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**Studies on Wood Components Affecting the Behavior of
Termites**

WAKAKO OHMURA

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INTRODUCTION

Termites make a large contribution to the decomposition and the recycling of dead woods and various plant litter in the ecosystem. Some species cause damages to cultivation plants and/or wooden structures, so they are recognized 'economically' important pest as well as 'ecologically' important biorecycling organizer.

Termites belong to the order 'Isoptera', and form highly developed societies (*i.e.* eusociality) which generally consist of the different castes, that is, reproductives (queen, king and nymph), workers, and soldiers. Although there are many characteristics in common with ants, they are closely related to cockroaches 'Blattariae' according to the phylogenetic systematics. Among the seven families of 'Isoptera' (Mastotermitidae, Kalotermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae, Termitidae, Serritermitidae), members of Termitidae are called the 'higher termites,' and those of the other families are called 'lower termites' (Abe, 1989). One of the differences between the 'lower termites' and the 'higher termites' is in the symbiotic microorganism. The formers possess both protozoa and bacteria in their guts, whereas the latter lack protozoa.

In general, termite diet of plant materials have high carbon and low nitrogen contents. The ratio of carbon to nitrogen (C/N) in wood is 350-1000 (LaFage and Nutting, 1978), whereas that of termite tissues is 4-12 (Matsumoto, 1976). In order to overcome the nitrogen scarcity of their diet, termites need to feed on a large amount of plant materials and digest them with the help of symbionts of protozoa and/or bacteria in their guts. Furthermore, some species can obtain nitrogen by nitrogen-fixation bacteria, and release excess of carbon as carbon dioxide by digestion and methane by methanogenic bacteria in their guts to solve the unbalance problem of C/N between termite tissue and their diet (French *et al.*, 1976; Potrikus and Breznak, 1977; Higashi *et al.*, 1992).

Macrotermitinae species, which belong to the 'higher termites', cultivate *Termitomyces* spp. (basidiomycetous fungi), and maintain fungus-combs in the internal chambers (fungus-gardens) of the nests. So, they are called 'fungus-growing termites'. The roles of fungus-combs are thought to be as follows (Abe, 1989) : (1) nutrient source (especially of nitrogen) (Matsumoto, 1983) (2) decomposer of plant materials (3) cellulase sources (Martin and Martin, 1978). Other wood-eating termites used to feed on woods decaying and decayed by wood-rotting fungi. Other insects, for example, leaf-cutting ants (*Atta* spp. and *Acromyrmex* spp.), ambrosia beetles and wood wasps are also well-known for cultivating fungi (Redfern, 1989). Leaf-cutting ants form the fungus-combs in their nests similar to fungus-growing termites (Weber, 1966, 1979). Ambrosia beetles bore into the woods for oviposition and introduce the spores of wood-staining fungi for the diet of hatched larvae. The females of the beetles have mycangiums in their bodies for keeping the spores. After the fungal hyphae spread in the boring tunnels, newly emerged larvae feed on the hyphae (Matsumoto, 1992). Wood wasps also introduce the spore of basidiomycetous fungi when they bore into the woods for ovipositions. The larvae grow up to eat the wood decayed by the introduced fungi. In the case of wood wasps, the fungi are considered to be not only their foods but also their sources of digestive enzyme for degrading the wood components (Sagara, 1989). Thus, insect lives are closely related to microorganism including fungi.

Defensive action of plants against insects attacks are also well known since older times. We mankind sometimes utilize these insect-plant relationships in our life. For example, pyrethrin from *Chrysanthemum cinerariaefolium* Bocquillon, camphor from *Cinnamomum camphora* Sieb. and nicotine from *Nicotiana* spp. have been used for pesticides. Natural compounds which control the behaviors of insects are generically called 'semiochemicals'. According to the classification of the term by Nordlund and Lewis (1976), 'semiochemicals' are roughly divided

into two groups; 'pheromone' and 'allelochemicals'. 'Allelochemicals' are further divided into four groups; 'allomones', 'kairomones', 'synomones' and 'apneumones'. In the explanation of insect-plant interaction, 'allomones' and 'kairomones' are defined as follows by Beck and Reese (1976):

(1) Allomone : a chemical or a mixture of chemicals that is released from plants, and act as the repellents and deterrents which are recognized as unsuitable diets for insects.

(2) Kairomones : a chemical or a mixture of chemicals that is released from plants, and work as the attractants and stimulants which are accepted as favorable food sources by insects

Another way to categorize chemicals in terms of the types of insect behavior were designated by Dethier *et al.* (1960) as follows:

(1) Locomotor stimulant : a chemical which cause, by a kinetic mechanism, insects to disperse from a region more rapidly than if the area did not contain the chemical. The effect may be to increase the speed of locomotion, to cause the insects to carry out avoiding reactions, or to decrease the rate of turning.

(2) Arrestant : a chemical which causes insects to aggregate in contact with it, the mechanism of aggregation being kinetic or having a kinetic component. An arrestant may slow the linear progression of the insects by reducing actual speed of locomotion or by increasing turning rate.

(3) Repellent : a chemical which causes insects to make oriented movements away its source.

(4) Attractant : a chemical which causes insects to make oriented movements toward its sources.

(5) Feeding, mating, or ovipositional deterrent : a chemical which inhibits one of these behavioral reactions when present in a place where insects would do in its absence.

(6) Feeding, mating, or ovipositional stimulant : a chemical which elicits one of these behavioral reactions.

The term "feeding preference" is sometimes used synonymous with "feeding stimulant". Although, in this dissertation, the author uses the term when a substrate containing a chemical is more consumed than an untreated one under the two-choice bioassay.

It should be noticed that the same chemical may reveal more than one of foregoing reactions.

Various kinds of secondary compounds are produced in trees as 'allomone' for the prevention of xylophagous insects attack. Some of the common tree species seen today in some regions have been regarded as a resultant selection by termites (Pearce, 1997). From 1940's to 1960's, many antitermitics in the tropical woods were isolated (Wolcott, 1947, 1951, 1953). Sanderman and Dietrichs (1957) classified these substances into three structural types : stilbenes, quinones and pyran derivatives. In Japan, Kondo *et al.* (1963) isolated triterpenoid saponins from *Kalopanax septemlobum* Koidz. as termiticidal substances. Triterpenoid saponins from *Ternstroemia japonica* Thunb., *Camellia japonica* L. var. *hortensis* Mak., and *Thea sinensis* L. also exhibited termiticidal activities (Watanabe *et al.*, 1966; Saeki *et al.*, 1966, 1968). Although the investigations on naturally occurring antitermitic substances have been still continued, the relationships between their chemical structures and activities have not been explained sufficiently yet. Recently, limonoids from *Citrus* spp. have been paid an attention as antifeedants, repellents, and/or insecticides to several species of insects (Harborne, 1993), and some of them were reported as effective agents against the lower termite, *Reticulitermes speratus* (Kolbe) (Serit *et al.*, 1992).

Turning to the termite 'kairomone', Esenther *et al.* (1961) reported that wood decayed by a brown-rot fungus, *Gloeophyllum trabeum* (Pers. ex. Fr.) Murr. attracted the lower termite, *Reticulitermes flavipes* (Kollar). The fungus

produced the same substance as a trail-following pheromone, (Z,Z,E)-3,6,8-dodecatrien-1-ol (DTE-OH), which is common to most of rhinotermitids (Matsumura *et al.*, 1969). Watanabe (1963) tested many compounds of known structure for attractancy to *R. flavipes*, and concluded that the propenyl and styryl radical, for example, cinnamyl alcohol and isosafrole were active. Furthermore, Becker (1964) tested the termite-attractant activity of eight kinds of aromatic compounds which were formed from enzymic degradation of softwood lignin by wood-rotting fungi (Ishikawa *et al.*, 1962, 1963) using five species of termites. The results indicated that the attitude of the termites toward the compounds varied with the species, and that the acidic compounds such as vanillic acid and protocatechuic acid showed the attractant activity, whereas aldehydes such as vanillin showed weak attractant or repellent activity. Recently, it was reported that the termite, *Neotermes bosei* Snyder showed attraction towards some of the carbohydrates, fatty acids, amino acids and organic solvents (Mishra 1992), and that the subterranean termite, *Coptotermes formosanus* Shiraki most preferred filter papers impregnated with D-aspartic acid and L-glutamic acid among the 21 amino acids tested (Chen and Henderson 1996).

Clément *et al.* (1988) reviewed the biochemical and ecological relationships between the four European *Reticulitermes* spp. that the termites perceived some secondary plant compounds as allomones at high levels and did them as kairomones at low levels. Thus, termites have developed complex chemical relationships with secondary plant compounds.

After World War II, our excessive dependence upon synthetic chemicals has caused serious environmental pollutions all over the world. Also, quite recently, "endocrine disrupters" are casting a serious problem in our life. 'DDT (dichlorodiphenyltrichloroethane)', 'BHC (benzene hexachloride)' and 'CHL (chlordan)' which had been used for insecticides for many years and their intermediate derivatives, are regarded as the strong endocrine disrupters as well as

other organochlorines such as 'dioxins' because of their long persistence in the environment (Tanabe, 1998). They act as endocrine disrupters in extremely small quantities. Furthermore, their effects are realized only when the human being grew up sexually long after the uptake during babyhood.

Therefore, a concept of 'integrated pest management (IPM)' is more and more important for the tomorrow's pest control. It is not simply depending upon the man-made chemical applications, but also utilizing various control methods in combination with the chemicals to keep pest population as low as possible (Saito, 1986). To establish a reliable technique for protecting wooden constructions from termite attacks, physiological and ecological features of termites must be more deeply understood.

Methods of termite controls for buildings can be roughly divided into two aspects; "prevention" and "eradication" of termite attacks (Creffield, 1991). "Prevention of termite attack" is to protect buildings from termite attack by treatment of woodworks and/or soils by chemical or physical techniques. For the wood treatment, various water-borne and oil-borne chemicals are applied by pressure and superficial treatments. CCA (copper-chromium-arsenate) is one of the most important and prevailed wood preservatives nowadays. Copper and arsenic are toxic to fungi and insects, respectively. Chromium(III), which is converted from chromium(VI) during conditioning after impregnation, acts to fix copper and arsenic into woods, thus, long-years performance of the CCA-treated wood is achieved (Takahashi, 1986). However, there have been fears concerning the yields of toxic gaseous arsenate and carcinogenic chromium(VI), resulting from the incineration disposal of the CCA-treated wood after use.

Soils beneath and/or around the buildings are also treated using chemicals such as organophosphates and synthetic pyrethroids, because subterranean termites are used to invade into buildings through the soils. Such a

chemical soil treatment sometimes causes a water pollution by an unsuitable operation.

In recent years, the health and environmental impacts associated with the applications of such conventional preservatives tend to restrict their use and to demand us for the development of new safer treatment procedures (Richardson, 1993). Some physical barriers using basaltic particles (Tamashiro *et al.*, 1987, 1991) and stainless steelmesh (Lenz, 1994) have been developed as substituents or supplement to chemical soil treatment. Further, the improvement of crawl space ventilation by using humidity-controlling materials has been applied to Japanese housing constructions (Takahashi, 1997).

On the other hand, 'eradication of termite attack' is to destruct termite colonies by using chemical or biological agents. As for the biological agents, some entomologogenous fungi (Yoshimura *et al.*, 1992; Jones *et al.*, 1996) and nematodes (Danthanarayana and Vitarana, 1987) have been examined for their applicability as termite control methods. The idea of bait-toxicant method was born to enhance the effectiveness of the termiticides. The first trial was done in the USA using decayed wood and a slow-acting toxicant, mirex (Esenther and Beal, 1974; 1978). Thus, baits must be more attractive than the surrounding available food sources, and the chemicals should be slow-acting so as to be transferred throughout the colony. Recently, the effective bait-toxicant systems are developed extensively by use of the specific insect growth regulators (Pearce, 1997).

In this dissertation, the effects of wood components on the behavior of termites were investigated using the two economically important termites in Japan, *R. speratus* and *C. formosanus*. Based on the results obtained, the application of the wood components for termite controls were evaluated in laboratory. Part I deals with saponins and flavonoids, which are known as antitermitics. The structure-regulated substances were synthesized or isolated, and served for the termiticidal and/or antifeedant bioassays. Through the results of the assays, the structure-

activity relationships were discussed. In PART II, feeding-preferences and trail-following substances were tried to isolate from woods deteriorated by steam treatment or fungal decay. Interaction between the wood components and the behavior of termites were investigated. PART III deals with the application of some wood components to termite controls. Polyphenols which are abundant in woods were used with heavy metal salts, and the hot-water extracts of steamed Japanese larch (*Larix leptolepis* (Sieb. et Zucc.) Gord.) heartwood were used with boric acid for bait-toxicant technique.

PART I

EFFECTS OF WOOD COMPONENTS ON THE MORTALITY AND FEEDING OF TERMITE

**Chapter 1 : Termiticidal and Antifeedant Activities of
Triterpenoid Saponins Against the Two Subterranean
Termites, *Reticulitermes speratus* (Kolbe) and
Coptotermes formosanus Shiraki**

**Chapter 2 : Antifeedant Activity of Flavonoids and Related
Compounds Against *Coptotermes formosanus* Shiraki**

Chapter 1 : Termiticidal and Antifeedant Activities of Triterpenoid Saponins Against the Two Subterranean Termites, *Reticulitermes speratus* (Kolbe) and *Coptotermes formosanus* Shiraki

1.1 Introduction

Saponins are known to have various biological activities. They are divided into two groups according to their structure of aglycon: 'triterpenoid saponins' and 'steroid saponins'. As to the termiticidal activities of triterpenoid saponins, Kondo and others in a study of extractives from harigiri (*Kalopanax septemlobum* Koidz.) showed that the most active substance was a saponin consists of one mol of oleanolic acid and two moles each of glucose and arabinose (Kondo *et al.*, 1963). Subsequently, several other saponins from mokkoku (*Ternstroemia japonica* Thunb.), tsubaki (*Camellia japonica* L. var. *hortensis* Mak.) and cha-no-ki (*Thea sinensis* L.) were also investigated for their termiticidal activities (Watanabe *et al.*, 1966; Sacki *et al.*, 1966, 1968). The result of biological testing revealed that a saponin on acid hydrolysis gave the corresponding aglycon in a yield near 50% and exhibited the greatest termiticidal activity against *Coptotermes formosanus* Shiraki. However, the sugar moieties of saponins used in these investigations were not determined chemical structurally, and their aglycon moieties also were not made clear except for *T. japonica* (Watanabe *et al.*, 1966). Therefore, further study is necessary to explain the antitermitic activities of saponins.

In this chapter, several monodesmosidic saponins with methyl oleanolate as an aglycon were synthesized to obtain structurally regulated saponins. Then, these saponins were submitted to termiticidal and antifeedant bioassays against *Reticulitermes speratus* (Kolbe). The antifeedant activity against *R. speratus*

was compared with that against *C. formosanus*. The purpose of this chapter is to discuss the relationships between the sugar chain structures of these saponins and their antitermitic activities (Ohmura *et al.*, 1997).

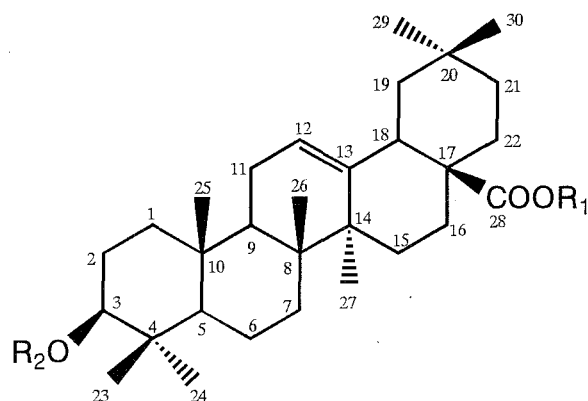
1. 2 Materials and Methods

Synthesized saponins were identified by electron impact mass spectroscopy (EI-MS), fast atom bombardment-mass spectrometry (FAB-MS), proton-nuclear magnetic resonance (^1H -NMR) spectroscopy, and/or carbon-13-nuclear magnetic resonance (^{13}C -NMR) spectroscopy. The EI-MS and FAB-MS spectra were obtained using a JEOL DX-303 spectrometer. The measurements by FAB-MS were made by adding sample solutions to the glycerol matrix. The ^1H - and ^{13}C -NMR spectra were recorded on both JEOL GSX-400 and JEOL ALPHA-500 spectrometers. Pyridine- d_5 was used as the solvent. The ^1H - ^1H COSY (homonuclear chemical shift correlation spectroscopy), ^1H - ^{13}C COSY (heteronuclear chemical shift correlation spectroscopy), ^1H - ^1H NOESY (nuclear Overhauser enhancement spectroscopy), HOHAHA (homonuclear Hartmann-Hahn spectroscopy) and HMBC (heteronuclear multiple bond connectivity) experiments were made to assist in assigning the spectra.

1.2.1 Synthesis of methyl oleanolate glycosides

1.2.1.1 Isolation of oleanolic acid

Oleanolic acid (**1**) (Fig. 1-1) was isolated from CH_2Cl_2 extracts of the outer bark of shirakamba (*Betula platyphylla* Sukatchev var. *japonica* Hara) and identified from its ^{13}C -NMR data and reference to the literature (Ohara *et al.*, 1986).



	R_1	R_2
(1):	H	H
(2):	CH_3	H
(3):	CH_3	1β G1
(4):	CH_3	1β G1 (4,1) β G2
(5):	CH_3	1β G1 (4,1) β G2 (4,1) α G3
(6):	CH_3	1β G1 (4,1) β G2 (4,1) α G3 (4,1) α G4
(7):	CH_3	1β G1 (4,1) β G2 (4,1) α G3 (4,1) α G4 (4,1) α G5

Fig. 1-1. Structures of oleanolic acid (1), methyl oleanolate (2), and synthesized methyl oleanolate glycosides (3)-(7).

Notes : Gn (n = 1, 2, 3, 4, 5) indicates a glucosyl unit. G1 is an innermost unit binding to methyl oleanolate. G2 is a second one from G1, G3, G4 and G5 are third, fourth and fifth ones from G1, respectively. G5 is an outermost unit.

1.2.1.2 Synthesis of methyl oleanolate

Oleanolic acid (1) was methylated with a diazomethane ether solution at 0 °C for three hours to give methyl oleanolate (2) (Fig. 1-1).

Methyl oleanolate (2) EI-MS m/z : 470 (M^+). ^{13}C -NMR (pyridine- d_5): δ (chemical shift) 15.7(C-25), 16.6(C-24), 17.2(C-26), 18.8(C-6), 23.8(C-11), 23.8(C-30), 23.8(C-16), 26.2(C-27), 28.1(C-2), 28.1(C-15), 28.8(C-23),

30.9(C-20), 33.2(C-22), 33.2(C-29), 33.3(C-7), 34.1(C-21), 37.4(C-10), 39.0(C-1), 39.4(C-4), 39.7(C-8), 41.8(C-18), 42.0(C-14), 46.2(C-19), 46.9(C-17), 48.1(C-9), 51.6(COOCH₃), 55.9(C-5), 78.0(C-3), 122.9(C-12), 144.1(C-13), and 177.8(C-28).

1.2.1.3 Synthesis of methyl oleanolate-3-yl β -D-cellobioside

Methyl oleanolate-3-yl β -D-cellobioside (**4**) (Fig. 1-1) was synthesized by condensing hepta-*O*-acetyl α -D-cellobiosyl bromide with (**2**) according to the Königs-Knorr type condensation (Ohara and Hishiyama, 1994). The purification of (**4**) was performed by silica-gel column chromatography using *n*-hexane/acetone = 9/4 as an eluent. Methyl oleanolate-3-yl β -D-glucoside (**3**) (Fig. 1-1) also was synthesized by the same procedure as was (**4**).

1.2.1.4 Enzymic reaction

Methyl oleanolate-3-yl β -D-cellobioside (**4**) (0.17 g), α -cyclodextrin (1.5 g) and sodium dodecyl sulfate (0.5 g) (Wako Pure Chemical Industries. Ltd.) were suspended in 0.1 M acetate buffer (pH 5.5, 62.5 ml). To the suspension, cyclodextrin glucosyltransferase (CGTase) from *Bacillus stearothermophilus* (400 THU/ml, 0.4 ml) was added, and the mixture was incubated at 65 °C for 18 h. At the end of the reaction, the mixture was extracted with *n*-BuOH. The extracts were chromatographed on a silica-gel column using CHCl₃/MeOH/H₂O = 10/4/1 as an eluent to obtain methyl oleanolate oligoglycosides (**5**)-(7) (Fig. 1-1).

1.2.2 Antitermitic bioassay

1.2.2.1 Termites

A colony of *R. speratus* was collected in the precincts of the Forestry and Forest Products Research Institute, Tsukuba, Ibaraki-Pref. Only undifferentiated mature larvae (workers) were used.

In the case of *C. formosanus*, externally undifferentiated mature larvae (workers) were collected from a laboratory colony. The colony was maintained in the dark at 28 ± 2 °C and approximately 80 % R.H. for more than fifteen years at Forestry and Forest Products Research Institute.

1.2.2.2 Test chamber

A test chamber was made of a plastic cup (rim diameter ; 60 mm, bottom diameter ; 50 mm, height ; 50 mm) with a 20 mm diameter hole in the bottom. Hard plaster powder (5 g) was mixed with 4 ml of deionized water, and the suspension was poured into the bottom of the chamber. After the plaster had set, the cup was layered with 10 g of sea sand (15-20 mesh: Junsei Chemical Co., Ltd.), and moistened with 2 ml of deionized water. A dampened cotton pad was spread under the chamber so as to maintain the humidity.

1.2.2.3 Termiticidal bioassay

Thirty mg of (2)-(7) was dissolved in MeOH or in CHCl_3 . One g of cellulose powder was treated with one of these test solutions, dried at room temperature, and put on a plastic saucer (1.5 cm diameter). The plastic saucer was put on the bottom of the test chamber (Fig. 1-2) and fifty workers were introduced into the test chamber. Untreated cellulose powder was used as a control. In addition, the test under complete starvation (water supply only) was also evaluated. The 3-week trial was conducted in a dark room at 26 °C and 75 % R. H. Three

duplicates were undertaken for each sample, and the number of surviving workers were counted every two days.

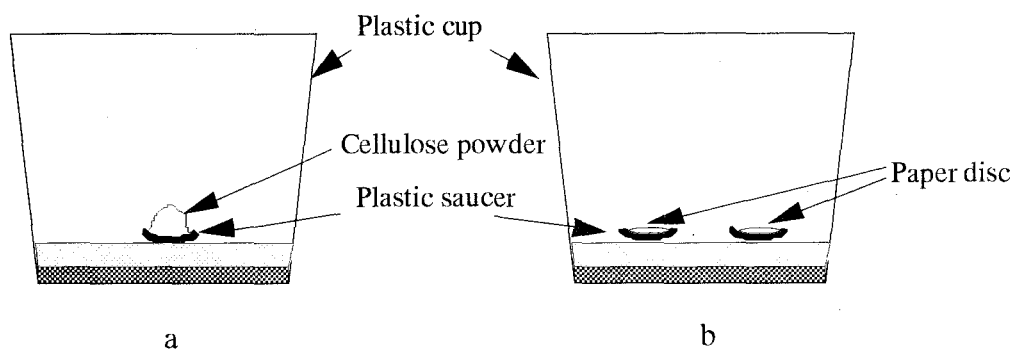
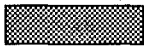


Fig. 1-2. Antitermitic bioassay methods.

Notes: a: No choice bioassay method for testing termiticidal activity.
b: Two-choice bioassay method for testing antifeedant activity.

Legend:  : sea sand.
 : hard plaster.

1.2.2.4 Antifeedant bioassay

For the bioassay against *R. speratus*, paper discs (Toyo Seisakusho Co., Ltd.) with 8 mm diameter were used after treating with 15 μ l of the MeOH or CHCl_3 solutions containing 0.5% (w/w) of (2)-(7). In the case of *C. formosanus*, paper discs with 13 mm diameter (Whatman International Ltd.) were used after treating with 60 μ l of the MeOH or CHCl_3 solutions containing 0.5 % (w/w) of each test material. After air-drying, they were dried in a vacuum desiccator for one day. The untreated control discs were dried in the same manner. Two discs treated with different sample solutions were put on plastic saucers, and they were placed diagonally 12 mm away from the center of the test chamber (Fig. 1-2). After three

days, the discs were removed, dried at 40 °C for 6 h, and dried in a vacuum desiccator for one day. The weight loss of each disc was determined. Fifty workers were used for the bioassays against *R. speratus* and one hundred workers were used for those against *C. formosanus*. Three duplicates were made for each sample.

1.3 Results and Discussion

Structures of (1)-(7) and a part of the ^{13}C -NMR spectral data for (3)-(6) are shown in Table 1-1 and Fig. 1-1, respectively.

Table 1-1. A part of ^{13}C -NMR data of synthesized saponins (3)-(6).

