIVITY

| Metal | Metal concn 10 mM Methylation rate (cpm) | Relative rate (%) | Metal concn 0·5 mM Methylation rate (cpm) | Relative rate (%) |
|---|---|---|---|---|
| None | 22 500 | 93 | 8500 | 85 |
| $MgCl_2$ | 24 300 | 100 | 10 000 | 100 |
| $BaCl_2$ | 22 800 | 95 | | |
| $CoCl_2$ | 3400 | 14 | 5700 | 57 |
| $ZnCl_2$ | 3200 | 13 | 1800 | 18 |
| $CdCl_2$ | 1000 | 4 | 500 | 5 |
| $NiCl_2$ | 1000 | 4 | | |

The assay conditions are as described in the text. Metal concentration: Exp. 1, 10 mM; Exp. 2, 0·5 mM.

Pine OMT exhibits maximal activity at pH 7·5, which is a little lower than the optimal pH of 8·0 determined for bamboo OMT.[2] Magnesium ion seemed to stimulate the enzyme activity. On the other hand, Zn, Cd, Ni, and Co ions considerably inhibited the activity (Table 2). These results are quite similar to those obtained with mammalian OMT.[19] It is interesting, however, to notice that 'Nerine OMT' catalyzing *para*-methylation of norbelladine does not require any divalent metals.

TABLE 3. INHIBITION EXPERIMENT

| Reagent (concn) | Methylation rate (cpm) | Relative rate (%) |
|---|---|---|
| None | 28 000 | 100 |
| Thiourea (5 mM) | 27 700 | 99 |
| α,α′-Dipyridyl (5 mM) | 23 600 | 84 |
| EDTA (5 mM) | 2300 | 8 |
| Monoiodoacetate (5 mM) | 900 | 3 |
| PCMB (0·5 mM) | 8100 | 29 |
| NaCN (0·5 mM) | 27 000 | 97 |
| $NaN_3$ (10 mM) | 28 300 | 101 |

The assay conditions are as described in the text.

Effects of chelating agents and SH-inhibitors were examined (Table 3). SH-inhibitors such as monoiodoacetic acid and PCMB also potently inhibited the enzyme reaction, which indicates a possible function of an SH-group in the active centre of the enzyme molecule. The inhibition by EDTA supported the requirement for Mg ion as indicated above. Fe and Cu ions may not be involved in this enzymic reaction, since no significant inhibition by NaCN, α,α′-dipyridyl, $NaN_3$ or thiourea was observed.

[18] J. D. MANN, H. M. FALES and S. H. MUDD, *J. Biol. Chem.* **238**, 3820 (1963).
[19] J. AXELROD and R. TOMCHIC, *J. Biol. Chem.* **233**, 702 (1968).

Table 4 shows how pine OMT acts on a number of phenolic substances. The enzyme preferred CA as the methyl group acceptor to any other phenolics tested. Protocatechuic aldehyde and 3,4-dihydroxyphenylacetic acid served as fairly good substrates. Pyrocatechol, which is an excellent substrate for rat liver OMT,[19,20] was hardly methylated by this enzyme.

TABLE 4. SUBSTRATE SPECIFICITY OF PINE OMT

| Substrate | Methylated product | Relative methylation (%) |
|---|---|---|
| Caffeic acid | Ferulic acid | 100 |
| Caffeic acid | isoFerulic acid | 0 |
| 5-Hydroxyferulic acid | Sinapic acid | 5 |
| 3,4,5-Trihydroxycinnamic acid | 5-Hydroxy-ferulic acid | 25 |
| Chlorogenic acid | Feruloylquinic acid | 10 |
| isoFerulic acid | 3,4-Dimethoxycinnamic acid | 0 |
| *p*-Coumaric acid | *p*-Methoxycinnamic acid | 0 |
| D-Catechin | 'Methylated catechin' | 0 |
| Protocatechuic aldehyde | Vanillin | 68 |
| 5-Hydroxyvanillin | Syringaldehyde | 0 |
| Protocatechuic acid | Vanillic acid | 20 |
| Gallic acid | 5-Hydroxyvanillic acid | 0 |
| Pyrocatechol | Guaiacol | 3 |
| 3,4-Dihydroxyphenylacetic acid | 3-Methoxy-4-hydroxyphenylacetic acid | 54 |
| 3,4-Dihydroxymandelic acid | 3-Methoxy-4-hydroxymandelic acid | 0 |
| Pinosylvin | Pinosylvin monomethylether | 10 |
| Catechylglycerol-β-guaiacyl ether | Guaiacylglycerol-β-guaiacyl ether | 0 |

The assay conditions are as described in the text. The identification of the products are described in the text, except that feruloylquinic acid, vanillic acid, 3-methoxy-4-hydroxyphenylacetic acid, and pinosylvin monomethyl ether were presumed to be formed from the methylation rate determined.

