J Wood Sci (2004) 50:82–86
DOI 10.1007/s10086-003-0530-1

ORIGINAL ARTICLE

Takeshi Nagasaki · Norihisa Osada · Mika Sato
Seiichi Yasuda · Koetsu Takahashi · Takanori Imai

Studies on the immunolabeling of heartwood extractives: characterization of anti-agatharesinol antiserum

Received: November 18, 2002 / Accepted: February 14, 2003

Abstract The reactivity of previously prepared antiserum against agatharesinol, a norlignan, was determined by competitive inhibition–ELISA (enzyme-linked immunosorbent assay) using several natural compounds that are representative of plant extractives as competitive inhibitors. The antiserum strongly reacted with some norlignans (i.e., agatharesinol, hinokiresinol, and metasequirin C), each of which has a common chemical-structural unit. This result suggests that the antiserum recognizes the specific chemical-structural unit composing those norlignans. Moreover, differences in the reactivities of the antiserum with agatharesinol derivatives and synthetic chalcones propose the *trans*-3-*p*-hydroxyphenyl-1-phenylpropene structural unit as the major antigenic determinant for the antiserum.

Key words Antibody · Agatharesinol · Norlignan · Competitive inhibition–ELISA

Introduction

When heartwood is treated with organic solvents, low-molecular weight substances called "heartwood extractives" are obtained. The extractives are specific for tree species and affect wood characteristics such as color, resistance against decay, and so on. Although the formation of heartwood extractives has been studied from both biochemical[1–4] and cytological[5–7] perspectives, questions concerning their biosynthesis, transfer, accumulation, and distribution are still open to investigation. To study the biosynthesis of heartwood extractives histochemically, we have introduced a method that had not been applied in this research field, namely immunolabeling.[8]

In immunological studies, a particular compound can be surveyed and characterized according to the selectivity of an antigen-antibody reaction. By applying immunolabeling to microscopic sections of plant tissues, various plant polymers have been localized in situ and their formation examined.[9–11] Therefore, immunolabeling must be an effective method by which to study the formation of heartwood extractives in situ.

In our previous paper, anti-agatharesinol polyclonal antibodies were prepared by immunizing a rabbit with an agatharesinol-*p*-aminohippuric acid-bovine serum albumin conjugate and partially affinity-purified.[8] The preliminary studies on the recognition selectivity of the antiserum by competitive inhibition–ELISA (enzyme-linked immunosorbent assay) using sugi (*Cryptomeria japonica*) heartwood extractives and some phenylpropanoids as competitive inhibitors indicated that the antiserum could immunolabel norlignans, especially agatharesinol in sugi extractives,[8] and agatharesinol was localized immunohistochemically in sugi heartwood tissue.[12]

In the present study, the antiserum was characterized in further detail. The reactivity of the antiserum was examined by competitive inhibition–ELISAs using several natural compounds that are representative of plant extractives, agatharesinol derivatives, and synthetic chalcones as competitive inhibitors. This examination of the reactivity of the antiserum led to a proposal of the antigenic determinant for the antiserum on the basis of the relations between the reactivities of the antiserum with the inhibitors and their chemical structures.

T. Nagasaki · N. Osada · M. Sato · T. Imai (✉)
Laboratory of Biomass Resources Utilization, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan
Tel. +81-52-789-4161; Fax +81-52-789-4012
e-mail: takaimai@agr.nagoya-u.ac.jp

S. Yasuda
Laboratory of Forest Chemistry, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

K. Takahashi
Section of Forest Resources, Department of Bioenvironment, Faculty of Agriculture, Yamagata University, Tsuruoka 997-8555, Japan

Part of this paper was presented at the 45th Lignin Symposium, Matsuyama, October 2000 and the 51th Annual Meeting of the Japan Wood Research Society, Tokyo, April 2001

Experimental

Competitive inhibition–ELISA

The amount of artificial antigen for plate coating and the concentration of the antiserum to be used in a competitive inhibition–ELISA were determined by checkerboard titrations, as described elsewhere.[8,13] The assays were performed using the artificial antigen coating concentration of 2000 ng/100 μl with the antiserum diluted 1:5000. The optimal concentration of competitive inhibitors (10^{-3} M) was chosen from a standard curve for agatharesinol competitive inhibition–ELISA because the enzyme activity (extent of the antigen-antibody reaction) showed a linear relation in the agatharesinol (competitive inhibitors) concentration range of 10^{-2}–10^{-5} M.[8,14]

