A New Class of Biflavonoids: 2′-Hydroxygenistein Dimers from the Roots of White Lupin

Mitsuyoshi Sakasai[a], Hiroki Fukui[a], Hideyuki Yamane[a], Aung Naing Kyaw[a], and Satoshi Tahara[a,b,*]

[a] Department of Applied Bioscience, Faculty of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, Japan
[b] CREST of Japan Science and Technology Corporation. Fax: +81-11-706-4182. E-mail: tahara@abs.agr.hokudai.ac.jp
* Author for correspondence and reprint requests

Two novel isoflavonoid dimers presumably originating from 2′-hydroxygenistein, 5,7,4′-trihydroxycoumaranochroman-4-one-(3→5‴)-5″,7″,2‴,4‴-tetrahydroxyisoflav-one (**1**, lupinalbisone A) and 5,7,4′-trihydroxycoumaranochro-man-4-one-(3→6″)-5″,7″,2‴,4‴-tetrahydroxyisoflavone (**2**, lupinalbisone B) were isolated from the roots of *Lupinus albus* L., and their structures involving relative stereochemistry were elucidated by spectroscopic methods. Using horse radish peroxidase and 2′-hydroxygenistein (**3**) as the substrate revealed the formation of these dimers together with 5,7,4′-trihydroxycoumaronochromone (**4**, lupinalbin A). Dimerization of **3** caused a remarkable increase of antifungal activity.

Introduction

The historical, biological and chemical aspects of naturally occurring flavonoid dimers have been well reviewed (Geiger, 1994; Porter, 1994; Bohm, 1994; Dewick, 1994). The first biflavonoids, flavone-flavone, was isolated in 1929 from *Ginkgo biloba* L. and named ginkgetin (Nakazawa, 1962). Since that time, more than 200 dimeric flavonoids (in a broad sense) have been isolated. However, almost all of them are flavonoid (in a narrow sense) dimers, and dimeric flavonoids consisting of one or two isoflavonoid units are relatively rare in nature. Thirteen dimers (5 isoflavan-isoflavans, 1 isoflavan-isoflavone, 1 isoflavene-isoflavone, 1 isoflavan-flavone, 2 isoflavone-flavone, 2 isoflavone-flavene and 1 isoflavan-chalcone) are listed in a review article which includes our knowledge until the end of 1990 (Dewick, 1994). A few dimers, for example, isoflavanone-benzofuranone (Bekker *et al.*, 1998), 2 isoflavone-isoflavones (Hakamatsuka *et al.*, 1992), 3 isoflavone-isoflavanones (Moreira *et al.*, 1994), and 12a-hydroxyrotenoid-isoflavan (Rastrelli *et al.*, 1999) have been reported. Diverse biological activities of flavonoid dimers have been observed (Lin *et al.*, 1997).

Here we report the isolation and structure elucidation of a new class of isoflavone dimers, lupinalbisones A (**1**) and B (**2**), consisting of 2,3-dihydro-lupinalbin A and 2′-hydroxygenistein linked through a single C-C bond (Fig. 1). These compounds were found in the white lupin roots, as well as in a reaction mixture of horse radish peroxidase with 2′-hydroxygenistein (**3**) and H_2O_2 present. It has been shown that dimerization of **2** resulted in a remarkable increase in fungitoxicity.

Results and Discussion

Two novel biflavonoids i.e., lupinalbisones A (**1**) and B (**2**) were isolated from the roots of white lupin collected in middle August at the late seed-ripening state. Interestingly, enzymatic dimerization of 2′-hydroxygenistein (**3**) catalyzed by horse radish peroxidase in the presence of H_2O_2 yielded exactly the same compounds with **1** and **2**. The new isoflavonoid dimers **1** and **2** were characterized through detailed spectroscopic studies.

Lupinalbisone A (**1**)

Peracetylation of lupinalbisone A (**1**) yielded a single product showing ESI-MS peaks at *m/z* 903 (12%) and 887 (100%), respectively corresponding to the mass units of [starting material – 7H + $CH_3CO \times 7$ + K] and [starting material – 7H +
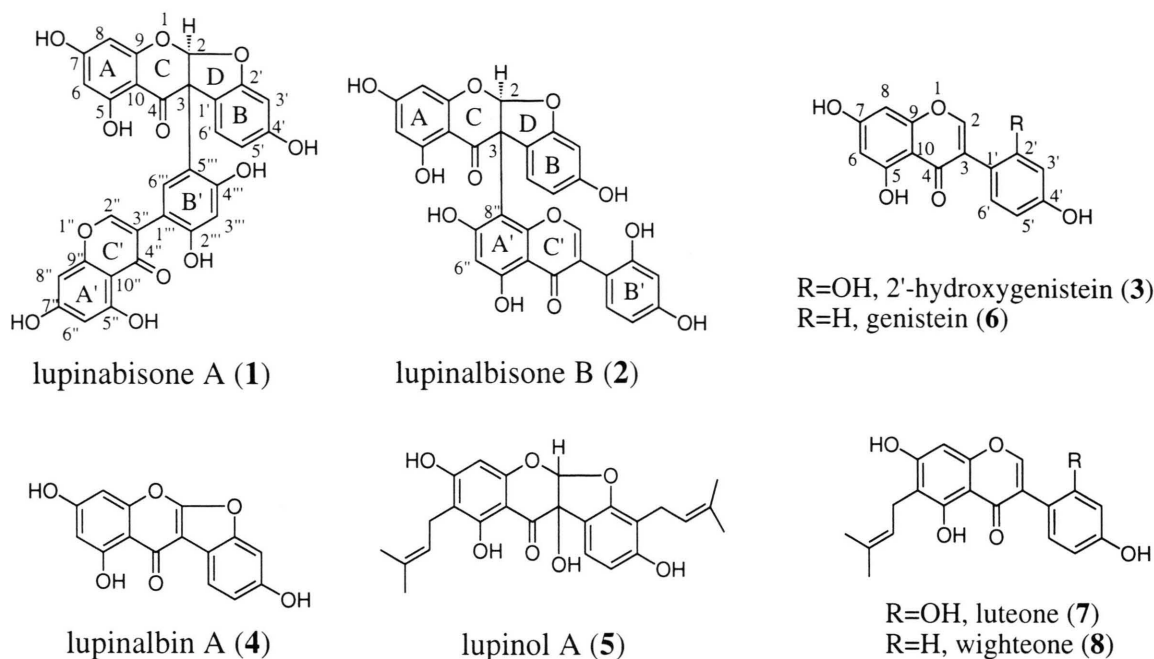
lupinabisone A (**1**)   lupinalbisone B (**2**)   R=OH, 2'-hydroxygenistein (**3**)
                                                                R=H, genistein (**6**)

