Biosynthesis of Lignans

Part I. Biosynthesis of Arctiin (3) and Phillyrin (5)

By Joachim Stöckigt and Martina Klischies

Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität Bochum, D-4630 Bochum, Germany

Keywords
Lignans
Arctiin
Phillyrin
Biosynthesis
Synthesis
of C_6-C_3-precursors
Dimerisation
GC-MS
Forsythia suspensa

Biosynthesis of Lignans. Part I. Biosynthesis of Arctiin (3) and Phillyrin (5)

Summary

Lignans constitute a class of naturally occuring phenolic compounds, widely distributed in higher plants. They are formally composed of two phenylpropanoid units, stereospecifically joined at the β-carbon atoms of their side chains. Their biosynthesis has as yet not been investigated. To see, if these plant phenolics originate from simple phenylpropanes, various radioactively labelled, putative precursors were fed to *Forsythia* shoots. Chemically synthezised arylpropane derivatives, such as $^3H/^{14}C$-glucoferulic acid, -glucoferulic aldehyde, and -coniferin were incorporated into the lignans arctiin (3) and phillyrin (5) while ^3H-3,4-dimethoxycinnamic acid was not incorporated. From these results it may be concluded that the hydroxylated compounds are direct precursors of these dimeric phenylpropanes and are incorporated through a dimerisation step without degradation of the C_6-C_3 skeleton.

Schlüsselwörter
(Sachgebiete)
Lignane
Arctiin
Phillyrin
Biosynthese
Synthese
von C_6-C_3-Vorstufen
Dimerisierung
GC-MS
Forsythia suspensa

Biosynthese von Lignanen. Teil I. Biosynthese von Arctiin (3) und Phillyrin (5)

Zusammenfassung

Lignane sind eine Klasse natürlich vorkommender phenolischer Verbindungen, die in höheren Pflanzen weit verbreitet sind. Sie bestehen formal aus zwei Phenylpropaneinheiten, die stereospezifisch an den β-C-Atomen verbunden sind. Ihre Biosynthese ist bisher nicht untersucht worden. Um zu prüfen, ob diese pflanzlichen Phenole aus einfachen Phenylpropanen aufgebaut werden, wurden verschiedene radioaktiv markierte präsumptive Vorstufen an Sproßspitzen von Forsythien gefüttert. Die chemisch synthetisierten Arylpropanderivate ($^3H/^{14}C$-Glucoferulasäure, -Glucoferulaaldehyd und -Coniferin) wurden mit Ausnahme von ^3H-3,4-Dimethoxyzimtsäure in die Lignane Arctiin (3) und Phillyrin (5) eingebaut.
Aus diesen Ergebnissen kann geschlossen werden, daß die hydroxylierten Verbindungen direkte Vorstufen dieser dimeren Phenylpropane sind und ohne Veränderung des C_6-C_3 Kohlenstoffskeletts über einen Dimerisierungsschritt eingebaut werden.

Introduction

Lignans are a class of phenylpropanoids, which have a widespread occurrence in many different plant species and can be isolated from roots, leaves, bark, wood and resinous exudates. It is uncertain, whether they have an essential function in the plants in which they occur (Erdtman 1955). Apart from the group of neolignans, investigated by Gottlieb (1972), more than one hundred of these natural products have now been found, their structures elucidated and syntheses developed (see Devon and Scott 1975). With the exception of some special types, such as sesamolin (Haslam and Haworth 1955) or the phrymarolins (Taniguchi 1972), lignans (Fig. 1) can be classified on the basis of carbon skeleton (1) into simple diols e. g. secoisolariciresinol (2), lactones, e. g. arctiin (3), tetrahydrofuran derivatives, e. g. lariciresinol (4), tetrahydrofuranofurans, e. g. phillyrin (5), or tetrahydronaphthalene compounds, e. g. conidendrin (6).