An ELISA was carried out according to the method of Hassan et al.[13] with slight modification. All the procedures described below were performed at room temperature. Artificial antigen (2000 ng) dissolved in 100 μl phosphate-buffered saline (PBS) was applied to the wells of a 96-well microtitration plate (Iwaki, Japan), and the plate was allowed to stand for 2 h. The solutions were then removed, and all the wells were washed three times with PBS (400 μl), following all which the wells were blocked with PBS-OA (5% (w/v) ovalbumin and 5% (w/v) sucrose in PBS) (400 μl). After the wells were washed three times with PBS, the antiserum, diluted 1:4500 (final concentration 1:5000) in PBS (90 μl) with or without competitive inhibitors dissolved in methanol (10 μl), were applied to the wells. After 2 h the wells were washed thoroughly with PBS, and then goat:anti-rabbit immunoglobulin G (IgG)–alkaline phosphatase conjugate (Sigma, Japan) diluted 1:3000 in PBS (100 μl) was applied to the wells. After 1 h the wells were washed thoroughly with PBS. Color development was performed using an alkaline phosphatase substrate kit (Bio-Rad, Japan) containing *p*-nitrophenylphosphate as the substrate according to the instruction manual.

The reactivities of analytes (competitive inhibitors) with the antiserum were calculated as follows.

$$R(\%) = 100(A_0 - A_{ana})/(A_0 - A_{aga})$$

where R is the relative reactivity of the antiserum with the analyte to agatharesinol, and A_0, A_{ana}, and A_{aga} are absorbances at 405 nm (enzyme activity) of the reaction mixtures reacted without analyte, with analyte, and with agatharesinol, respectively.

Preparation of competitors

Extractives and related compounds

Agatharesinol,[15] sequirin C,[15] sugiresinol,[16] and ferruginol[17] were isolated from sugi (*Cryptomeria japonica*) heartwood. *trans*-Hinokiresinol[18] and hinokinin[19] were isolated from hinoki (*Chamaecyparis obtusa*) heartwood. *cis*-Hinokiresinol[20] was isolated from *Anemarrhena asphodeloides*. Athrotaxin[21] and metasequirin C[22] were isolated from metasekoia (*Metasequoia glyptostroboides*) heartwood. The chemical structures of all these extractives were identified by reference to previous reports. Coniferyl alcohol was a kind gift of Dr. K. Fukushima (Nagoya University, Japan). *p*-Coumaric acid, guaiacyl glycerol-β-guaiacyl ether, and catechin were purchased from Tokyo Kasei Kogyo (Japan). Hinokitiol was purchased from Wako (Japan). The chemical structures of those compounds are shown in Fig. 1.

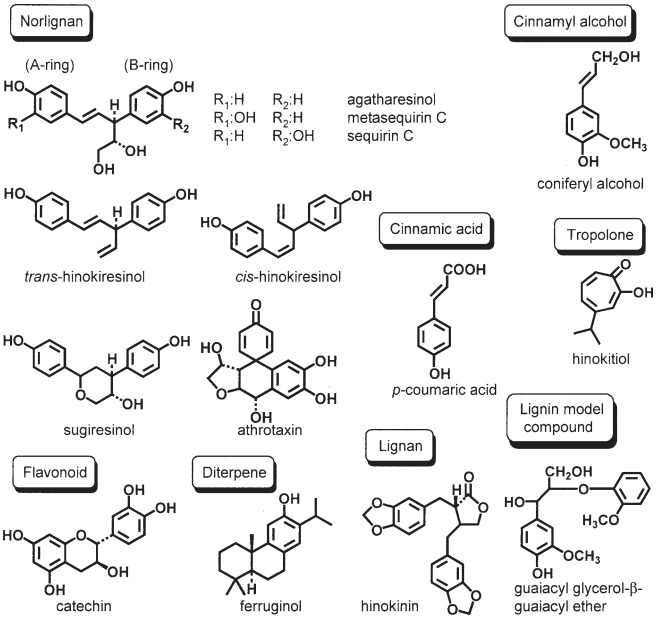

Fig. 1. Structural formulas of the compounds used to determine the reactivity of antiserum against agatharesinol

Agatharesinol derivatives

Three agatharesinol derivatives were prepared by the methods described below. The purities of the derivatives were determined by gas chromatography after trimethylsilylation, and the chemical structures (Fig. 2) were confirmed by ^{1}H,^{13}C-NMR spectroscopy.