Moieties	C	(3)	(4)	(5)	(6)
Aglycon	3	88.8	88.9	88.9	88.9
	28	178.0	178.0	178.0	178.0
G1	1	106.9	106.4	106.5	106.5
	2	75.8	74.8	75.3	75.2
	3	78.8	76.8	76.3	76.8
	4	71.8	81.5	81.0	80.8
	5	78.3	76.2	76.3	76.3
	6	63.0	62.3	62.6	62.1
G2	1		104.9	104.6	104.5
	2		75.2	75.3	74.1
	3		78.1	76.7	77.3
	4		71.4	81.0	81.5
	5		78.3	75.4	76.6
	6		62.2	62.2	61.6
G3	1			103.1	102.9
	2			74.4	73.8
	3			77.5	74.8
	4			71.7	81.4
	5			75.3	73.6
	6			61.7	61.7
G4	1				103.1
	2				74.3
	3				75.3
	4				71.8
	5				75.2
	6				62.6

Note : G1, G2, G3 and G4 are the same as in Fig. 1-1.

1.3.1 Structures of methyl oleanolate glycosides

Oleanolic acid (**1**) has two reactive functional groups (3-hydroxyl and 28-carboxyl) for glycosylation. In this study, only the latter group was protected to synthesize the monodesmosidic saponins. The treatment of (**1**) with diazomethane at 0 °C for 3 h gave methyl oleanolate (**2**) almost quantitatively.

At first, methyl oleanolate-3-yl β -D-cellobioside (**4**) was synthesized to obtain the adequate acceptor for transglucosylation by CGTase. FAB-MS, ^1H -NMR, and ^{13}C -NMR were used to explain the structure of (**4**). The FAB-MS spectrum showed m/z : 817 ($\text{M}^+ + \text{Na}$) that was consistent with the proposed structure. In the ^{13}C -NMR spectrum of (**4**), the signal of C-3 was observed at 88.9 ppm. Compared with the corresponding carbon of (**2**), the signal was shifted to a 10.9 ppm lower magnetic field. The ^1H - ^1H NOESY spectrum provided the evidence needed for the assignment of anomeric protons. Resonance at 4.88 ppm was due to the H-1 of the G1 glucosyl residue (Fig. 1-1) by connectivity to the H-3 at 3.33 ppm. The coupling constant $J_{1,2}$ of G1 was 8.0 Hz, indicating the β configuration. From these analytical data, the structure of (**4**) was confirmed.

Furthermore, methyl oleanolate oligoglycosides were synthesized from α -cyclodextrin as a donor and (**4**) as an acceptor by transglucosylation of CGTase. By silica-gel column chromatography of the *n*-BuOH extracts, three compounds were isolated, and their structures were determined. From the data of ^1H -NMR, ^{13}C -NMR, and FAB-MS, three compounds were identified as methyl oleanolate-3-yl β -D-(4-*O*- β -D-maltosyl)-glucoside (**5**), methyl oleanolate-3-yl β -D-(4-*O*- β -D-maltotriosyl)-glucoside (**6**), and methyl oleanolate-3-yl β -D-(4-*O*- β -D-maltotetraosyl)-glucoside (**7**), respectively. Details of the identifications are as follows: The ^1H -NMR spectrum of (**5**) was very complicated. However, assignment of each signal was accomplished easily by consideration of the

HOHAHA spectrum. Furthermore, the ^1H - ^1H -NOESY experiment was useful in assigning the anomeric protons. Resonance at 5.86 ppm was due to the H-1 of the G3 glucosyl residue (Fig. 1-1) by connectivity to the H-4 of the G2 glucosyl residue (Fig. 1-1) at 4.25 ppm. The $J_{1,2}$ of G3 was 4.0 Hz, indicating the α configuration. The ^1H - ^{13}C COSY experiment then permitted the assignment of the carbon signal at 81.0 ppm [C-4 of the G2 glucosyl residue, (Fig. 1-1)]. The signal was shifted to a 9.6 ppm lower magnetic field compared to the corresponding carbon of (4). These NMR data indicated that (5) was formed by the α -(1,4) transglucosylation to the non-reducing end of (4). From these data, the structure of (5) was confirmed. In addition, the FAB-MS spectra showed m/z : 979 ($\text{M}^+ + \text{Na}$). In the same manner as above, the structures of (6) and (7) were also confirmed.

1.3.2 Termiticidal activities against *R. speratus*

Methyl oleanolate (2) and synthesized methyl oleanolate glycosides (3)-(7) were submitted to no-choice bioassays for termiticidal activities. Results of the bioassays are shown in Table 1-2 and Fig. 1-3. Termites under complete starvation had an average survival of 0.7 % after the 3-week test period, compared with 80 % in the control. Survival rate of termites exposed to cellulose powder treated with aglycon (2) was 83%. In the case of the cellulose powder treated with methyl oleanolate glycosides (3)-(7), survival rates were less than that treated with aglycon (2). However, differences in termite survival rates were not observed at a significant level among (3)-(7) indicating that there was no clear correlation between the length of sugar chains of methyl oleanolate glycosides and their termiticidal activities. In addition, all of (3)-(7) did not exhibit such low survival rates as observed under the complete starvation.

From these results of no-choice bioassay, it is suggested that termiticidal activities of (3)-(7) were not acute and that they could be caused by

antifeedant activities of (3)-(7). Therefore, antifeedant activities were also examined.

Table 1-2. Survival rates of the workers of *R. speratus* at the end of the test period of the termiticidal bioassay.

	Mean $\pm$ SE (%)
Cellulose	79.3 $\pm$ 0.67 a
Starvation	1.3 $\pm$ 1.33 c
(1)	84.0 $\pm$ 2.4 a
(2)	86.7 $\pm$ 0.67 a
(3)	42.0 $\pm$ 7.02 b
(4)	38.7 $\pm$ 5.21 b
(5)	26.7 $\pm$ 12.8 bc
(6)	38.0 $\pm$ 19.2 bc
(7)	45.3 $\pm$ 5.46 b

Notes: These values indicate the averages of survival rates $\pm$ standard errors (N=3). Survival rates followed by the same letter (a, b, c) are not statistically significant at $P = 0.05$.

1.3.3 Antifeedant activities against *R. speratus*

Disc weight losses due to termite feeding on discs treated with (2)-(7) were examined by the two-choice bioassay method. Differences in weight losses between two methyl oleanolate glycosides with different glucocyl unit were compared (Table 1-3 and Fig. 1-4). (2) showed antifeedant activity, and (3) showed greater antifeedant activity than (2). No difference was observed between (3) and (4). Also, the activities decreased with increases in the number of sugar residues. Consequently, it was found that (3) and (4) had the greatest antifeedant activities among the compounds tested.

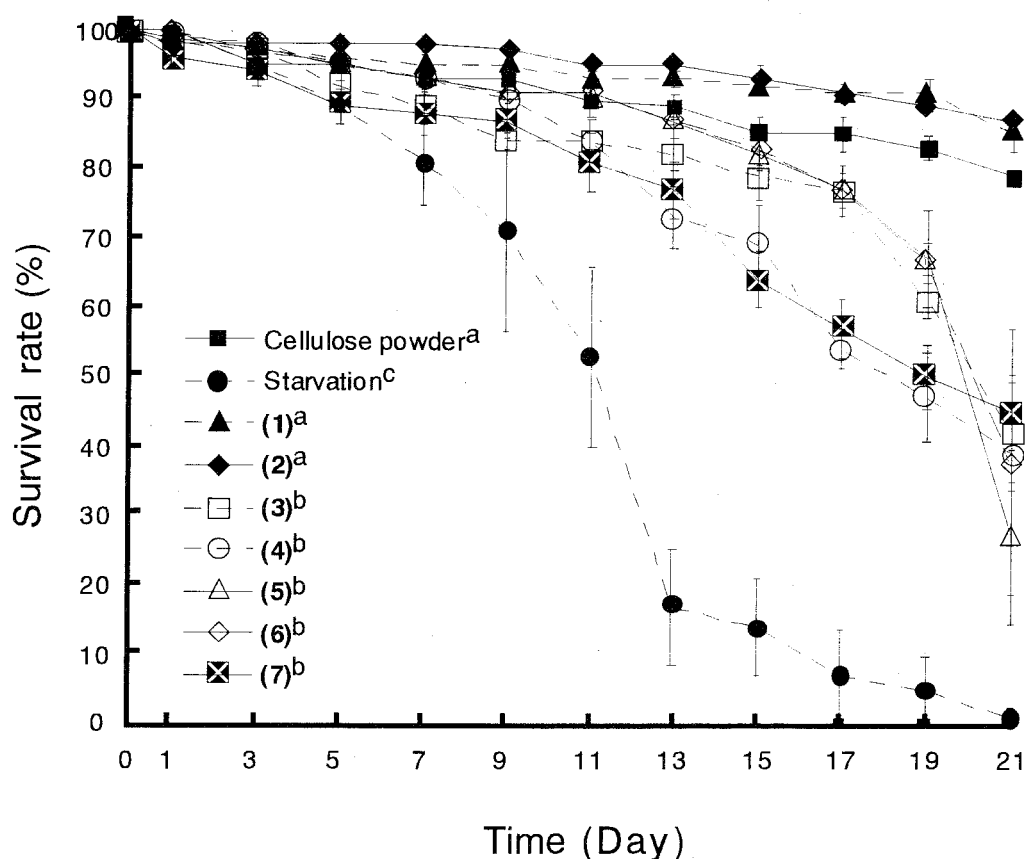


Fig. 1-3. Results of termiticidal bioassay of *R. speratus*.

Notes : At the end of the test period, survival rates followed by the same letter (a, b, c) are not significant statistically at $P = 0.05$. Error bars indicate $\pm$ standard errors ($N = 3$).

Two factors are considered to be related to the greatest antifeedant activities of (3) and (4). One is the configurations of the glycosidic linkages, and another is the adequate polarities of triterpenoid saponins. (3) and (4) have β -glycosidic linkages only in their molecules, but other saponins, (5)-(7), have both α - and β -ones. As the author found that *R. speratus* eat cellulose and starch equally (Ohmura, unpublished data), it may be said that the former factor is not so important. On the other hand, molecular hydrophilicity was found to be related closely to human sweet- or bitter- tastes (Acton and Stone, 1976).

Table 1-3. Differences in weight losses of the discs (mg) treated with methyl oleanolate (2) and methyl oleanolate glycosides (3)-(7) in the two-choice bioassays for *R. speratus*.

	Mean $\pm$ SE (mg)
Cont. – Cont.	0.9 $\pm$ 0.25
Cont. – (2)	7.38 $\pm$ 0.16*
(2) – (3)	6.67 $\pm$ 0.22*
(3) – (4)	0.4 $\pm$ 2.69
(4) – (5)	-4.73 $\pm$ 0.72*
(5) – (6)	-5.63 $\pm$ 0.28*
(6) – (7)	-5.6 $\pm$ 0.91*

Notes : These values indicate the averages of differences in weight losses of the discs $\pm$ standard errors (N=3). Differences were calculated by subtracting the amounts of weight losses of the discs treated with right side compounds from those treated with left side compounds. Thus, positive values indicate that right side compounds have higher antifeedant activity than left side compounds. Cont. means control disc.

* means significantly different from Cont.-Cont. at the 0.05 level.

As aglycon (2) is a hydrophobic compound, and glucose is a hydrophilic one, it is regarded that the molecular hydrophilicities of triterpenoid saponins increase with the increasing numbers of glucose residues. Therefore, the above results of the greatest antifeedant activities of (3) and (4) are considered attributable to the adequate molecular polarities of both compounds. From these viewpoints, it may be assumed that adequate molecular polarity is an important factor for termite-antifeedant activities of triterpenoid saponins.

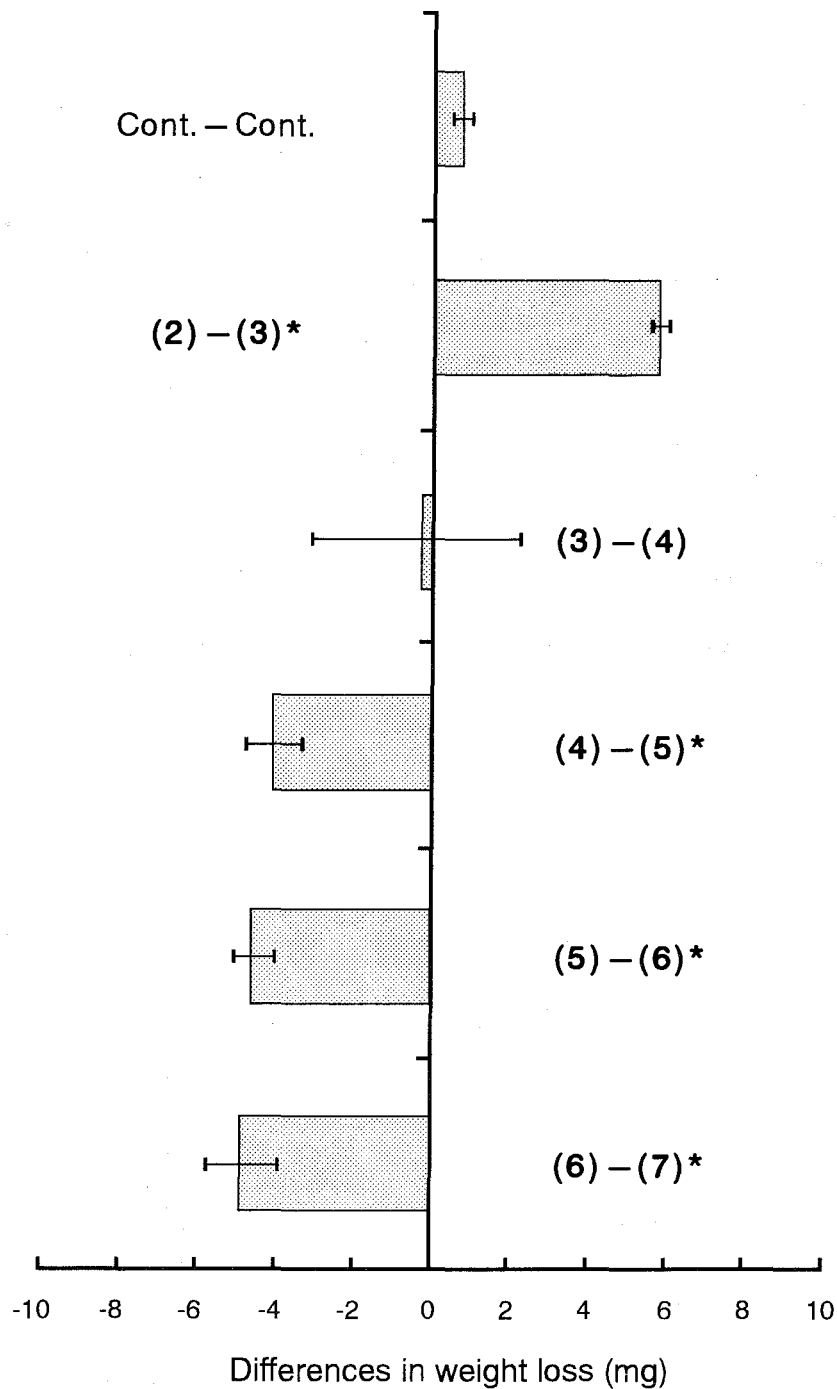


Fig. 1-4. Differences in weight losses of the discs treated with methyl oleanolate (2) and methyl oleanolate glycosides (3)-(7) in the choice bioassay with *R. speratus*.

Notes : Differences were calculated by subtracting the amounts of weight losses of the discs treated with right side compounds from those treated with left side compounds. Cont means a control disc. Error bars indicate $\pm$ standard errors (N = 3). * means significantly different from Cont.-Cont. at the 0.05 level.

1.3.4 Antifeedant activities against *C. formosanus*

Table 1-4 and Fig. 1-5 show the results of the choice tests against *C. formosanus*. (2) also showed antifeedant activity against *C. formosanus*, but there was no significant difference in weight loss in each test from that in the control test at the 0.05 level excepting tests for untreated control versus (2), and (4) versus (5). Thus, the results of antifeedant activity against *C. formosanus* were not clear compared to those against *R. speratus*. Higher retention of test compounds on discs might be needed for *C. formosanus* to show statistically significant differences between the difference in weight loss obtained from each test and that from the control test.

Table 1-4. Differences in weight losses of the discs (mg) reated with methyl oleanolate (2) and methyl oleanolate glycosides (3)-(6) in the two-choice bioassay for *C. formosanus*.

	Mean $\pm$ SE (mg)
Cont. – Cont.	3.83 $\pm$ 0.85
Cont. – (2)	22.7 $\pm$ 1.70*
(2) – (3)	-15.6 $\pm$ 6.21
(3) – (4)	9.07 $\pm$ 2.34
(4) – (5)	-13.6 $\pm$ 2.15*
(5) – (6)	-11.2 $\pm$ 2.52

Notes : These values indicate the averages of differences in weight losses of the discs $\pm$ standard errors (N=3). Differences, Cont. and * are the same as in Table 1-3.

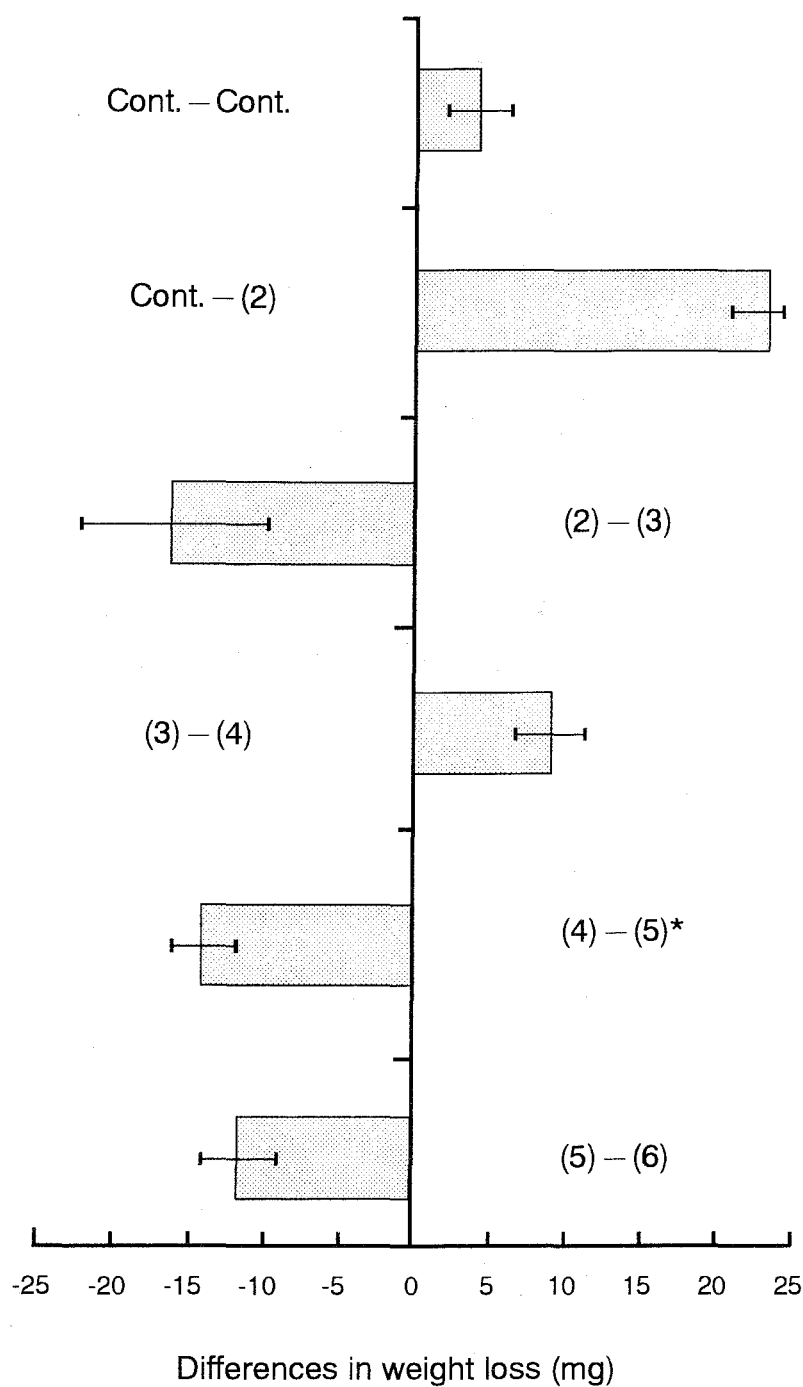


Fig. 1-5. Differences in weight losses of the discs treated with methyl oleanolate (2) and methyl oleanolate glycosides (3)-(6) in the choice bioassay with *C. formosanus*.

Notes : Differences were calculated by subtracting the amounts of weight losses of the discs treated with right side compounds from those treated with left side compounds. Cont. means control disc. Error bars indicate $\pm$ standard errors (N = 3). * means significantly different from Cont.-Cont. at the 0.05 level.

1.4 Summary

Some methyl oleanolate glycosides were synthesized by Königs-Knorr type condensations and enzymic reactions to investigate the relationships between the structures and the antitermitic activities of triterpenoid saponins. Synthesized saponins were subjected to termiticidal and antifeedant bioassays with *R. speratus*. In the termiticidal bioassays, all synthesized saponins demonstrated greater activities than their aglycon, methyl oleanolate, although no clear correlation was found between the sugar-chain structures and the termiticidal activities. On the other hand, methyl oleanolate-3-yl β -D-glucoside and methyl oleanolate-3-yl β -D-cellobioside showed the greatest antifeedant activity on *R. speratus*, and the activity decreased according to the lengthening of the chain of the sugar moiety. Because the molecular hydrophilicity increases with the increasing amounts of sugar residues, it is assumed that adequate polarity is necessary to reveal the antitermitic activities of triterpenoid saponins. The results of antifeedant activity against *C. formosanus* were not clear compared to those of *R. speratus*. Higher retention of test compounds on discs might be needed for *C. formosanus* to show statistically significant differences between the difference in weight loss obtained from each test and that from the control test.

Chapter 2 : Antifeedant Activity of Flavonoids and Related Compounds Against the Subterranean Termite, *Coptotermes formosanus* Shiraki

2.1 Introduction

Flavonoids are widely distributed in plants, and it is assumed that they are related to the resistance toward attacks by insects and fungi in several plant species. For instance, taxifolin and aromadendrin show antifungal activity against several wood-rotting fungi (Malterud *et al.*, 1985). Also, taxifolin inhibits the growth of *Heliothis zea* larvae (Elliger *et al.*, 1980), and exhibits antitermitic activity against the West Indian drywood termite, *Cryptotermes brevis* (Walker) (Wolcott, 1953) and Formosan subterranean termite, *C. formosanus* (Chapter 3).

Many pest control agents have been developed from phytochemical lead compounds (Arnason *et al.*, 1993). They are generally non-persistent and show the mild modes of action. Thus, plant extracts may be used as promising alternatives for pest control agents in future. In Chapter 2, the antifeedant effects of some flavonoids and their related compounds against *C. formosanus* were investigated to obtain the better understanding of antitermitic activity of flavonoids. Both choice tests and no-choice tests were used to assess the potency of each compound as an antifeedant (Ohmura *et al.*, 1999a, to be published).

2.2 Materials and methods

Instruments used in this study were the same as described in Chapter 1.

2.2.1 Preparation of test materials

Kaempferol and catechin were purchased from Wako Pure Chemical Industries Ltd., and fisetin, phloretin and myricetin were from Extrasynthese (France).

Quercetin was isolated from Japanese larch heartwood according to the procedure described by Takehara and Sasaya (1979). Its NMR and FAB-mass spectra coincided with those in the literature.

Eriodictyol, genistein, sakuranetin and isosakuranetin were isolated from the woods of *Prunus* species by the method described by Hasegawa and Shirato (1957). These NMR spectral data were in agreement with those published (Pelter and Ward, 1978; Mizuno *et al.*, 1987; Liu *et al.*, 1992).

Taxifolin, aromadendrin and naringenin were isolated from Japanese larch wood as follows. One kg of Japanese larch wood meal passing 1 mm screen mesh was extracted with 4 L of MeOH for 48 h at room temperature. The MeOH extracts were dried on a rotary evaporator, and dissolved in 2-butanone. The 2-butanone solution was evaporated under reduced pressure, and the residue was sequentially extracted with *n*-hexane and ethyl acetate. The ethyl acetate soluble fraction was separated by chromatography on a silica-gel (70-230 mesh, Merck) column eluted with benzene/acetone (9/1, v/v). Taxifolin, aromadendrin and naringenin were isolated by repeating recrystallization from a mixture of benzene and tetrahydrofuran. These compounds were identified by comparison of FAB-mass spectra and melting points with authentic samples (Takehara and Sasaya, 1979).

There are possibly several isomers as to the stereochemistry of flavonoids, but only one isomer has been identified so far as a natural product. The absolute configuration of the 2-position of flavanones is commonly 2*S*, and that of the enzymically derived 3-hydroxyflavanones is designated 2*R*. (Heller and Forkmann, 1988; Stafford, 1990).

Catechinic acid was prepared by a base-catalyzed reaction of catechin at pH 12, 100 °C (Sears *et al.*, 1974) and purified by Sephadex LH-20 column chromatography using ethanol as an eluent. The ¹H-NMR spectrum of the methyl ether derivative was in agreement with that published (Sears *et al.*, 1974).

These compounds were submitted to the following bioassays. Their chemical structures are shown in Fig. 2-1.