At present, a number of theories exist on the biosynthesis of these phenylpropanoids, including the proposed dimerisation of two phenylpropane units through coupling between the β-carbon atoms of the side chains (Erdtman 1955; Neish 1966), however direct experimental evidence is lacking. Thus, it is not known, whether simple arylpropanes, such as cinnamic acids and their derivatives, are direct precursors of lignans.

In this paper, the first investigation of the biosynthesis of the lignans, arctiin (3) and phillyrin (5) in *Forsythia* is reported.

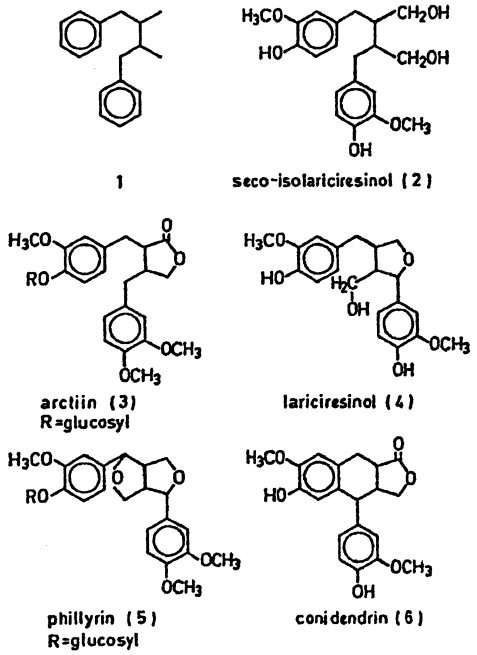

Fig. 1. Main types of lignans

Results and Discussion

The structures of arctiin (3) and phillyrin (5) suggest that ferulic acid and coniferyl alcohol might serve as biosynthetic precursors of these compounds.

To investigate the possible incorporation of these compounds into (3) and (5), we used the glucose deri-

vatives of ferulic acid, coniferyl alcohol and coniferyl aldehyde. In addition, 3,4-dimethoxycinnamic acid was also tested as a precursor. The glucose derivatives were used to protect the phenolic groups of the cinnamyl compounds against oxidation and also to facilitate better solubility and efficient uptake into the plant material.

Synthesis of precursors

Since the glucoside derivatives of these cinnamyl compounds were not commercially available, each was synthesized and doubly labelled with either ^{14}C, and ^{3}H (see experimental for synthesis details). In these syntheses, the benzaldehyde (9) is the key compound for preparation of the cinnamyl derivatives, since each is ultimately derived from this intermediate (Fig. 2, 3). The Knoevenagel reaction with malonic acid and (9) yielded acetylated glucoferulic acid (10) almost quantitatively. The acid was de-acetylated to glucoferulic acid (11) according to published methods (Fuchs 1955). In this earlier study, however, it was reported that upon reduction of the acid chloride (12) with sodium trimethoxyborohydride at —80° C, only aldehyde was produced. However, by varying the temperature we were able to effect both aldehyde and alcohol production and at —18° C equimolar yields were obtained. Therefore, the temperature is critical for reduction reaction.

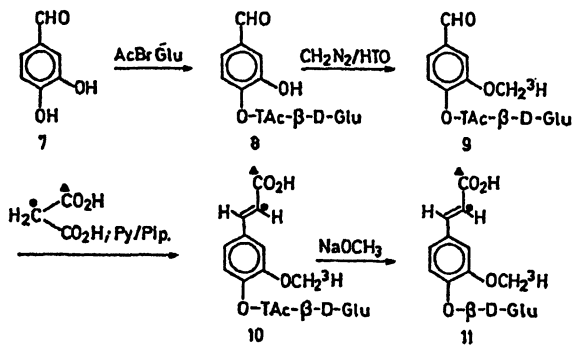

Fig. 2. Synthesis of $^{3}H/^{14}C$-β-D-glucoferulic acid. (● = β-^{14}C, ▲ = γ-^{14}C)

