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O-METHYLTRANSFERASES INVOLVED IN THE BIOSYNTHESIS OF LIGNINS

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Abstract—The substrate specificity of *S*-adenosylmethionine–catechol *O*-methyltransferase (*EC* 2.1.1.6) extracted from shoots of bamboo and poplar was compared with that of the *O*-methyltransferase present in the sieve tissue of gymnosperms. The bamboo and poplar *O*-methyltransferases were found to be *meta*-specific for 3,4-dihydroxy-, 3,4,5-trihydroxy- and 3,4-dihydroxy-5-methoxycinnamic acids. The rate of formation of sinapic acid from 5-hydroxyferulic acid in the *in vitro* reaction was found to be greater than that of ferulic acid from caffeic acid. On the other hand, sieve tissue of *Ginkgo biloba*, one of the gymnosperms, did not methylate 3,4,5-trihydroxycinnamic and 3,4-dihydroxy-5-methoxycinnamic acids but only 3,4-dihydroxycinnamic acid.

INTRODUCTION

THE INCORPORATION of the methyl group of methionine into the methoxyl groups of lignin was first demonstrated by Byerrum *et al.*[1] using barley plants. Since then, the occurrence of *S*-adenosylmethionine–catechol *O*-methyltransferase (E.C. 2.1.1.6) in higher plants has been reported by several workers.[2–6] The present authors have investigated the role of the *O*-methyltransferase catalysing the formation of ferulic and sinapic acids during lignification in growing bamboo.[7]

In connexion with the biosynthesis of lignins of different plants, interest has also been focused on the fact that the lignins of angiosperms largely contain guaiacyl units whereas the lignins of angiosperms contain both syringyl and guaiacyl groups. However, it has not yet been determined what factors determine these differences. The present paper describes the enzyme-catalysed methylation of various hydroxycinnamic acids and the substrate specificity of *O*-methyltransferases from bamboo, poplar and ginkgo in relation to the metabolic differences between angiosperms and gymnosperms.

RESULTS

Figure 1 shows the time course for formation of *O*-methylated hydroxycinnamic acids by *O*-methyltransferase from bamboo shoot. The rate of formation of sinapic acid (SA) from 5-hydroxyferulic acid (5-HFA) was found to be much greater than that for ferulic acid (FA) from caffeic acid (CA). 3,4,5-Trihydroxycinnamic acid (TCA) was also found to be efficient

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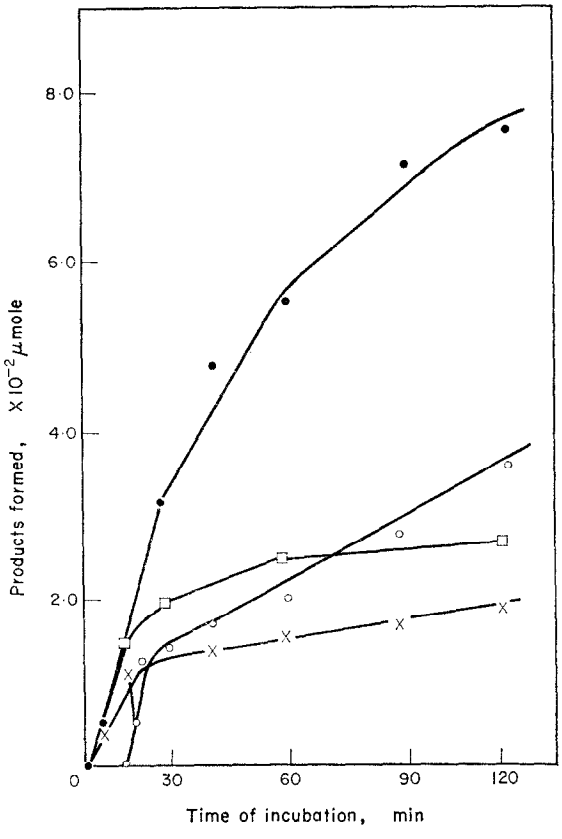
8·0
6·0
4·0
2·0
0
30
60
90
120
Products formed, X 10^{-2} μmole
Time of incubation, min

FIG. 1.