lupinalbin A (**4**)   lupinol A (**5**)   R=OH, luteone (**7**)
                                                         R=H, wighteone (**8**)

Fig. 1. Structures of isoflavonoids mentioned in the text.

Lupinalbisones A (**1**) and B (**2**)
M⁺ *m/z* 570

a *m/z* 286   **b** *m/z* 284

c *m/z* 153   **d** *m/z* 134   **e** *m/z* 176   **f** *m/z* 255
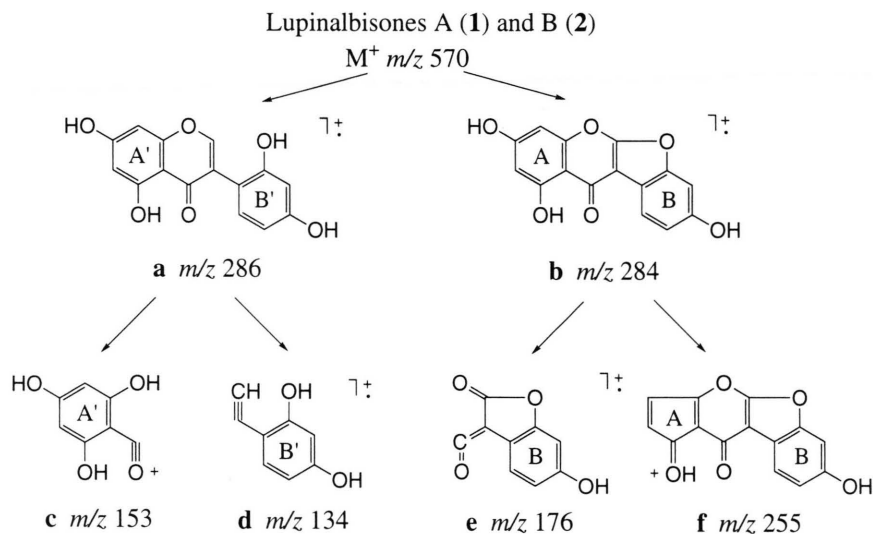
Fig. 2. Major mass fragments of lupinalbisones A (**1**) and B (**2**).

CH₃CO × 7 + Na]. The number of hydroxyl groups in **1** was thus confirmed to be seven reduced by one during the process of dimerization of the tetrahydroxylated isoflavone **3**.

The FD-MS spectrum of **1** gave a molecular ion at *m/z* 570 (M⁺, 100%) assignable to the molecule $C_{30}H_{18}O_{12}$ (=2′-hydroxygenistein × 2 – 2H) which was also supported by ¹H and ¹³C NMR spectroscopy (Table I). In addition to the molecular ion, the FD-MS fragmentation yielded further two prominent fragment ions, **a** at *m/z* 286 (17%) and **b** at *m/z* 284 (13%), respectively corresponding to

Table I. Proton and carbon-13 NMR assignments of two 2′-hydroxygenistein dimers with reference to those of 2′-hydroxygenistein (δ_C with the same symbol: interchangeable).

C No.	Lupinalbisone A (1)		Lupinalbisone B (2)		2′-Hydroxygenistein (3)	
	^{1}H (HMBC correld.C)	^{13}C	^{1}H (HMBC correld.C)	^{13}C	^{1}H	^{13}C
2	6.43 s (1′,2′,3,5‴,9)	111.5	6.53 s (2′,3,8″,9)	111.9	8.16 s	156.5
3	–	61.0	–	58.8	–	122.3
4	–	194.7	–	193.3	–	182.2
5	–	165.3	–	165.2	–	163.6
6	5.96 d (5,7,8,10)	97.3	5.97 s (5,7,8,10)	97.5	6.31 d	100.0
7	–	167.1	–	167.0	–	165.2
8	5.98 d (6,7,9,10)	95.9	6.02 d (6,7,9,10)	96.3	6.46 d	94.5
9	–	160.9	–	160.5	–	159.0
10	–	100.9	–	101.0	–	106.0
1′	–	116.3	–	117.5	–	110.9
2′	–	161.4	–	160.9	–	157.7
3′	6.50 d (1′,2′,5′)	99.2	6.44 d (1′,4′,5′)	98.5	6.49 d	104.7
4′	–	156.6	–	157.2*	–	160.0
5′	6.57 dd (1′,3′,4′)	110.5	6.51 dd (1′,3′)	110.1	6.44 dd	108.2
6′	7.19 d (2′,3)	128.1	7.18 d (1′,2′,3)	128.2	7.13 d	132.9
5-OH	12.15 s (5,6,10)	–	12.03 s	–	12.78 s	–
2″	8.04 s (1‴,3″,4″,9″)	156.3	7.56 s	155.2		
3″	–	122.1	–	122.0		
4″	–	181.9	–	182.2		
5″	–	163.6	–	163.3#		
6″	5.97 s (5″,7″,8″,10″)	100.0	6.39 s (5″,7″,8″,10″)	100.1		
7″	–	165.2	–	163.2#		
8″	6.41 d (6″,7″,9″,10″)	94.5	–	105.3		
9″	–	159.0	–	160.1″		
10″	–	105.9	–	106.2		
1‴	–	110.7	–	110.3		
2‴	–	157.8	–	157.8*		
3‴	6.58 s (1‴,2‴,4‴,5‴)	104.8	6.45 d (1‴,5‴)	104.4		
4‴	–	160.6	–	159.9″		
5‴	–	120.3	6.40 dd (1‴,3‴)	108.1		
6‴	6.59 s (1‴,2‴,3″,3)	132.7	7.05 d (2‴,3″,4‴)	132.8		
5″-OH	12.70 s (5″,6″,10″)		13.25 s			