## DISCUSSION

It was suggested previously[8,16,17] that the substrate specificity of plant OMT participating in formation of lignins is an important biochemical factor determining the types of the units of angiosperm and gymnosperm lignins.

Pine OMT exhibits *meta*-specificity to 3,4-dihydroxycinnamic acid, which is consistent with bamboo OMT. It is interesting to notice, however, that 'Nerine OMT'[18] is strictly *para*-specific in the methylation of norbelladine, a precursor of the Amaryllidaceae alkaloids and also that the *para*-methylations take place during biosynthesis of anethole[21] and 4'-methoxyflavonoids[22,23] in some plants. In spite of the natural occurrence of such *para*-methoxylated compounds, the finding that OMT's from pine, ginkgo,[17] poplar,[8] and bamboo shoots, and callus tissues[16] are all functionally *meta*-specific to lignin precursors is in harmony with the fact that plant lignins consist of exclusively *meta*-methoxylated constituents, i.e. the guaiacyl and/or the syringyl units.[10]. In addition to such orientation specificity, pine OMT has a fairly narrow substrate specificity of utilizing CA in preference to the other phenolics. *p*-Coumaric acid, (+)-catechin, 5-hydroxyvanillin, gallic acid, pyrocatechol, and

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[21] K. KANEKO, *Chem. Pharm. Bull.* **10**, 1085 (1962).
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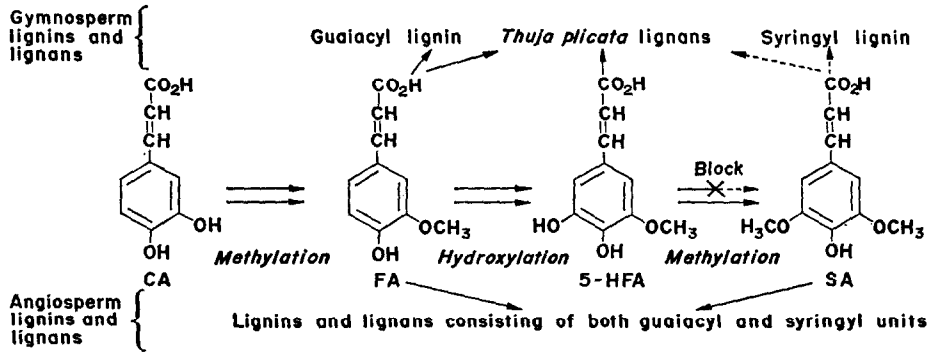
[23] J. EBEL, W. BARZ and H. GRISEBACH, *Phytochem.* **9**, 1529 (1970).

catechylglycerol-β-guaiacyl ether (a dimeric lignin model compound) were not methylated. The observation that this lignin model compound did not serve as a methyl acceptor supports the assumption that the methoxylation of lignins must take place at the stage of the hydroxycinnamate monomers prior to polymerization of coniferyl and sinapyl alcohols. Pinosylvin was only slightly methylated, although both pinosylvin and its monomethyl ether occur in heartwoods of *Pinus* species.[24]




SCHEME 1. POSSIBLE MECHANISM OF BIOSYNTHETIC DIFFERENTIATION OF ANGIOSPERM- AND GYMNOSPERM-LIGNINS AND LIGNANS.

It is surprising, however, that 5-HFA, which is an important precursor for the syringyl units, was hardly methylated by the pine enzyme. THC was a more efficient substrate, yielding 5-HFA as a predominant product, but was less effective than CA. Thus, the effectiveness of the hydroxycinnamates for the pine enzyme is in decreasing order: CA, THC, and 5-HFA. Thus, the presence of a hydroxyl or a methoxyl group at the 5-position of CA causes a steric hindrance to the formation of 'ES-complex' and, consequently, slows down the methylation rate. On the other hand, bamboo and poplar OMT's are able to methylate both CA and 5-HFA equally without undergoing such a steric hindrance. Therefore, the lesser ability of gymnosperm OMT to catalyze the methylation of 5-HFA results in the blocking of the biosynthesis of the syringyl lignin, even though the necessary substrate is provided from FA by hydroxylation.[15] This view is consistent with the occurrence of the syringyl units in minute amounts in gymnosperm lignins. Also, the blocking mechanism by gymnosperm OMT may also be related to the abundance of lignans such as plicatic acid, plicatin, and thujaplicatins[25] with a methyl pyrogallol nucleus (corresponding to 5-HFA) and the scarcity in amount of the lignans, thujaplicatin methyl ethers (TME's) with the syringyl nucleus, both types of which co-occur in heartwood of western red cedar (*Thuja plicata*, gymnosperm). This can be compared with the situation in the angiospermous elm, *Ulmus thomasii*, in which lignans with syringyl nuclei thomasic acid[26] are abundant whereas the former class of lignans are minor components. Although the biosynthesis of such lignans has not yet been elucidated, 5-HFA may well play an important role as a precursor (see Scheme 1). In conclusion, the differences in the methoxyl patterns between the angiosperm and gymnosperm lignins as well as the above-mentioned lignans can be universally