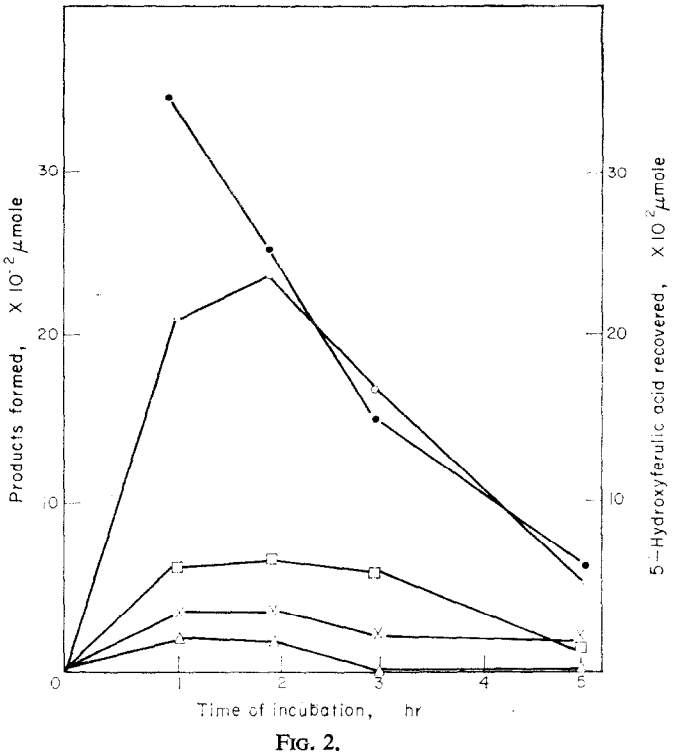
30
20
10
0
1
2
3
4
5
Products formed, X 10^{-2} μmole
5-Hydroxyferulic acid recovered, X 10^{-2} μmole
Time of incubation, hr

FIG. 2.

as a substrate and gave both 5-HFA and SA; for the first 10 min after the start of the enzymic reaction only 5-HFA was formed from TCA. However, when SA formation begins, the rate is higher than that of 5-HFA. Thus, the lag phase observed in the formation of SA suggests that the two *meta*-hydroxyl groups of TCA are not methylated at the same time, but that 5-HFA is an intermediate. The amount of SA formed from TCA was greater than that of FA from CA after 90 min of incubation.

Figure 2 shows the time course for methylation of 5-HFA and TCA by sliced tissue from bamboo shoot. 5-HFA administered to the sliced tissue decreased as SA was formed. The amount of SA reached a maximum after 2 hr and then decreased. TCA incubated with the tissue was converted to both SA and 5-HFA, which is consistent with the results described above. The amount of SA formed from 5-HFA was much greater than that from TCA, which is also similar to the results from the *in vitro* experiments.

Table 1 shows the results of experiments on the methylation of hydroxycinnamic acids by *O*-methyltransferases extracted from shoots of bamboo and poplar. Again it can be seen that the amounts of SA formed from 5-HFA in the bamboo and the poplar enzyme systems

TABLE 1. METHYLATION OF HYDROXYCINNAMIC ACIDS BY *O*-METHYLTRANSFERASES FROM POPLAR AND BAMBOO

Substrate	Product formed	Amount*	
		Poplar	Bamboo
Caffeic acid	Ferulic acid	100	100
5-Hydroxyferulic acid	Sinapic acid	335	200
3,4,5-Trihydroxycinnamic acid	5-Hydroxyferulic acid	33	35
3,4,5-Trihydroxycinnamic acid	Sinapic acid	31	45

* The amounts of products are expressed as relative value based on the amount of ferulic acid formed. The amounts of ferulic acid formed in the poplar and the bamboo enzyme systems were found to be 6·4 μg and 4·3 μg/30 min of incubation. The enzymatically formed products were determined as described in the text.

are twice and three times, respectively, greater than the amount of FA formed from CA. On the other hand, the amounts of SA and 5-HFA from TCA in both systems are much less.