^{1}H J values (Hz):
1: H-6, H-8, J=2.0; H-3′, J=2.2; H-5′, J=8.2, 2.2; H-6′, J=8.2; H-6″, H-8″, J=2.0. **2:** H-6, H-8, J=2.2; H-3′, J=2.2; H-5′, J=8.4, 2.2; H-6′, J=8.4. H-3‴ J=2.2; H-5‴, J=8.4, 2.2; H-6‴, J=8.4. **3:** H-6, H-8, J=2.2; H-3′, J=2.5; H-5′, J=8.4, 2.5; H-6′, J=8.4.
NOE correlations: **1:** between H-5′ and H-6′; between H-2″ and H-6‴.

molecular ions of 2′-hydroxygenistein (**3**), and lupinalbin A (**4**). These two ions were also detected in the EI-MS spectrum of **1** as intense fragments and the fragment **a**, *m/z* 286 (43%) was further decomposed via retro-Diels-Alder (RDA) fission to yield **c** *m/z* 153 (26%) from A-ring and **d**, *m/z* 134 (13%) from B-ring (Mabry and Markham, 1975). The latter ion **b**, *m/z* 284 (100%) gave weak ions, **e** at *m/z* 255 (5%) and **f** at *m/z* 176 (7%) characteristic of mass fragments of simple coumaronochromones (Hanawa *et al.*, 1991) (Fig. 2).

The presence of two building blocks (coumaranochromanone-type, I and isoflavone-type, II) in lupinalbisone A (**1**) estimated by mass spectros-

copy was also confirmed by NMR analyses. For coumaranochromanone-type building block (I), 15 carbons: 5 oxygenated, 5 hydrogen bearing and 2 quaternary aromatic carbons; one carbonyl carbon (C-4, δ_C 194.7); one sp^2 quaternary carbon (C-3, δ_C 61.0); and a lower field resonating sp^3 carbon (δ_C 111.5) bearing a hydrogen atom (δ_H 6.53) assignable to an acetal group (see the relevant data for lupinol A, **5**: Tahara *et al.*, 1991), and 7 protons (except for two phenolic protons of C-7-OH and C-4′-OH) were correlated each other by 2D NMR techniques (H-H COSY, HMQC, HMBC and NOESY) and unambiguously assigned as shown in Fig. 3.
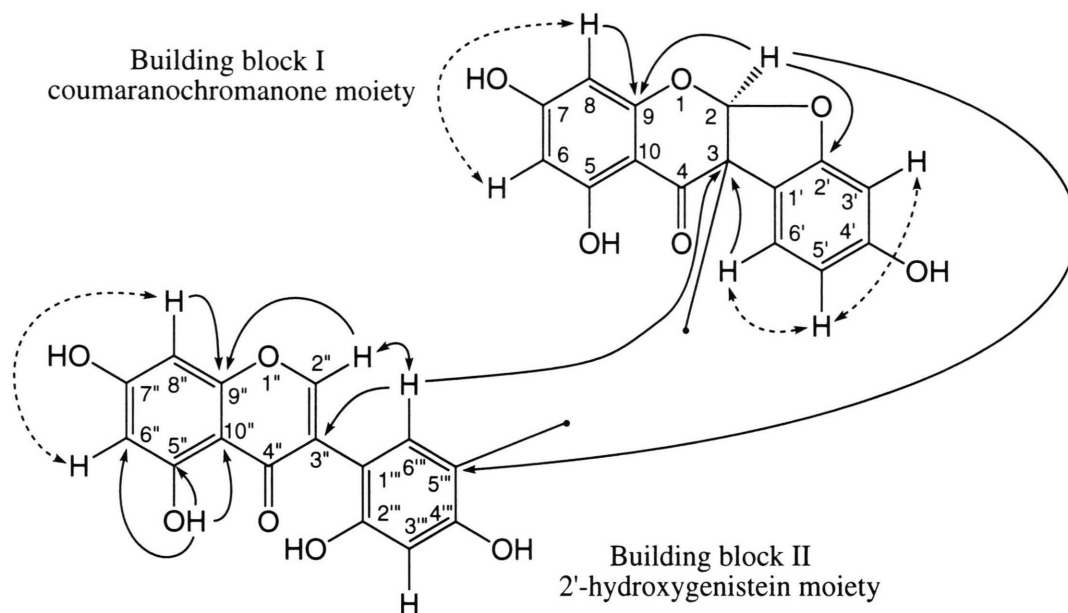
Fig. 3. 2D NMR correlations found on lupinalbisone A (**1**). Observed correlations: ←----→ H–H COSY; → HMBC (selected): ↔ NOESY.

The 2′-hydroxygenistein building block in **1** was more easily feasible in comparison of the ^{1}H and ^{13}C NMR data with those of authentic 2′-hydroxygenistein (**3**) (Table I). The ^{1}H and ^{13}C chemical shifts for A' and C' rings are quite close to those of **3**, whilst the δ_C value for C-5‴ (corresponding to C-5′ in **3**) was shifted to lower field by 12 ppm due to C-C bond formation which resulted in the change of coupling pattern of aromatic protons in B'-ring from 1,2,4-trisubstituted benzene-type (=2′-hydroxygenistein) to 1,2,4,5-tetrasubstituted benzene-type (=B'-ring in the building block II).