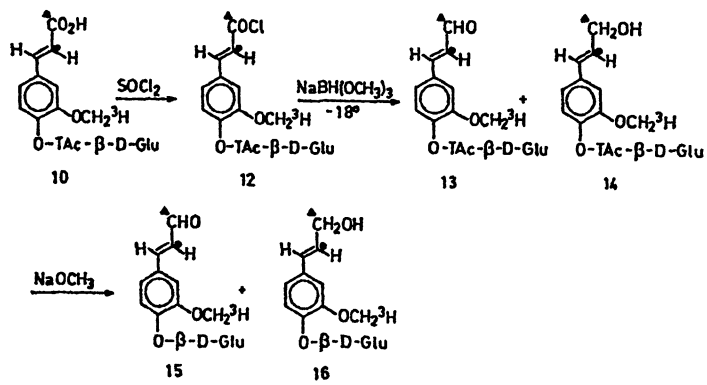

Fig. 3. Synthesis of $^{3}H/^{14}C$-β-D-glucoferulic aldehyde and -coniferyl alcohol. (● = β-^{14}C, ▲ = γ-^{14}C)

Feeding experiments

Young *Forsythia* shoots which already contain the lignans, arctiin (3) and phillyrin (5), in their leaves (Thieme and Winkler 1968) were used for feeding experiments. From aqueous solutions all precursors (11, 15, 16 and 18) were found to be taken up rapidly through the cut ends of the stems. After a feeding time of 20 hours, the plant material was extracted and chromatographed on TLC plates. Fig. 4 shows the distribution of radioactivity among metabolic products derived from glucoferulic acid (11) after chromatography on Silicagel. As can be seen, a good incorporation into (27,8% of the radioactivity which could be extracted with methanol) arctiin (3) and phyllirin (5) was obtained. Feeding experiments with glucoferulic aldehyde (15) and coniferin (16) gave similar distributions of radioactivity. After purification of both lignans to constant specific activity (for unequivocally identification of arctiin (3) and phillyrin (4), see Table 2 and GC-MS analysis in the experimental section) by chromatography and separation on acetylated polyamide the specific incorporation of the precursors (11, 15, 16) into the lignans (see Table 1) changed from 1.9 to 0.7% for arctiin (3) and 1.4 to 0.4% for phillyrin (5). Highest incorporation was achieved with glucoferulic acid, but merely on the basis of the incorporation data it cannot be determined at which oxidation level the most effective incorporation into lignans occurs. Also there is a likehood that the precursors might be differentially translocated and metabolized, e. g. into lignin, since 53% of the radioactivity could not be extracted from the plant material.

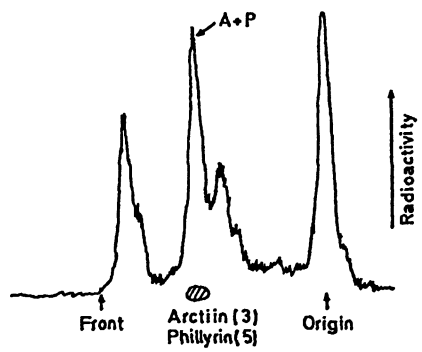

Fig. 4. Purification of plant-MeOH extracts (Precursor: Glucoferulic acid-$^{3}H/^{14}C$)

Recent studies in *Forsythia* and other higher plants have shown the presence of an enzyme system capable of the sequential reduction of ferulic acid to coniferyl alcohol via the aldehyde (Mansell et. al. 1972, 1974; Gross et al. 1973). Since reduction involves three different cinnamyl compounds it is possible that our substrates were first de-glucosylated by an endogenous β-glucosidase then reduced to the aldehyde or alcohol just prior to incorporation into the lignans.

Feeding experiments with doubly labelled precursors indicated no significant change in the $^{3}H/^{14}C$ ratios (Table 1). These results show unequivocally that the precursors are incorporated without degradation of the carbon skeleton and indicate the direct dimerization of the two arylpropane units to the corresponding lignans.