In order to know in more detail about substrate specificity of the bamboo *O*-methyltransferase, the effectiveness of various phenolic compounds as methyl group acceptors was examined. R_f values and other data were used as criteria for identification. Purification of the enzyme was attempted but no satisfactory results were obtained because the activity was readily lost.

Table 3 shows how the various phenolic compounds were methylated. CA, 5-HFA and TCA are effective as substrates as described above. Chlorogenic acid (caffeoylquinic acid) was also indirectly methylated, giving feruloylquinic acid. On the other hand, *p*-hydroxycinamic acid and *iso*-FA (3-hydroxy-4-methoxycinnamic acid) were not methylated. Similarly, neither *iso*-FA nor 3,4-dimethoxycinnamic acid was formed from CA. Other phenolic compounds listed in Table 3 were not utilized as methyl group acceptors.

In order to examine the participation of *O*-methyltransferase in formation of gymnosperm lignin precursors, attempts were made to prepare cell-free extracts of the enzyme from various gymnosperms including Ginkgo biloba but without success. Therefore, sliced tissues of *Podocarpus macrophylla*, *Pinus strobus*, *Cryptomeria japonica* and shoots of *G. biloba* were used directly. It was shown by radioautography using either CA-2-^{14}C or methionine-$^{14}CH_3$

TABLE 2. R_f VALUES AND COLOUR REACTIONS OF AUTHENTIC COMPOUNDS

Compounds	R_f values			Colours		
	(1)*	(2)*	(3)*	In u.v.	(A)†	(B)†
p-Coumaric acid	0·04	0·53	0·14	dark violet	grey	red-brown
p-Methoxycinnamic acid	0·83	0·71	—	dark grey		
Caffeic acid	0	0·28	0·50	blue	yellow-brown	brown
Ferulic acid	0·35	0·39	0·97	bright blue	blue	red-violet
Isoferulic acid	0·15	0·27	—	blue-violet	violet	red-brown
3,4-Dimethoxycinnamic acid	0·72	0·43	—	faint blue		
3,4,5-Trihydroxycinnamic acid	0	0·17	0	blue	yellow-brown	yellow-brown
5-Hydroxyferulic acid	0	0·21	0·21	light blue	grey	dark brown
Sinapic acid	0·25	0·27	0·98	green-blue	grey-brown	pink
	(1)*	(4)*	(5)*	In u.v.	(B)†	(C)†
p-Hydroxybenzaldehyde	0·27	0·35	—			yellow-brown
Anisaldehyde	0·96	0·89	—	dark		bright yellow
Protocatechualdehyde	0	0·01	0·28			yellow-brown
Vanillin	0·84	0·78	0·84			yellow-brown
Isovanillin	0·59	0·67	0·78	light blue	yellow	yellow-brown
Veratraldehyde	0·80	0·92	0·92	blue	bright yellow	yellow-brown
5-Hydroxyvanillin	0·08	—	0	dark		brown-orange
Gallaldehyde	0	—	0	dark		brown-orange
Syringaldehyde	0·80	—	0·10	dark		brown-orange
	(4)*		(5)*	In u.v.	(B)†	(D)†
p-Hydroxybenzoic acid	0·01		0·28			bright yellow
Anisic acid	0·74		0·84			bright yellow
Protocatechuic acid	0		0·02		red-blue	grey-green
Vanillic acid	0·08		0·42		yellow	yellow-brown
Veratric acid	0·67		0·78			yellow
Gallic acid	0		—		bright yellow	grey-blue
Syringic acid	0·88		—		blue-grey	grey-brown
Pinosylvin	0·27		—	light blue	orange-yellow	
Pinosylvin monomethyl ether	0·90		—	light blue	yellow	

* Solvents used for paper chromatography: (1) toluene–AcOH–H_2O (4:1:5, organic layer); (2) 2% AcOH; (3) $CHCl_3$–AcOH–H_2O (2:1:1, organic layer); (4) benzene–HCOOH–H_2O (500:1:49, organic layer); (5) benzene–MeCOEt–HCOOH–H_2O (450:50:1:49, organic layer).