The connection of the two building blocks between C-3 and C-5‴ was deduced by the HMBC correlations between H-2 and C-5‴, and between H-6‴ and C-3 (Fig. 3). Thus the total structure of **1** was established as shown by Fig. 1.

*Stereochemistry of lupinabisone A (**1**)*

The relative stereostructure of lupinalbisone A (**1**) was deduced from the results of NMR experiments.

(A) The intensities of cross peaks between H-2 and C-5‴ ($^3J_{C,H}$) observed by two HMBC experiments at 5 and 10 Hz were same. As reported by

Schaufelberger *et al.*, (1991) and Rodriguez (1992), the long range spin coupling constants $^3J_{C,H}$ depends on the dihedral angle, and anti-(*trans*)-type $^3J_{C,H}$ and sin(*cis*)-type $^3J_{C,H}$ have been empirically determined to be 7–8 Hz and 1–3 Hz, respectively. According to this rule, the $^3J_{C,H}$ of our compound **1** should be between 5 and 10 Hz which suggested the *trans*-type ring fusion of lupinalbisone A at C-2 and C-3 (Fig. 4).

(B) In the NOESY spectrum, no NOE between protons in Block I and those of block II were observed. When these two blocks bind to give a *cis*-type ring fusion, H-2 and H-6‴ can approach each other close enough to give an NOE. The result of NOE experiment also suggests a *cis*-fusion of the relevant ring junction.

Calculation of a stable conformer for lupinalbisone A (**1**) by a molecular modeling software Chem3D-Pro (version 3.5.1, CambridgeSoft Co.) revealed that *trans* configuration (fixed anti-conformation) was the most stable one. Optical purity of isolated dimers has not yet been determined, however, lupinalbisones A (**1**) and B (**2**) were laevo- and dextro-rotatory, respectively, and showed complicated CD curves indicative of their optically selective formation in lupin roots.

Part structure I
trans (anti): $^3J_{C,H} = 7\text{-}8$ Hz

Part structure II
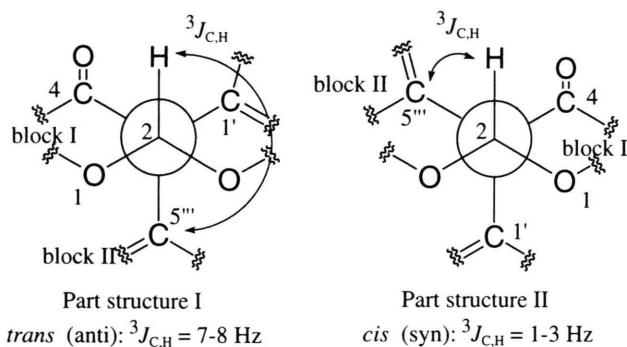*cis* (syn): $^3J_{C,H} = 1\text{-}3$ Hz

Fig. 4. Long range carbon-proton coupling constants ($^3J_{C,H}$) for couples of nuclei in *trans*(anti)- and *cis*(syn)-configurations.
Part structure I: The HMBC correlation intensity $^3J_{C\text{-}5,H\text{-}2}$ measured at 5 Hz is approximately equal to that measured at 10 Hz.
Part structure II: The intensity measured at 5 Hz must be stronger than that measured at 10 Hz.

Lupinalbisone B (2)

The structure of second isolate, lupinalbisone B (2) was elucidated in a similar manner to that applied to the first isolate (1). The FD-MS, EI-MS and NMR spectroscopic detection of the two building blocks related to 5,7,4′-trihydroxycoumaranochromanone and 2′-hydroxygenistein revealed 2 to be a regio-isomer of 1. In contrast to 1, lupinalbisone B (2) possessed intact B- and C-rings for the isoflavone moiety, whilst only one aromatic proton resonating at δ_H 6.39 as a singlet was detected for the isoflavone A-ring. Therefore, only the connectivity of the two building blocks, from C-3 of the coumaranochromanone moiety to a carbon (C-6″ or C-8″) in the isoflavone moiety remained.

In case of lupinalbisone B (2), HMBC correlation was obserbed from H-2 in the coumaranochromanone moiety to a carbon in isoflavone A-ring at δ_C 105.3 which was assigned to C-8″ because of its appropriate alkyl substitution effect on the C-8 carbon of 2′-hydroxygenistein (3) [$\Delta\delta = (\delta_C$ for C-8″ in 2) $- (\delta_C$ for C-8 in 3) $= 10.8$ ppm; usually $\Delta\delta = 11\text{-}13$ ppm, (Agrawal and Bansal, 1989); and in case of lupinalbisone A, $\Delta\delta = (\delta_C$ for C-5‴ in 1) $- (\delta_C$ for C-5′ in 3) $= 12.1$ ppm]. Thus the connection between C-3 in the coumaranochromanone moiety and C-8″ in the isoflavone moiety resulted in the complete structure of lupinalbisone B as shown 2. The detection of HMBC correlations between the A-ring singlet proton ($\delta_H = 6.39$) to C-5″, C-7″, C-8″ and C-10″ also supported the proposed structure (Fig. 1).

The relative stereochemistry of 2 has been tentatively deduced *trans* like that of 1, due to the similarity of the NMR data for proton and carbons around the juncture in the coumaranochromanone moiety.