Since 3,4-dimethoxycinnamic acid was not incorporated in lignans (Table 1) and can not be reduced by enzymes involved in lignin biosynthesis (Gross and Zenk 1974, Gross et al. 1975) it seems that the dimerization of the precursors occurs after the reduction to the appropriate aldehyde or alcohol. Moreover, there

Table 1
Incorporation of ^{3}H and $^{3}H/^{14}C$ labelled ferulic acid derivatives into the lignans arctiin (3) and phillyrin (5)

Precursor	Amount taken up (μmole)	^{3}H: ^{14}C	Arctiin (3) incorp. absol. (%)	Arctiin (3) incorp. spec. (%)	^{3}H: ^{14}C	Phillyrin (5) incorp. absol. (%)	Phillyrin (5) incorp. spec. (%)	^{3}H: ^{14}C
Glucoferulic acid								
(β-^{14}C; -$OCH_2{}^{3}H$) . .	2.43	10.65 : 1	3.66	1.9	10.37 : 1	0.68	1.40	10.11 : 1
(-$OCH_2{}^{3}H$)	2.74	—	6.27	5.1	—	1.81	3.47	—
(γ-$^{14}CO_2H$; -$OCH_2{}^{3}H$)	—	9.90 : 1	—	—	9.98 : 1	—	—	9.98 : 1
Glucoferulic aldehyde . .								
(β-^{14}C;-$OCH_2{}^{3}H$) . . .	0.41	12.80 : 1	1.37	1.2	11.63 : 1	0.20	0.40	11.27 : 1
Coniferin								
(β-^{14}C; -$OCH_2{}^{3}H$) . .	1.41	12.90 : 1	0.74	0.7	11.48 : 1	0.16	0.40	12.25 : 1
3,4-*Dimethoxycinnamic acid*								
(4-O-$CH_2{}^{3}H$)	0.50	—	0	0	—	0	0	—

are some indications, from feeding experiments with doubly labelled coniferin-$^{14}C^{3}H_2OH$, that there is no re-oxidation to ferulic acid and subsequent dimerization at this oxidation level (Klischies and Stöckigt, unpublished). Therefore, we propose that coniferyl alcohol is the direct precursor and secoisolariciresinol (2) would thus be the first dimeric intermediate in lignan biosynthesis and after simple biochemical reactions, such as, dehydration and O-methylation, or after oxidation of one alcohol group to a carboxy group and splitting off H_2O, phillyrin (5) and arctiin (3) could be synthesized. Since plant lignans are optically active, these dimerization reactions should proceed by an enzymatic process and not a free radical reaction as proposed for the polymerization of coniferyl alcohol during biosynthesis of optically inactive lignin (Geissman and Crout 1969).

Experimental

Syntheses of labelled precursors

To obtain high labelled various precursors, tritiated methoxybenzaldehydes with high specific activity were synthesized.

a) Synthesis of $^{3}H/^{14}C$-β-D-glucosidoferulic acid (11)

4-(tetra-O-acetyl-β-D-glucosyl)-protocatechualdehyde (8):

This aldehyde was prepared according to the procedure of Helferich et al. (1955). Yield: 40%; mp 179.5 —180° (ref. mp 180°).

4-(tetra-O-acetyl-β-D-glucosyl)-vanillin-$OCH_2{}^{3}H$ (9):

Under water free conditions, the aldehyde could be synthesized from compound (8) after an exchange reaction (1.5 hours) with HTO (spec. act. 5 Ci/ml) in absolute dioxane and by methylation with an excess of dry diazomethane in a yield of 71% and a spec. act. of 34 μCi/μmole.