† Colour reagents: (A) diazotized *p*-nitroaniline; (B) diazotized sulphanilic acid; (C) 2,4-dinitrophenylhydrazine in 2 N HCl solution; (D) 2% $FeCl_3$.

that *O*-methyltransferase is present in all of the gymnosperms tested and that FA-2-^{14}C and FA-O^{14}CH$_3$ were formed respectively. Formation of FA-O^{14}CH$_3$ suggests that the methyl group of methionine-^{14}CH$_3$ was transferred to the *meta*-hydroxyl group of CA. The methylation did not require addition of ATP or *S*-adenosylmethionine to the incubation medium. When 5-HFA-2-^{14}C, or TCA and methionine-^{14}CH$_3$ were incubated with the sliced tissue of shoots of *G. biloba*, neither radioactive SA nor 5-HFA was formed. It was found that CA-2-^{14}C was selectively methylated yielding only FA-2-^{14}C when radioactive CA and 5-HFA were simultaneously incubated in the same reaction medium. If any SA had been formed, it would have been detected between FA and the origin on the chromatogram.

TABLE 3. SPECIFIC METHYLATION BY BAMBOO *O*-METHYLTRANSFERASE

Substrate	Expected product	Result
Caffeic acid	Ferulic acid	+
5-Hydroxyferulic acid	Sinapic acid	+
3,4,5-Trihydroxycinnamic acid	5-Hydroxyferulic acid	+
3,4,5-Trihydroxycinnamic acid	Sinapic acid	+
Chlorogenic acid*	Feruloylquinic acid	+
p-Coumaric acid	*p*-Methoxycinnamic acid	−
Caffeic acid	Isoferulic acid	−
Isoferulic acid	3,4-Dimethoxycinnamic acid	−
p-Hydroxybenzoic acid	Anisic acid	−
Protocatechuic acid	Vanillic acid	−
Protocatechuic acid	Veratric acid	−
Gallic acid	Syringic acid	−
p-Hydroxybenzaldehyde	Anisaldehyde	−
Protocatechu-aldehyde	Vanillin	−
Protocatechu-aldehyde	Isovanillin	−
Isovanillin	Veratraldehyde	−
5-Hydroxyvanillin	Syringaldehyde	−
Gallaldehyde	5-Hydroxyvanillin	−
Gallaldehyde	Syringaldehyde	−
Pinosylvin	Pinosylvin monomethylether	−

* The reaction mixture containing chlorogenic acid (1 μmole) as a substrate was incubated for 30 min at 30° as described in the text. After the addition of 0·5 ml of 10% HCl, the mixture was extracted with 20 ml EtOAc. The EtOAc fraction was divided into two equal portions. One was submitted to paper chromatography in toluene–AcOH–H_2O (4:1:5, upper layer) in order to determine free ferulic acid originally contained in the reaction mixture; however, none was present. The other portion was submitted to HCl-hydrolysis and ferulic acid liberated from any enzymatically formed feruloylquinic acid was determined as described in the text. The amount of ferulic acid after hydrolysis was found to be 2·2 μg.