Methylated agatharesinol. Agatharesinol (150 mg) was dissolved in methanol (1 ml), and freshly prepared diazomethane in diethyl ether (30 ml) was added to the solution. The mixture was allowed to stand for 2 h in the dark at room temperature. After residual diazomethane and diethyl ether were evaporated, another fresh diazomethane in diethyl ether (30 ml) was added. This procedure was repeated five times. The residue was then subjected to silica gel column chromatography [acetone-*n*-hexane (1:2)] to purify methylated agatharesinol.

^{1}H-NMR ($CDCl_3$); δ 3.42–3.67 (2H, H-5 × 2), 3.74 (6H, s, OCH_3 × 2), 3.82–3.97 (2H, H-3, 4), 6.15 (1H, dd, J = 8.9 and 15.7, H-2), 6.37 (1H, d, J = 15.9, H-1), 6.80 (2H, d, J = 8.4, aromatic H), 6.85 (2H, d, J = 8.1, aromatic H), 7.19 (2H, d, J = 8.4, aromatic H), 7.24 (2H, d, J = 8.4, aromatic H).

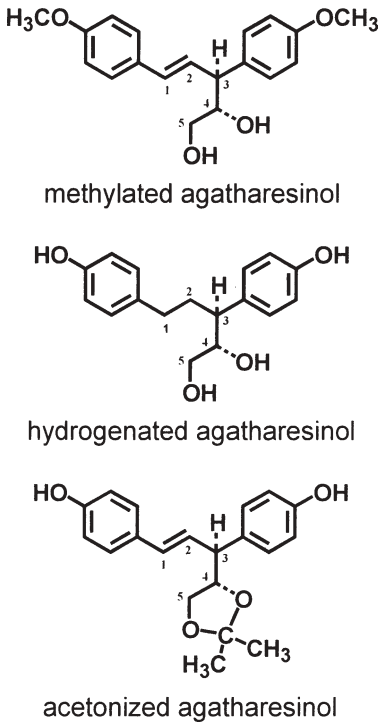

Fig. 2. Preparation of agatharesinol derivatives

^{13}C-NMR ($CDCl_3$); δ 52.1, 55.3, 64.8, 74.76, 114.0, 114.4, 127.5, 127.7, 129.5, 130.1, 131.1, 133.1, 158.6, 159.2.

Hydrogenated agatharesinol. Agatharesinol (150 mg) was dissolved in ethanol (30 ml), and 5% palladium carbon (45 mg) was added to the solution. The flask containing the mixture was evacuated, followed by packing with H_2. Then, after removing the palladium carbon by filtration, the filtrate was evaporated to give hydrogenated agatharesinol.

^{1}H-NMR (acetone-d_6); δ 2.03 (2H, H-2 × 2), 2.35 (1H, ddd, J = 4.7 and 7.1, H-1), 2.70 (1H, ddd, J = 4.7 and 7.1, H-1), 3.28 (1H, dd, J = 7.3 and 10.9, H-5), 3.46 (1H, dd, J = 4.3 and 11.1, H-5), 3.58 (1H, q, J = 7.1, H-3), 3.82 (1H, m, H-4), 6.72 (2H, d, J = 8.7, aromatic H), 6.78 (2H, d, J = 8.7, aromatic H), 6.94 (2H, d, J = 8.4, aromatic H), 7.12 (2H, d, J = 8.7, aromatic H). ^{13}C-NMR (acetone-d_6); δ 32.5, 34.4, 46.7, 64.5, 74.7, 114.6, 114.9, 129.1, 130.1, 132.3, 136.5, 155.3, 155.7.