4-(tetra-O-acetyl-β-D-glucosyl)-ferulic acid-($OCH_2{}^{3}H$, or γ-^{14}C) (10):

The acetylated acid was prepared by a Knoevenagel reaction of (2-^{14}C) or (1-^{14}C) malonic acid (diluted with unlabelled acid to a $^{3}H/^{14}C$ ratio of approximately 10 : 1), 18.4 mg (177 μmole; spec. act. 1.41 μCi/μmole) and the aldehyde (9), 85.65 mg (177.7 μmole; spec. act. 15.02 μCi/μmole) by standard procedures in 0.1 ml anhydrous pyridine/piperidine (10 : 0.2; v/v), temperature 75—85 °C, reaction time 3.5 hours. After dilution of the mixture with 1.0 ml H_2O and 0.05 ml acetic acid, the acid (10) crystallized. Recrystallization was done from 95% alcohol; yields ranged from 85—95%.

$^{3}H/^{14}C$-β-D-glucosidoferulic acid (11):

The deacetylation (Fuchs 1955) was done with 5% sodium methoxide in a yield of 83%, spec. act. ^{3}H : 15.02 μCi/μmole; ^{14}C : 1.41 μCi/μmole; $^{3}H/^{14}C$ = 10.65 : 1.

b) Synthesis of $^{3}H/^{14}C$-β-D-glucosidoferulic aldehyde (15) and $^{3}H/^{14}C$-coniferin (16) was done according to the method described by Fuchs (1955).

4-(tetra-O-acetyl-β-D-glucosyl)-ferulic aldehyde (13) and 4-(tetra-O-acetyl-β-D-glucosyl)-coniferyl alcohol (14):

84 mg (160 μmole) of the acid (10) were converted to the acid chloride by reaction with $SOCl_2$ in absolute benzene at 75°C for 2.5 hrs. The organic solvent was evaporated and the residue dried under vacuum over KOH for 16 hrs. The chloride was dissolved in 1.5 ml dry tetrahydrofuran and reduced with 81 mg (633 μmole) sodium trimethoxyborohydride (Brown and Mead 1953) at —18°C for 20 min, then at room temperature for 25 min.

After hydrolysis with 1 ml H_2O, the organic solvent was evaporated, the aqueous phase extracted with benzene (3 × 3 ml), the benzene solution dried over Na_2SO_4, and evaporated.

$^{3}H/^{14}C$-β-D-glucosidoferulic aldehyde (15) and $^{3}H/^{14}C$-coniferin (16):

The residue was dried, dissolved in 0.3 ml anhydrous $CHCl_3$ and deacetylated as described above. Both compounds (15) and (16) were purified by chromatography on silicagel GF_{254} plates (1.5 mm) developed in $CHCl_3$/MeOH = 7 : 1.7;

(15) yield 16%, spec. act. 12.16 μCi/μmole ^{3}H
0.95 μCi/μmole ^{14}C

(16) yield 15%, spec. act. 12.78 μCi/μmole ^{3}H
0.99 μCi/μmole ^{14}C

c) Synthesis of 3,4-dimethoxycinnamic acid — (4-$OCH_2{}^{3}H$) (18)

3,4-dimethoxybenzaldehyde-(4-$OCH_2{}^{3}H$) (17):

2 g (13.16 mmole) vanillin were dissolved in 15 ml absolute dioxane and 100 μl HTO (5 Ci/ml) added. After an exchange time of 2.5 hrs. the aldehyde was methylated with diazomethane under the above conditions. The colorless reaction mixture was evaporated under vacuum for 16 hrs. The yield of the aldehyde was determined by scanning the radioactivity: yield 75%, spec. act. 13.6 μCi/μmole.

207 mg (1.36 mmole) aldehyde (17) and 145 mg (1.39 mmole) malonic acid were dissolved in 0.3 ml absolute pyridine/piperidine (10 : 0.2 v/v) and heated at 100° C for 3.5 hrs. After

hydrolysis with 3 ml 3 n hydrochloric acid, the cinnamic acid could be isolated in a yield of 75%, spec. act. 13.6 μCi/μmole.

The Rf value on silicagel plates, developed in benzene/ethyl-acetate/acetic acid = 85 : 14 : 1, the UV and mass spectra of the methylester were identical with authentic material.