DISCUSSION

It is well known that angiosperm lignins consist of guaiacyl, syringyl and *p*-hydroxyphenyl units whereas gymnosperm lignins contain mostly guiacyl units with small amounts of *p*-hydroxyphenyl units.[8–11] It has been reported, however, that some conifers exceptionally contain considerable amounts of syringyl units.[9] Although formation of the syringyl nucleus is assumed to correlate with biochemical evolution of higher plant lignins, the mechanism of

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[11] I. Kawamura and T. Higuchi, *J. Japan Wood Res. Soc.* **11**, 19 (1965).

its formation has not yet been elucidated. Higuchi and Brown[12] showed that labelled FA and 5-HFA were efficiently incorporated into the syringyl nucleus of wheat lignin, indicating that hydroxylation of FA occurred at the 5-position and the subsequent methylation of 5-HFA to SA. Hess demonstrated the transformation of FA-O$^{14}CH_3$ into SA-O$^{14}CH_3$ in seedlings of red cabbage.[13] However, evidence for the occurrence of 5-HFA and "FA-5-hydroxylase" in plants has not yet been obtained, although plant *O*-methyltransferases methylate 5-HFA to SA.[6-7] The moiety of 5-HFA ring occurs naturally in forms such as petunidin[14] and plicatic acid.[15] If one assumes that 5-HFA is a natural precursor for syringyl groups in lignin, it is suggested that one of the reasons for difficulty in its detection is possibly due to its being bound to the *O*-methyltransferase. 5-HFA formed from TCA was rapidly further methylated yielding SA even when the level of 5-HFA was quite low,

Figure 1 and Table 1 show that a larger amount of SA was formed as compared with FA and suggest that SA should accumulate in considerable amounts in plant tissue. However, SA could not be detected as easily as FA in growing bamboo, perhaps because SA is rapidly incorporated into lignin in the tissue. Figure 2 shows, however, that SA accumulated in the tissue only when a sufficient amount of 5-HFA was supplied. This suggests that the reaction rate observed *in vitro* would not necessarily occur in the living plant. Table 3 confirms the previous results[7] that bamboo *O*-methyltransferase works only with the 3- or 5-hydroxyl group in 3,4-dihydroxy- and 3,4,5-trihydroxycinnamic acids. On the other hand, hydroxybenzoic acids, hydroxybenzaldehydes and other phenolics were not effective as methyl group acceptors. However, Hess[5] reported that protocatechualdehyde, protocatechuic and gallic acids, and esculetin were methylated by cell-free extracts from *Petunia* and Finkle *et al.* observed a similar methylation reaction in a grass. Although Finkle *et al.*[3] and Hess[5] recognized *para*-methylation patterns and *para*-methylated substances, such as anethole[16] and Nerine,[17] occur in some plants, no *para*-methylation was observed with the bamboo enzyme. As for methylation of 3,4-dihydroxycinnamic acid, Finkle[18] found that it was methylated only at the 3-position, which is consistent with the present results. It is not clear what factors are involved in determination of *meta*- and *para*-methylation. One of the factors may be a requirement for magnesium ion, which could affect enzyme-substrate orientation by cross-linking between *p*-hydroxyl group of catechols and *S*-adenosylmethionine, as proposed by Senoh *et al.*[19] Consequently, the *meta*-hydroxyl group is substituted by the methyl group of *S*-adenosylmethionine yielding *meta*-methylated products. Variations in the pH value of the reaction medium can also influence the ratio of *para*- and *meta*-methylated products in some compounds.[19] The differences in substrate specificities between animal and plant *O*-methyltransferases have been summarized by Daly.[20]

From these results, it is clear that *O*-methyltransferases involved in the biosynthesis of lignin precursors such as ferulic and sinapic acids do not act upon other phenolic compounds such as alkaloids and flavonoids. Moreover, the results with sliced tissue of ginkgo shoots