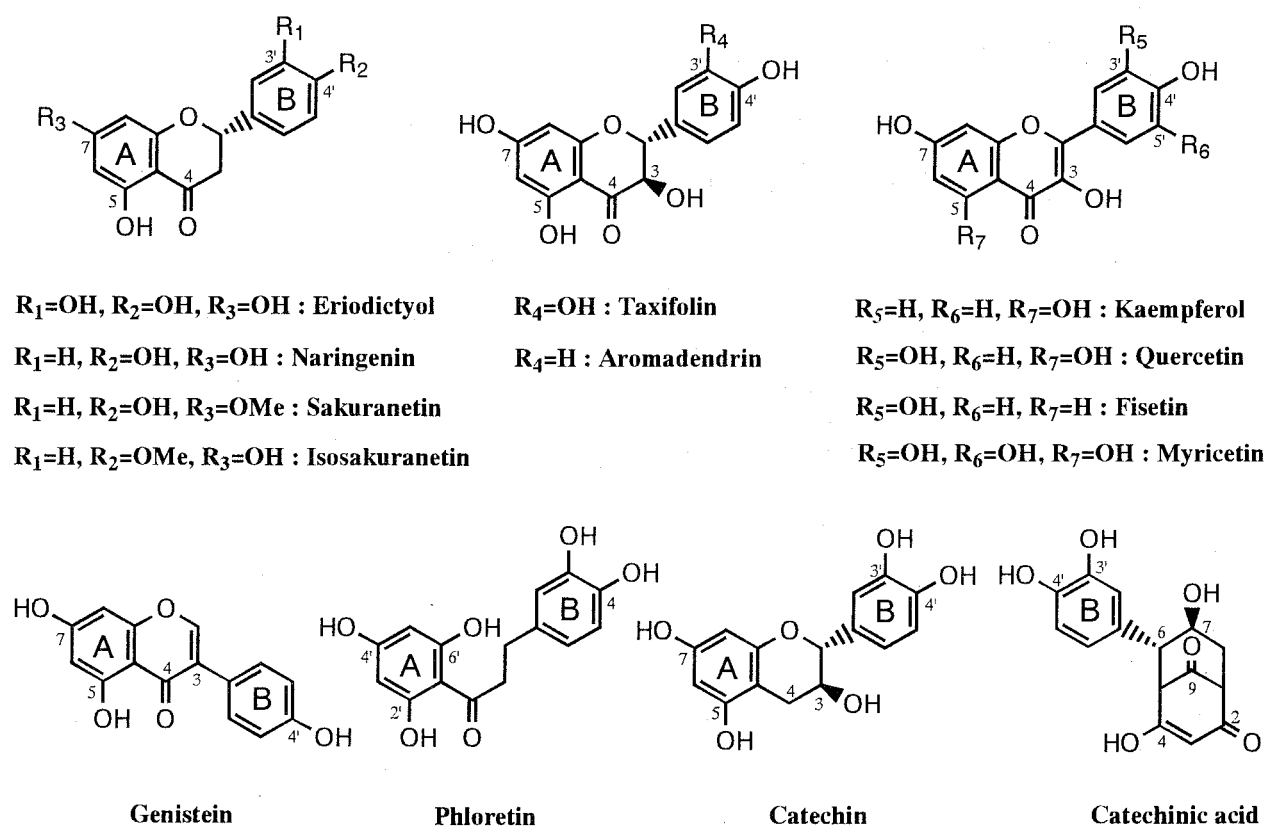


Fig. 2-1. Structures of tested compounds.

2.2.2 Termite used for bioassays

The test termite, *C. formosanus* was collected from a laboratory colony maintained in the Forestry and Forest Products Research Institute. The colony has

been reared on wood pieces in the dark at 28 ± 2 °C, 80 % R. H. for more than 15 years. Only pseudoergites (workers) above third instar were used in the bioassays.

2.2.3 Antifeedant bioassay

No-choice and two-choice bioassays were conducted in this study. A test container was made of a plastic cup (rim diameter; 6 cm, bottom diameter; 5 cm, height; 5 cm) with a hard plaster bottom. The bottom was covered with 10 g of sea sand (30-50 mesh: Junsei Chemical Co., Ltd.), and moistened with 2 ml of deionized water. Paper discs (diameter ; 13 mm, Whatman International Ltd.) were permeated with 60 µl of the MeOH solutions containing each of the test compounds. The treatment retentions was 1.0 % (w/w) per disc. The control discs were untreated. The discs were dried at 60 °C for 12 h followed by drying in a vacuum desiccator for one day. In the no-choice test, a 15 mm diam. plastic saucer holding a disc was placed on the center of the test container, and 50 termites were introduced into the container. In the two-choice test, the two plastic saucers supporting a disc permeated with a sample solution and a control disc, respectively, were placed diagonally 12 mm away from the center of the test container, and 100 termites were introduced. After three days, the discs were taken out and dried in the same manner mentioned above to determine the weight loss of each disc. Three replications were made for each test compound in the two methods of bioassays.

On the basis of the weight losses of the discs, the indices of the activity of the test compounds were calculated (Daniewski *et al.*, 1995). In the no-choice bioassay, the absolute coefficient of antifeedancy (A) was obtained by the following equation.

$$A = [(KK-EE)/(KK+EE)] \times 100 (\%) \quad (1)$$

where KK and EE mean the weight losses of the control and treated discs, respectively.

In the two-choice bioassay, the relative coefficient of antifeedancy (R) was calculated by the following equation.

$$R = [(K-E)/(K+E)] \times 100 (\%) \quad (2)$$

where K and E represent the weight losses of the control and treated discs, respectively.

The total coefficient of antifeedancy (T) is;

$$T = A + R \quad (3)$$

Thus, negative values of T indicate that the compounds have feeding-preference activity, and positive ones values of T indicate the compounds have antifeedant activity. The maximum value of T reaches 200 for a complete antifeedant. All compounds tested were classified into the following six classes according to their T values;

feeding preference : $T < 0$

class I : $0 \leq T < 50$

class II : $50 \leq T < 100$

class III : $100 \leq T < 150$

class IV : $150 \leq T < 200$

class V : $T = 200$

2.3 Results and Discussion

2.3.1 Antifeedant activity of test compounds

As shown in Table 2-1, all compounds tested except catechinic acid showed antifeedant activity. Four flavonoids obtained from Japanese larch wood (taxifolin, aromadendrin, quercetin and naringenin) showed the high activities. According to the classification due to the T values of test compounds, quercetin, taxifolin, and naringenin were classified into class IV, and aromadendrin was

classified into class III. Other flavonoids were classified into class III, except for catechin. Catechin was classified into class II.

Table 2-1. Antifeedant activity and functional groups of test compounds

Compounds	T value ^c	class ^d	Hydroxylation			C-4 carbonyl group ^e	Other substituents
			A-ring	B-ring	Pyran ring		
Quercetin	162.6	IV	5, 7	3', 4'	3	○	
Taxifolin	154.2	IV	5, 7	3', 4'	3	○	
Naringenin	153.6	IV	5, 7	4'		○	
Isosakuranetin	144.1	III	5, 7			○	4'-methoxyl (B-ring)
Aromadendrin	137.3	III	5, 7	4'	3	○	
Phloretin^a	134.3	III					
Myricetin	132.7	III	5, 7	3', 4', 5'	3	○	
Sakuranetin	131.9	III	5	4'		○	7-methoxyl (A-ring)
Eriodictyol	118.5	III	5, 7	3', 4'		○	
Genistein	117.5	III	5, 7	4'		○	
Fisetin	111.6	III	7	3', 4'	3	○	
Kaempferol	100.9	III	5, 7	4'	3	○	
Catechin	98.4	II	5, 7	3', 4'	3		
Catechinic acid^b	-32.1	feeding preference		3', 4'			

Notes : ^{a,b} The numbering systems are different from those of the other compounds (See Fig. 2-1).

^c T is the total coefficient of antifeedancy which is equal to A plus R. A is the absolute coefficient of antifeedancy, $[(KK-EE)/(KK+EE)] \times 100$ (%) of test compounds in the no-choice bioassay, where KK and EE represent the weight losses of the control and treated discs, respectively. R is the relative coefficient of antifeedancy, $[(K-E)/(K+E)] \times 100$ (%) in the two-choice bioassay, where K and E represent the weight losses of the control and treated discs, respectively.

^d feeding preference (T<0), class I (0≤T<50), class II (50≤T<100), class III (100≤T<150), class IV (150≤T<200).

^e The mark (○) indicates the existence of C-4 carbonyl group in the molecule.

2.3.2 Effects of A-ring structures on the antifeedant activity

Methylation of hydroxyl groups at C-7 in the A-ring and at C-4' in the B-ring of naringenin gives sakuranetin and isosakuranetin, respectively. They showed antifeedant activity less than naringenin, although the latter had a higher activity than the former. These results suggest that hydroxyl groups at C-7 in A-rings have a larger effect on the activity than those at C-4' in B-rings, and that C-7 hydroxyl groups of A-rings are necessary for high antifeedant activity. Because quercetin showed antifeedant activity higher than fisetin, the hydroxyl group at C-5 in A-rings also plays an important role in the antifeedant activity.

Catechinic acid, which is a base-catalyzed reaction product of catechin revealed feeding-preference activity. It possesses no A-ring and pyran ring in the molecule, although the B-ring remains unchanged (Fig. 2-1). This supports the importance of A-ring or pyran ring for the antifeedant activity.

2.3.3 Effects of B-ring structures on the antifeedant activity

The growth of the corn earworm, *Heliothis zea* was inhibited by *ortho* hydroxylated flavonoids (Elliger *et al.*, 1980). Thus, naringenin and kaempferol which have a hydroxyl group only at C-4' in B-rings showed no antigrowth activity. In the present study, both naringenin and kaempferol showed antifeedant activity. Quercetin showed antifeedant activity higher than kaempferol, indicating that two vicinal phenolic hydroxyl groups on B-rings would be necessary for enhancing the activity. This was supported by the higher activity of taxifolin than aromadendrin. On the contrary, eriodictyol showed the lower activity than naringenin, although the former contains 3',4'-dihydroxylated B-ring and the latter contains 4'-hydroxylated B-ring. Both eriodictyol and naringenin possess no hydroxyl group at C-3 in pyran rings, whereas quercetin, kaempferol, taxifolin and

aromadendrin contain it in the molecules. Therefore, it is assumed that the presence of hydroxyl group at C-3 in pyran rings enhances the effect of the hydroxyl groups at C-3' in B-rings on the antifeedant activity.

Myricetin showed lower antifeedant activity than quercetin, but showed higher activity than kaempferol. This result shows that 3',4'-dihydroxylated B-rings are more effective than 3', 4', 5'-trihydroxylated ones, followed by 4'-hydroxylated ones

2.3.4 Effects of pyran ring structures on the antifeedant activity

A dihydrochalcone, phloretin, showed less antifeedant activity than naringenin. This compound has the same structure as naringenin except for the absence of the pyran ring. From this result, the pyran ring was found to be important for the antifeedant activity. Sanderman and Dietrichs (1957) classified the antitermitic substances into three structural types, *i.e.* stilbenes, quinones and pyran derivatives. The importance of the pyran ring of flavonoids for the activity confirmed in this study is in good agreement with their report.

Taxifolin with the carboxyl group at C-4 showed the higher activity than catechin with the methylene group at C-4. Furthermore, only catechin was classified into class II, whereas all other flavonoids tested, having the carbonyl group at C-4, were classified into class III or IV. Therefore, the carbonyl group in pyran rings is considered to be necessary for the high antifeedant activity.

2.4 Summary

Both the choice and no-choice tests were conducted in order to evaluate the antifeedant activity of some flavonoids and their related compounds against the subterranean termite, *C. formosanus*. All test compounds showed antifeedant

activity, whereas only catechinic acid, which possesses no A-ring and pyran ring in the molecule, showed feeding-preference activity. From the results, the following conclusions concerning structure-activity relationships have been obtained.

1. Both C-5 and C-7 hydroxyl groups in A-rings are necessary for the high activity.
2. The 3-hydroxyflavones and 3-hydroxyflavanones with 3',4'-dihydroxylated B-rings show higher activity than those with 4'-hydroxylated or 3',4',5'-trihydroxylated B-rings.
3. The presence of the carbonyl group at C-4 in pyran rings is essential for the high activity.
4. The cleavage of the ether linkage of the pyran ring decreases the activity.

These suggest that some flavonoids such as quercetin and taxifolin might be useful for termite control agents considering their abundance in plants.

PART II

PREFERENTIAL BEHAVIORS OF TERMITE ENHANCED BY COMPONENTS IN THE TREATED WOOD

**Chapter 3 : Components of Streamed and Non-Steamed Japanese
Larch (*Larix leptolepis* (Sieb. et Zucc.) Gord.)
Heartwood Affecting the Feeding Behavior of
Coptotermes formosanus Shiraki**

**Chapter 4 : Trail-following Substances Produced by Brown-rot
Fungi, *Gloeophyllum trabeum* (Pers. ex Fr.) Murr. and
Serpula lacrymans (Wulf. ex Fr.)**

**Chapter 3 : Components of Steamed and Non-Steamed Japanese
Larch (*Larix leptolepis* (Sieb. et Zucc.) Gord.)
Heartwood Affecting the Feeding Behavior of the
Subterranean Termite, *Coptotermes formosanus*
Shiraki**

3.1 Introduction

Steam treatment has often been applied to improve the penetrability, the color or the drying rate of lumbers and boards (Brauner and Conway 1964; Kubinsky and Ifju 1973; Chen 1975; Chen and Workman Jr. 1980). Recently, it was reported that when the steam-treated heartwood boards from Japanese larch (*Larix leptolepis* (Sieb. et Zucc.) Gord.) were used as interior walls of a house, they were severely attacked by *Coptotermes formosanus* Shiraki. This attractive effect depended on the water soluble fraction from the hot water extracts of steamed larch boards (Doi *et al.* 1998).

Heartwood extractives such as terpenoids, quinones, stilbenes and flavonoids are known to exhibit strong feeding-deterrence activity against termites (Scheffrahn 1991). These substances are closely related to the feeding resistance of woods to termites. On the other hand, it was reported that a termite species, *Neotermes bosei* Snyder was attracted by some kinds of carbohydrates, fatty acids, amino acids and organic solvents (Mishra 1992), and that a subterranean termite, *C. formosanus* preferably fed on some amino acids such as D-aspartic acid and L-glutamic acid (Chen and Henderson 1996).

In this chapter, the components of the steamed and the non-steamed Japanese larch heartwoods affecting the feeding-preference and feeding-deterrence activities to *C. formosanus* were investigated to obtain the better understanding of

the reasons why termites like the steamed larch wood (Ohmura *et al.*, 1999b, to be published).

3.2 Materials and methods

3.2.1 Hot water extraction of Japanese larch heartwood

Boards of Japanese larch measuring 150(tangential) × 20(radial) × 1200(longitudinal) mm were steamed at 170 °C for 60 min. in a 500 L autoclave. After steaming, the boards were air-dried and ground to obtain the wood meal passing 1 mm screen mesh. One hundred grams of wood meal of steamed larch was extracted with 1000 ml of deionized water under reflux for 8 h. The extraction process was repeated five times and water was replaced for each process. The combined hot-water extracts were concentrated to approximately 300 ml under reduced pressure. Hot-water extracts from the non-steamed larch wood were also prepared by the same procedure as above. These extracts were stored below -20 °C for the fractionation processes and the bioassays.

3.2.2 Fractionation of the hot water extracts

Hot-water extracts from the steamed larch wood were extracted sequentially with *n*-hexane, diethyl ether and ethyl acetate. These three organic solvent-soluble fractions were dehydrated with anhydrous sodium sulfate and dried on a rotary evaporator to give the *n*-hexane extract (S-Hex), the diethyl ether extract (S-Ether) and the ethyl acetate extract (S-EtOAc). The residual water layer was freeze-dried to give the water-soluble fraction (S-Water). A part of the S-Water (1300 mg) was chromatographed on a Suprapak-2 (Supelco) column that was

eluted first with 3000 ml of deionized water (3 subfractions), and then with 500 ml of each of 25% MeOH, 50% MeOH and 100% MeOH.

Hot-water extracts from the non-steamed larch wood were also fractionated through the same procedure mentioned above to obtain the *n*-hexane extract (N-Hex), the diethyl ether extract (N-Ether), the ethyl acetate extract (N-EtOAc), and the residual water soluble fraction (N-Water).

3.2.3 NMR and MS spectroscopy

The NMR spectra of the fractions obtained were measured with JEOL LAMBDA-400 and ALPHA-500 spectrometers. Acetone- d_6 , CD_3OD and D_2O were used as the solvents. The MS spectra were obtained using a JEOL JMS-600H spectrometer.

3.2.4 Thin layer chromatography

A thin layer chromatography (TLC, silica gel 60 F₂₅₄, Merck), developed with toluene/ethyl formate/formic acid (5/4/1, v/v/v), was used to examine the extracts.

3.2.5 Isolation of taxifolin and aromadendrin

One kg of Japanese larch wood meal passing 1 mm screen mesh was extracted with 4 L of MeOH for 48 h at room temperature. The MeOH extracts were dried on a rotary evaporator, and dissolved in 2-butanone. The 2-butanone solution was evaporated under reduced pressure, and the residue was sequentially extracted with *n*-hexane and ethyl acetate. The ethyl acetate soluble fraction was separated by chromatography on a silica-gel (70-230 mesh, Merck) column eluted

with benzene/acetone (9/1, v/v). Taxifolin and aromadendrin were isolated by repeating recrystallization from a mixture of benzene and tetrahydrofuran. These compounds were identified by the comparison with the MS spectra and melting points of authentic samples (Takehara and Sasaya 1979).

3.2.6 Quantitative analyses of flavonoids

The ethyl acetate soluble fraction from the MeOH extracts of the non-steamed larch wood (N-MeOH/EtOAc) was subjected to a high performance liquid chromatography (HPLC) for the quantification of flavonoids. Waters μ Bondasphere 5 μ C₁₈ 100 Å column (150 × 3.9 mm i.d., Waters) was employed for the determination of flavonoids: eluent: MeOH/0.2% H₃PO₄ (40/60, v/v); flow rate: 0.5 ml/min.; Detector: UV 280 nm. The amounts of taxifolin and aromadendrin were determined using the calibration curves of these compounds which were isolated from larch wood.

3.2.7 Quantitative analyses of 5-hydroxymethylfurfural

The quantitative analyses of 5-HMF in the S-Hex, the S-Ether, the S-EtOAc and the S-Water were made by means of a HPLC method. The HPLC was carried out using a Shimadzu CLC-ODS(H) column (250 × 4.6 mm i.d.) and a detector of UV 283 nm. The eluent of H₂O/CH₃CN (99/1, v/v) flew at a rate of 1.0 ml/min. The content of 5-HMF was calculated from a calibration curve determined using a commercial reagent (Wako Pure Chemical Industries Ltd.).

3.2.8 Quantitative analyses of coniferyl aldehyde and vanillin

The quantitative analyses of coniferyl aldehyde and vanillin in the S-Hex, the S-Ether, the S-EtOAc and the S-Water were made by means of a HPLC method. The HPLC was carried out using a Shimadzu CLC-ODS(H) column (250 × 4.6 mm i.d.) and a detector of UV 280 nm. The mobile phase was a gradient mixture of 0.086 % (v/v) H₃PO₄ (A) and MeOH/CH₃CN (1/1, v/v) (B) flew at a rate of 1.0 ml/min. A correct separation was obtained with the following elution gradient : 0-20 min. (A/B=95/5), 20-50 min. (from A/B=95/5 to A/B=80/20), 50-80 min. (A/B=80/20). The content of each compound was calculated from a calibration curve determined using a commercial reagent (Wako Pure Chemical Industries Ltd.).

3.2.9 Sugar analyses

Sugar compositions of the hydrolysate of the hot-water extracts of the steamed and the non-steamed larch woods were determined by a HPLC technique according to the methods of Effland (1977) and Pettersen *et al.* (1984). An Aminex HPX-87P column (300 × 7.8 mm i.d., Bio-Rad) was employed for the determination of monosaccharides. The column was maintained at 85 °C. The flow rate of water as an eluent was 0.5 ml/min. *Meso*-erythritol was used as an internal standard.

3.2.10 Two-choice tests using *C. formosanus*

Two-choice feeding test was carried out using termites collected from a laboratory colony of *C. formosanus*, that was maintained at 28 ± 2 °C and 80 % relative humidity (RH) at the Forestry and Forest Products Research Institute, Tsukuba, Japan. Only pseudoergites (workers) above third instar were used for the feeding tests.

A test chamber was made of a plastic cup (rim diam.; 60 mm, bottom diam.; 50 mm, height; 50 mm) with a 20 mm diam. hole each in the bottom. Five grams of hard plaster powder was mixed with 4 ml of deionized water, and the suspension was poured into the bottom of the chamber. After the plaster hardened, the cups were layered with 10 g of sea sands (30-50 mesh: Junsei Chemical Co., Ltd.) and moistened with 2 ml of deionized water.

Each fraction obtained from the hot-water extracts of the steamed or the non-steamed larch woods was dissolved in 100 ml of CHCl_3 , MeOH or deionized water. Paper discs (13 mm in diam., Whatman International Ltd.) were permeated with 60 μl of each of the solutions, and dried at 60 °C for 12 h followed by vacuum drying for 24 h. The control disc was untreated, but dried in the same procedure.

In the two-choice feeding test, one disc treated with a sample solution and one control disc were put on the separate saucers in the chamber. For control test, two control discs were put into the separate saucer. These plastic saucers were placed on diagonally opposite sides of the chamber. One hundred workers were introduced per chamber. The chambers were maintained in the dark at 26 ± 2 °C and 75 % RH for 3 d. Three to five replicates were set for each test. Two flavonoids (taxifolin and aromadendrin) and arabinogalactan, which are main components of the extracts of the non-steamed larch heartwood, were also subjected to the two-choice tests. The treatment retentions of flavonoids were 0.1, 0.5 and 1.0 % (w/w) per disc, and those of arabinogalactan were 1.0, 5.0 and 10.0 % (w/w) per disc. Vanillin was assayed at 0.01, 0.1 and 1.0 % (w/w) per disc, and 5-HMF and coniferyl aldehyde were also assayed at 0.2 and 0.02 % (w/w) per disc, respectively. Arabinogalactan used was isolated from larch wood (Tokyo Kasei Kogyo Co., Ltd.).

After the test period, the paper discs were weighed to determine the weight losses by the termite attack. Preference or deterrence in feeding was evaluated by getting positive or negative value of the difference in weight losses

between treated and control discs. Then, a feeding index (FI) was calculated using the following equation:

$$FI (\%) = (S - C_S) / (C_A + C_B) \times 100$$

where S is the average weight loss of treated discs (mg); C_S is the average weight loss of control discs (mg); C_A and C_B are the average weight losses of control discs when using two control discs in a chamber (mg).

Differences between the difference in weight loss obtained from each test and that from the control test were statistically analyzed using the *t*-test.

3.3 Results and Discussion

3.3.1 Components of non-steamed larch wood affecting the feeding behavior of *C. formosanus*

The non-steamed larch woods extracted with hot water were preferred by *C. formosanus*, whereas the non-steamed, unextracted ones were hardly attacked (Doi *et al.* 1998). This indicates the hot-water extracts of the non-steamed larch wood included the feeding-deterrence substances. In the present study, each fraction obtained from the hot-water extracts of the non-steamed larch wood was subjected to the two-choice tests using *C. formosanus*. The N-Ether showed the feeding-deterrence activity ($P < 0.01$), while the N-Water showed the feeding-preference activity ($P < 0.05$). The other two fractions (N-Hex and N-EtOAc) showed neither feeding-preference nor feeding-deterrence activities significantly ($P > 0.05$) (Table 3-1 and Fig. 3-1). Japanese larch heartwood is rich in flavonoids (Demachi *et al.* 1968; Sasaya *et al.* 1980). The results of the ^1H - and ^{13}C -NMR analyses of the N-Ether indicated the presence of taxifolin and aromadendrin, which agreed with those of the TLC analysis preliminarily conducted.

