Inhibition of Tyrosinase by Flavonoids, Stilbenes and Related 4-Substituted Resorcinols: Structure-Activity Investigations

Kuniyoshi Shimizu, Ryuichiro Kondo*, and Kokki Sakai

Department of Forest Products, Faculty of Agriculture, Kyushu University, Fukuoka, Japan

Received: March 18, 1999; Accepted: June 27, 1999

Abstract: **Several flavonoids, stilbenes and related 4-substituted resorcinols, obtained from *Artocarpus incisus* and other plants or synthesized, were tested for their inhibitory activity against tyrosinase. The structure-activity relationships suggested that specific natural or synthesized compounds having the 4-substituted resorcinol skeleton have potent tyrosinase inhibitory ability. Kinetic studies have indicated that specific compounds having the 4-substituted resorcinol skeleton exhibit competitive inhibition of the oxidation of DL-β-(3,4-dihydroxyphenyl)alanine (DL-DOPA) by mushroom tyrosinase. These findings could lead to the design and discovery of new tyrosinase inhibitors.**

Key words: **4-Substituted resorcinols, tyrosinase inhibitors, *Artocarpus incisus*, Moraceae, structure-activity relationship.**

Introduction

The color of mammalian skin and hair is determined by a number of factors, the most important of which is the degree and distribution of the melanin pigmentation. Melanin biosynthesis inhibitory compounds are useful not only as skin-whitening agents used in cosmetics but also as a remedy for disturbances in pigmentation. Tyrosinase (phenol oxidase) is known to be a key enzyme for melanin biosynthesis in plants, microorganisms and mammalian cells (1). Therefore, many tyrosinase inhibitors have been tested in cosmetics and pharmaceuticals as a way of preventing overproduction of melanin in epidermal layers. Also, tyrosinase is one of the most important key enzymes in the insect molting process, and investigation on its inhibitors may be important in finding alternative insect control agents. Melanin formation is considered to be deleterious to the color quality of plant-derived food. This broadens the possible use of tyrosinase inhibitors as food additives, in addition to insect control agents and whitening agents. These observations led us to search for naturally occurring tyrosinase inhibitors. In our previous screening of the mushroom tyrosinase inhibitor, seven compounds from the heartwood extracts of *Artocarpus incisus* L. f. (Moraceae) showed potent inhibitory activity (2). This paper deals with the inhibitory activity of flavonoids, stilbenes and their congeners, including some related 4-substituted resorcinols.

Planta Medica 66 (2000) 11 – 15

ISSN: 0032-0943

Materials and Methods

Chemicals

The compounds [(−)-pinocembrin (**2**) (3), (+)-aromadendrin (**4**) (4), (±)-fustin (**5**) (5), (±)-taxifolin (**6**) (6), (+)-dihydromyricetin (**2**) (7), chrysin (**11**) (8), kaempferol (**13**) (9), quercetin (**14**) (5), myricetin (**15**) (10), pinosylvin (**21**) (11), oxyresveratrol (**22**) (12) and bis(2,4-dihydroxyphenyl)methane (**34**) (13)] were provided by the Laboratory of Wood Chemistry, Department of Forest Products, Faculty of Agriculture, Kyushu University in Japan, and their purities and identification had been confirmed by comparison with references. The following reagents were purchased: [(±)-flavanone (**1**), flavone (**10**), 2,4-dihydroxybenzaldehyde (**26**), 2,4-dihydroxy-*N*-(2-hydroxyethyl)benzamide (**29**), 2,4-dihydroxybenzophenone (**30**), 4-hexylresorcinol (**38**) and 4-dodecylresorcinol (**39**) from Aldrich Chem. Co.], [(±)-naringenin (**3**) and morin (**17**) from Sigma Chem. Co.], [2,4-dihydroxyacetophenone (**27**), 2,4-dihydroxybenzoic acid (**28**), resorcinol (**32**), L-tyrosine and DL-β-(3,4-dihydroxyphenyl)alanine (DL-DOPA) from Wako Pure Chemical Industries, Ltd.)], [4-(2-pyridylazo)resocrinol (**31**), 4-(2-thiazolylazo)resorcinol (**33**) from Dojindo Laboratories] and [4-chlororesorcinol (**35**), 4-ethylresorcinol (**40**) from Tokyo Kasei Kogyo Co., Ltd.]. The reagents (+)-dihydromorin (**8**), (+)-norartocarpanone (**9**), apigenin (**12**), artocarpin (**16**), artocarpesin (**18**), isoartocarpesin (**19**), 4-prenyloxyresveratrol (**23**), chlorophorin (**24**), artocarbene (**25**) (2) and (−)-angolensin (**20**) (14) were isolated previously.