[12] T. HIGUCHI and S. A. BROWN, *Can. J. Biochem. Physiol.* **41**, 613 (1963).
[13] D. HESS, *Planta* **60**, 568 (1964).
[14] G. M. ROBINSON and R. ROBINSON, *Konstitution und Vorkommen der Organischen Pflanzenstoffe* (edited by W. KARRER), p. 685, Birkhäuser-Verlag (1958).
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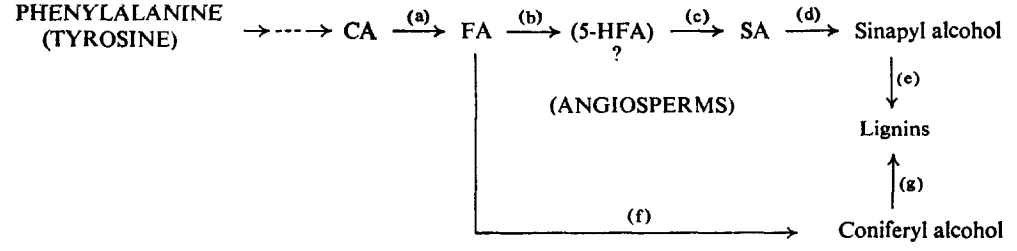
show that gymnosperm *O*-methyltransferase selectively methylates CA in the mixture of CA and 5-HFA, and indicate one reason for the metabolic differences between gymnosperms and angiosperms during lignification. For the formation of syringyl nuclei in angiosperm lignins, FA must be first hydroxylated at the 5-position, giving 5-HFA, which is subsequently methylated to SA. This process can be considered as a diverging step which differentiates angiosperms from gymnosperms and is hence related to the biochemical evolution of plant lignins.

However, if SA or sinapyl alcohol is artificially supplied to gymnosperms, they might be incorporated into gymnosperm lignins. Reznik,[21] on the other hand, did not observe any incorporation of syringin into spruce lignin. In view of his observation and the present result it is suggested that syringyl compounds are not necessary for the formation of gymnosperm lignins. Furthermore, as shown in Scheme 1, gymnosperms may not contain the enzyme systems participating in formation of syringyl lignin found in angiosperms. The metabolic differences between angiosperms and gymnosperms may be as follows:

(1) Gymnosperms may lack one or more of the series of the enzymes including (b), (c), (d), or (e) (Scheme 1). Gymnosperm *O*-methyltransferase may methylate only CA.
(2) Angiosperm *O*-methyltransferase may consist of two enzymes; one for CA and another for 5-HFA.
(3) The enzyme system, (f) in gymnosperms may not reduce SA to sinapyl alcohol.
(4) Peroxidases and phenoloxidases in gymnosperms may differ from those in angiosperms in that the former utilizes only coniferyl alcohol and that the latter both coniferyl alcohol and sinapyl alcohol. However, this possibility seems to be ruled out because peroxidases and phenoloxidases generally have a broad spectrum of substrate specificity.

Accordingly, gymnosperms may regulate their metabolism in order to utilize only guaiacyl precursors (ferulic acid, coniferyl alcohol, etc.) even when syringyl compounds and 5-HFA are administered to the plants. This may correspond with a tendency of cells to synthesize only necessary precursors and to convert unnecessary substances in other ways.



SCHEME 1. METABOLIC PATHWAY OF LIGNINS IN ANGIOSPERMS AND GYMNOSPERMS.
(a), (c), *O*-Methyltransferase; (b), FA-5-hydroxylase; (d), (f), enzyme systems involved in reduction of the hydroxycinnamic acids; (g), peroxidases or phenoloxidases.

EXPERIMENTAL

Samples and Reagents

Fresh bamboo shoots of *Phyllostachys pubescens* (Mōsō) and *P. reticulata* (Madake), young shoots of *Populus nigra* were used as representatives of angiosperms for the preparation of *O*-methyltransferase.

[21] H. REZNIK, *Biochemistry of Wood* (*Proceedings of the IVth International Congress of Biochemistry, Vienna*) (edited by K. KRATZL and G. BILLEK), p. 70, Pergamon Press, Oxford (1958).

Young shoots of the maidenhair tree (*Ginkgo biloba*) and *Podocarpus macrophylla* Maki and seedlings of *Cryptomeria japonica* and *Pinus strobus* were employed as representatives for gymnosperms.

Caffeic, ferulic, 5-hydroxyferulic, sinapic and 3,4,5-trihydroxycinnamic acids were synthesized from the corresponding benzaldehydes and malonic acid according to the method of Neish.[22] Labelled cinnamic acids were prepared in the same way by use of malonic acid-2-^{14}C.