Characteristic responses of lupinalbisones A (1) and B (2) to Gibbs reagent

The peculiar coloration of lupinalbisone A (1) (genistein-like slow and white-blue colorization), and lupinalbisone B (2) (2′-hydroxygenistein-like rapid and reddish purple coloration) toward Gibbs reagent are also consistent with the proposed structures. In 1, Gibbs-positive hydroxyl groups (*para*-unsubstituted phenolic OH, King *et al.*, 1957) are only C-5-OH and C-5″-OH, both strongly H-bonded to carbonyl groups, which slowly give a white-blue pigment on silica gel plates as with genistein (6), when sprayed with Gibbs reagent and weakly fumed with NH$_3$ gas (Nakahara *et al.*, 1986). The formation of coumaranochromanone ring system blocked one of Gibbs-positive 2′-OH group and the connection between C-3 in the coumaranochromanone moiety and C-5‴ in the isoflavone moiety changed a Gibbs positive 2‴-OH originally present in the isoflavone monomer into a *para*-substituted Gibbs-negative OH in 1. In contrast, 2 contains both H-bonded Gibbs-positive C-5-OH and C-5″-OH, and C-2‴-OH free from H-bonding. The latter hydroxyl group (C-2‴-OH) yields a reddish purple pigment rapidly after spraying with Gibbs reagent followed by fuming with weak NH$_3$ gas, as with 3 (Nakahara *et al.*, 1986).

Fungitoxic activity of lupinalbisones A (1) and B (2)

Initially we found a remarkable increase of fungitoxicity when 2′-hydroxygenistein (**3**) was subjected to enzymatic oxidation by horse radish peroxidase. The direct detection of fungitoxins in the enzymatic reaction products on silica gel thin-layer plates revealed that the fungitoxic activity was mainly due to the dimeric fraction. The results of a fungitoxic experiment on thin-layer plates using purified lupinalbisones A (**1**) and B (**2**) are shown in Fig. 5. These results are compared with those of isoflavone momomers **3** and genistein (**6**), and a representative lupin fungitoxin luteone (**7**). Both, **1** and **2** exhibited strong antifungal activities against the test fungus *Cladosporium herbarum*, and they were approximately as potent as **7**, under the conditions where the monomeric simple isoflavones (**3** and **6**) are inactive at the dose amount of 1000 μg/14 mm i.d. circular zone of silica gel layer with 0.25 mm thickness.

Naturally occurring biflavonoidal fungitoxins including **1** and **2** are supposed to be produced in biologically and/or mechanically injured or stressed plants by the aid of peroxidase-like enzyme in the relevant plants as proposed previously (Hakamatsuka *et al.*, 1992; Geiger *et al.*, 1987; and Kobayashi *et al.*, 1994) and partly demonstrated in the present study. Lupinalbisones A (**1**) and B (**2**) were also found in the reaction medium consisting of horse radish peroxidase and 2′-hydroxygenistein (**3**) as the substrate, which was composed as a model system of the injured lupin. Interestingly, dimerization of **3** caused a remarkable increase in fungitoxicity. Therefore, this kind of oxidative *trans*formation of plant secondary metabolites to yield antifungal substances probably belonging to the post-inhibitins (Ingham, 1973), may play a role in the defence system. It may occur in an emergency when plants are mechanically or biologically injured, especially during the lag phase prior to *de novo* biosynthesis of induced fungitoxins known as phytoalexins.

It is, therefore, reasonable to say that the fungitoxic compounds **1** and **2** are post-inhibitin-like defence substances (Ingham, 1973) in lupins which are armed, to our knowledge, with prohibitins (prenylisoflavones like luteone and wighteone, **7** and **8**, Harborne *et al.*, 1976) and phytoalexins (prenylisoflavones in phytopathogen-challenged seedlings, Ingham and Dewick, 1980).
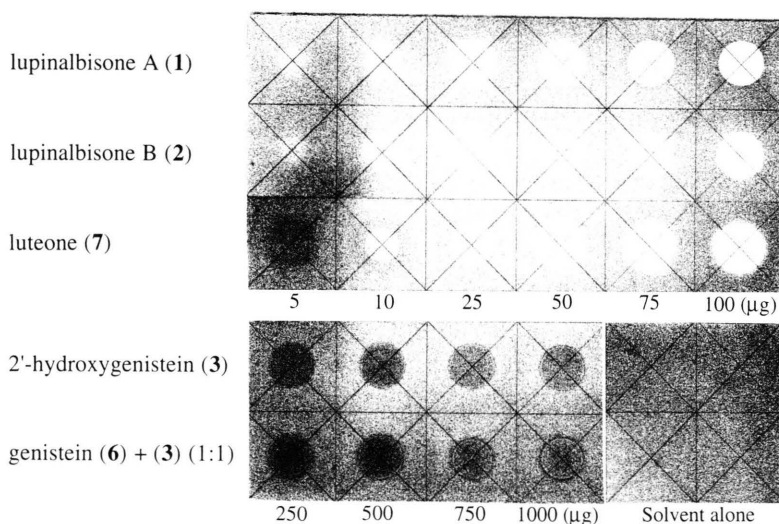