Acetonized agatharesinol. Agatharesinol (150 mg) was dissolved in acetic anhydride (15 ml), and sulfuric acid (0.3 ml) was added to the solution. The mixture was allowed to stand for 40 h in the dark at room temperature. Then the mixture was neutralized with 6 N NaOH. After removing acetic anhydride by evaporation, the aqueous mixture was extracted with diethyl ether. The ether fraction was evaporated, and the residue was subjected to silica gel column chromatography [acetone-*n*-hexane (2:5)] to purify acetonized agatharesinol.

^{1}H-NMR (acetone-d_6); δ 1.26 (3H, s, CH_3), 1.30 (3H, s, CH_3), 3.49 (1H, t, J = 8.0, H-5), 3.74 (1H, t, J = 8.0, H-5), 4.02 (1H, dd, J = 6.4 and 8.2, H-3), 4.42 (1H, m, H-4), 6.23 (1H, dd, J = 8.2 and 15.8, H-2), 6.42 (1H, d, J = 16.0, H-1), 6.76–6.83 (4H, aromatic H), 7.18 (2H, d, J = 8.4, aromatic H), 7.24 (2H, d, J = 8.8, aromatic H). ^{13}C-NMR (acetone-d_6); δ 25.9, 27.1, 53.1, 68.5, 79.5, 109.5, 115.9, 116.1, 127.8, 128.2, 129.7, 130.2, 131.6, 133.4, 156.7, 157.7.

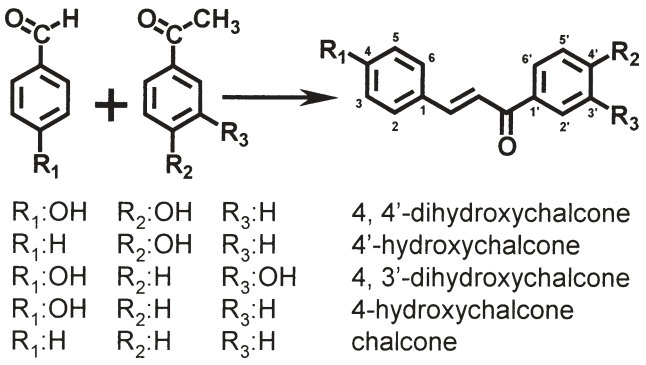

Fig. 3. Synthesis of the chalcones with various hydroxylation patterns

Synthetic chalcones

4,4′-Dihydroxychalcone was synthesized by condensing *p*-hydroxyacetophenone and *p*-hydroxybenzaldehyde in the presence of aqueous KOH (aldol condensation) according to the method of Ohashi et al.[23] Other chalcones varying in the hydroxylation pattern were synthesized in a manner similar to the above, using acetophenone and benzaldehyde hydroxylated at specific positions on the aromatic rings as starting materials (Fig. 3).

Results and discussion

To characterize the previously prepared anti-agatharesinol antiserum, first the reactivities of the antiserum with several natural compounds that are representative of plant extractives were examined (Table 1). The analytes were chosen because of their chemical-structural relation to agatharesinol and their representability. They are classified as norlignan, lignan, cinnamyl alcohol, cinnamic acid, lignin model compound, flavonoid, diterpene, and tropolone (for structures, see Fig. 1).

Compounds classified other than norlignan that are biosynthesized via biosynthetic pathways different from that of norlignan resulting in distinct chemical structures from agatharesinol showed much lower reactivity with the antiserum than agatharesinol. Even among norlignans, the reactivity of the antiserum varies with the chemical structure of each norlignan; that is, the antiserum showed strong reactivities with some norlignans chemical-structurally close to agatharesinol. These results indicated that the antiserum recognizes a specific chemical-structural unit composing agatharesinol, and an antigenic determinant for the antiserum can be proposed on the basis of the relations between reactivities of the antiserum with norlignans and their chemical structures.