Feeding experiments

Plant material: *Forythia suspensa* var. *fortunei* was grown in a phytotron under 16 hrs. photoperiod, 25—20° C day-night temperature cycle and a rel. humidity of 70%. For feeding experiments, the precursors were dissolved in 0.5 ml H_2O and fed for 20 hrs. through the cut end of 3.5 cm long *Forsythia* shoots.

Isolation and purification of the labelled lignans

After feeding, the plant material was cut into pieces, extracted twice with hot methanol, filtered, concentrated and chromatographed on Silicagel GF_{254} (1.5 mm, in $CHCl_3$/MeOH = 6.5 : 1). The chromatogramm was scanned for radioactivity and the lignan-band (Rf = 0.53) (Fig. 4), which contained arctiin (3) and phillyrin (5), eluted with ethanol and further purified by rechromatography in the same solvent system. Both lignans were then separated and isolated using TLC on acetylated Polyamide (Macherey and Nagel), developed in 1% formic acid (arctiin: Rf = 0.3; phillyrin: Rf = 0.22). The specific activity was determined spectrophotometrically (λmax = 280 nm) and by scintillation-counting, (incorporation data and spec. act. are summarized in Table 1 and 2).

Identification of arctiin (3) and phillyrin (5)

The isolated labelled lignans were diluted with inactive authentic arctiin (3) and phillyrin (5), obtained from Prof. Thieme (Leipzig) or were isolated from *Forsythia* plants and compared with the authentic material in three different solvent systems; A ($CHCl_3$/MeOH = 10 : 1), B ($CHCl_3$/EtOH = 55 : 5), C (CH_2Cl_2/THF/EtOH = 30 : 20 : 5); arctiin: Rf = 0.12 (A), 0.34 (B), 0.25 (C); phillyrin: Rf = 0.11 (A), 0.32 (B), 0.24 (C).

They were recrystallized three times from methanol (arctiin) and H_2O (phillyrin) (data see Table 2). After deglucosidation with β-glucosidase (Fa. Roth, 300 u/mg) (25 mg of the lignan with 1500 u) at pH 4.5, 30° C and 16 hrs. incubation time, and isolation the specific activities of the aglycones phillygenin and arctigenin were redetermined (Table 2). For identification the labelled undiluted aglycones were investigated by GC-MS-analysis. GC-MS-conditions: a glass column (1.5 m × 2 mm i. d.) packed with 3% SE 30 on supelcoport was used. Carrier gas was He (20 ml/min). Samples were injected at 300° C and the temperature raised from 260° C—300° C at a rate of 8° C per min (arctigenin Rt = 7.7 min, phillygenin Rt = 8.5 min). The mass spectra were set to 80 eV. The Rt values and fragmentation patterns were identical with authentic samples. MS: Arctigenin; 372 (38)M^+, 177 (15), 151 (65), 137 (100) m/e. MS: Phillygenin; 372 (23)M^+, 177 (39), 165 (38), 151 (100), 137 (42) m/e.

Table 2

Identification of the labelled lignans, arctiin (3) and phillyrin (5) from feeding experiments

(arctiin was recrystallized from MeOH, phillyrin from H_2O)

Crystallization after dilution with unlabelled lignans	Arctiin (3) spec. act. (dpm/μmole)	Phillyrin (5) spec. act. (dpm/μmole)
1.	4.6×10^4	1.0×10^4
2.	4.4×10^4	1.1×10^4
3.	4.2×10^4	0.9×10^4
4.*)	4.5×10^4	0.9×10^4

*) after β-glucosidase treatment.

Beckman model 24 double beam spectrophotometer was used for UV, radioactivity was determined by scintillation counting in a betaszint, BF 5000 and the mass spectra were done on a Varian Mat 111.

Acknowledgements

The technical assistance of Miss B. Ries and Mr. L. Andert are gratefully appreciated. We thank Prof. Dr. Thieme (Leipzig, GDR), for samples of the lignans.