4-Methylresorcinol (**36**), 4-(phenylmethyl)resorcinol (**37**) and 4-propylresorcinol (**41**) were prepared by reduction of **26**, **30** and 2′,4′-dihydroxypropiophenone (Aldrich Chem. Co.) with $NaBCH_3CN$ (Aldrich Chem. Co.), respectively. EIMS, *m/z*: 36 (M^+: 124), **37** (M^+: 200), **41** (M^+: 152).

Enzyme assays

Mushroom tyrosinase [EC 1. 14. 18. 1] activity was determined by using L-tyrosine or DL-DOPA as the substrate. L-Tyrosine oxidation assay was done as described previously (2). DL-DOPA oxidation assay of 0.1 ml of mushroom tyrosinase solution (625 U/ml, Wako Pure Chemical Industries, Ltd.): 0.7 ml of DL-

DOPA buffer solution (2.0 mM), 0.1 ml of McIlvain buffer (pH 6.8) and 0.1 ml of DMSO with or without sample were mixed and incubated at 25 °C. A control reaction was conducted without the test sample. The absorbance was measured at 475 nm before and after incubation. The percentage of inhibition of tyrosinase was calculated as follows: tyrosinase inhibition (%) = (A – B)/A × 100, where A represents the difference in the absorbance of the control sample between the incubation time of 0.35 and 0.45 min, and B represents the difference in the absorbance of the test sample between the incubation time of 0.35 and 0.45 min. The results were from the three concurrent readings and each S.D. was usually within 2% of the mean. Kojic acid (Tokyo Kasei Kogyo Co., Ltd.) was used as a positive standard.

Results and Discussion

To study structure-activity relationships, several flavonoids and stilbenes were tested for their inhibitory activity on tyrosinase (substrate: L-tyrosine) by measuring the concentration required to effect a 50% inhibition of enzyme activity (IC_{50}). Nine compounds (**8**, **9**, **12**, **16**, **18**, **19**, **23**, **24** and **25**) isolated from *A. incisus* were previously examined for their inhibitory activity towards mushroom tyrosinase (2). Seven of these (**12** and **16** were not included) exhibited potent tyrosinase inhibitory activity [Table **1**, kojic acid (positive standard, substrate: L-tyrosine): IC_{50} = 8.66 μM (2)]. Interestingly, the active compounds had 4-substituted resorcinol as a common skeleton (Fig. **1**). This brief structure-activity relationship could mean that the 4-substituted resorcinol skeleton is important for revealing the tyrosinase inhibitory activity. In addition, it should be noted that artocarpin (**16**) did not show inhibitory activity, in spite of having a 4-substituted resorcinol skeleton at ring B. Therefore, to clarify which substructure is important to reveal the tyrosinase inhibitory effect, further structure-activity relationships were examined in detail. The test compounds were flavonoids and stilbenes isolated from various plants, synthesized or commercially available. The results were summarized in Table **1** and Fig. **2**.