Table 1. Reactivities of the antiserum with natural compounds

Class	Substance	*R* (%)
Norlignans	Agatharesinol	100[a]
	trans-Hinokiresinol	206.9[b]
	cis-Hinokiresinol	95.1
	Metasequirin C	97.4
	Sequirin C	62.3[b]
	Sugiresinol	64.1[b]
	Athrotaxin	42.8
Lignan	Hinokinin	44.2[b]
Cinnamyl alcohol	Coniferyl alcohol	33.6[b]
Cinnamic acid	*p*-Coumaric acid	34.2[b]
Lignin model compound	Guaiacyl glycerol-β-guaiacyl ether	25.1[b]
Flavonoid	Catechin	34.1
Diterpene	Ferruginol	22.5
Tropolone	Hinokitiol	38.3

Data represent means for three independent measurements
[a] Reactivity of agatharesinol with the antiserum was defined to be 100%
[b] Previously reported data[10]

Table 2. Reactivities of the antiserum with agatharesinol derivatives

Substance	*R* (%)
Agatharesinol	100[a]
Methylated agatharesinol	54.4
Hydrogenated agatharesinol	72.1
Acetonized agatharesinol	96.9

Data represent means for three independent measurements
[a] Reactivity of agatharesinol with the antiserum was defined to be 100%

Table 3. Reactivities of the antiserum with chalcones

Substance	*R* (%)
Agatharesinol	100[a]
4,4′-Dihydroxychalcone	100.4
4′-Hydroxychalcone	88.6
4,3′-Dihydroxychalcone	89.9
4-Hydroxychalcone	78.1
Chalcone	51.1

Data represent means for three independent measurements
[a] Reactivity of agatharesinol with the antiserum was defined to be 100%

First, although both metasequirin C and sequirin C are monohydroxylated derivatives of agatharesinol on their aromatic rings (Fig. 1), the reactivity of our antiserum with metasequirin C was much higher than that with sequirin C (Table 1). Metasequirin C is hydroxylated at the R_1 position on the A-ring, whereas sequirin C is that hydroxylated at the R_2 position on the B-ring (Fig. 1). This result indicates that the hydroxylation pattern on the B-ring is much more sensitive to recognizing agatharesinol by the antiserum than is the pattern on the A-ring (Fig. 1). Second, the lower reactivity of the antiserum with sugiresinol, an isomer of agatharesinol of which the dihydroxypentene chain is cyclized, and with athrotaxin, which has a side-chain skeleton different from that of agatharesinol, indicates the importance of the side chain for recognition by the antiserum. Third, the high reactivity of the antiserum with *trans*-hinokiresinol, an analogue of agatharesinol in which the 1,2-ethanediol structural unit in the side chain is substituted with an ethene structural unit, indicates the lesser importance of the 1,2-ethanediol structure in the side chain for antibody recognition. Unexpectedly, *trans*-hinokiresinol had much stronger reactivity than agatharesinol, but the reason for the stronger reactivity is not well understood. The strong reactivity of the antiserum with *trans*-hinokiresinol must be due to its significant chemical-structural similarity to agatharesinol (Fig. 1). Moreover, much higher reactivity of the antiserum with *trans*-hinokiresinol than with *cis*-hinokiresinol indicates that the antiserum clearly distinguishes the *trans*form from the *cis* form of the pentene structural unit in the side chain, and the *trans*-form significantly participates in the recognition.

In summary, the *trans*-3-*p*-hydroxyphenyl-1-phenylpropene structural unit is proposed as the major antigenic determinant for the antiserum (Fig. 4). Thus, the antiserum preferentially recognizes specific norlignans by distinguishing slight differences in their chemical structures.

To confirm the above proposal, three agatharesinol derivatives (methylated, hydrogenated, and acetonized agatharesinol, for structure, Fig. 2) and synthetic chalcones (Fig. 3) were prepared, and the reactivities of the antiserum with those compounds were examined (Table 2, 3). Hydrogenated agatharesinol, with the side chain saturated, showed decreased reactivity with the antiserum compared with agatharesinol, confirming that the propene unit in the side chain participates in recognition by the antiserum. Acetonized agatharesinol, with the alcoholic hydroxyl groups in the side chain modified, reacted with the antiserum as strongly as agatharesinol, whereas methylated agatharesinol, with the phenolic hydroxyl groups modified, showed much lower reactivity with the antiserum than agatharesinol. These results verify the less importance of the 1,2-ethanediol structural unit and the importance of the hydroxyphenyl structure for recognition. To confirm the greater importance of the hydroxylation pattern on the B-ring than that on the A-ring (Fig. 4) for recognition, the reactivities of the antiserum (with the chemically synthesized chalcones varying systematically in their hydroxylation pattern on their aromatic rings; Fig. 3) were examined (Table 3). Because chalcone has a 1,3-diphenylpropene structural unit, as does agatharesinol, they would be suitable model compounds for determining the importance of the substitution pattern on the aromatic rings. As expected,