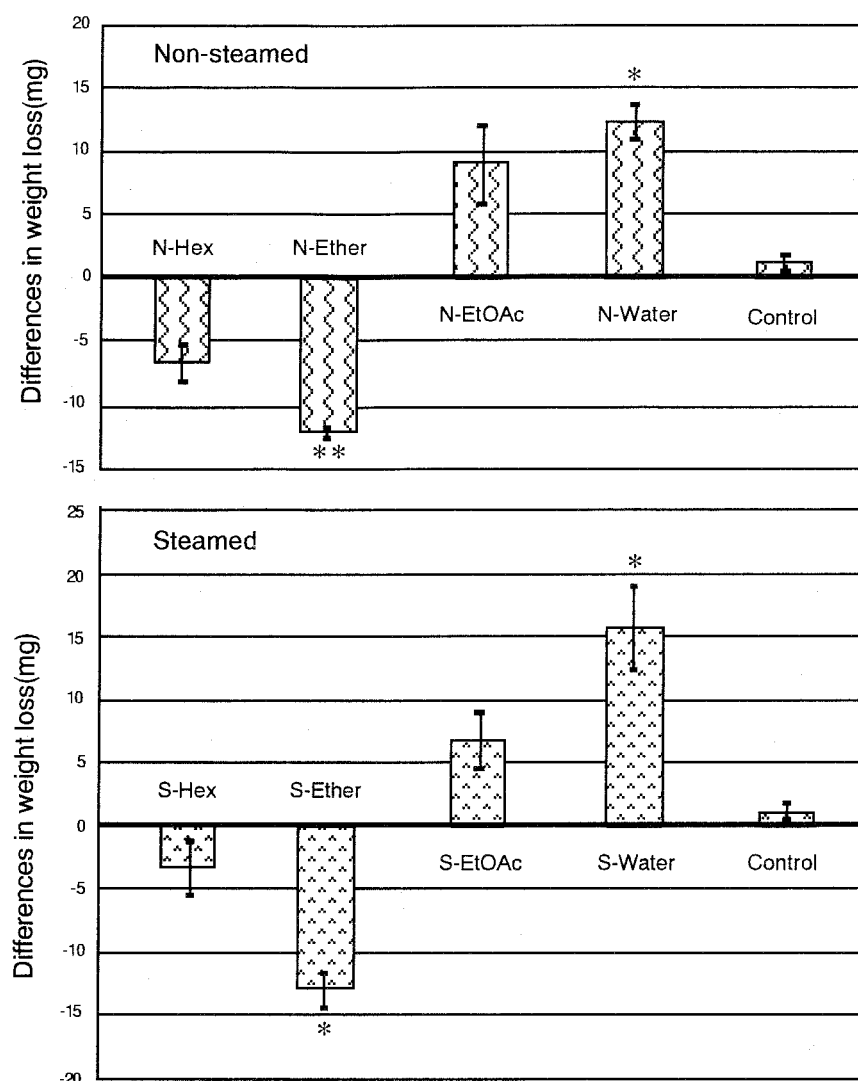


Fig. 3-1. Differences in weight loss (mg) of the paper discs permeated with each fraction obtained from the hot-water extracts of the non-steamed and the steamed larch woods in the two-choice feeding tests.

Notes : N-Hex, N-Ether, N-EtOAc, and N-Water are the *n*-hexane extract, the diethyl ether extract, the ethyl acetate extract, and the residual water soluble fraction from the non-steamed larch wood, respectively. S-Hex, S-Ether, S-EtOAc, and S-Water are the *n*-hexane extract, the diethyl ether extract, the ethyl acetate extract, and the residual water soluble fraction from the steamed larch wood, respectively. Differences were calculated by subtracting the amounts of weight losses of control discs from those of treated discs. In the control two-choice tests, differences between two control discs were calculated. Error bars indicate $\pm$ standard errors (N=3). ** and * mean significantly differences from the control two-choice tests at the 0.01 and 0.05 levels, respectively.

Table 3-1. Feeding indices (%) of the paper discs permeated with each fraction obtained from the hot-water extracts of the non-steamed and the steamed larch wood samples in the two-choice feeding tests.

Fraction	Non-steamed sample	Steamed sample
<i>n</i> -Hexane	-27.4	-13.9
Diethyl ether	-49.6	-52.4
Ethyl acetate	36.5	28.0
Water	49.6	63.4

The N-MeOH/EtOAc was analyzed with the HPLC in order to determine these flavonoids. The HPLC showed two main peaks, which was confirmed to be taxifolin and aromadendrin due to the coincidence of retention times with authentic samples. The contents of taxifolin and aromadendrin were 43.5 and 8.7 % on the N-MeOH/EtOAc, respectively, and these values corresponded to 1.76 and 0.35 % on wood, respectively. Then, they were subjected to the termite-bioassays because these compounds possibly effect the termite feeding. Taxifolin showed the feeding-deterrence activity at the retention of 1.0 % ($P < 0.05$) in the two-choice tests (Table 3-2 and Fig. 3-2), suggesting that taxifolin content was high enough to exhibit the feeding-deterrence activity for the non-steamed larch wood. On the other hand, aromadendrin did not show the significant feeding-deterrence activity at the tested retentions.

It is well known that *Larix* species contain appreciable amounts of arabinogalactan, usually within the wide range of 5-30 % (Côté *et al.* 1966). Aspinall *et al.* showed that Japanese larch heartwood contained 4.8 % of arabinogalactan (1968). Then, this polysaccharide was also subjected to the two-choice feeding tests because this was possibly contained in the N-Water that showed the feeding preference activity. As expected, there were distinct feeding-preference activities at 1-10 % retentions of arabinogalactan ($P < 0.01$) (Table 3-2

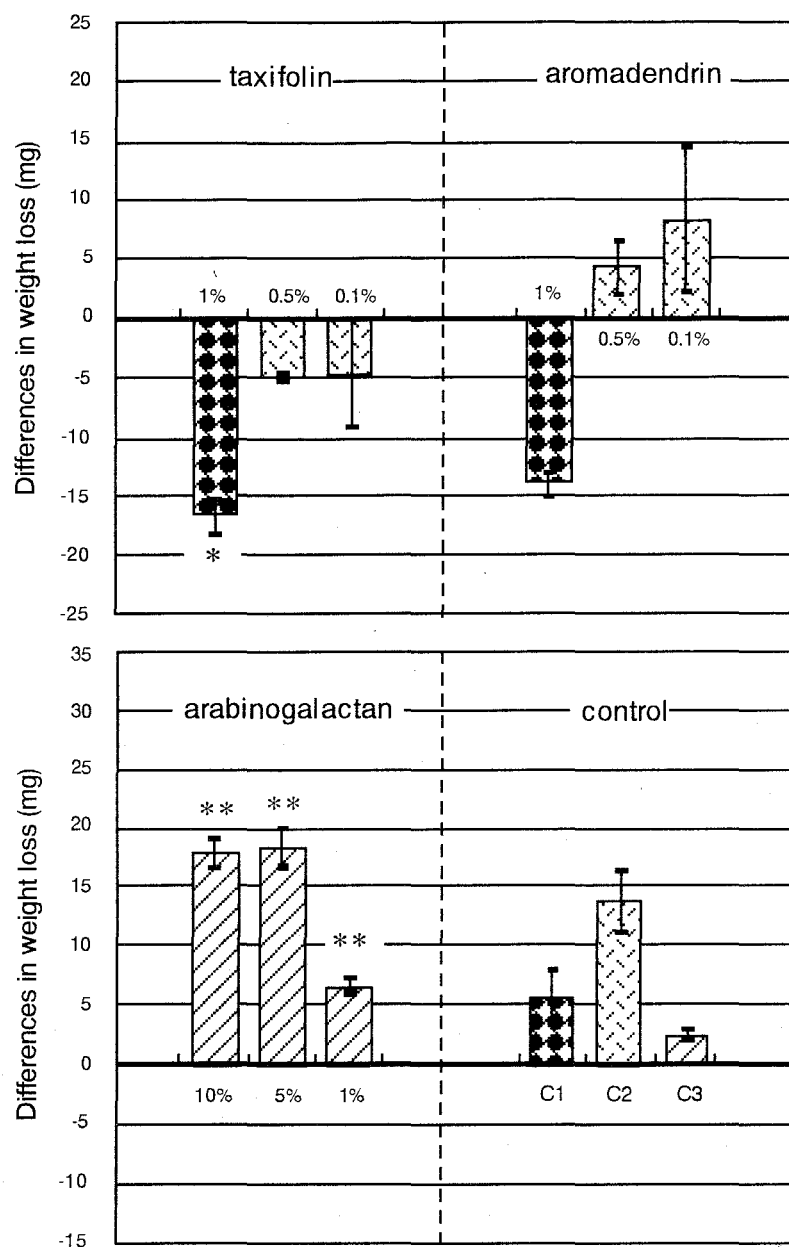


Fig. 3-2. Differences in weight loss (mg) of the paper discs permeated with taxifolin, aromadendrin and arabinogalactan in the two-choice feeding tests.

Notes : C1 means the results of the control two-choice tests of taxifolin and aromadendrin at 1.0 % retention. C2 means the results of the control two-choice tests of taxifolin and aromadendrin at 0.5 and 0.1 % retentions. C3 means the results of the control two-choice tests of arabinogalactan. Differences were calculated by the same way as in Fig. 3-1. Error bars, ** and * are the same as in Fig. 3-1.

Table 3-2. Feeding indices (%) of taxifolin, aromadendrin and arabinogalactan in the two-choice feeding tests.

Tested sample	Retention (%w/w)	Feeding index (%)
Taxifolin	0.1	-9.33
	0.5	-23.1
	1.0	-77.8
Aromadendrin	0.1	37.3
	0.5	18.7
	1.0	-65.3
Arabinogalactan	1.0	33.3
	5.0	91.4
	10.0	88.9

and Fig. 3-2). Thus, this is one of the contributors to the feeding-preference activity of the termite to Japanese larch wood.

3.3.2 Components of steamed larch wood affecting the feeding behavior of *C. Formosans*

The steamed larch woods extracted with hot water were hardly attacked by *C. formosanus*, whereas the steamed, unextracted ones were severely attacked (Doi *et al.* 1998). These results suggest the hot-water extracts of the steamed larch include the feeding-preference substances. The S-Water from the steamed larch wood showed the feeding-preference activity ($P<0.05$) while the S-Ether showed the feeding-deterrence activity ($P<0.05$) (Table 3-1 and Fig. 3-1). In the ^{13}C -NMR spectrum of the S-Water, many resonances characteristic of saccharides were observed in the 60-105 ppm region. The resonances at 80-90 ppm, which were assigned to sugar carbons linked to other anomeric carbons by ether bonds, were not so high as the other carbons. These results indicate that the main components of

the S-Water are not polysaccharides but monosaccharides and low-molecular weight oligosaccharides. Furthermore, several signals due to acetyl groups were observed, suggesting that the S-Water contains partially acetylated saccharides. As

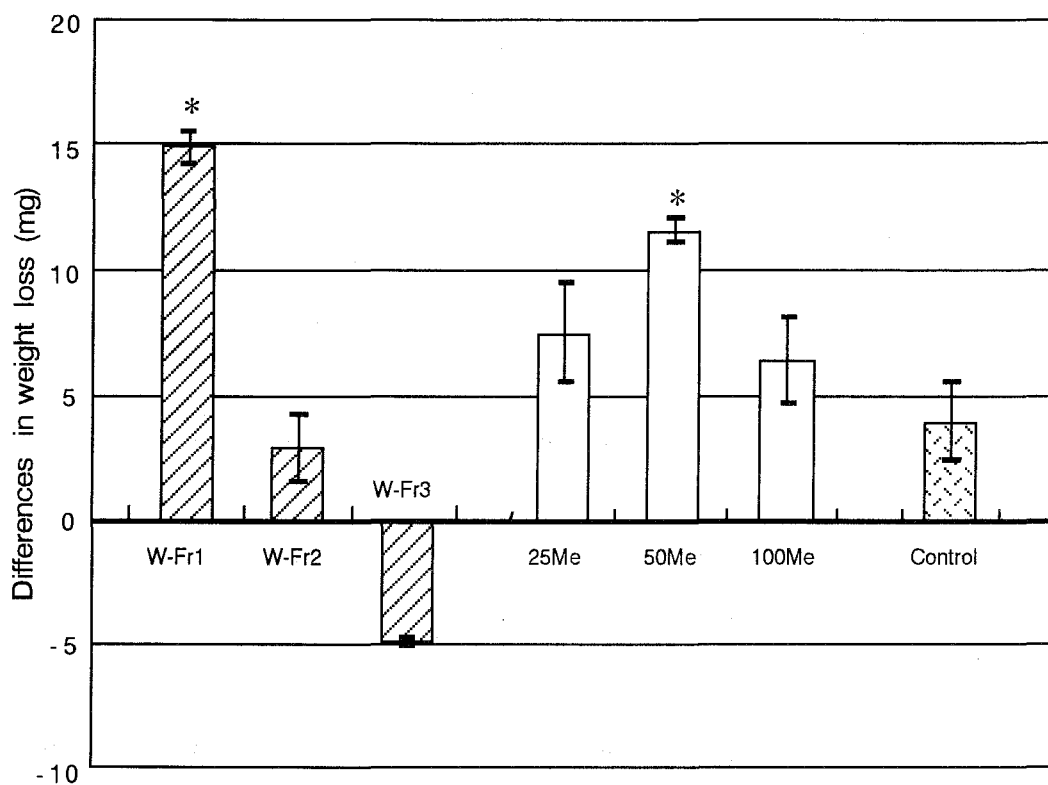


Fig. 3-3. Differences in weight loss of the paper discs permeated with each fraction obtained from the S-Water in the two-choice feeding tests.

Notes : The S-Water was the water soluble fraction from hot-water extract of steamed larch wood. W-Fr1, W-Fr2 and W-Fr-3 are subfractions of water eluates, and 25Me, 50Me and 100Me are the 25% MeOH eluate, 50% MeOH eluate and 100% MeOH eluate from the S-Water using the Superpak-2 column, respectively. Error bars indicate $\pm$ standard errors (N=3). Differences were calculated by the same way as in Figs. 3-1 and 3-2. * means significantly difference from the control tests at the 0.05 level.

Table 3-3. Feeding indices (%) of vanillin, coniferyl aldehyde and 5-hydroxymethylfurfural in the two-choice feeding tests.

Tested sample	Retention (%w/w)	Feeding index (%)
Vanillin	0.01	9.06
	0.1	61.1
	1.0	-145
Coniferyl aldehyde	0.02	-1.33
5-Hydroxymethylfurfural	0.2	-11.5

the S-Water also contained the yellow-colored substances, this fraction was chromatographed with a Superpak-2 resin to eliminate the colored substances as well as to obtain pure saccharides. Results of bioassays showed that both the first fraction of the water eluates and 50 % MeOH eluate had significant feeding-preference activity ($P < 0.05$, Fig. 3-3). This suggests that several constituents contribute to the feeding-preference activity of the steamed larch.

The S-Ether showed the feeding-deterrence activity although taxifolin and aromadendrin were not detected by the TLC method. Furthermore, in the ^{13}C -NMR spectrum of the S-Ether, any signals characteristic of flavonoid skeletons were not observed. These results suggest that flavonoids undergo some degradation reaction during steaming of larch wood. On the other hand, coniferyl aldehyde, vanillin and 5-HMF were detected by the HPLC analysis of the S-Ether and other fractions. The content of vanillin, coniferyl aldehyde and 5-HMF on the steamed larch wood was 0.02, 0.02 and 0.2 %, respectively. Vanillin and coniferyl aldehyde might be caused by the degradation of lignin. As to 5-HMF, it is well known that furfural and its condensates are formed by the steaming of hardwood (Ishihara *et al.*, 1996). In this study, it has been confirmed that 5-HMF is formed also from larch wood steamed at 170 °C. The results of two-choice tests of vanillin, 5-HMF and coniferyl aldehyde are shown in Table 3-3 and Fig. 3-4. Vanillin

tended to show the feeding-preference activity at 0.01 % retention, whereas coniferyl aldehyde and 5-HMF tended to show feeding-deterrence activity at 0.02 and 0.2 % retention, respectively, although they were not statistically significant at those retentions ($P>0.05$). From these results, it is assumed that the S-Water makes the largest contribution to the feeding behavior of *C. formosanus*.

Results of sugar analyses of hot-water extracts from the non-steamed and the steamed larch woods are shown in Fig. 3-5. The main sugars in the non-steamed larch wood were arabinose and galactose, which suggests that arabinogalactan is a main carbohydrate of the hot-water extracts. In the steamed larch wood, the ratios of arabinose and galactose decreased and those of mannose and xylose increased. These results suggest that arabinogalactan was converted to other substances such as 5-HMF during the steaming of larch wood, and that a part of hemicellulose came to be easily extracted by hot-water extraction.

The steamed Japanese larch heartwood showed the largest consumption by *C. formosanus* among seven wood species (*L. leptolepis*, *Cryptomeria japonica*, *Pinus densiflora*, *Thujopsis dolabrata*, *Picea jezoensis*, *Abies sachalinensis*, and *Fagus crenata*) after the steam treatment (Doi *et al.* 1999). Japanese larch heartwood contains flavonoids in enough amounts to reveal the feeding-deterrence activity as well as arabinogalactan as the feeding-preference substance. As stated above, taxifolin is considered to play an important role in the protection of larch wood against termite attack. Therefore the decomposition of taxifolin during the steam treatment seems to be closely related with the attraction of the steamed larch wood to the termite. The feeding-deterrence substances such as taxifolin in the non-steamed larch wood might be decomposed more easily than those of the other wood species.

On the reaction behaviors of larch wood components during the steam treatment, it has been confirmed that the following three reactions occur; 1) the decomposition of taxifolin, 2) the conversion of arabinogalactan to other

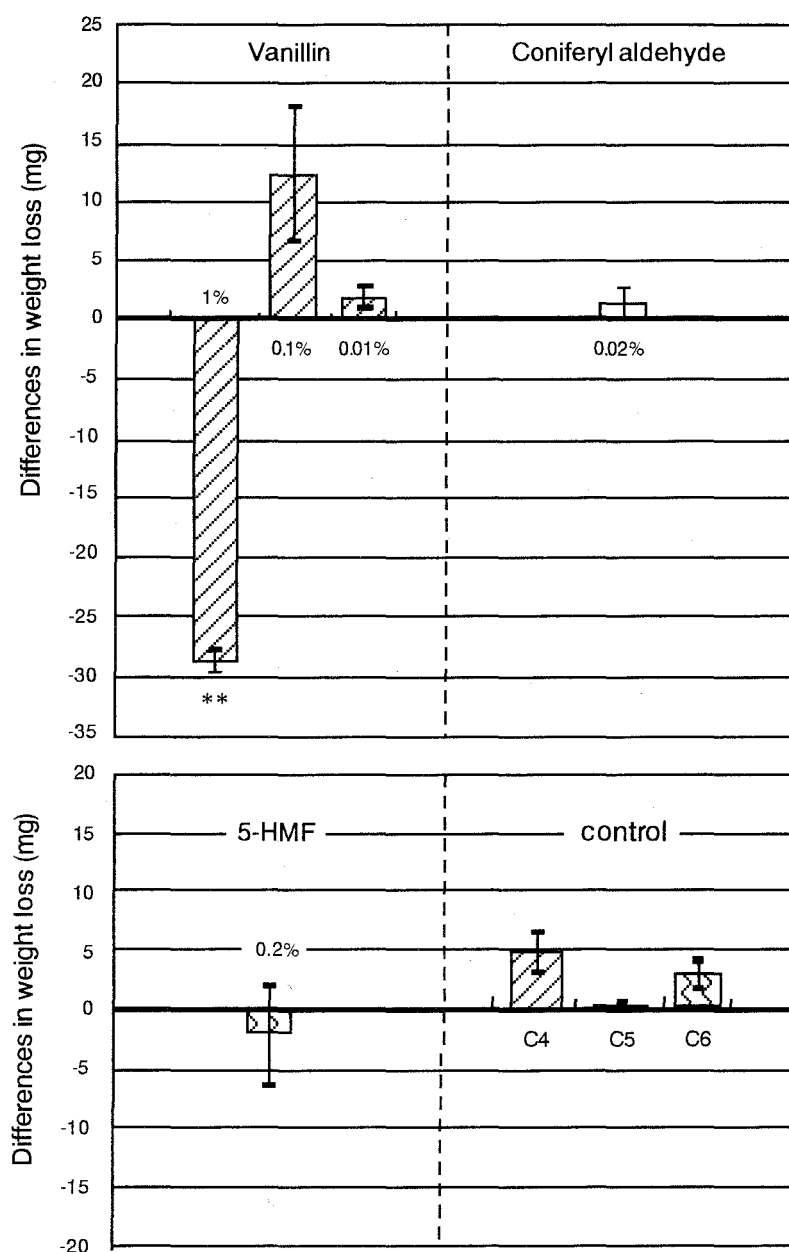


Fig. 3-4. Differences in weight loss (mg) of the paper discs permeated with vanillin, coniferyl aldehyde and 5-hydroxymethylfurfural (5-HMF) in the two-choice feeding tests.

Notes : C4 means the results of the control two-choice tests of vanillin at 1.0, 0.1 and 0.01 % retention. C5 and C6 mean the results of the control two-choice tests of coniferyl aldehyde and 5-HMF at 0.02 and 0.2 % retentions, respectively. Differences were calculated by the same way as in Fig. 3-1. Error bars and ** are the same as in Fig. 3-1.

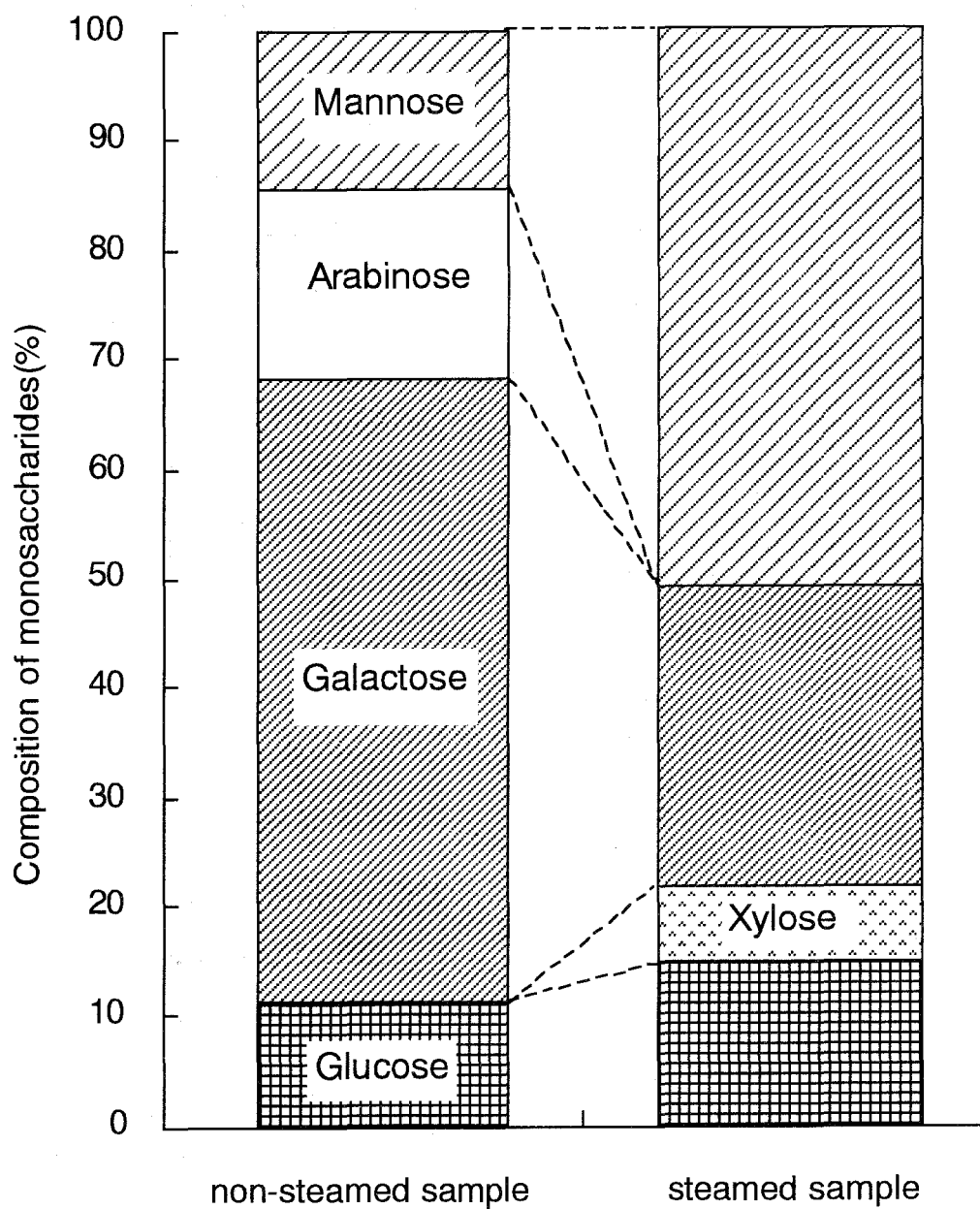


Fig. 3-5. Sugar compositions of the hot-water extracts of the non-steamed and the steamed larch wood samples.

Notes : Mannose (▨), Arabinose (□), Galactose (▩), Xylose (▤), Glucose (▧).

substances, 3) the formation of 5-HMF. It was reported that catechin was converted to *ent*-epicatechin when it was steamed at 220 °C for 20 min. (Ohara 1998). In another experiment, the author found that when taxifolin suspended in 0.2 M acetate buffer (pH=3.46) was heated at 170 °C for 60 min. in a stainless autoclave, it was converted to alphononin in a yield of *ca* 50 %. Alphononin is a kind of auronols which have furan rings in their molecules. The remaining taxifolin was found to be isomerised at both 2- and 3-positions. Aromadendrin was also converted to an auronol, maesopsin, although neither naringenin nor quercetin was converted to their corresponding auronols. Comparing the reaction behaviors of these flavonoids, it was supposed that carbonyl groups at the 4-positions of flavonoid compounds might be necessary to form auronols during steam treatment. Pyran rings of taxifolin and aromadendrin are thought to be opened to form furan rings during steaming. No flavonoid has been detected in the steamed larch, suggesting that further reaction might be occurred with other wood components during steam treatment when the pyran rings of flavonoids were opened.