Among five stilbenes (**21 – 25**), four (**22 – 25**) having a 4-substituted resorcinol skeleton showed potent tyrosinase inhibitory activity, but one (**21**) did not. These results can be explained by the fact that hydroxylation of **21**, resulting in **22**, increases its inhibitory activity dramatically. Also, the addition of isoprenyl chain (prenyl or geranyl) to the stilbenes having a 4-substituted resorcinol skeleton slightly increased their inhibitory activities (**22**: IC_{50} = 0.98 μM → **23**: IC_{50} = 0.66 μM → **24**: IC_{50} = 0.26 μM). Resveratrol (3,4′,5-trihydroxystilbene), 3,5-dihydroxy-4′-methoxystilbene, 3,4′-dimethoxy-5-hydroxystilbene, trimethylresveratrol and piceid (4-*O*-β-D-glucosylresveratrol) showed much lower inhibitory effects than oxyresveratrol (**22**) on dopa oxidase activity of mushroom tyrosinase (15). Therefore, in the case of stilbenes, the 4-substituted resorcinol skeleton must be the most important feature for revealing potent tyrosinase inhibition.

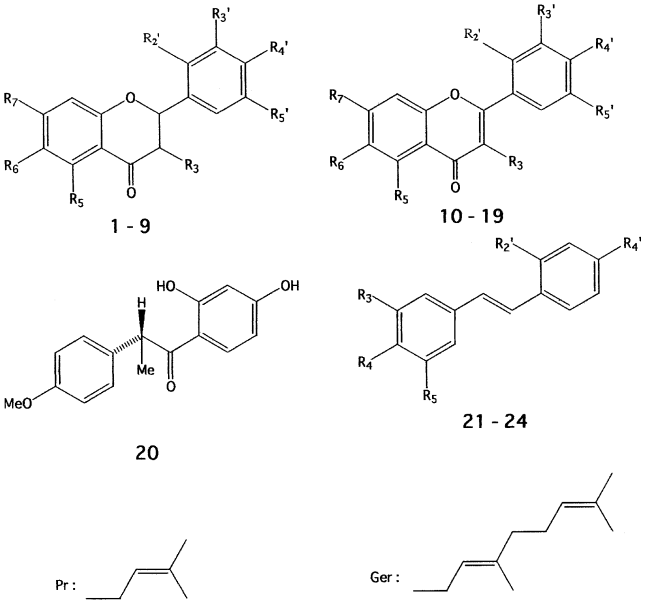


Fig. 2 The chemical structures of **1 – 24**.

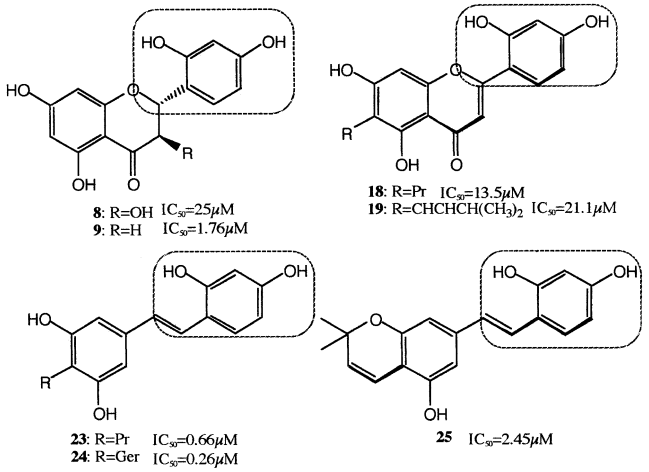


Fig. 1 The chemical structures and IC_{50} of active components from *A. incisus*. The boxed part: 4-substituted resorcinol skeleton.