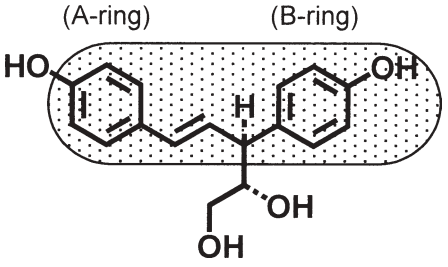

Fig. 4. Chemical-structural unit as an antigenic determinant

4,4′-dihydroxychalcone, with the same hydroxylation pattern as agatharesinol, had high reactivity with the antiserum, whereas chalcone, with no hydroxyl group on its aromatic rings, had much less reactivity with the antiserum. These results indicate that hydroxylation on the aromatic rings is essential for recognition. The reactivity of the antiserum with 4′-hydroxychalcone was higher than that with 4-hydroxychalcone, confirming that the hydroxylation pattern on the B-ring is more critical for recognition than the pattern on the A-ring, as indicated by the difference in the reactivity between sequirin C and metasequirin C. Although reactivity of the antiserum with 4,3′-dihydroxychalcone was relatively high, taking the much lower reactivity with sequirin C (where the B-ring is hydroxy-substituted at the 3′-position) into consideration, the lower reactivity of the antiserum with 4,3′-dihydroxychalcone than with 4,4′-dihydroxychalcone indicates the importance of the 4′-hydroxylation pattern on the B-ring. All these results support and strengthen the above proposal of the 3-*p*-hydroxyphenyl-1-phenylpropene structural unit as the major antigenic determinant for the antiserum (Fig. 4).

That the reactivity of the antiserum with 4-hydroxychalcone was higher than that with chalcone suggests that the A-ring also appears to participate in the antigen-antibody reaction. Polyclonal antibodies recognize the antigen from various directions, so the A-ring may be recognized as a minor antigenic determinant.

We previously reported that antiserum selectivity recognizes norlignans, especially agatharesinol in sugi extractives,[8] and that agatharesinol was localized immunoshistochemically in sugi heartwood tissue.[12] In the plant kingdom, in addition to sugi, certain plants produce norlignan; and plants containing extractives that cross-react with the antiserum may also be present. The antiserum can be applied to the immunolabeling of norlignan in plant species other than sugi if the recognition properties of the antiserum, as characterized in this study, well understood.

Conclusions

The relations between reactivities of antiserum with structurally related compounds and their chemical structures suggest that *trans*-3-*p*-hydroxyphenyl-1-phenylpropene structural unit is the major antigenic determinant for the antiserum.

Acknowledgments We are grateful to Dr. K. Fukushima at Nagoya University for coniferyl alcohol. This research was partially supported by the Ministry of Education, Culture, Sports, and Technology with a Grant-in-Aid for Young Scientists (B) (14760111, 2002).