Fig. 5. Fungitoxicity of 2′-hydroxygenistein dimers and related isoflavones on silica gel plates.
Dose amount: μg/14 mm i.d. circular zone of silica gel layer with 0.25 mm thickness.
White and dark (black) zones are, respectively, showing the silica gel free from fungal growth caused by fungitoxicity of test compounds, and that covered with the grown fungus owing to insufficient doses or inactiveness of test compounds and background areas.
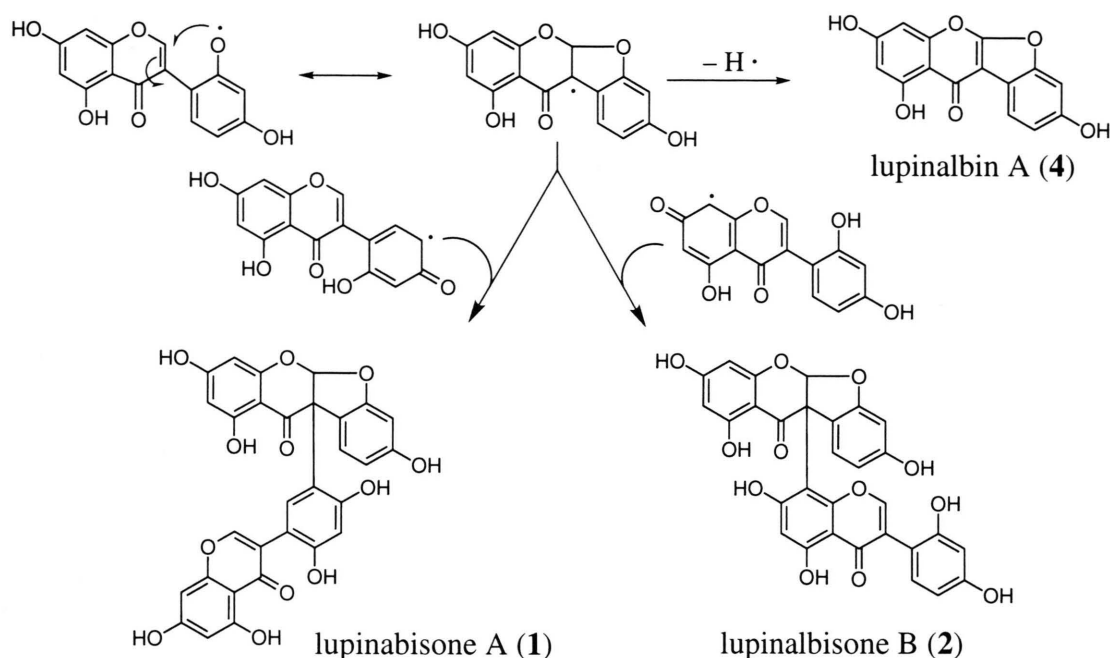
2'-Hydroxygenistein (**3**) + H₂O₂/2 $\xrightarrow{\text{POD}}$ 2'-Hydroxygenistein radical + H₂O

Fig. 6. Proposed pathways for lupinalbisones A (**1**) and B (**2**) through 2'-hydroxygenistein radicals.

A proposed pathway for 2'-hydroxygenistein dimerization in the reaction mixture catalyzed by horse radish peroxidase is shown in Fig. 6. Reactions approximately similar to those in Fig. 6 may occur in senescent and/or injured lupin roots.

Hakamatsuka and colleagues (1992) have isolated two new isoflavone dimers, kudzuisoflavones A and B together with daidzein from yeast extract-treated cell suspension cultures of *Pueraria lobata* L. (Leguminosae). The role of those isoflavone dimers was not clarified and the antifungal activities of the isoflavone dimers could not be examined due to their low yields, even though they were apparently to be produced as phytoalexins. Those compounds have been suggested to be the artificial by-products of a peroxidase-related reaction such as lignification activated by yeast extract as the elicitor. The authors also described that the production of those isoflavone dimers were observed, when daidzein was incubated with horse radish peroxidase in the presence of H₂O₂, whilst neither details nor a further report regarding the enzymatic dimerization have appeared.

Experimental

General

Analytical and preparative thin-layer separations were carried out on Merck pre-coated silica gel plates (F$_{254}$, layer thickness 0.25 or 0.50 mm). Wako-gel C-60 (silica gel) was used for column chromatography. ^{1}H and ^{13}C NMR spectra (including 2D NMR spectroscopy) were determined in acetone-d_6 at 500 MHz (int. standard TMS: ^{1}H δ 0.00) and at 125 MHz (int. standard acetone-d_6, ^{13}C δ 30.6), respectively on a Bruker AMX500. Mass and UV spectra were recorded on a JEOL JMS-SX 102A or JMS-AX500, and a Hitachi U-3210, respectively.

Chemicals and reagents

2'-Hydroxygenistein (**3**) and other isoflavones (**6–8**) used in the present study were all isolated from lupin roots as previously reported (Tahara *et al.*, 1984a). Horse radish peroxidase (POD, 100 u/mg) was purchased from Wako Pure Chem. Ind.

Gibbs reagent: Commercially available 2,6-dichloro-4-(chloroimino)-2,5-cyclohexadien-1-one was dissolved in CHCl$_3$ (200 mg/100 ml).

Enzyme reaction, products and characterization

To the mixture of 2′-hydroxygenistein (**3**, 20 mg/0.7 ml of MeOH), POD (1.63 mg/0.4 ml H$_2$O), and 184 ml of 50 mM phosphate buffer pH 5.8, was added, 16 ml of 30% H$_2$O$_2$ diluted in 16 ml of the buffer over 30 min. The mixture was allowed to stand at 25 °C with gentle stirring for 5 hr. The reaction mixture was then extracted twice with EtOAc and the organic fraction concentrated under reduced pressure was subjected to PTLC in CHCl$_3$-MeOH (=10:1, v/v) to yield lupinalbin A (**4**, 0.5 mg; *Rf* 0.6–0.7; under UV365 nm light, bright yellow; Gibbs test, slow, white-blue and changed dark blue after several hours), and a dimer fraction (*ca.* 1.2 mg; *Rf* 0.2–0.3).

Lupinalbin A (**4**) (*Rf* 0.17 in CHCl$_3$-MeOH= 50:3, v/v) from the present enzymatic reaction was identified by direct comparison of TLC, MS, and ^{1}H NMR data with those of the authentic compound isolated from lupin roots previously (Tahara *et al.*, 1985).

The dimer fraction gave two major spots with other impurities, which were finally isolated by preparative TLC as with the lupin dimers (see below). The FD-MS spectra of upper and lower running products, respectively, agreed with those of lupinalbisones A (**1**) and B (**2**), and the ^{1}H NMR spectrum of the upper running one was indistinguishable from that of lupinalbisone A (**1**), whilst the lower one has not compared with **2** because of the insufficiency of isolated amount. Together with these spectroscopic properties, both enzymatic products coincide well with **1** and **2** in their behaviours on thin-layer plates, *Rf* in EtOAc-MeOH-H$_2$O-conc. ammonia water (=60:10:3:1, v/v) and CHCl$_3$-MeOH-HCOOH (=60:10:1), and responses to Gibbs reagent; coloration speed and quality of colours.