Among 20 flavonoids, only 4 flavonoids (**8**, **9**, **18** and **19**), which have the 4-substituted resorcinol skeleton at ring B, showed potent tyrosinase inhibitory activity. Glabridin (one of the isoflavans) (16), kurarinone (flavanone), kushenol N (dihydroflavonol) and kosamol A (dihydroflavonol) (17) were reported as potent tyrosinase inhibitors that have a common 4-substituted resorcinol skeleton at ring B. In contrast, **16**, **17** and **20** did not show tyrosinase inhibitory activity, in spite of having a 4-substituted resorcinol skeleton at ring B. These results indicate that for flavonoids not only a 4-substituted resorcinol skeleton but also additional structural factors are necessary to reveal tyrosinase inhibitory activity.

In the case of flavonoids having a 4-substituted resorcinol skeleton, except for **20** (which belongs to the α-methyldeoxybenzoins), the flavanone type compounds (flavanones and their C3 substituted derivatives) were more potent inhibitors than the flavone type compounds (flavones and their C3 substituted derivatives), e.g., **8** showed a stronger inhibitory effect than the corresponding flavone **17**. Introduction of a C3 substituent to the flavanone (**9** → **8**) and flavone type (**18** and **19** → **16** and **17**) dramatically decreased their activity. Thus, even in flavonoids having a 4-substituted resorcinol skeleton,

Table 1 Inhibitory activity of flavonoids and stilbenes on tyrosinase (substrate: L-tyrosine).

No	Name	R_3	R_5	R_6	R_7	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	(C2, C3)	IC_{50} (μM)
1	(±)-flavanone	H	H	H	H	H	H	H	H	2*S*,2*R*	>200
2	(−)-pinocembrin	H	OH	H	OH	H	H	H	H	2*S*	>200
3	(±)-naringenin	H	OH	H	OH	H	H	OH	H	2*S*,2*R*	>200
4	(+)-aromadendrin	OH	OH	H	OH	H	H	OH	H	(2*R*,3*R*)	lag time decrease[a]
5	(±)-fustin	OH	H	H	OH	H	OH	OH	H	(2*R*,3*R*),(2*S*,3*S*)	lag time decrease[a]
6	(±)-taxifolin	OH	OH	H	OH	H	OH	OH	H	(2*R*,3*R*),(2*S*,3*S*)	lag time decrease[a]
7	(+)-dihydromyricetin	OH	OH	H	OH	H	OH	OH	OH	(2*R*,3*R*)	lag time decrease[a]
8	(+)-dihydromorin	OH	OH	H	OH	OH	H	OH	H	(2*R*,3*R*)	25[b]
9	(+)-norartocarpanone	H	OH	H	OH	OH	H	OH	H	2*S*	1.76[b]
10	flavone	H	H	H	H	H	H	H	H		>200
11	chrysin	OH	OH	H	H	H	H	H	H		>200
12	apigenin	H	OH	H	OH	H	H	OH	H		>185[b]
13	kaempferol	OH	OH	H	OH	H	H	OH	H		103
14	quercetin	OH	OH	H	OH	H	OH	OH	H		lag time decrease[a]
15	myricetin	OH	OH	H	OH	H	OH	OH	OH		lag time decrease[a]
16	artocarpin	Pr	OH	CHCHCH $(CH_3)_2$	OCH_3	OH	H	OH	H		>228[b]
17	morin	OH	OH	H	OH	OH	H	OH	H		>330
18	artocarpesin	H	OH	Pr	OH	OH	H	OH	H		13.5[b]
19	isoartocarpesin	H	OH	CHCHCH $(CH_3)_2$	OH	OH	H	OH	H		21.1[b]
20	(−)-angolensin										>200
		R_3	R_4	R_5	$R_{2'}$	$R_{4'}$					
21	pinosylvin	OH	H	OH	H	H					>46
22	oxyresveratrol	OH	H	OH	OH	OH					0.98
23	4-prenyloxyresveratrol	OH	Pr	OH	OH	OH					0.66[b]
24	chlorophorin	OH	Ger	OH	OH	OH					0.26[b]
25	artocarbene[c]										2.45[b]

[a] means promotion effect which could act as cofactor like diphenol (1).

[b] Obtained from data of Ref. (2).