3.4 Summary

The attraction of steamed Japanese larch heartwood to the subterranean termite, *C. formosanus* was investigated. Hot-water extracts of the steamed and the non-steamed larch woods were sequentially extracted with *n*-hexane, diethyl ether, and ethyl acetate. Furthermore, the residual water soluble fraction of the steamed larch wood was fractionated by column chromatography using a Superpak-2 resin. Preference or deterrence in feeding was assessed in the two-choice tests method using paper discs permeated with each fraction. The diethyl ether extracts of the non-steamed larch wood showed the feeding-deterrence activity, and this fraction was the most important one affecting the feeding behavior of the termite. Taxifolin, which was the main component of the diethyl ether extracts, was found to have the

feeding-deterrence activity. In the case of the steamed larch wood, taxifolin was not detected in any fractions, and the residual water-soluble fraction showed the feeding-preference activity. Furthermore, it was found that several constituents in the residual water-soluble fraction contributed to the feeding-preference activity of the steamed larch. From these results, the degradation of taxifolin and the formation of new hydrophilic substances during the steam treatment were considered to be important factors of the attraction of steamed larch wood to the termite.

Chapter 4 : Trail-Following Substances Produced by Brown-rot Fungi, *Gloeophyllum trabeum* (Fr.) Murr. and *Serpula lacrymans* (Wulf.: Fr.) Schroet.

4.1 Introduction

Chapter 3 deals with a termite-steamed wood interaction. Chapter 4 deals with an interaction between termite and wood decayed by brown-rot fungi. Esenther *et al.* (1961) found that the North American subterranean termite, *Reticulitermes flavipes* (Kollar) was easily attracted by wood decayed by *Gloeophyllum trabeum* (Fr.) Murr. The fungus produced substances analogous to a trail-following pheromone, (Z,Z,E)-3,6,8-dodecatrien-1-ol (DTE-OH) commonly found in most rhinotermitids (Matsumura *et al.*, 1969). A brown-rot fungus *Serpula lacrymans* (Wulf.: Fr.) Schroet. was reported to have a termite-attractant activity similar to *G. trabeum* (Becker, 1965). Matsuo and Nishimoto (1973, 1974) who worked on the involvement of wood decayed by some fungi in terms of termite attraction and feeding activity of termites, concluded that a couple of chemical substances of *S. lacrymans*-decayed wood were related to termite arrestant activity. However, it was not determined whether one of the substances was identical with DTE-OH. Recently, Sugamoto *et al.* (1990) showed that *S. lacrymans* was more attractive to *C. formosanus* than *G. trabeum* in feeding termites when using the wood decayed at approximately 15 % of weight losses.

In this chapter, chemical substances originated from wood decayed by *S. lacrymans* and *G. trabeum* were used for the determination of their termite trail-following activities by bioassay. Various chemical analyses were concurrently conducted to relate the extracts to the known trail-following pheromone, DTE-OH. Another series of experiments were designed for *G. trabeum* to chemically confirm the production of DTE-OH by the fungus, because the precise identification had not

been made yet except for the comparative demonstration with a synthesized compound with regard to trail-following activity (Ohmura *et al.*, 1995).

4.2 Materials and Methods

4.2.1 Test fungi

Two brown-rot fungi, *Gloeophyllum trabeum* (Fr.) Murr. (FFPRI 6268) and *Serpula lacrymans* (Wulf.: Fr.) Schroet. (IFO 8697) were used to prepare microbially attacked wood and mass of mycelia.

4.2.2 Wood substrate

Wood chips were prepared from sapwood of Akamatsu (*Pinus densiflora* Sieb. et Zucc.) and served for fungal exposure.

4.2.3 Test termite

Externally undifferentiated mature larvae (workers) of *C. formosanus* were obtained from a laboratory colony. The colony was originally collected in Wakayama Prefecture and maintained in the dark at 28 ± 2 °C and approximately 80 % R.H. for more than 10 years at the Wood Research Institute of Kyoto University.

4.2.4 Preparation of *S. lacrymans*-decayed wood

Wood chips of *P. densiflora* (80 g) were first sterilized with ethylene oxide gas and put into a glass jar containing 400 g quartz sand and 110 ml nutrient

solution as medium. Nutrient solution contained 2.0 g $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, 3.0 g KH_2PO_4 , 25.0 g glucose, 10.0 g malt extracts and 5.0 g pepton in 1000 ml distilled water (nutrient solution A).

After inoculation with a monoculture of *S. lacrymans*, the glass jars were incubated at $20 \pm 2^\circ\text{C}$ for 4 months. Total amount of 16 kg of solid wood chips was used for decay.

4.2.5 Preparation of *G. trabeum* mycelia

A glass filter (Whatman GF/A, 70 mm in diameter) was similarly sterilized and placed on the 2 % agar medium containing nutrient solution A in a petri dish. After inoculation, the dishes were incubated at $26 \pm 2^\circ\text{C}$ for 2 weeks so that 12 g of dried mycelia was finally obtained.

4.2.6 Trail-following bioassay

A modified open-field bioassay technique (Tokoro *et al.*, 1991) was used with a few further amendments.

A 15 cm-long circle line was drawn on a fine quality paper with a pencil for the later chemical application (Fig. 4-1). Two μl of a sample solution from extracts and fractions following the processes as described below, was applicated along the circle line using a 5 μl -micropipette. After evaporation of solvent, a plastic ring (1.5 cm i.d. and 1 cm in height) with two openings was placed on the pencil line so that the openings were positioned on the circle. Furthermore, a petri dish lid wrapped up in red cellophane was placed above the test circle in order to minimize the influence of air blow and light. A worker termite was introduced into the plastic ring and its walking distance along the circle line was measured. Two circles were drawn for a sample and five termites each were

served for assay. The trail-following activities were compared on the basis of the minimum effective concentration (MEC). In the present bioassay, MEC was defined as dry mass of wood before decay or that of mycelia per one ml of solvent. When the mean walking distance of ten termites was above 7.5 cm (semicircle), the sample was regarded as active. All bioassays were carried out at 26 ± 2 °C and approximately 60 % R.H.

4.2.7 Extraction

Three quarters of *S. lacrymans*-decayed wood and all of *G. trabeum* mycelia were extracted with *n*-hexane (Hex) for 5-7 days. Another *S. lacrymans*-decayed wood was extracted with dichloromethane for 5-7 days in order to compare the trail-following activity between the two extracts.

The extracts were dried with anhydrous sodium sulfate and concentrated for the next bioassay and/or fractionation.

4.2.8 Isolation

The extracts were fractionated by silica-gel column chromatography with Hex /ethyl acetate (EtOAc), successively increasing the polarity as shown in Figs. 4-2a and 4-2b.

Subsequently, the active fractions were further fractionated by argentation silica-gel column chromatography containing 20 % (w/w) AgNO₃ in the similar manner to normal silica-gel column chromatography. In case of *S. lacrymans*-decayed wood extraction, the active fractions were prefractionated by HPLC in the same manner as mentioned below before this operation (Fig. 4-2a).

The active fractions were finally fractionated by normal phase high performance liquid chromatography (HPLC) with diethyl ether (Er)/Hex. The

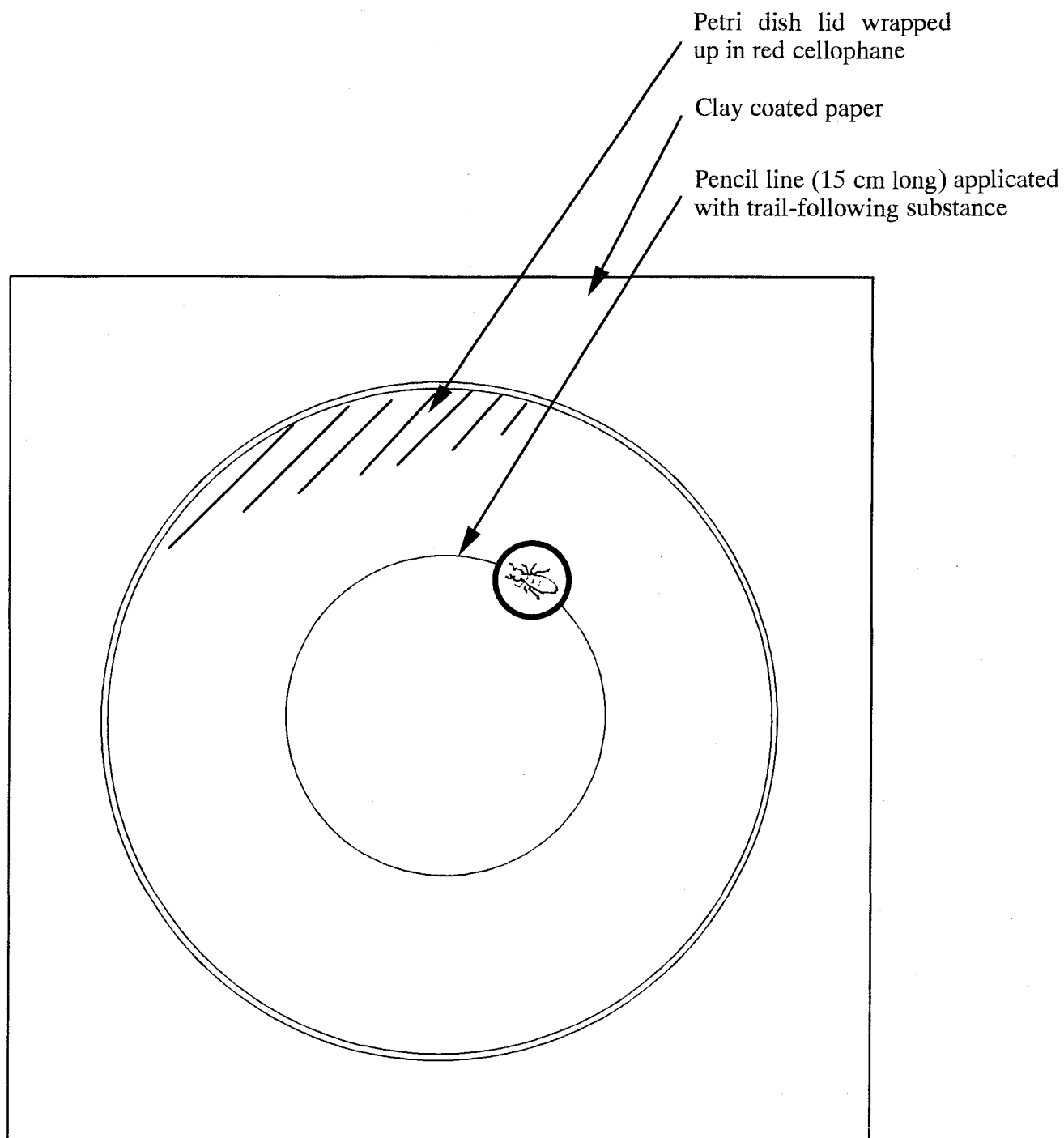


Fig. 4-1. Trail-following bioassay.

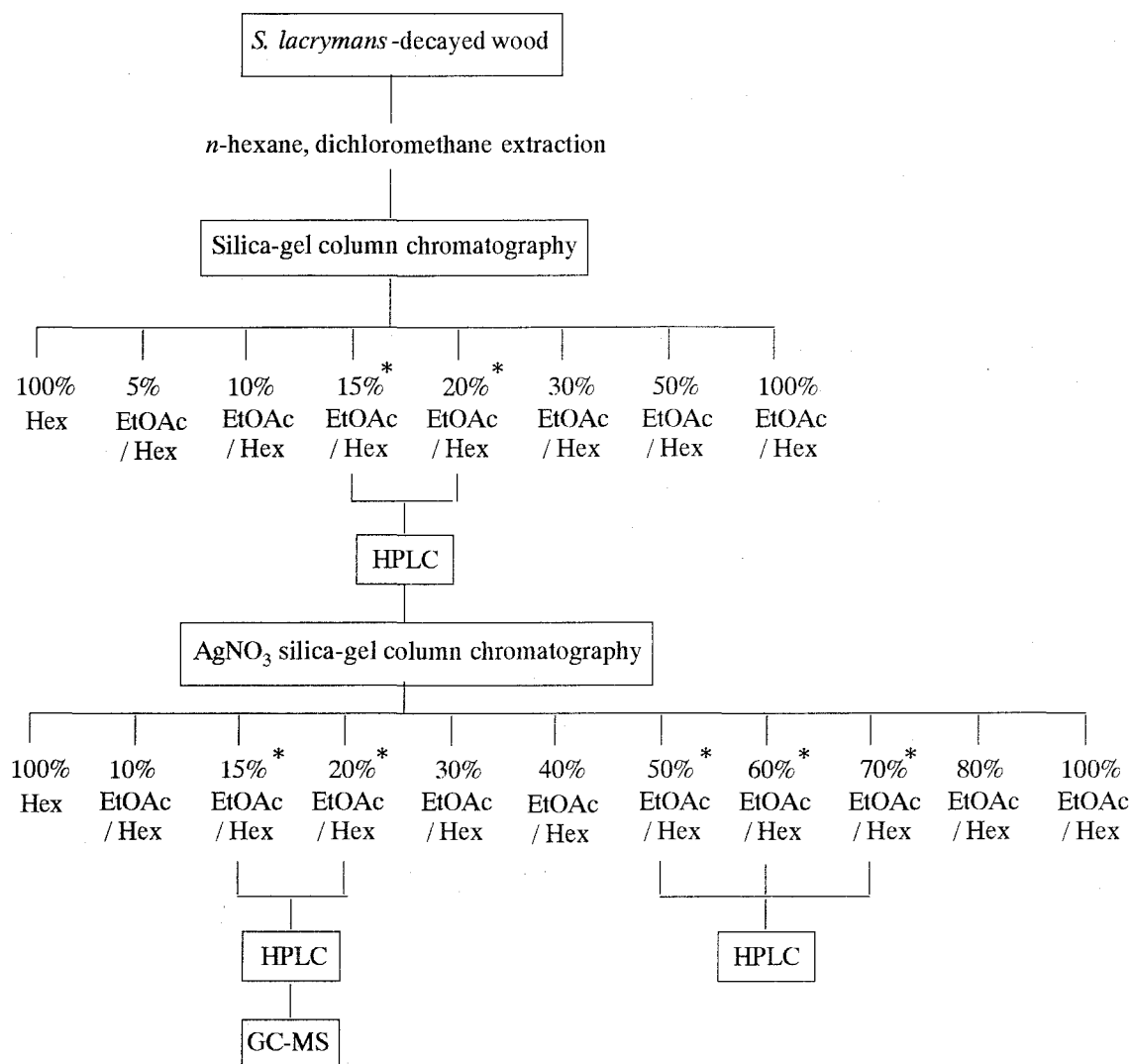


Fig.4-2a. Isolation of trail-following substances from *S. lacrymans* decayed wood.

* : active fraction in bioassay.

apparatus was equipped with both a Cosmosil 5SL column for separation and a Cosmosil 10SL guard column. The conjugated double bond of the substances was detected at 234 nm using UV-VIS spectrophotometer. All fractions were served for bioassays.

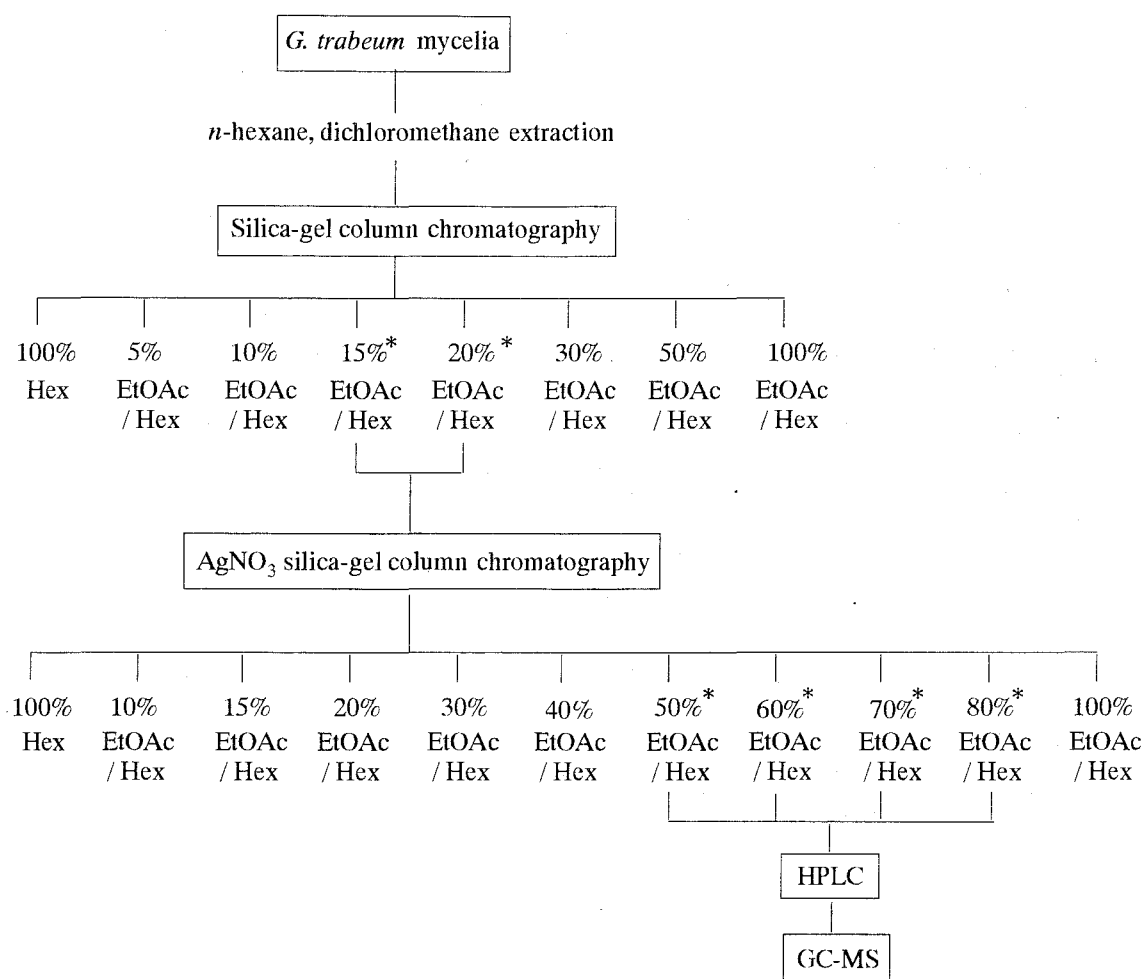


Fig. 4-2b. Isolation of trail-following substances from *G. trabeum* mycelia.

* : active fraction in bioassay.

4.2.9 Identification

The gas chromatographic separations were performed on Shimadzu GC-15A equipped with a flame ionization detector (FID) under the following conditions: fused-silica capillary column, CBP1-W12 (12.5 m × 0.53 mm i.d. Nacalai Tesque); injection port temperature, 250 °C; detector temperature, 350 °C; carrier gas, helium (velocity : 60 cm/sec.). Column temperature was kept at 60 °C for 1 min., programmed from 60 °C to 315 °C at 20 °C/min. and then kept at 315 °C for 10 min.

The capillary gas chromatography mass spectrometry (GC-MS) analysis was carried out on Shimadzu GC-14A connected with QP 1000EX mass spectrometer. Analytical conditions were as follows: fused-silica capillary column, CBP20-M (12.5 × 0.20 mm i.d., Shimadzu Corp.); column temperature, gradient from 60 °C to 220 °C at 20 °C/min.; injection port temperature, 250 °C. The ionization voltage was 70 eV and the ion-source temperature was 200 °C. The GC-MS-SIM analysis was carried out to confirm the existence of DTE-OH. SIM monitors were set at m/z 180, molecular ion of DTE-OH, and m/z 79, 91 and 105 corresponding to the diagnostic fragment ions of it.

4.3 Results and Discussion

4.3.1 Trail-following activity

4.3.1.1 Crude extracts

In order to compare the trail-following activity of each extract, the minimum effective concentration (MEC) was measured. MEC of Hex extracts of *S. lacrymans*-decayed wood was 3.7 g/ml and that of *G. trabeum* mycelia was 2.4×10^{-5} g/ml. MEC of dichloromethane extracts was as high as that of Hex extracts. These two extracts were mixed and used for the next silica-gel column chromatographic analyses.

4.3.1.2 Fractions by silica-gel column chromatography

Both extracts of *S. lacrymans*-decayed wood and *G. trabeum* mycelia showed the trail-following activity in the range of 15-20 % EtOAc/Hex fractions (Figs. 4-2a and 4-2b). As alcohols and sterols were usually eluted in 15-20 %

EtOAc/Hex fractions, the trail-following substances might be alcohol and/or sterol compounds.

4.3.1.3 Fractions by AgNO_3 silica-gel column chromatography

After fractionation by silica-gel column chromatography, the active fractions were further fractionated by AgNO_3 silica-gel column chromatography. Those of *G. trabeum* mycelia were eluted in 60-80 % EtOAc/Hex fractions. It showed that the active compounds of *G. trabeum* might have conjugated double bonds as described earlier (Matsumura *et al.*, 1969). In case of *S. lacrymans*, the active compounds were eluted in both range of 15-20 % EtOAc/Hex and 50-70 % EtOAc/Hex fractions. The 15-20 % EtOAc/Hex fractions were ten times as active as 50-70 % EtOAc/Hex ones. The activity reduced to 1/5 - 1/50 by AgNO_3 silica-gel column chromatography. Though the substances which have conjugated double bonds might exist in the 50-70 % fractions of *S. lacrymans*, the main activity was not due to the substances of these fractions.

4.3.1.4 Fractions by normal phase HPLC

The active fractions of *S. lacrymans* were separated into about 150 subfractions by HPLC using 15 % Et/Hex as a mobile phase in order to get precise fractions, and finally 5 active fractions were given. Their retention time ranged from 24 to 43 min. The retention time of synthesized DTE-OH was 39.5 min. Subsequently, these five fractions were further separated with 10 % Et/Hex. The retention time of synthesized DTE-OH was 64.4 min. with 10 % Et/Hex. A fraction with retention time of 46-53 min was determined most active (Fig. 4-3a). The active fraction of *G. trabeum* mycelia was also fractionated with 10 % Et/Hex.

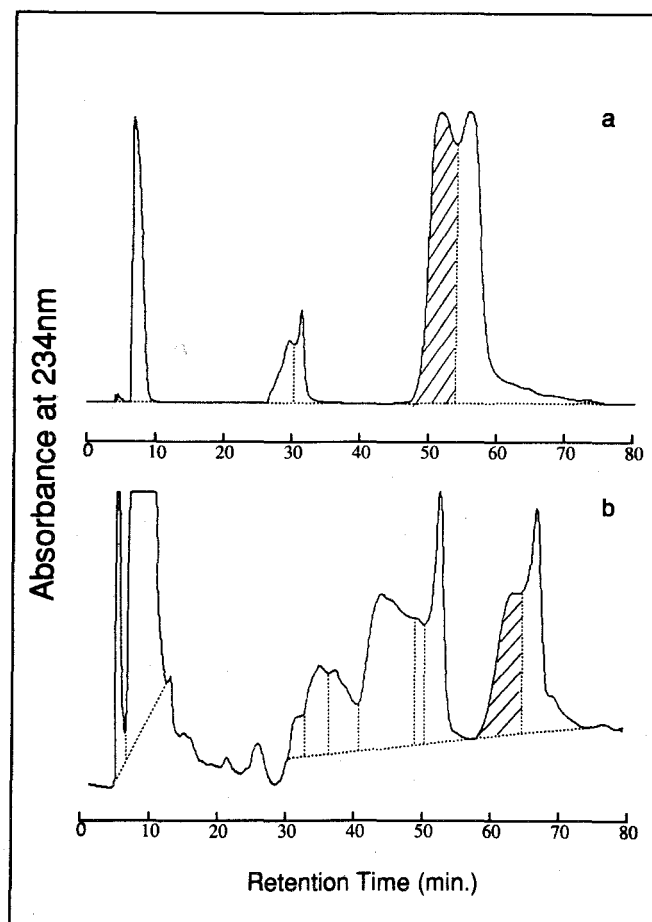


Fig. 4-3. Results of high performance liquid chromatography.

a : Active fraction of *S. lacrymans*-decayed wood.

b : Active fraction of *G. trabeum* mycelia.

Note : Shaded portions include active ranges in bioassay.

The active fractions were at the retention time around 63.4 min. and 66.9 min. (Fig. 4-3b). The former was more active than the latter.

4.3.2 Trail-following substances

The most active fraction of *S. lacrymans*-decayed wood was first analyzed by GC using non-polar capillary column (Fig. 4-4). There were two major and many minor peaks and some of the minor peaks appeared near the

retention time of synthesized DTE-OH (6.5 min.). The fraction was further analyzed by capillary GC using polar column. Results of GC-MS analyses of synthesized DTE-OH, the active fractions of *S. lacrymans*-decayed wood and *G. trabeum* mycelia were shown in Figs. 4-5, 4-6 and 4-7. The retention time of DTE-OH was 11.3 min. (Scan No.333) in total ion chromatogram (TIC) and its molecular ion certainly existed at m/z :180 in the mass spectrum (MS) (Fig. 4-4). In *S. lacrymans* fraction, there was neither a peak near the retention time of DTE-OH in TIC nor an ion of m/z :180 in MS (Fig. 4-6). These results suggest that the trail-following activity of *S. lacrymans*-decayed wood is not due to DTE-OH. Several minor peaks near the Scan No.330 are considered as analogues of DTE-OH.