Plant materials and extraction

The roots of white lupin (*Lupinus albus* L. cv. Kievskij Mutant) collected in middle August, *ca* 1.5 kg, were extracted with MeOH (7 liters × 3). The concentrated extract was partitioned between EtOAc and H$_2$O, and the organic layer was re-duced to dryness (12.0 g). The residue was subjected to silica gel (300 g) column chromatography to yield *ca* 600 mg of isoflavone dimer fraction eluted with 700 ml of a mixture of CHCl$_3$-MeOH (=20:3, v/v) after washing the column with 2500 ml of the same solvent. The isoflavone dimer fraction was reinforced by 700 mg of another isoflavone dimer fraction similarly prepared from 1.7 kg of lupin roots harvested in early August. The combined isoflavone dimer fraction (1.3 g) was partly purified by rechromatography under conditions similar to those of the first column to yield 690 mg of the crude dimer mixture, which was further subjected to a Sephadex LH-20 (90 ml) column with MeOH as an eluting solvent to give three fractions as follows: (1) the initial 210 ml containing no isoflavone dimer; (2) continuing 110 ml of the eluate containing LB-4 and lupinalbisone A (**1**); and (3) continuing 145 ml of the eluate containing **1** and lupinalbisone B (**2**), which were detected at *Rf* 0.09 and 0.08, respectively, on thin-layer plates developed in EtOAc-MeOH-H$_2$O-conc. NH$_4$OH. Compounds **1** and **2** were further purified through silica gel column chromatography and multiple development preparative TLC (PTLC) using a mixture of EtOAc-MeOH-H$_2$O-conc. NH$_4$OH (= 60:10:3:1, v/v) as an eluting and developing solvent system. Finally, **1** and **2** were subjected to re-PTLC in CHCl$_3$-MeOH-HCOOH (=60:10:1, v/v), to give highly pure substances, **1** (*Rf* 0.37; 21.4 mg; Gibbs test, slow, white-blue and changed dark blue in several hours) and **2** (*Rf* 0.36; 7.3 mg; Gibbs test, rapid, reddish purple). The amount of LB-4 was too small for further structural elucidation experiments.

Bioassay

In addition to the thin-layer chromatogram method reported by Homans and Fuchs (1970), a modified antifungal assay on silica gel plates (Tahara *et al.*, 1984b) was conducted by using *Cladosporium herbarum* Fr. AHU 9262 supplied from the Laboratory of Applied Microbiology, Graduate School of Agriculture, Hokkaido University. The specified amount of pure isolate and reference compounds were dissolved in acetone. The solution (25 µl) was spotted on a thin layer plate (Merck, Silica Gel 60 F$_{254}$, 0.25 mm thickness) by a microsyringe to give a circular zone (*ca* 14 mm

i.d.) of the homogeneously adsorbed test material. After the acetone had evaporated, a spore suspension of C. herbarum in a medium was sprayed onto the plate and incubated in a moist atmosphere for 2–3 days at 25 °C according to Homans and Fuchs (1970).

Physicochemical properties of lupin isoflavone dimers 1 and 2

Lupinalbisone A (**1**): Pale yellow powder. Gibbs test: slow, white-blue and dark blue after several hours (genistein-type). $[\alpha]_D$ −24.3° (c=1.48, acetone). FD-MS *m/z* (%): 571 ([M+1]$^+$, 43), 570 ([M]$^+$, 100), 286 (17), 284 (13). EI-MS *m/z* (%): 286 (43), 285 (25), 284 (100), 269 (11), 255 (5), 200 (7), 176 (7), 153 (26), 134 (13), 126 (17), 69 (15). UV λmax (MeOH), nm: 261, 292; + NaOMe, 270, 329; + AlCl$_3$, 269, 307; + AlCl$_3$/HCl, 269, 307; + NaOAc, 272, 331; + NaOAc/H$_3$BO$_3$, regenerated the MeOH spectrum. ^{1}H and ^{13}C NMR data are summarized in Table I.

Lupinalbisone B (**2**): Pale yellow powder. Gibbs test: rapid, reddish purple (2'-hydroxygenistein-type). $[\alpha]_D$ +6.6° (c=0.38, acetone). FD-MS *m/z* (%): 571 ([M+1]$^+$, 39), 570 ([M]$^+$, 100), 286 (56), 284 (53). EI-MS *m/z* (%): 286 (36), 284 (100), 269 (8), 255 (5), 228 (5), 200 (6), 176 (7), 153 (21), 134 (10), 126 (7), 69 (11). UV λmax (MeOH), nm: 264, 290; + NaOMe, 281sh, 329; + AlCl$_3$, 271, 307; + AlCl$_3$/HCl, 271, 309, 361; + NaOAc, 284, 331; + NaOAc/H$_3$BO$_3$, regenerated the MeOH spectrum. ^{1}H and ^{13}C NMR data are summarized in Table I.

Lupinalbisone A hepta-acetate

Minute amounts of lupinalbisone A (**1**, 0.5 mg) were acetylated in acetic anhydride/pyridine (1:1, v/v) at room temperature for 12 h and processed as usual to yield the corresponding hepta-acetate. ESI-MS *m/z* (%): 903 ([M+K]$^+$, 12), 887 ([M+Na]$^+$, 100), 845 ([M-42+Na]$^+$, 23).

Agrawal P. K. and Bansal M. C. (1989), Isoflavonoids. In: Carbon-13 NMR of Flavonoids (P. K. Agrawal, ed.) Elsevier, Amsterdam, pp. 183–235.

Bekker R., Brandt E. V. and Ferreira D. (1998). Structure and stereochemistry of the first isoflavone-benzofuranone biflavonoids. Tetrahedron Lett. **39**, 6407–6410.

Bohm, B. A. (1998). Introduction to Flavonoids. Harwood Academic Publ., Singapore, pp. 62–68.