[c] See Fig. **1**.

introduction of a C3 substituent decreased the inhibitory activity, probably because of its steric hindrance.

Compound **20** did not show tyrosinase inhibitory activity, in spite of having a 4-substituted resorcinol skeleton. To clarify which substructure causes inactivity of **20**, we examined the effects of different C4 substituents on the tyrosinase inhibitory activity of the 4-substituted resorcinols (Table **2**). Table **2** demonstrates the powerful influence of the C4 substituent on the potency of these compounds. Surprisingly, introduction of a carbonyl substituent (**26 – 30**) decreased the inhibitory activity dramatically. Also, compounds having an azo substituent (**31**, **33**) showed much lower inhibitory activity than **22 – 25**, in spite of having a similar shape to stilbenes concerning the double bond. Introductions of chlorine (**35**), alkyl (**36 – 41**) or phenylmethyl (**34**) substituents at C4 resulted in potent inhibitory activities. The non-substituted **32** did not show a potent inhibitory activity.

Kinetic studies were carried out with the five active compounds (**8**, **9**, **23 – 25**) from *A. incisus*, as well as the related compounds (**22**, **32** and **40**). The Lineweaver-Burk plot of **23** for DL-DOPA as a substrate is shown in Fig. **3**. The mode of inhibition of tyrosinase by **23** was competitive. In addition, similar results were given by **9**, **22**, **24**, **25** and **40** (Table **3**). Compounds **8** and **32** did not show typical inhibitory patterns. Interestingly, these compounds (**8** and **32**) exhibited some stimulatory activity to the enzyme at low concentration, similar to a previous report (18). The results obtained so far suggest that (a) **8** and the corresponding flavanone **9** not possessing a C3-hydroxy group affect mushroom tyrosinase in different ways, and that (b) **32** and the corresponding 4-substituted resorcinols (**9**, **22 – 25** and **40**) affect mushroom tyrosinase in different ways. However, further work is needed to clarify the inhibitory mechanism of **8** and **32**.

Thus, the C4-substituents of resorcinol derivatives and the C3-substituents of flavonoids that have a 2′,4′-dihydroxyphenyl skeleton seem to significantly affect tyrosinase activity.

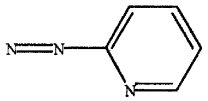
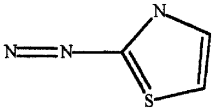
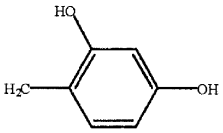
The tyrosinase inhibitory effects (IC_{50}, Ki and inhibition type) of representative 4-substituted resorcinols using DL-DOPA as a substrate are shown in Table **3**. Compound **40** showed a stronger inhibitory activity than that of kojic acid, using both L-tyrosine and DL-DOPA as a substrate but the inhibitory effects of **9** and **22 – 24** were weaker than that of kojic acid using DL-DOPA as a substrate, in spite of showing much stronger

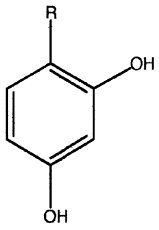
inhibitory activity using L-tyrosine as a substrate. Thus, the order of inhibitory effects of these compounds having a 4-substituted resorcinol skeleton were different depending on whether L-tyrosine or DL-DOPA was used as a substrate, in comparison with kojic acid.

Oxyresveratrol (**22**) showed a competitive inhibitory type activity in this study, although it was recently reported as a non-competitive inhibitor on mushroom tyrosinase with L-DOPA as a substrate (15). The difference may be explained as follows. It was reported recently that 4-substituted resorcinols such as 4-ethylresorcinol, 4-hexylresorcinol and 4-dodecylresorcinol could be classified as slow-binding competitive inhibitors of mushroom tyrosinase (19). Therefore, the difference in the inhibitory type of oxyresveratrol against tyrosinase between us and Shin et al. (15), seems to be due to estimated tyrosinase inhibitory activity by different limited reaction times. To characterize the behavior of these inhibitors completely, a further kinetic study must be needed in order to determine the kinetic parameters (K_I, K'_I and k_6) according to (19). However, in this study, the results of IC_{50} by using assays with limited reaction time are a worthy and valid parameter for understanding the structure-activity relationships.