TIC and MS of *G. trabeum* mycelia (Fig. 4-7) indicated that many kinds of substances existed around the Scan No.330 and MS of Scan No.330-333 showed the peak of m/z :180, corresponding to molecular ion of DTE-OH. However, the peak was not exactly identified with DTE-OH.

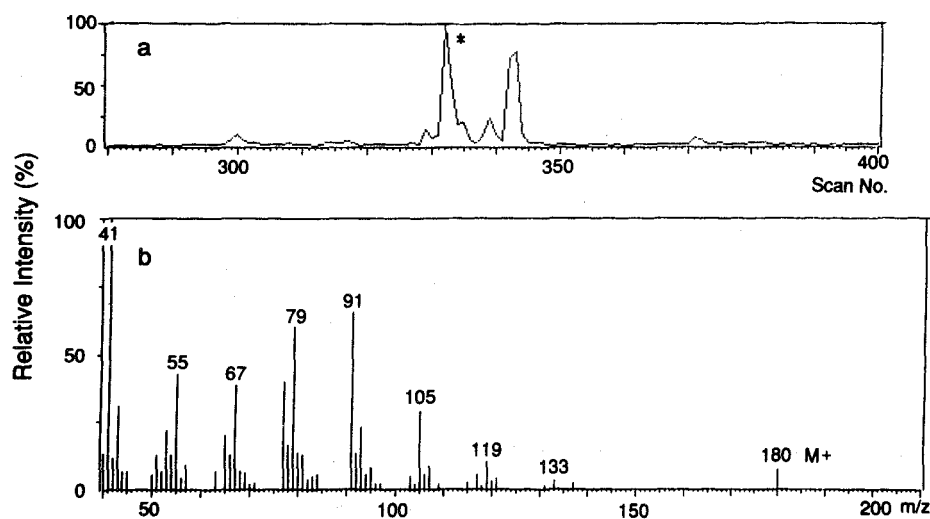


Fig. 4-4. Results of capillary gas chromatography.

a : Active fraction of *S. lacrymans*-decayed wood.

b : Synthesized (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (DTE-OH).

* : Retention time = 6.5 min.

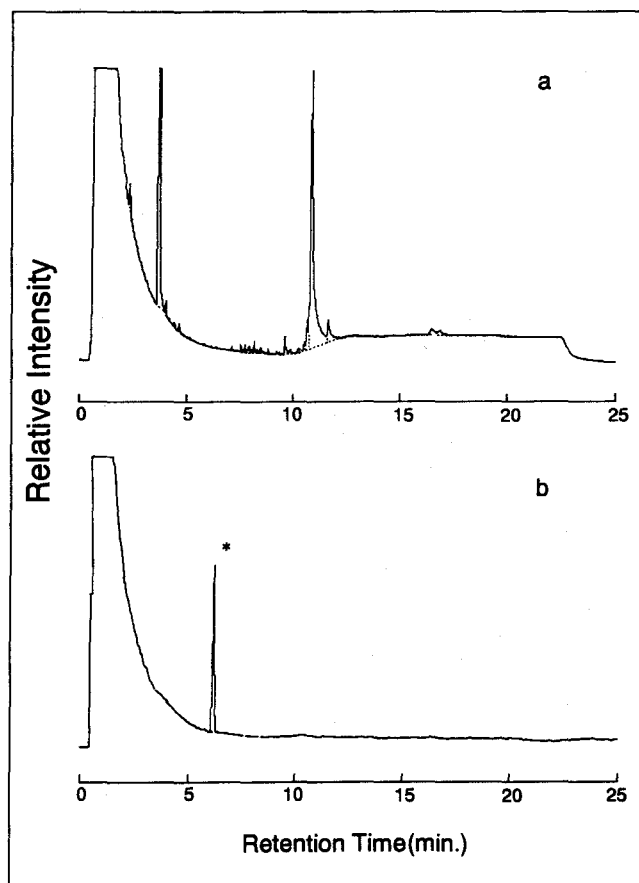


Fig. 4-5. Capillary gas chromatography mass spectrometry of synthesized (Z,Z,E)-3,6,8-dodecatrien-1-ol (DTE-OH).

a : Total ion chromatogram of DTE-OH.

b : Mass spectrum at Scan No. 333.

Finally, the active fractions of *S. lacrymans*-decayed wood and *G. trabeum* mycelia were analyzed again using SIM method in order to confirm the existence of DTE-OH. Results of the analyses were shown in Fig. 4-8. As numbers on the chromatograms mean ion intensity at a full scale, ion intensity of each Scan No. can be roughly calculated. Comparing the chromatograms of *G. trabeum* extract (Fig. 4-8b) with that of DTE-OH (Fig. 4-8c), there were peaks at the same retention time (11.3 min.) and the ratio of the ion intensities of these

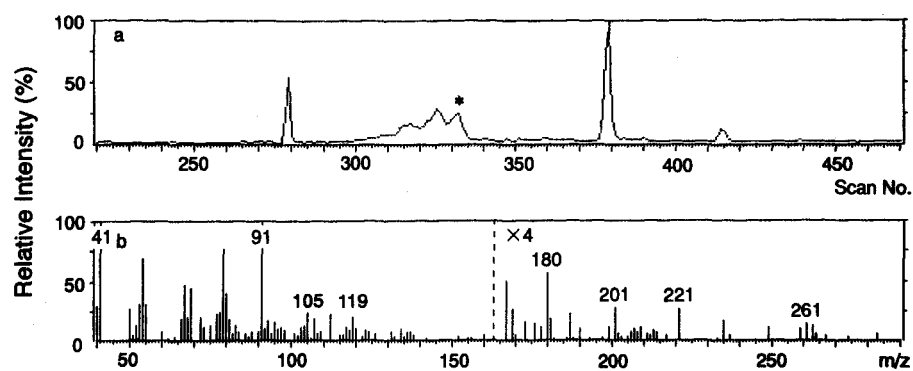


Fig. 4-6. Capillary gas chromatography mass spectrometry of *S. lacrymans*-decayed wood.

a : Total ion chromatogram of active fraction.

b : Mass spectrum at Scan No. 333.

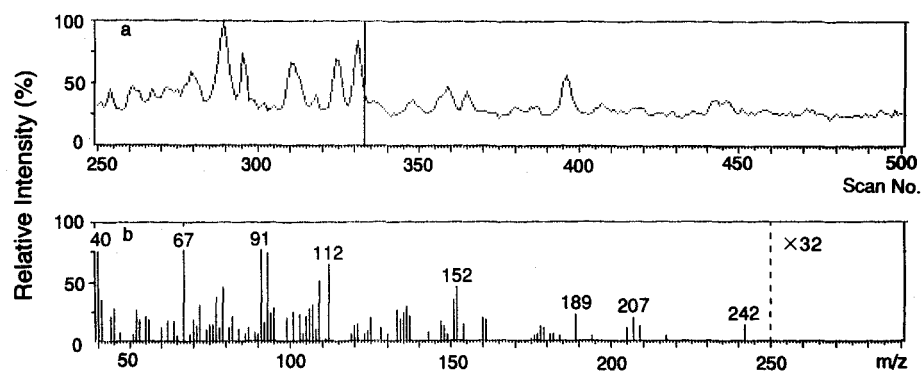


Fig. 4-7. Capillary gas chromatography mass spectrometry of *G. trabeum* mycelia.

a : Total ion chromatogram of active fraction.

b : Mass spectrum at Scan No. 333.

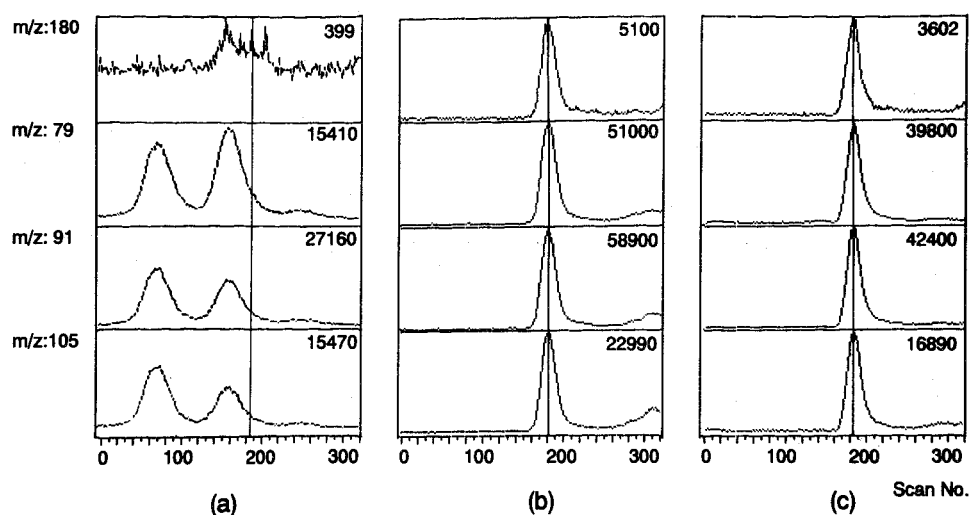


Fig. 4-8. Results of GC-MS-SIM analyses.

(a) : *S. lacrymans*-decayed wood.

(b) : *G. trabeum* mycelia.

(c) : Synthesized (Z,Z,E)-3,6,8-dodecatrien-1-ol (DTE-OH).

* : Vertical line is identical with Scan No. of DTE-OH.

** : Each number on the graph is ion intensity at a full scale.

peaks due to the molecular ion ($m/z:180$) and the diagnostic fragment ions of DTE-OH ($m/z: 79,91$ and 105) were nearly equal. Therefore, we regarded these two substances as the same chemical substance. On the other hand, in *S. lacrymans* extract, there was no peak at 11.3 min. Furthermore, the ratio of the ion intensities of the molecular ion and the diagnostic fragment ions at the retention time of 11.3 min. were quite different from that of DTE-OH (Fig. 4-8a). These results indicate that the main trail-following activity of *S. lacrymans*-decayed wood extracts do not come from DTE-OH. It is known that some kinds of substances which are different from DTE-OH also show the trail-following activity. Several analogues of dodecatrienols including DTE-OH were synthesized and their trail-following activities were compared by Tai *et al.* (1971). They found that not only the position of alcoholic hydroxyl group and double bonds but also stereochemistry of

dodecatrienols greatly related to the activity. For example, an ink of ball point pen which contains diethylene glycohol monomethyl ether and diethylene glycohol monoethyl ether has trail-following activity, but the activity level is 1/100,000 of trail-following pheromone, DTE-OH. The case of *S. lacrymans* seemed to be the same as this example. Considering the result of AgNO₃ silica-gel column chromatography, cooperation of several substances might cause the trail-following activity.

4.4 Summary

Chemical substances prepared from wood decayed by *S. lacrymans* and mycelia of *G. trabeum* were used for the determination of their termite trail-following activities by bioassay. The trail-following activity of *G. trabeum* mycelia was 105 times as high as that of *S. lacrymans* decayed wood, although further isolations resulted in the decrease of activity. GC-MS-SIM analysis showed that *G. trabeum* mycelia peaks corresponded with DTE-OH that was involved in termite trail-following activity.

Trail-following active compound in the *S. lacrymans* decayed wood was not DTE-OH. It is possibly a type of alcohol, although to confirm this further work is needed.

PART III

APPLICATION OF WOOD COMPONENTS TO TERMITE CONTROL

**Chapter 5 : Improvement of the Antitermitic Activities of
Polyphenols by Combination with Heavy Metals**

**Chapter 6 : Bait-Toxicant Application of Steamed Larch
Heartwood Extracts Incorporating Boric Acid
Impregnation**

Chapter 5 : Improvement of Antitermitic Activity of Polyphenols by Combination with Heavy Metals

5.1 Introduction

Plant polyphenols are known as "natural preservatives" because of their various bioactive abilities. Tannins are defined as kinds of polyphenols that have protein-precipitation capacity (Bate-Smith and Swain, 1962). From the biological point of view, the importance of tannins in plants lies in their effectiveness as repellents to predators. The relevant property is 'astringency' rendering the tissues unpalatable by precipitating proteins or, by immobilizing enzymes, impeding the invasion of the host by the parasite (Bate-Smith 1973). Thus, dietary tannins can reduce growth and fecundity of some insects (Schultz 1988), and inhibit β -glucosidase and other cellulose-degrading enzymes (Ozawa 1987; Mitsunaga *et al.* 1998). In respect of chemical structures, tannins are divided into two major groups: condensed tannins and hydrolysable tannins. Catechin is an abundant monomer unit of condensed tannins, and exists relatively common in nature. As to the toxicity of catechin shows toxicity to insects, Guerra *et al.* (1990) have reported that catechin inhibits the development of bollworm, *Heliothis zea* (Boddie) larvae, and shows antifeedancy against *C. formosanus* (Chapter 2).

Heavy metals are known to be toxic to our environment. Among them, nickel causes the natural soil pollution around serpentine area. There exist Ni hyperaccumulating plants, nearly half of which are in the family of Brassicaceae, and they have more than 1000 mg Ni kg⁻¹ dry weight (Brooks *et al.* 1990). Plants grown on Ni-amended soil showed greater survival and yield than plants on unamended soil when they were subjected to herbivory by lepidopteran larvae, so Ni hyperaccumulation may be an effective chemical defense of plant against herbivores (Martens and Boyd 1994). As for the effect of Ni on termites, Ni-plated

wood caused 100 % mortality of test termites, *C. formosanus*, within two weeks (Hasegawa *et al.* 1994). On the other hand, organometallic or inorganic compounds containing such as Cu and Zn have been used as wood preservatives.

This chapter deals with possible improvement of antitermitic activity of polyphenols when they are combined with some heavy metal salts. Cellulose powder was treated with condensed tannins (TA) or catechin (CA) mixed with three kinds of heavy metal salts (NiCl_2 , ZnCl_2 , CuCl_2), respectively, and subjected to the no-choice bioassay to test their termiticidal and antifeedant activities against *C. formosanus*. Protein-precipitation capacity was also measured to know the relationships between astringency and antitermitic activity of tested compounds.

Furthermore, preventive effects of the tunneling activity of *C. formosanus* was assessed using zeolite gravels treated with CA, Cu(II) or CA/Cu(II) comparing with untreated zeolite gravels and untreated sea sand (Ohmura and Ohara 1999c, to be published).

5.2 Materials and Methods

5.2.1 Chemicals

Three kinds of heavy metal salts, *i.e.* nickel(II) chloride hexahydrate ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$), zinc(II) chloride anhydride (ZnCl_2) and copper(II) chloride dihydrate ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$) were purchased from Wako Co. Ltd. Catechin (CA) was purchased from Nacali Tesque Co. Ltd.

5.2.2 Preparation of condensed tannin

Seventy % acetone extract from the bark meal of *Acacia mearnsii* De Wild. (6 g) was extracted with *n*-hexane and ethyl acetate, successively. The ethyl

acetate soluble fraction was dried to give ethyl acetate extract (2.73 g). The ethyl acetate extract (TA) was used in this study. It contains mixtures of proanthocyanidin oligomers, and has 2,3-*trans* heterocyclic rings, prorobinetinidin extender units, and procyanidin or prodelfphinidin branched terminal units (Ohara *et al.* 1994).

5.2.3 Termite bioassay

5.2.3.1 Termite

Termites used in this study were pseudoergites (workers) above third instars from the laboratory colony of *C. formosanus* maintained in Forestry and Forest Products Research Institute, Tsukuba as described in Chapter 2.

5.2.3.2 Termiticidal bioassay

The test solutions (solvent: MeOH) were prepared for mixing 3 % (w/w) [percentage of tannin monomer unit to cellulose powder] of TA and equimolar of metal salts (TA/Ni(II), TA/Cu(II), TA/Zn(II)). Furthermore, CA was also used to prepare the similar test solutions mixing with these metal salts (CA/Ni(II), CA/Cu(II), CA/Zn(II)). Single solutions of TA, CA, Ni(II), Cu(II) and Zn(II) were also tested for comparison.

One gram of cellulose powder (Toyo Roshi Kaisha, Ltd.) was treated with 3 ml of each test solution, dried at 60 °C for removing solvent, and then put on a plastic saucer. It was introduced into a test chamber which were made according to the procedure as described in Chapter 1 with fifty workers of *C. formosanus*. Untreated cellulose powder was used as a control. In addition, the test under complete starvation (water supply only) was also evaluated. Workers were checked

every three days for mortality during the 21-day bioassay. Three replicates were performed. These test chambers were placed in the dark at 26 ± 2 °C and 75 % relative humidity.

In order to know the effects of the test materials on the feeding behavior of termites, a paper disc (13 mm diameter, Toyo Roshi Kaisha, Ltd.) treated with each test solution was forced to eat instead of the treated cellulose powder. The weight losses of the discs eaten by fifty termites for first 3 days were measured. Three replicates were run under the same conditions as termiticidal bioassay.

5.2.3.3 *Protein-precipitation assay*

The method of Makkar *et al.* (1987) was used with slight modification. A hundred mg of CA were dissolved in 25 ml of MeOH/H₂O (1/1, v/v). Each metal salt was dissolved in 25 ml of MeOH/H₂O (1/1, v/v), and mixed with the CA solution to give a final concentration of metal of 0.02 % (w/v) (in metal equivalent). Fifty ml of bovine serum albumin (BSA) solution (2 mg/ml of acetate buffer (pH=5.0) containing 10 mg of sodium chloride) was added to the mixed solution, and the suspension was shaken sufficiently and kept at room temperature for 20 min. For comparison, 0.2 % (w/v) of CA and TA solutions, and 0.02 % (w/v) (in metal equivalent) of each metal salts solution were also assayed individually. The reaction mixture was centrifuged at 3,600 r.p.m. for 20 min., and the supernatant was discarded. The precipitate was weighed after drying at 105 °C for 12 hr. This assay was continued for 14 days after mixing the CA with each metal salt.

5.2.3.4 *UV spectral analysis*

A UV spectral analyzer, Shimadzu UV-VISIBLE Recording Spectrophotometer (UV-250) was employed to measure the UV spectra of an equimolar (1.72 mM) mixture of CA and each metal salt solution (solvent : MeOH). The changes of the absorbance of the CA/metal solutions were measured at room temperature for 14 days.

5.2.3.5 Tunneling bioassay

Particles of zeolite (particle size; 1.5-3.0 mm) were treated with MeOH containing 3 % (w/v) CA, 0.66 % (w/v) CuCl₂ or the mixture of them, and after dried, they were served for horizontal and vertical tunneling tests to know the effects of the test materials on the tunneling behavior of termites. The tunneling tests were performed according to the procedure described by Sornnuwat *et al.* (1995) and Grace *et al.* (1993), respectively, after several modifications (Figs. 5-1 and 5-2).

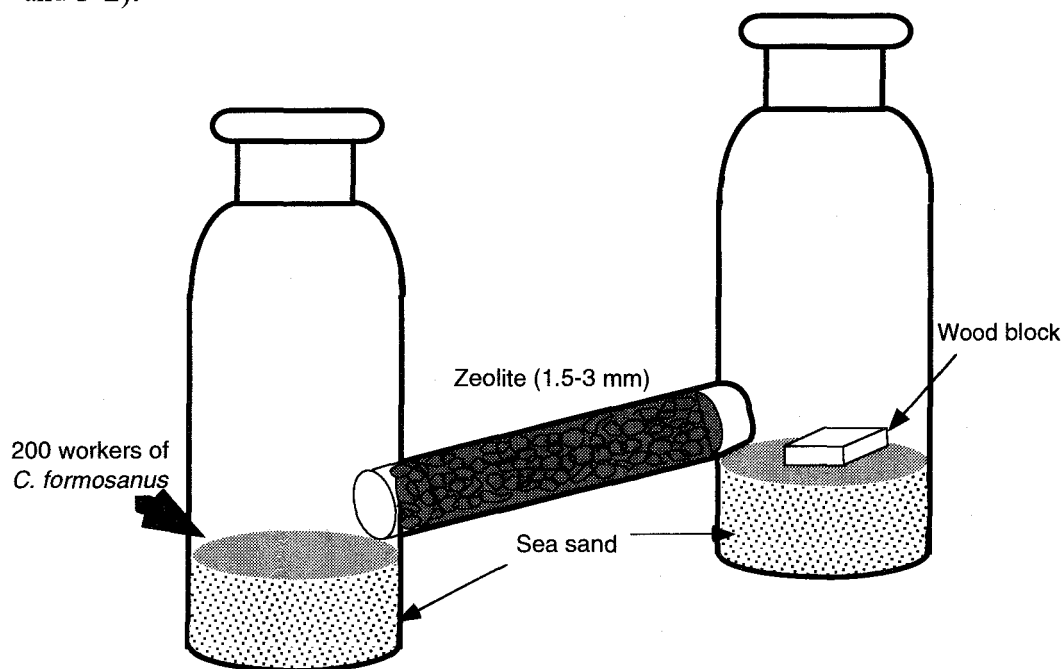


Fig. 5-1. Test unit for horizontal tunneling test.

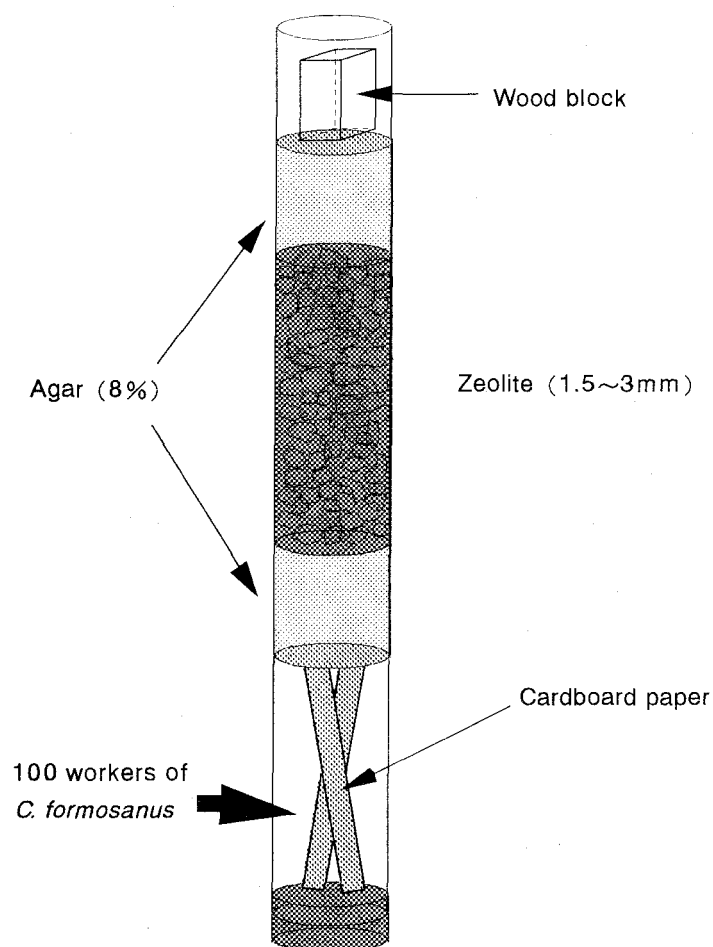


Fig. 5-2. Test glass tube for vertical tunneling test.

For horizontal tunneling tests, a glass unit which are designated in the Japan Wood Preserving Association Standard 13, were used (Fig. 5-1). Thirty g of sea sand (15-20 mesh, Junsei Co. Ltd.) were put into the two bottles each of which has one hole on the side wall, and moistened with 5 ml of distilled water. Sixty hundred mg of the workers of *C. formosanus* (about 200 individuals) were introduced into one bottle. A wood block of *Pinus densiflora* Sieb. et Zucc. (15 (tangential) $\times$ 15 (radial) $\times$ 5 (longitudinal) mm) was placed in another bottle. These two bottles were jointed with a glass tube (inner diameter : 15 mm)

containing 50-mm treated zeolite gravel (particle size : 1.5 - 3 mm). Top of the two bottles were covered with cotton wool to keep moisture contents in the test units.

For vertical tunneling tests, a glass tube (inner diameter, 12 mm; length, 160 mm) was used (Fig. 5-2). About 50-mm treated zeolite gravel sandwiched between 8 % (w/v) agar was stuffed to the tube. Two pieces of cardboard paper (50 mm × 5 mm) were placed in the bottom of the tube with 300 mg of the workers (about 100 individuals) of *C. formosanus*. The bottom end of the tube was capped with a plastic plug. A wood block of *P. densiflora* (15 (tangential) × 5 (radial) × 5 (longitudinal) mm) was placed on the top side of the agar as food. The top of the tube was sealed with Parafilm™ to keep moisture content in the test tube.

Untreated zeolite gravel and untreated sea sand were also subjected to these tunneling bioassays for control tests. The day spent for penetrating the 50-mm test substrate or the total distance (mm) tunneled through the test substrate for 21-day experimental period was recorded. Three replicates per treatments were performed.

5.3 Results and Discussion

5.3.1 Termiticidal activity

Mortality of termites fed on TA after 21 days was almost same as that of CA, but lower than starvation (Table 5-1 and Fig. 5-3). Weight losses of the discs treated with TA and CA were quite lower than that of untreated control (Table 5-2). These results demonstrated that TA and CA had the antifeedant activity against termite rather than the termiticidal toxicity.

Table 5-1. Average mortalities (%) of workers of *C. formosanus* fed on cellulose powder treated with catechin (CA), *A. mearnsii* tannins (TA), Cu, Ni, Zn, CA/Cu, CA/Ni, CA/Zn, TA/Cu, TA/Ni, TA/Zn, untreated (Control) and starvation at the end of the test period of the termiticidal bioassay.