Extraction of O-*Methyltransferase from Plants*

Extraction of *O*-methyltransferase from bamboo shoots were carried out as described in the previous paper.[7] *O*-Methyltransferase in young shoots of *Populus nigra* was extracted as follows; 100 g of young shoots were cut into small pieces, homogenized at about 4° in a Waring blender with an equal weight of 0·1 M Tris buffer solution (pH 7·3) containing dithiothreitol (10 mg) and $NaBH_4$ (40 mg). The homogenate was filtered through gauze and the filtrate was centrifuged at 5500 *g* for 20 min. To the supernatant solution solid $(NH_4)_2SO_4$ was added to 0·8 saturation. The precipitate was dissolved in a small amount of 0·01 M Tris buffer solution (pH 7·5) and the solution was passed through a Sephadex G-25 column to remove $(NH_4)_2SO_4$. The eluate containing enzyme protein was collected and used for the assay of *O*-methyltransferase activity.

Assay of O-*Methyltransferase*

The reaction mixture usually contained the following components: 0·1 ml each of 0·01 M of sodium caffeate (1 μmole), 0·04 M of $MgCl_2$ (4 μmoles), 0·04 M sodium ascorbate (4 μmoles), 0·005 M *S*-adenosylmethionine iodide (0·5 μmole), 0·2 ml of 1 M Tris buffer solution (pH 8·0) and 1–2 ml of the enzyme solution. The reaction mixture was incubated at 30° for 30 min. After the enzymic reaction was stopped by addition of 0·5 ml of 10% HCl, the mixture was extracted with 3 × 5 ml portion of Et_2O. The Et_2O was removed and the residue was chromatographed on paper in toluene–AcOH–H_2O (4:1:5, upper layer). The reaction product, i.e. ferulic acid in this case, was detected in u.v. light. The fluorescent band on the chromatogram was cut out and the product eluted with 5 ml of 95% EtOH at 50° for 10 min. The amount of eluted ferulic acid was determined by the measurement of absorbancy at 323 nm. When 5-hydroxyferulic acid (5-HFA) was used as a substrate, sinapic acid (SA) was formed and was separated by paper chromatography and determined at 325 nm. When 3,4,5-trihydroxycinnamic acid was used as a substrate, the quantities of the components and the enzyme solution for the methylation reaction were doubled and at the end of incubation the reaction mixture was divided into two equal portions. One was used for the determination of SA and the other was submitted to paper chromatography in $CHCl_3$–AcOH–H_2O (2:1:1, lower layer) and eluted 5-HFA was determined by the measurement of absorbancy at 326 nm.

Examination of O-*Methyltransferase Activity with Plant Tissue*

Sliced tissue (5 g) of young shoots of bamboo, *Podocarpus macrophylla* (Maki) and *Ginkgo biloba* and seedlings of *Cryptomeria japonica* and *Pinus strobus* were incubated at room temp. for 1 hr with a reaction medium (1 ml), containing 0·5–1 mg of the substrate (hydroxycinnamic acids, 0·30–0·5 μc, but non-labelled hydroxycinnamic acids were used when incubated with the tissue of bamboo shoot) and 0·25–0·5 μmole of *S*-adenosylmethionine or methionine-$^{14}CH_3$ (1 μc) instead. At the end of incubation the product was extracted with hot 80% EtOH, and after paper chromatography it was identified and determined as described above.

Identification of Methylated Products

FA, 5-HFA and SA were identified and determined as described above. Their R_f values are given in Table 2. However, when the other phenolic compounds were used as substrates, as shown in Table 2 the expected methylated products were checked by comparison of their R_f values, fluorescence under u.v. lamp and colour reactions with those of the authentic compounds. When a radioactive methylated product was obtained, it was identified by autoradiography with the chromatogram scanner (Aloka PCS-4).

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