Some 4-substituted resorcinols have been reported as inhibitors of enzymatic (polyphenol oxidase) browning in food and beverages (20). However, their structure-activity relationships have been poorly understood. Therefore, our identification of specific compounds having the 4-substituted resorcinol skeleton as potent inhibitors, as outlined above, and the notion that hydrophobic and less bulky substituents were important for controlling the tyrosinase inhibitory effect, may lead to the design and discovery of new tyrosinase inhibitors (Fig. **4**). The natural products and synthesized chemicals having 4-substituted resorcinol skeletons should be reinvestigated with regard to their roles as tyrosinase inhibitors. Furthermore, from the chemotaxonomic point of view, specific extracts of plants known as having flavonoids, stilbenes or other types with 4-substituted resorcinol skeleton, for example Moraceae (2) or Leguminosae (16), (17), are candidates for tyrosinase inhibitory materials. Finally it should be noted that these compounds not only inhibit the tyrosinase but also have other properties, such as resveratrol derivatives which act as an antioxidant, an antimutagen and a cancer chemopreventive agent (21).

Table 2 Inhibitory activity of 4-substituted resorcinols on tyrosinase (substrate: L-tyrosine).

No.	substituent (R)	IC_{50} (μM)
26	CHO	>200
27	$COCH_3$	>200
28	COOH	>200
29	$CONHCH_2CH_2OH$	>200
30	COC_6H_5	>200
31		436
32	H	227
33		185
34		58.0
35	Cl	13.0
36	CH_3	12.0
37	$CH_2C_6H_5$	2.80
38	$CH_2(CH_2)_4CH_3$	1.98
39	$CH_2(CH_2)_{10}CH_3$	1.63
40	CH_2CH_3	1.10
41	$CH_2CH_2CH_3$	0.91



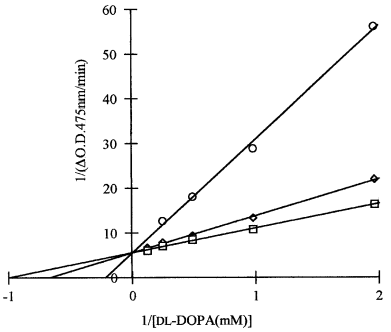


Fig. 3 Lineweaver-Burk plots of mushroom tyrosinase and DL-DOPA in the absence or presence of 4-prenyloxyresveratrol; □ Control, ◇ 4.8 μM 4-prenyloxyresveratrol, ○ 16 μM 4-prenyloxyresveratrol.

Table 3 The tyrosinase inhibitory effects of representative 4-substituted resorcinols tested in reaction using DL-DOPA as a substrate.

Compound	IC_{50} (μM)	Ki (μM)	Type of inhibition
9	90.4	47.8	Competitive
22	20.8	9.24	Competitive
23	17.6	8.70	Competitive
24	19.2	13.4	Competitive
25	6.35	8.49	Competitive
40	3.80	5.39	Competitive
Kojic acid	17.2	11.8	Mix

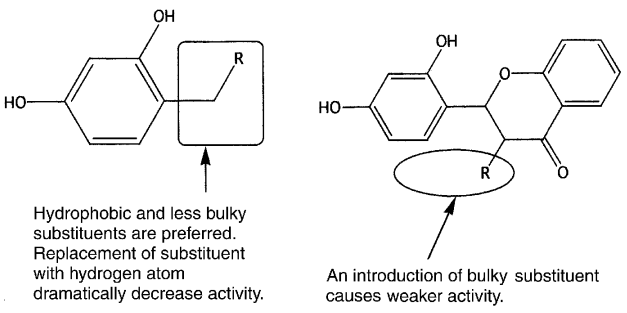


Fig. 4 Summarized structure – activity relationships of compounds having 4-substituted resorcinol skeleton.