Treatment	Mortality (%)
	Mean $\pm$ SE
CA	23.5 $\pm$ 1.64 cd
TA	12.0 $\pm$ 1.54 d
Cu	21.3 $\pm$ 1.76 cd
Ni	58.7 $\pm$ 2.40 b
Zn	31.3 $\pm$ 4.37 c
CA/Cu	23.3 $\pm$ 3.71 cd
CA/Ni	100 $\pm$ 0 a
CA/Zn	26.7 $\pm$ 4.37 d
TA/Cu	15.3 $\pm$ 3.71 cd
TA/Ni	84.0 $\pm$ 5.29 a
TA/Zn	10.0 $\pm$ 2.00 d
Control	15.6 $\pm$ 2.32 cd
Starvation	32.2 $\pm$ 2.32 c

Notes : These values indicate the average of mortalities (%) $\pm$ standard error (N=3). Values followed by the same letter (a, b, c, d) are not statistically different at the 0.01 level according to Tukey-Kramer HSD test.

Among the three metal salts in single application, Ni(II) showed the highest termiticidal activity (Table 5-1 and Fig. 5-4). Mixing effect of polyphenols and metals varied with their combination markedly. Mortalities of termites fed on CA/Ni(II) and TA/Ni(II) were higher than those of CA, TA or Ni(II) (Table 5-1 and Figs. 5-3, 5-4, 5-5 and 5-6). Especially on CA/Ni(II), drastic rise of the mortality was observed from 12 days, reaching 100 % at the end of the test period. On the other hand, only less than 30 % mortalities were obtained after 21 days

when Cu(II) or Zn(II) was added to TA or CA. Thus, it was found that Cu(II) and Zn(II) had little additive effects on CA or TA on the termiticidal activity. These results suggest that the toxicity of polyphenols/metal salts was not acute but slow-acting one, that is, their accumulation in termite bodies might effect on the toxicity.

With respect to the disc consumption, addition of metal salts to TA or CA tended to enhance the consumption when comparing with the single application of these polyphenols (Table 5-2). Difference in consumption between Ni(II) and CA/Ni(II) was too small to show the statistically significant difference during first 3 days of test period, although, difference in accumulation of Ni(II) in termite bodies between the two was thought to increase with time to result in the significant difference in mortality after 18 days. As a result, it was assumed that the interaction between polyphenols and metals resulted in decreasing the antifeedant activity of polyphenols but increasing the termiticidal activity by increased consumption of treated samples.

5.3.2 Protein-precipitation capacity

Polyphenols are characteristic of the chemical defenses of apparent plants and act as quantitative-dosage dependent barriers, even to insects which normally feed on leaves containing them (Feeny, 1975, 1976). Tannins are defined as kinds of polyphenols that have protein-precipitation capacity. Due to the capacity, tannins in plants possibly render the tissues unpalatable and impede the invasion by the parasite. These suggest that the astringent property of tannins in plants are closely related to their toxicity to the parasite. Therefore, the protein-precipitation capacity of CA/metal mixtures was measured whether astringency also relates to the toxicity to a termite, *C. formosanus*. In order to determine the protein-precipitation capacity of CA/metal mixtures, BSA was added to the test solutions and the precipitates formed were estimated.

Table 5-2. Average weight losses of the discs (mg) treated with catechin (CA), *A. mearnsii* tannins (TA), Cu, Ni, Zn, CA/Cu, CA/Ni, CA/Zn, TA/Cu, TA/Ni, TA/Zn and untreated (Control) after three days' exposure to fifty workers of *C. formosanus*.

Treatment	Weight loss (mg)
	Mean $\pm$ SE
CA	1.17 $\pm$ 0.58 ef
TA	2.57 $\pm$ 0.38 def
Cu	0.53 $\pm$ 0.27 f
Ni	4.43 $\pm$ 0.35 def
Zn	5.90 $\pm$ 0.80 cde
CA/Cu	0.83 $\pm$ 0.64 ef
CA/Ni	5.80 $\pm$ 0.49 cde
CA/Zn	7.50 $\pm$ 1.94 bcd
TA/Cu	1.27 $\pm$ 0.76 ef
TA/Ni	9.73 $\pm$ 1.18 abc
TA/Zn	14.0 $\pm$ 0.64 a
Control	9.67 $\pm$ 0.58 ab

Notes : These values indicate the average of the weight losses of the discs $\pm$ standard error (N=3). Values followed by the same letter (a, b, c, d, e, f) are not statistically different at the 0.01 level according to Tukey-Kramer' HSD test.

TA yielded 138 mg of precipitates with BSA, whereas CA did not. This was in good agreement with the results reported by Kawamoto *et al* (1991). Among the three metal salts, only Cu(II) yielded 40 mg of precipitates.

Fig. 5-7 shows the relationships between the test periods and the weight of the precipitates yielded by each CA/metal mixture. CA/Ni(II) has formed no precipitate for 14 days. On the other hand, CA/Cu(II) formed precipitates soon

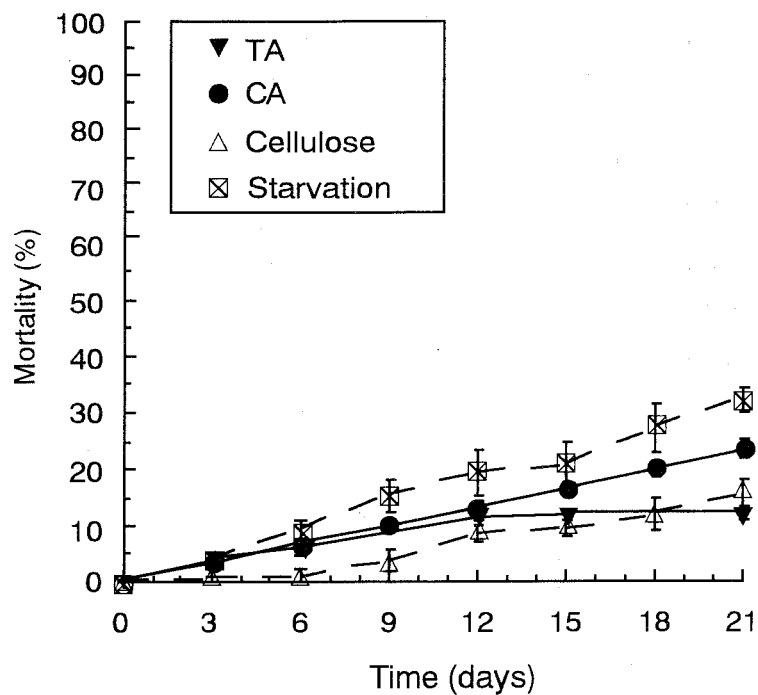


Fig. 5-3. Mortalities (%) of termites fed on cellulose powder treated with *A. marnsii* tannins (TA), catechin (CA), untreated (control) and starvation. Error bars indicate $\pm$ standard error (N=3).

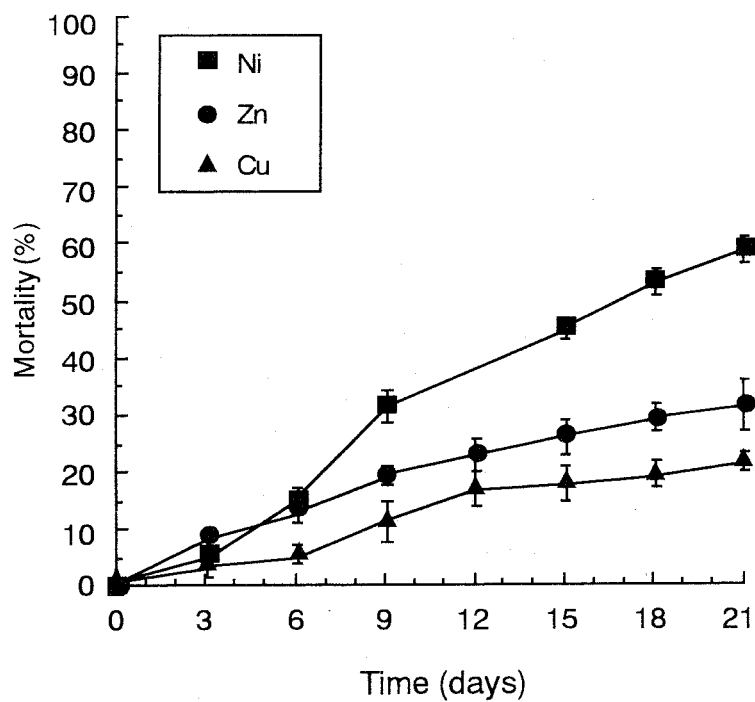


Fig. 5-4. Mortalities (%) of termites fed on cellulose powder treated with Ni, Zn and Cu. Error bars indicate $\pm$ standard error (N=3).

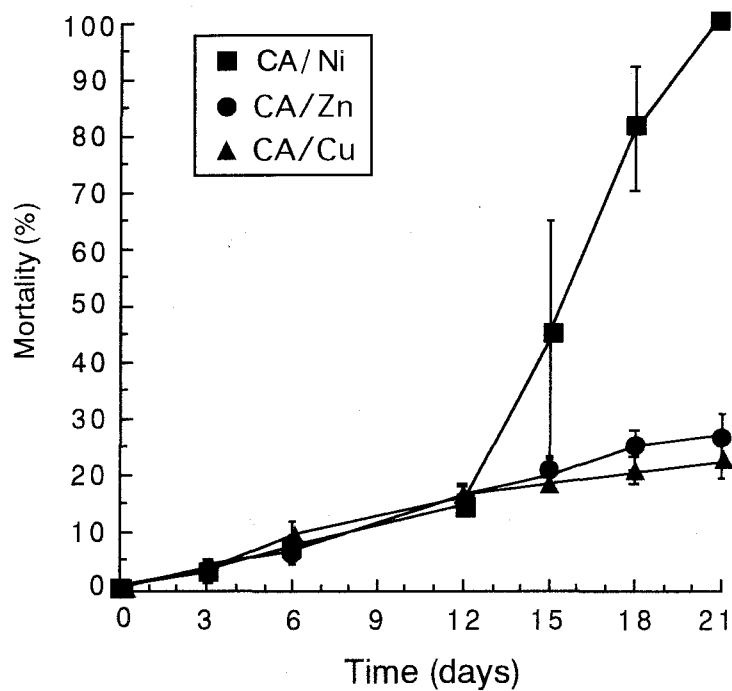


Fig. 5-5. Mortalities (%) of termites fed on cellulose powder treated with CA/Ni, CA/Zn and CA/Cu. Error bars indicate $\pm$ standard error (N=3).

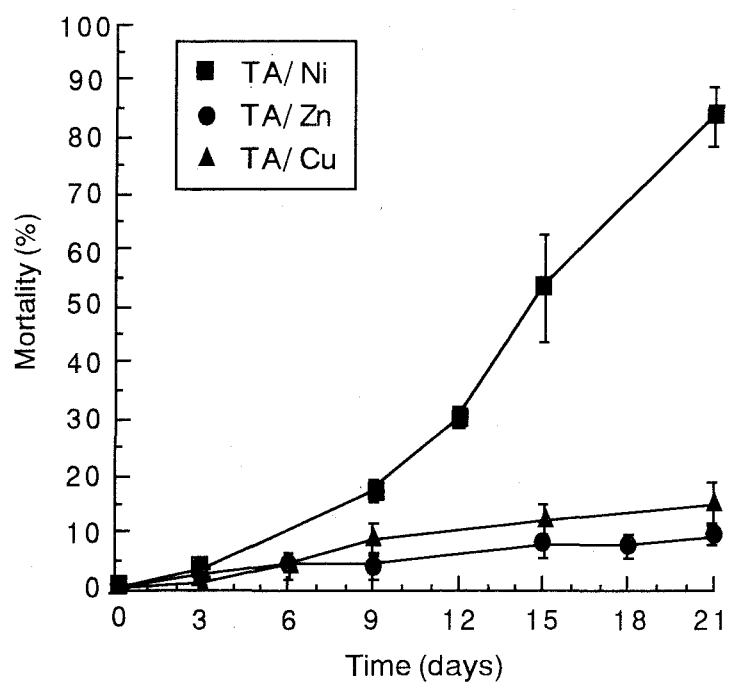


Fig. 5-6. Mortalities (%) of termites fed on cellulose powder treated with TA/Ni, TA/Zn and TA/Cu. Error bars indicate $\pm$ standard error (N=3).

after preparing the CA/Cu(II) solution, and the weight of precipitates gradually increased with time. After 7 days, the gain of the precipitates formed by CA/Cu(II) amounted to approximately 150 mg, which was as much as the weight of precipitates yielded by TA. CA/Zn(II) has not formed precipitates for the first 3 days, but on the 4th day after preparing the solution, it yielded 26 mg of precipitates. The weights of precipitates continued to increase slightly, and reached 52 mg on 14 days. Thus, astringent property of CA was found to be improved by combination with Cu(II) or Zn(II).

These results suggested that the structure of CA was changed by these metal salts. In order to confirm the structural change of CA, UV-absorbance of the CA/metal solutions were measured for 14 days. UV spectra showed that CA had the maximal absorption wavelength (λ_{max}) at 278 nm in MeOH (Fig. 5-8). CA/Ni(II) showed little absorption increase during 21 days after mixing. It was thought that Ni(II) had little interaction with CA. On the other hand, CA/Cu(II) and CA/Zn(II) solutions showed marked UV-absorption increase with time. In the case of CA/Cu(II), absorbance at 278 nm increased sharply compared to CA/Zn(II). Interaction of CA and Cu(II) is thought to be stronger than that of CA and Zn(II). When the CA/Cu(II) solution was treated with helium gas in order to substitute dissolved oxygen, the absorbance after 1 day became lower than that of untreated solutions. This is a good evidence to show the oxidation of CA in the solution. The results of UV spectra gave good agreement with the results of protein-precipitation capacity.

Relationships between the protein-precipitation capacity and the degree of polymerization of condensed tannin have been reported (Bate-Smith, 1973; Haslam 1974; Kawamoto *et al.* 1991; Ohara *et al.* 1994). Considering the increase in the weight of the precipitates, it was assumed that polymerization of CA occurred in the CA/Cu(II) and CA/Zn(II) solutions. The polymerization in the CA/Cu(II) solution was thought to occur earlier than that in the CA/Zn(II) solution.

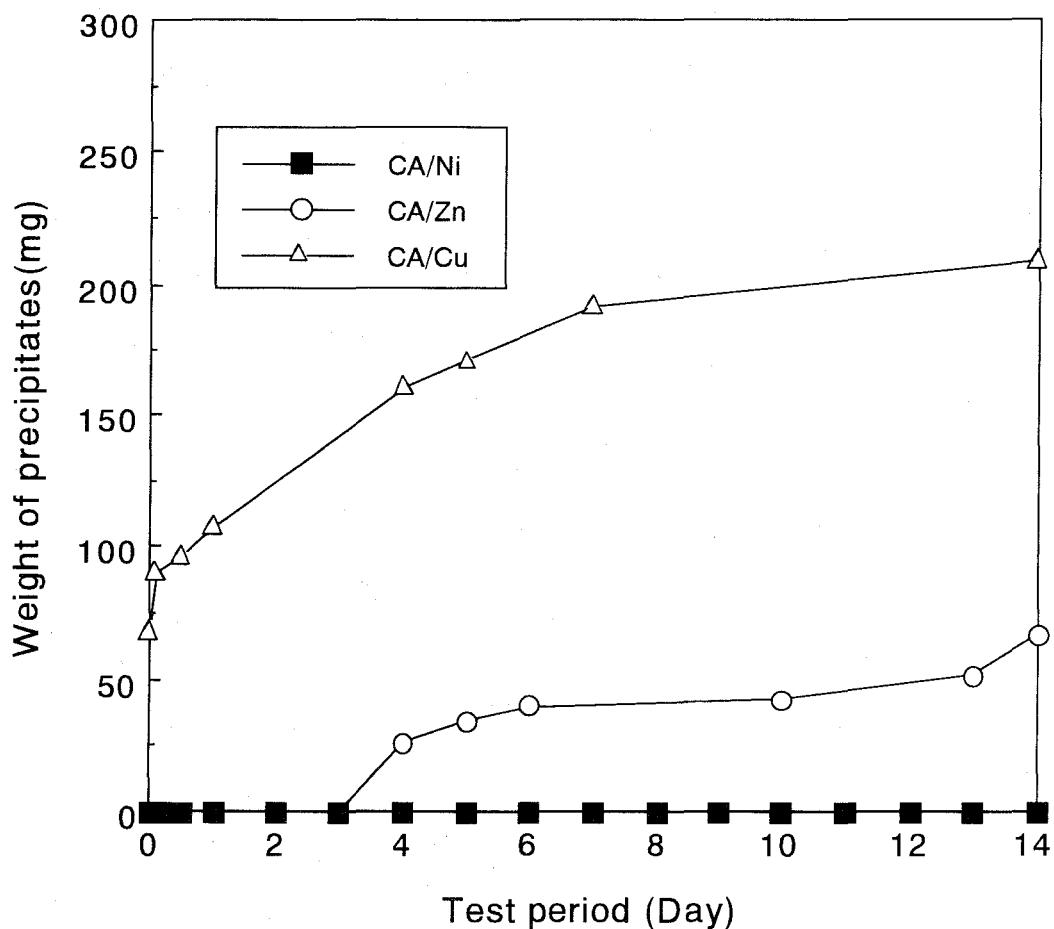


Fig. 5-7. The weight of bovine serum albumin(BSA)-precipitates of catechin (CA)/Ni, CA/Zn and CA/Cu.

To explain the polymerization of CA, two possibilities are supposed. One is the polymerization by forming complex which binds through metal atoms. The other is the co-polymerization by oxidation.

Weber (1990) reported in his study of the interaction of CA and Cu(II) using liquid chromatography, that CA/Cu(II) complex was detected 30 min. after mixing, but 10 hr after mixing, unknown species which do not contain Cu appeared. As CA/Cu(II) decreased with time, it may be assumed that CA/Cu(II) complex was an intermediate to form the unknown species. On the basis of these results, it was suggested that metal catalyzed oxidation and/or polymerization of

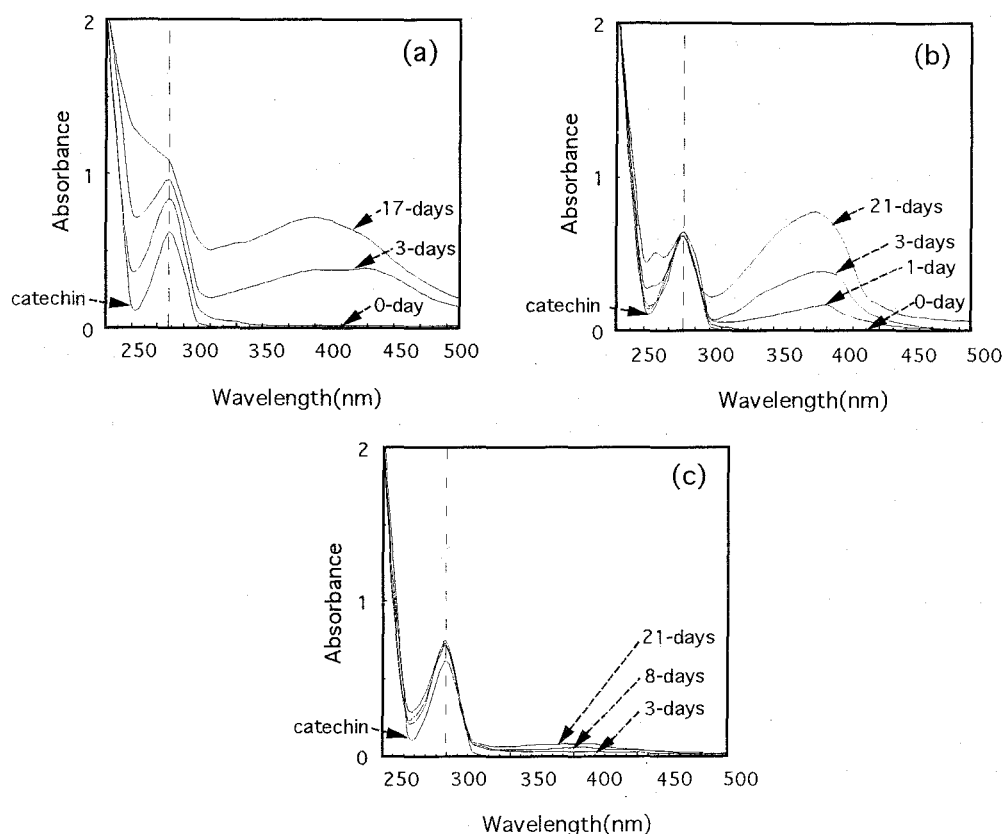


Fig. 5-8. Changes of UV absorption of catechin(CA) and (a) CA/Cu (b) CA/Zn and (c) CA/Ni.

catechin occurred in the CA/Cu(II) and CA/Zn(II) solutions, and the protein-precipitation capacity of the mixed solutions increased by the formation of the reaction products. Further study is needed to investigate the structures of the oxidation and/or polymerization products of catechin.

From these results, it was found that termiticidal activity negatively correlated with the BSA-precipitation capacity. As polyphenols exhibit different affinities for different proteins (Hagerman and Bulter, 1981), protein-precipitation assay using digestive enzymes or proteins associated with taste reception of termite might give different results obtained in this research.

5.3.3 Tunneling activity

It was found that CA had little termiticidal activity (Table 5-1 and Fig. 5-3), but showed the high feeding-deterrence activity (Table 5-2). Thus, CA was expected to enhance the prevention of the tunneling activity of zeolite gravel.

CA-treated zeolite gravel strongly prevented the penetration by *C. formosanus* in both vertical and horizontal tests. This result suggested that other flavonoids tested in Chapter 2 were possibly effective on the prevention of tunneling activity as well (Table 5-3 and Figs. 5-9 and 5-10). Cu(II) slightly

Table 5-3. These values indicate the average of mortalities (%) standard error (N=3). Values followed by the same letter (a, b, c, d) are not statistically different at the 0.01 level according to Tukey-Kramer HSD test.

		Horizontal		Vertical	
		Penetration length (mm)	Penetration time (Day)	Penetration length (mm)	Penetration time (Day)
CA	1	40	-	10	-
	2	30	-	10	-
	3	0	-	10	-
Cu	1	25	-	10	-
	2	40	-	25	-
	3	50	5	20	-
CA/Cu	1	50	5	50	9
	2	50	2	30	-
	3	50	10	50	12
Untreated (zeolite)	1	40	-	20	-
	2	45	-	50	11
	3	50	5	20	-
Untreated (sea sand)	1	50	1	50	3
	2	50	2	50	3
	3	50	1	50	6

Notes : (-) means no penetration during the test period of 21 days.



Fig. 5-9. Horizontal tunneling test using catechin (CA) treated zeolite.

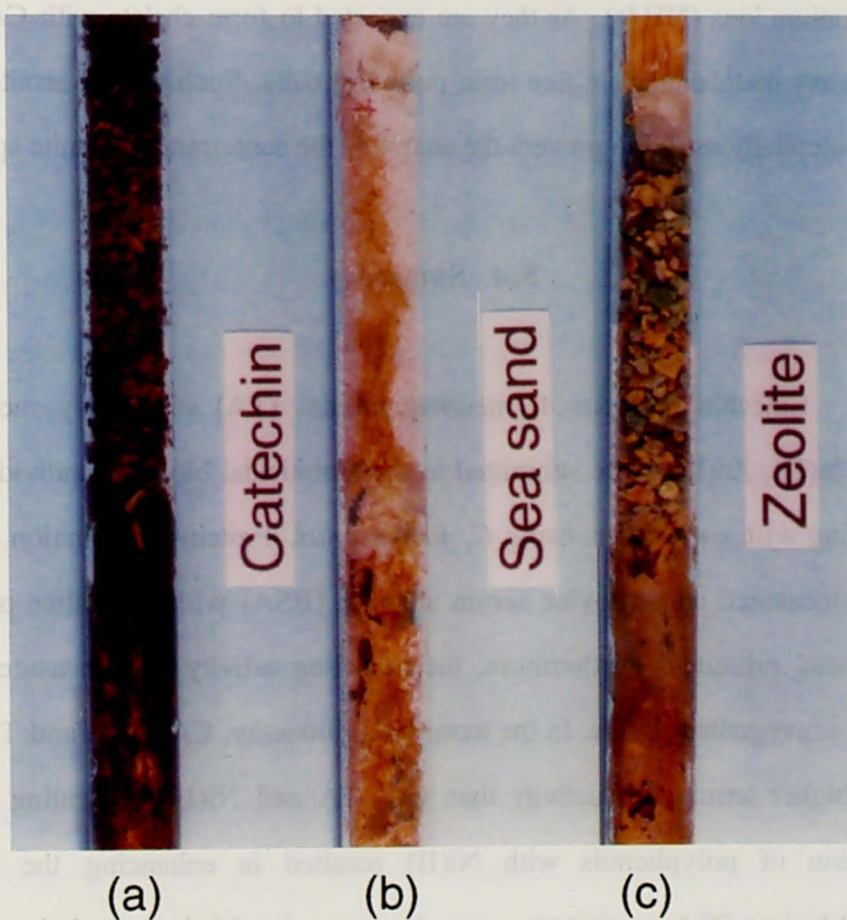


Fig. 5-10. Vertical tunneling tests using (a) catechin (CA) treated zeolite, (b) sea sand and (c) untreated zeolite.

enhanced the prevention of the tunneling activity, however, CA/Cu(II)-treated zeolite was penetrated easily, indicating that the addition of Cu(II) decreased the preventive effects of CA. Plugged CA/Cu(II)-treated zeolite particles in a glass tube apparently went to bind each other to make gaps. So, termites might easily penetrate Cu/CA-treated zeolite barrier.