Dewick P. M. (1994), Isoflavonoids. In: The Flavonoids: Advances in Research since 1986 (J. B. Harborne ed.) Chapman & Hall, London, pp. 197–202.

Gomez-Garibay F., Calderon J., Quijano L., Tellez O., Olivares M. d. S. and Rios T. (1997), An unusual prenyl biflavanol from *Tephrosia tepicana*. Phytochemistry **46**, 1285–1287.

Geiger H., Stein W., Mues R. and Zinsmeister H. D. (1987), Bryoflavone and heterobryoflavone, two new isoflavone-flavone dimers from *Bryum capillare*. Z. Naturforsch. **42c**, 863–867.

Geiger H. (1994), Biflavonoids and triflavonoids. In: The Flavonoids: Advances in Research since 1986 (J. B. Harborne ed.) Chapman & Hall, London, pp. 95–115.

Hakamatsuka T., Shinkai K., Noguchi H., Ebizuka Y. and Sankawa U. (1992). Isoflavone dimers from yeast extract-treated cell suspension cultures of *Pueraria lobata*. Z. Naturforsch. **47c**, 177–182.

Hanawa F., Tahara S. and Mizutani J. (1991). Retro-Diels-Alder fragmentation of coumaronochromones and complete assignments of 13C NMR data for lupinalbin A and ayamenin B. Heterocycles **32**, 1563–1570.

Homans A. S. and Fuchs A. (1970), Direct bioautography on thin layer chromatograms as a method for detecting fungitoxic substances. J. Chromatogr. **51**, 327–329.

Harborne J. B., Ingham J. L., King L. and Payne M. (1976), The isopentenyl isoflavone luteone as a pre-infectional antifungal agent in the genus *Lupinus*. Phytochemistry **15**, 1485–1488.

Ingham J. L. (1973), Desiase resistance in plants: the concept of pre-infectional and post-infectional resistance. Phytopath. Z. **78**, 314–335.

Ingham J. L. and Dewick P. M. (1980), Sparticarpin: a pterocarpan phytoalexin from *Spartium junceum*. Z. Naturforsch. **35c**, 197–200.

King F. E., King T. J. and Manning L. C. (1957), The Gibbs reaction and the constitution of jacareubin. J. Chem. Soc. 563–566.

Kobayashi A., Koguchi Y., Kanzaki H., Kajiyama S. I. and Kawazu K. (1994), A new type of antimicrobial phenolics produced by plant peroxidase and its possible role in the chemical defense systems against plant pathogens. Z. Naturforsch. **49c**, 411–414.

Lin G.-Q. and Zhong M. (1997), The first enantioselective synthesis of optically pure (*R*)- and (*S*)-dihydroxy-4',4''',7,7''-tetramethoxy-8,8''-biflavone and the reconfirmation of their absolute configuration. Tetrahedron Lett. **38**, 1087–1090.

Lin Y.-M., Anderson H., Flavin M. T. and Pai Y.-H. S. (1997), In vitro anti-HIV activity of biflavonoids isolated from *Rhus succedanea* and *Garcinia multiflora.* J. Nat. Prod. **60**, 884–888.

Mabry T. J. and Markham K. R. (1975), Mass spectrometry of flavonoids. In: The Flavonoids (J. B. Harborne, T. J. Mabry and H. Mabry eds.), Chapman & Hall, London, pp.78–126.

Moreira I. C., Sobrinho D. C., De Carvalho G. M. and Braz-Filho R. (1994), Isoflavanone dimers hexaspermone A, B and C from *Ouratea hexasperma.* Phytochemistry **35**, 1567–1572.

Nakahara S., Tahara S., Mizutani J. and Ingham J. L. (1986), Transformation of the diprenylated isoflavone 2'-hydroxylupalbigenin by *Aspergillus flavus.* Agric. Biol. Chem. **50**, 863–873.

Nakazawa K. (1962), Chemical structure of ginkgetin. *Gifu Yakka Daigaku Kiyo* No. **12**, 1–18.

Porter L. J. (1994), Flavans and proanthocyanidins. In: The Flavonoids: Advances in Research since 1986 (J. B. Harborne, ed.) Chapman & Hall, London, pp. 23–55.

Rastrelli L., Berger I., Kubelka W., Caceres A., De Tommasi N. and De Simone F. (1999), New 12a-hydroxyrotenoids from *Gliricidia sepium* bark. J. Nat. Prod. **62**, 188–190.

Rodriguez A. D. (1992), Two-dimensional NMR spectra of asbestinin diterpenes isolated from *Briareum asbestinum* (Pallas.). Total stereochemical assignment by two-dimensional NMR techniques. Magn. Reson. Chem. **30**, 977–986.

Schaufelberger D. E., Chmurny G. N. and Koleck M. P. (1991), ^1H and ^{13}C NMR assignments of the antitumore machrolide bryostatin 1. Magn. Reson. Chem. **29**, 366–374.

Tahara S., Ingham J. L., Nakahara S., Mizutani J. and Harborne J. B. (1984a), Fungitoxic dihydrofuranoisoflavones and related compounds in white lupin, *Lupinus albus.* Phytochemistry **23**, 1889–1900.

Tahara S., Nakahara S., Mizutani J. and Ingham J. L. (1984b), Fungal transformation of the antifungal isoflavone luteone. Agric. Biol. Chem. **48**, 1471–1477.

Tahara S., Ingham J. L. and Mizutani J. (1985), New coumaronochromones from white lupin, *Lupinus albus* L. (Leguminosae). Agric. Biol. Chem. **49**, 1775–1783.

Tahara S., Moriyama M., Orihara S., Ingham J. L., Kawabata J. and Mizutani J. (1991), Naturally coccurring coumaranochroman-4-ones: a new class of isoflavonoids from lupins and Jamaican dogwood. Z. Naturforsch. **46c**, 331–340.