Acknowledgements

We thank Mr. T. Takeshima (Lab. of Polymer Science, Dep. of Forest Products, Fac. of Agriculture, Kyushu University) for providing bis(2,4-dihydroxyphenyl)methane (**34**). This work was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education (Research Fellowships of the Japan Society for the Promotion of Science for Young Scientist) and the Cosmetology Research Foundation (Tokyo, Japan).

References

[1] Sánchez-Ferrer A, Rodríguez-López JN, García-Cánovas F, García-Carmona F. Biochim. Biophys. Acta 1995; 1247: 1 – 11
[2] Shimizu K, Kondo R, Sakai K, Lee SH, Sato H. Planta Med. 1998; 64: 408 – 412
[3] Bick IRC, Brown RB, Hills WE. Aust. J. Chem. 1972; 25: 449 – 451
[4] Janes NF, Morgan JWW. J. Chem. Soc. 1960; 2560 – 2565
[5] Imamura H, Kurosu H, Takahashi T. Mokuzai Gakkaishi 1967; 13: 295 – 299
[6] Kondo T. J. Fac. Agric. Kyushu Univ. 1951; 10: 79 – 99
[7] Miller JM, Bohm BA. Phytochemistry 1979; 18: 1412 – 1413
[8] Harborne JB, Mabry TJ. The Flavonoids: Advances in Research,: p. 57, Chapmann and Hall, New York 1982
[9] Schultz TP, Harms WB, Fisher TH, McMurtrey KD, Minn J, Nicholas DD. Holzforschung 1995; 49: 29 – 34
[10] Mabry TJ, Markham KR, Thomas MB. The Systematic Identification of Flavonoids,: p. 309, Springer-Verlag, New York 1970
[11] Schultz TP, Boldin WB, Fisher TH, Nicholas DD, McMurtrey KD, Pobanz K. Phytochemistry 1992; 31: 3801 – 3806
[12] Malan E, Swinny E, Ferreira D, Hall AJ. Phytochemistry 1988; 27: 2309 – 2312
[13] Kim MG, Amos LW, Barnes EE. J. Polym. Sci., A. Polym. Chem. 1993; 31: 1871 – 1877
[14] Pilotti CA, Kondo R, Shimizu K, Sakai K. Mokuzai Gakkaishi 1995; 41: 593 – 597
[15] Shin NH, Ryu SY, Choi EJ, Kang SH, Chang IM, Min KR, Kim Y. Biochem. Biophys. Res. Commun. 1998; 243: 801 – 803
[16] Yokota T, Nishio H, Kubota Y, Mizoguchi M. Pigment. Cell. Res. 1998; 11: 355 – 361
[17] Lee SH, Park JS, Chung SR, Ro JS, Lee KS. International Symposium on Natural Medicines, Kyoto, Japan 1997: p. 182
[18] Kubo I, Kinst-Hori I, Yokokawa Y. J. Nat. Prod. 1994; 57: 545 – 551
[19] Jiménez M, García-Carmona F. J. Agric. Food Chem. 1997; 45: 2061 – 2065
[20] McEvily AJ, Iyengar R. Crit. Rev. Food Sci. Nutr. 1992; 32: 253 – 273
[21] Jang MS, Cai EN, Udeani GO, Slowing KV, Thomas CF, Beecher CWW, Fong HHS, Fong HHS, Farnsworth NR, Kinghorn AD, Mehta RD, Moon RC, Pezzuto JM. Science 1997; 275: 218 – 220

Dr. Ryuichiro Kondo

Department of Forest Products
Faculty of Agriculture
Kyushu University
Fukuoka, 812-8581
Japan
E-mail: kondo@agr.kyushu-u.ac.jp
Fax: +81-92-642-2989