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interpreted in terms of the substrate specificities of the OMT's that markedly differ from family to family, although distribution of gymnosperm OMT must be systematically surveyed further.

## EXPERIMENTAL

*Plant material.* Seeds of Japanese black pine (*Pinus thunbergii*) were germinated at 27° on wet vermiculite in the light of $2 \times 10^4$ lx for 14 days. The pine seedlings grown up to about 7 cm in length were harvested and used for the extraction of OMT after removal of the leaves.

*Chemicals.* Hydroxycinnamic acids were synthesized from the corresponding benzaldehydes and malonic acid.[27] 5-Hydroxyvanillin was prepared from vanillin according to the method of Erdtman.[28] AME-$^{14}CH_3$ (52·3 mCi/mM) and cold AME were procured from New England Nuclear and Boehringer Mannheim, respectively. The radioactive AME was diluted with cold one to the specific activity of 0·2 mCi/mM and used as a methyl group donor for the enzymic reaction. Other chemicals were purchased from Nakarai Chemicals Ltd.

*Extraction of OMT.* All manipulations were carried out at 0–4° unless otherwise stated. Roots and hypocotyls (130 g) from the seedlings were homogenized in a Waring blender with 10 g of Polyclar AT[29] added and 100 ml of 0·05 M phosphate buffer (pH 7·5) containing mercaptoethanol (5 mM). The homogenate was filtered and centrifuged at 14 000 rpm for 30 min. An aliquot of the supernatant solution (180 ml, 11 mg protein/ml) thus obtained was used for the first assay of OMT activity. After the addition of EDTA (5 mM) to the remaining solution (170 ml), the enzyme protein was precipitated with $Am_2SO_4$ between 0·25 and 0·60 saturation, followed by centrifugation to collect the precipitate, which was then used for the second assay after passing through Sephadex G25. The eluate usually contained about 5 mg protein/ml, which was determined spectrophotometrically.[30]

*Assay of OMT activity.* The assay is based on the transfer of $^{14}CH_3$ groups from AME to CA forming FA-O$^{14}CH_3$. The latter compound, but not AME, can be extracted into ether. The reaction mixture contained the following components; 0·5 ml of the enzyme solution, 0·1 ml each of CA or other substrates (0·5 μmol), the dil. AME-$^{14}CH_3$ (0·25 μmol), 0·1 M $MgCl_2$, 0·1 M Na-ascorbate, 0·1 M $NaN_3$, and 0·1 M phosphate buffer (pH 7·5). After 1 hr of the incubation at 30°, the reaction is stopped by the addition of 0·5 ml of 5% HCl. The radioactive product was extracted with ether (3 × 5 ml). After evaporation of the ether, the residue dissolved in 1 ml of dioxane was transferred in a vial test tube containing toluene scintillator (6 ml). Then, the amounts of the reaction products were calculated from the radioactivities determined with a Beckmann scintillation counter, on the basis of the finding that $10^4$ cpm is equivalent to 26·6 nmol of the products formed.

*Identification of the reaction products.* The enzymic formation of FA, 5-HFA, and SA was recognized by radiochromatography with a radiochromatogram scanner as previously described.[16] Alternatively, the product formed from CA was identified as FA by analysis of the mass spectrum obtained with the GC–MS spectrometer (Shimadzu-LKB 9000), after transforming the substrate and the product into the TMS-derivatives with TMS-reagents. The two derivatives were separated by GLC with SE-52 (3%) at 210°. In this case, the reaction mixture containing the components on 5-fold scale was incubated for 2 hr. Radioactive vanillin formed from protocatechuic aldehyde was observed by the radiochromatography with $H_2O$-saturated iso-propyl ether.[15]

*Acknowledgement*—The authors wish to appreciate Dr. W. D. Loomis of Oregon State University, U.S.A. for providing of Polyclar AT, and also to thank Mr. H. Nakatsubo in this division for the synthesis of catechylglycerol-β-guaiacyl ether. The present investigation was supported in part by the scientific fund from the Ministry of Education.

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