The support and valuable criticism of Prof. Dr. M. H. Zenk are gratefully acknowledged.

Financial support was provided by the Deutsche Forschungsgemeinschaft to Prof. Dr. M. H. Zenk.

References

Brown, H. C. and E. J. Mead. 1953. Addition Compounds of Alkali Metal Hydrides II. Sodium Trimethoxyborohydride as a Reducing Agent for Organic Compounds. J. Amer. Chem. Soc. 75: 6263—6265.

Devon, T. K. and A. J. Scott. 1975. Handbook of Naturally Occuring Compounds. Vol. I., Acetogenins, Shikimates and Carbohydrates, 71—84, Academic Press, New York and London.

Erdtman, H. 1955. Lignans, in: (Paech, K. and Tracy, M. V.) Modern Methods of Plant Analysis. Vol. III: 428—449, Springer Verlag, Berlin, Heidelberg, New York.

Fuchs, W. 1955. Anwendung radioaktiver Isotope bei der Erforschung des Lignins VI. Zur Synthese der Glukoside radioaktiver Oxyzimtalkohole. Chem. Ber. 88: 1825—1827.

Geissman, T. A. and D. H. G. Crout. 1969. Organic Chemistry of Secondary Plant Metabolism. p. 400. Freeman, Cooper and Company, San Francisco.

Gottlieb, O. R. 1972. Chemosystematics of the *Lauraceae*. Phytochem. 11: 1537—1570.

Gross, G. G., R. L. Mansell and M. H. Zenk. 1975. Hydroxycinnamate: Coenzyme A Ligase from Lignifying Tissue of Higher Plants. Some Properties and Taxonomic Distribution. Biochem. Physiol. Pflanzen 168: 41—51.

Gross, G. G., J. Stöckigt, R. L. Mansell and M. H. Zenk. 1973. Three novel Enzymes involved in the Reduction of Ferulic Acid to Coniferyl Alcohol in Higher Plants: Ferulate: CoA Ligase, Feruloyl-CoA Reductase and Coniferyl Alcohol Oxidoreductase. FEBS Letters 31: 283—286.

Gross, G. G. and M. H. Zenk. 1974. Isolation and Properties of Hydroxycinnamate: CoA Ligase from Lignifying Tissue of *Forsythia*. Eur. J. Biochem. 42: 453—459.

Haslam, E. and R. D. Haworth. 1955. The Constituents of Natural Phenolic Resins Part XXIII. The Constitution of Sesamolin. J. Chem. Soc., 827—833.

Helferich, B., H. E. Schreiber, R. Streeck and F. Vorsatz. 1955. Die Beeinflussung der fermentativen Spaltbarkeit von Phenol-β-D-Glukosiden durch Substitution im Benzolkern. Ann. 518: 211—225.

Mansell, R. L., G. G. Gross, J. Stöckigt, H. Franke and M. H. Zenk. 1974. Purification and Properties of Cinnamyl Alcohol Dehydrogenase from Higher Plants involved in Lignin Biosynthesis. Phytochem. 13: 2427—2435.

Mansell, R. L., J. Stöckigt and M. H. Zenk. 1972. Reduction of Ferulic Acid to Coniferyl Alcohol in a Cell Free System from a Higher Plant. Z. Pflanzenphysiol. 68: 286—288.

Neish, A. C. 1966. Coumarins, Phenylpropanes, and Lignin, in: (Bonner J. and J. E. Varner) Plant Biochemistry, 581—617. Academic Press, New York and London.

Taniguchi, E. 1972. The Mass Spectra of Phrymarolins, 1.2-Dioxygenated-3.7-dioxybicyclo (3.3.0) Octane Lignans. Agr. Biol. Chem. 36: 1497—1503.

Thieme, H. and H.-J. Winkler. 1968. Über das Vorkommen von Lignanglukosiden in der Gattung *Forsythia*. Isolierung von Phillyrin und Arctiin. Pharmazie 23: 402—403.