References

1. Hillis WE, Inoue T (1966) The formation of polyphenols in trees. III. The effect of enzyme inhibitors. Phytochemistry 5:483–490
2. Magel EA, Hillinger C, Wagner T, Holl W (2001) Oxidative pentose phosphate pathway and pyridine nucleotides in relation to heartwood formation in *Robinia pseudoacacia* L. Phytochemistry 57:1061–1068
3. Suzuki S, Umezawa T, Shimada M (2001) Norlignan biosynthesis in *Asparagus officinalis* L.: the norlignan originates from two non-identical phenylpropane units. J Chem Soc Perkin Trans 1:3252–3257
4. Suzuki S, Nakatsubo T, Umezawa T, Shimada M (2002) First in vitro norlignan formation with *Asparagus officinalis* enzyme preparation. Chem Commun 2002: 1088–1089
5. Wardrop AB, Cromshaw J (1962) Formation of phenolic substances in the ray parenchyma of angiosperms. Nature 193:90–92
6. Nobuchi T, Harada H (1985) Ultrastructural changes in parenchyma cells of sugi (*Cryptomeria japonica* D. Don) associated with heartwood formation. Mokuzai Gakkaishi 31:965–973
7. Kwon M, Davin LB, Lewis NG (2001) In situ hybridization and immunolocalization of lignan reductases in woody tissues: implications for heartwood formation and other forms of vascular tissue preservation. Phytochemistry 57:899–914
8. Nagasaki T, Yasuda S, Imai T (2001) Preparation of antibody against agatharesinol, a norlignan, using a hapten-carrier conjugate. Phytochemistry 58:833–840
9. Awano T, Takabe K, Fujita M (1998) Localization of glucuronoxylans in Japanese beech visualized by immunogold labeling. Protoplasma 202:213–222
10. Joseleau J-P, Ruel K (1997) Study of lignification by noninvasive techniques in growing maize internodes: an investigation by Fourier transform infrared cross-polarization-magic angle spinning ^{13}C-nuclear magnetic resonance spectroscopy and immunocytochemical transmission electron microscopy. Plant Physiol 114:1123–1133
11. Nakashima J, Awano T, Takabe K, Fujita M, Sakaki H (1997) Immunocytochemical localization of phenylalanine ammonia-lyase and cinnamyl alcohol dehydrogenase in differentiating tracheary elements derived from zinnia mesophyll cells. Plant Cell Physiol 38:113–123
12. Nagasaki T, Yasuda S, Imai T (2002) Immunohistochemical localization of agatharesinol, a heartwood norlignan, in *Cryptomeria japonica*. Phytochemistry 60:461–466
13. Hassan F, Rothnie NE, Yeung SP, Palmer MV (1988) Enzyme-linked immunosorbent assays for alkenyl glucosinolates. J Agric Food Chem 36:398–403
14. Chen T, Dwyre-Gygax C, Daniels CR, Breuil C (1998) Evaluation of an enzyme-linked immunosorbent assay (ELISA) for quantifying DDAC in industrial treating solutions. Wood Sci Technol 32:287–296
15. Takahashi K (1981) Heartwood phenols and their significance to color in *Cryptomeria japonica* D. Don. Mokuzai Gakkaishi 27:654–657
16. Kai Y, Shimizu M (1968) On the phenolic constituents from *Cryptomeria japonica* D. Don. IV. The structure of sugiresinol (2). Mokuzai Gakkaishi 14:425–429
17. Ogiyama K, Yasue M, Takahashi K (1983) Chemosystematic study on heartwood extractives of *Cryptomeria japonica* D. Don. Presented at the international symposium on wood and pulp: chemistry, Tsukuba, Japan. 1:101–106
18. Hirose Y, Oishi N, Nagai H, Nakatsuka T (1965) The structure of hinokiresinol. Tetrahedron Lett 6:3665–3668
19. Yueh-Hsiung K, Chia-Hsien C, Yun-Lian L (2002) New lignans from the heartwood of *Chamaecyparis obtusa* var. formosana. Chem Pharm Bull 50:978–980
20. Iida Y, Oh K-B, Saito M, Matsuoka H, Kurata H, Natsume M, Abe H (1999) Detection of antifungal activity in *Anemarrhena asphodeloides* by sensitive BCT method and isolation of its active compound. J Agric Food Chem 47:584–587
21. Enoki A, Takahama S, Kitao K (1977) The extractives of metasekoia, *Metasequoia glyptostroboides* Hu et Cheng. I. The isolation of metasequirin-A, athrotaxin and agatharesinol from the heartwood. Mokuzai Gakkaishi 23:579–586
22. Takahashi K (1997) Presented at the 47th annual meeting of the Japan Wood Research Society, Kouchi, Japan, p 404
23. Ohashi H, Ido Y, Imai T, Yoshida K, Yasue M (1988) 4,4′-Dihydroxychalcone from the heartwood of *Chamaecyparis obtusa*. Phytochemistry 27:3993–3994