A layer of zeolite gravel in horizontal test tended to be penetrated easier than that in vertical test (Table 5-3). The zeolite gravel in vertical test was apparently stuffed more tightly than that in horizontal test. This might be an effect of the gravity. As subterranean termites such as *C. formosanus* used to invade into houses from underground and to penetrate soils against the gravity, the vertical test is thought to be reflected the field condition more actually than the horizontal test.

Zeolites are hydrated aluminum-silicate minerals, and have an affinity for ammonium ions (NH_4^+). As they are expected to form chelate with CA and to adsorb heavy metals, they are free from polluting soils. Such treated zeolite barrier may be potentially useful to prevent the attack by the subterranean termite species.

5.4 Summary

Catechin (CA) or *A. mearnsii* tannins (TA) and heavy metal salts (Ni(II), Cu(II), Zn(II)) were submitted to the termiticidal bioassay individually or after mixing with each other, using *C. formosanus*. Protein-precipitation capacity was also measured using bovine serum albumin (BSA) with time after preparing the CA/metal mixtures. Furthermore, the tunneling activity was measured using CA/metal impregnated zeolite. In the termiticidal bioassay, CA/Ni(II) and TA/Ni(II) showed higher termiticidal activity than CA, TA and Ni(II), indicating that the combination of polyphenols with Ni(II) resulted in enhancing the activity. CA/Ni(II) had no BSA-precipitation capacity even after 16 days, and showed little increase in UV absorption. On the other hand, both CA/Cu(II) and CA/Zn(II)

exhibited the BSA-precipitation capacity and showed drastic changes in UV spectra after preparing CA/metal salts solutions. These results suggested that the structure and property of CA did not change by Ni(II) but change by Cu(II) or Zn(II). As CA/Cu(II) showed the higher capacity and the larger change in UV absorption than CA/Zn(II), CA/Cu(II) was applied to zeolite gravels to prevent the tunneling activity of termites more effectively. However, CA-treated zeolite gravel prevented the penetration of tunneling activity longer than Cu(II)- or CA/Cu(II)-treated zeolite. Thus, the additional effects of polyphenols on these activities were different with difference of the kind of metals.

Chapter 6 : Bait-Toxicant Application of Steamed Larch Heartwood Extracts Incorporating Boric Acid Impregnation

6.1 Introduction

Chemical treatments of wood and/or soil are major termite control methods in today's world to protect wooden structures from termite attack. Such treatments require a large amount of chemicals and cost. In the United States, about \$ 1.5 billion were spent for termite control per year (Su, 1991).

Baiting systems, which are usually constructed with a combination of bait substrates and toxicants, has been introduced to eradicate termites. The systems may use less one-thousandth pesticide than the current soil termiticide treatments according to a preliminary estimate (Su and Scheffrahn, 1990a). Various kinds of slow-acting and environmental acceptable poisons are applied to be spread throughout the colony. First trial of the system was carried out using a decayed wood and a slow-acting toxicant, mirex (Esenther and Beal, 1974; 1978). Recently, moulting inhibitors such as diflubenzuron and hexaflumuron (Su and Scheffrahn, 1990b; Su, 1994), boron compounds (Grace, 1991; Susan, 1991) were tried to use as bait-toxicants. Insect growth regulators (IGRs) such as methoprene, fenoxycarb, Ro-16-1295 and S-31183 may also be used in future because of their delayed activity (Su and Scheffrahn, 1990c). On the other hand, bait-substrates must be more attractive than the surrounding food sources (Pearce, 1997). In China, crushed bagasse, eucalypt bark or pine sawdust is used with mirex, and placed in termite infested area. (Li *et al.*, 1994).

As described in Chapter 3, the water-soluble fraction (S-Water) from the hot-water extracts of the steamed Japanese larch heartwood showed the feeding-preference activity to a termite, *C. formosanus*. Thus, the S-Water is a potential bait-additive in a baiting system. In this chapter, the additive effects of the

S-Water to accelerate the ingestion of paper discs permeated with boric acid were investigated. Boron contents in the termite bodies were also measured to know whether boron could be distributed to other individuals (Ohmura *et al.*, 1999d, to be published).

6.2 Materials and Methods

6.2.1 Preparation of the test substrates

The water-soluble fraction (S-Water) from the hot-water extracts of the steamed Japanese larch heartwood was prepared as described in Chapter 3.

The S-Water was dissolved in the 150 ml of deionized water. Paper discs (13 mm diameter, Whatman International Ltd. *ca.* 60 mg per disc) were permeated with 90 µl of the S-Water solution incorporating boric acid (SB). They were dried at 60 °C for 12 h followed by drying in a vacuum desiccator for one day. Discs treated only with boric acid (BA) were prepared by the same procedure as above. Boric acid retentions were 0, 0.01-2.0 % (w/w per disc) for both the SB and the BA series.

6.2.2 Termite bioassay

6.2.2.1 Termite

Termites used in this chapter were obtained from the laboratory colony of *C. formosanus* as described in the preceding chapter. Soldiers were also obtained from the same colony.

6.2.2.2 Test chamber

A test chamber was made of a plastic cup (rim diameter ; 60 mm, bottom diameter ; 50 mm, height ; 50 mm) with a hard plaster bottom. Three g of sea sand (15-20 mesh: Junsei Chemical Co., Ltd.) was layered in the center of the test chamber, and moistened with 0.5 ml of deionized water. A disc on a plastic saucer (diameter ; 15 mm) was put onto the sea sand so that termites could easily reach the disc (Fig. 6-1a). A dampened cotton pad was spread under the chamber to supply the water to the sea sand layer.

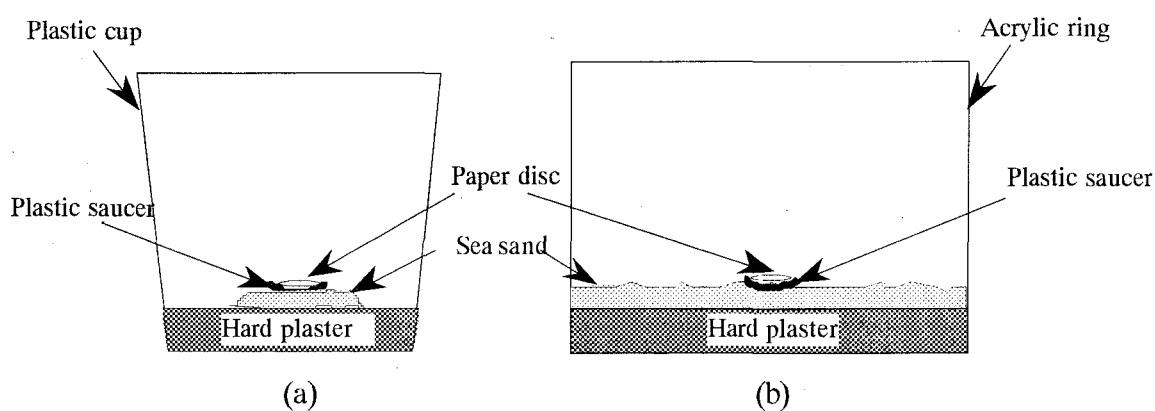


Fig. 6-1. Diagrams of test chambers for (a) no-choice bioassays and (b) boron analysis.

6.2.2.3 Short-term no-choice bioassay

A disc treated with the SB or BA at retentions ranging from 0 to 2.0 % boric acid was put on a saucer and 100 workers were introduced into a test chamber. Consumptions (weight loss in mg) of each three discs after 3 day were measured and averaged.

6.2.2.4 Long-term no-choice bioassay

Fifty workers were put into a test chamber, and forced to feed on each three disc treated with the SB or BA series with no boric acid (SB-0, BA-0), 0.1 % boric acid (SB-0.1, BA-0.1) or 2.0 % boric acid (SB-2, BA-2). Starvation (unfed) was also conducted. Termites were transferred into new discs every 3 days. Consumptions of the discs were also measured. Termites were checked every 3 days for mortality until the final 21 day.

6.2.3 Quantitative analysis of boron

6.2.3.1 Test chamber

An acrylic pipe (inner diameter ; 80 mm, height ; 50 mm) with a hard plaster bottom was used as a test chamber (Fig. 6-1b). Fifty g of sea sand (15-20 mesh: Junsei Chemical Co., Ltd.) was layered on the bottom of the test chamber, and moistened with 10 ml of deionized water. A disc on a plastic saucer (diameter ; 15 mm) was put on the center of the test chamber. A dampened cotton pad was also spread under the chamber to supply water into the sea sand layer.

6.2.3.2 Boron analysis

Two hundred workers with 30 soldiers were put into a test chamber, and forced to feed on each disc treated with the SB-0, SB-0.1, SB-2, BA-0, BA-0.1 or BA-2 for 7 days. Six chambers were prepared for each experiment. After that, 60 workers or 50 soldiers were picked up from one or two chambers, and put into a Teflon™ vessel.

On the other hand, 200 workers were put into a test chamber, and forced to feed as same as above. Three chambers were prepared for each experiment. After 7 days, 60 workers were picked up from a chamber, and put into a Teflon™ vessel.

The termites in each Teflon™ vessel were decomposed with HNO₃/H₂O₂ according to the literature (Haraguchi, 1992), and the obtained mixture was filtered through 0.45 µm diskfilter unit to get sample filtrate. Boron content of the filtrate was analyzed using inductively coupled plasma atomic-emission spectrometer (ICP-AES; Seiko SPS7700) at the wavelength of 249.773 nm after checking no interference of iron. Weight losses of the discs were also measured to estimate the uptake of boron by workers.

6.2.4 Statistical analysis

Statistical analyses were conducted with Tukey-Kramer HSD test using the computer software JMP (SAS Institute, 1989).

6.3 Results and Discussion

6.3.1 Effects of S-Water on the ingestion of boric acid

Consumptions of the discs in short-term no-choice bioassay are shown in Fig. 6-2. Consumptions of the discs were significantly decreased at 2.0 % retention in both the SB and BA treatments ($p < 0.01$). At the retentions of 0.01, 0.05 and 0.1 %, the termites consumed the SB discs larger than the BA discs at the same boric acid retentions and untreated control discs ($p < 0.01$). This suggests that termites could not detect the presence of below 0.1 % of boric acid in the discs by feeding during 3-day experiment.

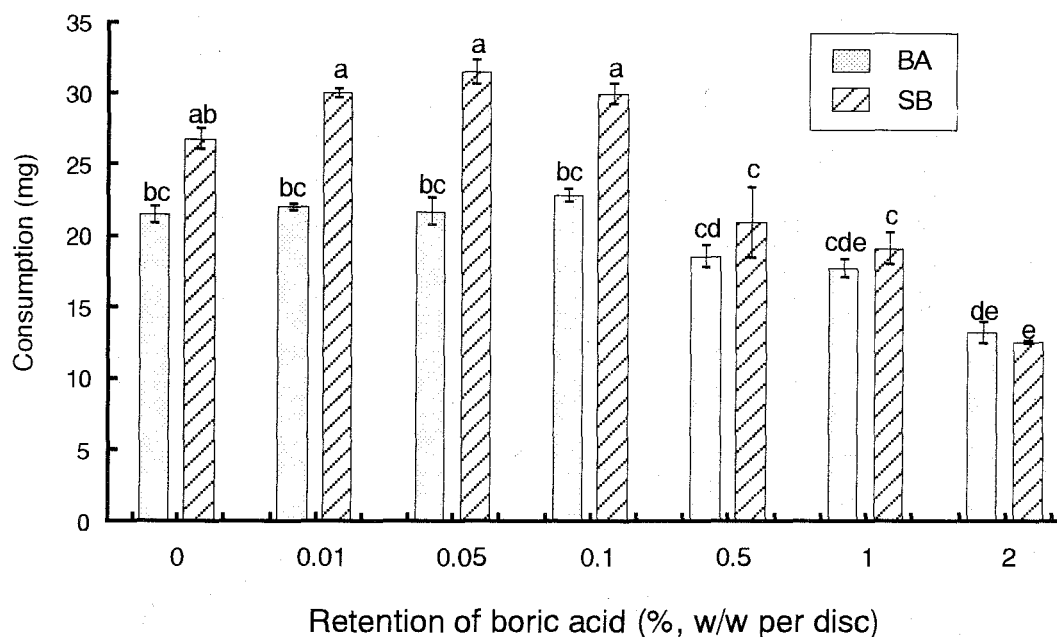


Fig. 6-2. Consumptions of the discs (mg) treated with boric acid (BA) or the S-Water plus boric acid (SB) series for 3-day exposure to 100 workers of *C. formosanus*.

Notes : The S-Water is the water-soluble fraction from hot-water extract of steamed larch heartwood. Error bars indicate $\pm$ standard errors (N=3). Different letters (a, b, c, d, e) denote significant differences at the 0.01 level.

On the other hand, the average consumptions of the SB discs were always larger than BA discs with the same retention of boric acid through 21 days in long-term no-choice bioassay (Fig. 6-3). Furthermore, the consumptions decreased with the time-passing in all discs irrespective of treatments. Tsunoda *et al.* (1993) reported that the rates of methane emission by the workers of *C. formosanus* decreased with the lapse of experimental time. Thus, the artificial culturing in the small test chambers may effect on the termite health conditions.

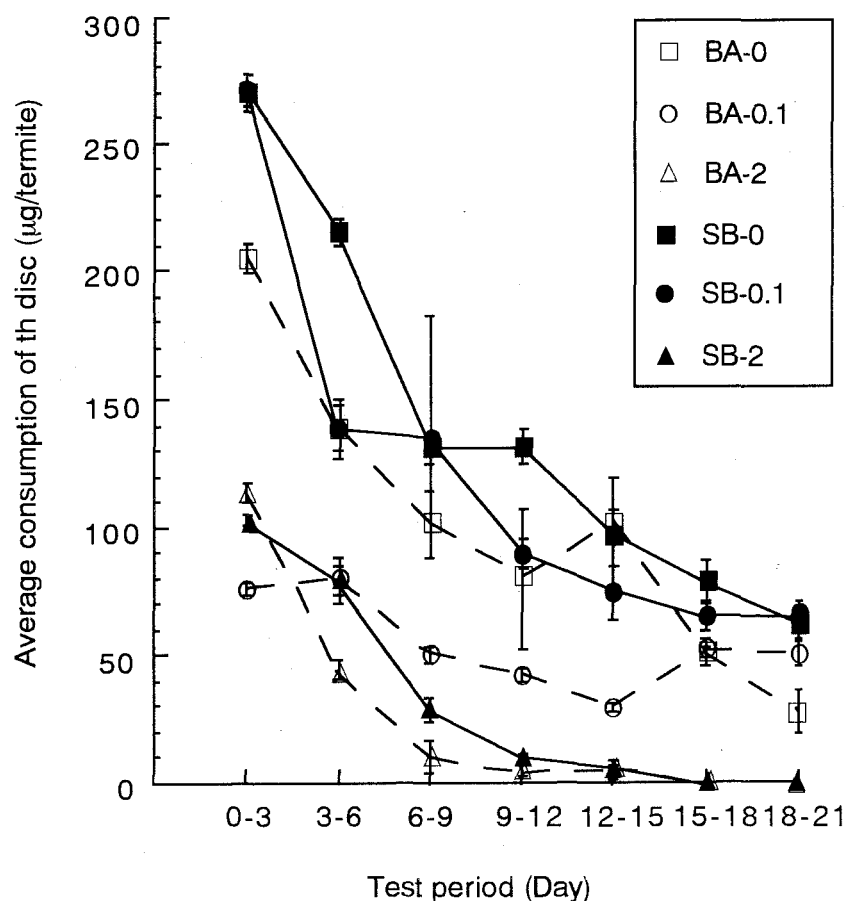


Fig. 6-3. Change of the average consumption of the discs ($\mu\text{g}/\text{termite}$) per 3 days by the workers of *C. formosanus* during the test period of 21 days.

Notes : BA-0, BA-0.1 and BA-2 are 0, 0.1 and 2.0 % (w/w per disc) boric acid retention, respectively. SB-0, SB-0.1 and SB-2 are the SB containing boric acid at retentions of 0, 0.1 and 2.0 % (w/w per disc), respectively. BA, SB and error bars are the same as in Fig. 6-2.

6.3.2 Accumulation of boron in termite bodies

In termite societies, soldiers play a role for defense while they depend on workers for feeding. Thus, soldiers were introduced into the test chambers

together with workers in order to know the transportation of boron from workers to soldiers through trophallaxis. Test chambers introducing only workers were also prepared for comparison.

At first, uptakes of boron by workers were calculated based on the consumptions of the discs (Table 6-1). Uptake of boron in the SB-0.1 disc was tended to be higher than that in the BA-0.1 disc regardless of the existence of soldiers, while there was no difference between the SB-2 and BA-2 discs. These results reflected the mortalities of termites shown in Fig. 6-3.

Table 6-1. Calculated boron uptake (ng/termite) by workers of *C. formosanus* for 7-day exposure.

Treatments	Uptakes of boron into workers (ng/termite)	
	With soldiers	Without soldiers
BA-0.1	94.6 $\pm$ 3.62 ab	90.2 $\pm$ 0.52 b
BA-2	671 $\pm$ 60.1 c	584 $\pm$ 5.33 c
SB-0.1	109 $\pm$ 3.13 a	108 $\pm$ 2.80 a
SB-2	689 $\pm$ 62.1 c	668 $\pm$ 30.5 c

Notes: BA-0.1, BA-2, SB-0.1 and SB-2 are the same as in Fig. 6-3. Values indicate the means of the uptakes of boron $\pm$ standard errors (N=3). Values followed by the same letters (a, b, c) are not significantly different at the 0.01 level.

These termites were subjected to the determination of boron contents in their bodies by ICP-AES. The results are shown in Table 6-2. Boron was detected even in soldier bodies. When the SB-2 and BA-2 discs were exposed to workers and soldiers, boron contents in workers were lower than those of the same experiment without workers. A part of boron ingested by workers were thought to be incorporated into soldiers. Almost the same contents of boron were detected in

Table 6-2. Boron content in termite bodies (ng/termite) detected by ICP-AES.

Treatments	Boron contents (ng/termite)		
	With soldiers		Without soldiers
	Soldiers	Workers	Workers
BA-0	3.06 ± 2.54 e	2.40 ± 1.02 e	4.92 ± 0.61 e
BA-0.1	7.32 ± 1.67 e	46.8 ± 6.92 de	44.0 ± 3.89 de
BA-2	115 ± 1.62 de	368 ± 32.5 c	476 ± 31.1 b
SB-0	0.00 ± 0.00 e	3.71 ± 0.60 e	0.65 ± 0.17 e
SB-0.1	10.6 ± 2.08 e	46.7 ± 10.4 de	43.3 ± 4.26 de
SB-2	146 ± 19.4 d	447 ± 39.8 bc	610 ± 29.0 a

Notes: BA-0, BA-0.1, BA-2, SB-0, SB-0.1 and SB-2 are the same as in Fig. 6-3.

Values indicate the means of the uptakes of boron ± standard errors (N=3).

Values followed by the same letters (a, b, c, d, e) are not significantly different at the 0.01 level.

the termites fed on the SB-0.1 and BA-0.1 discs, although the formers showed larger consumption of discs than the latters. Standard errors in the analysis data were too large for averages to obtain the significant difference between the two discs.

On the other hand, workers tested on the SB-2 discs had larger content of boron than those on the BA-2 discs (Table 6-2), although they ingested almost equal amount of boron (Table 6-1). It was assumed that the orally ingested boron was difficult to excrete or easy to accumulate in the termite bodies due to the presence of the S-Water. Boron forms complexes with almost all pentoses and hexoses (Chapelle and Verchere, 1988). As the main components of the S-Water are low-molecular weight saccharides (Chapter 3), the formation of boron-saccharide complexes might function to interrupt the excretion of boron from the termite bodies or to increase the accumulation in the bodies.

6.3.3 Effects of S-Water on the mortality

Fig. 6-4 shows the changes of the termite mortalities fed on the discs treated with the SB or BA containing boric acid at retentions of 0, 0.1 and 2.0 % (w/w). Unfed termite (starvation) reached an average mortality of 26 % at 21st day, compared with 12 % and 16 % mortalities in the treatments of the S-Water (SB-0) and untreated control (BA-0), respectively. The S-Water showed no significant effect on the mortality at each retention of boric acid, although the larger consumption was observed on the SB-0.1 discs in comparison with that of the BA-0.1 (Fig. 6-2). The mortalities on the SB-0.1 and BA-0.1 discs were almost the same as those of the SB-0 and BA-0 discs. Significant difference in mortalities between the SB-0.1 and BA-0.1 treatments might be occurred if the bioassay would be continued more than 21 days. In the application of the SB-2 and BA-2, the drastic increases of mortalities were observed after 12 days to reach approximately 100 % mortalities after 21 days. The same phenomenon was also observed when nickel (II) was applied to *C. formosanus* (Chapter 5). The reason for these sudden increases of mortality is not understood but some breaking of critical level of toxicant deposits could be supposed. The boron content in termite bodies are supposed to increase with time. When the accumulation of the toxicant exceeds the lethal accumulative dose, termites will be dead. The reason for no difference in mortalities between SB-0.1 and BA-0.1 discs might be that boron contents in termites fed on these discs did not reach the lethal accumulative dose during 21 days. As boron content in termite bodies fed on SB-0.1 would reach the lethal accumulative dose earlier than that of BA-0.1, significant difference in mortalities between the SB-0.1- and BA-0.1-treatments might occur if the bioassay would be continued more than 21 days. Toyoshima *et al.* (1997) suggested that boric acid forms complexes with biological substances having *cis*-hydroxyl groups in termite bodies, and these

complexes might cause the disruption of physiological functions of termite. On the other hand, it is assumed that the formation of boron complexes with saccharides in the S-Water results in delaying the effect of the toxicity. Such delayed toxicity of boric acid seems to be useful for the baiting system.

Thus, the S-Water incorporating boric acid was found to have potential as a bait-toxicant. Extended studies including field tests will be conducted before applying this bait-toxicant to a practical use.

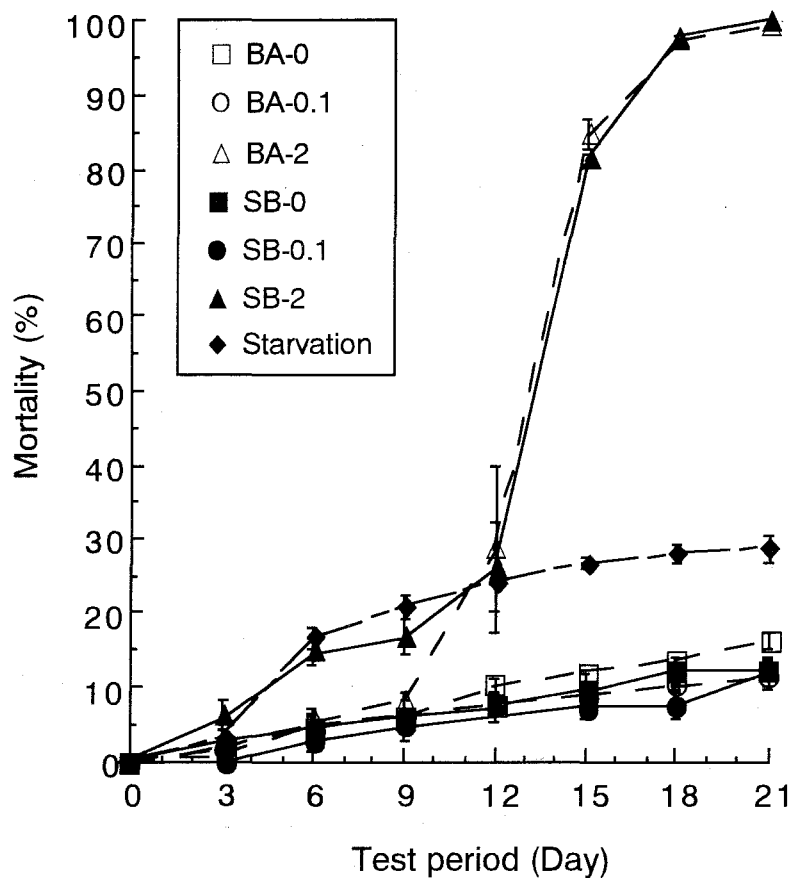


Fig. 6-4. Change of mortalities (%) of the workers of *C. formosanus* for 21 days.

Notes : BA, SB and error bars are the same as in Fig. 6-2. BA-0, BA-0.1, BA-2, SB-0, SB-0.1 and SB-2 are the same as in Fig. 6-3.

6.4 Summary

Laboratory tests were conducted to determine the efficacy of addition of extracts from the steamed larch heartwood for the uptake of boric acid against *C. formosanus*. Paper discs were treated with the water-soluble fraction obtained from the hot-water extracts of the steamed larch wood (S-Water) with or without boric acid at retention of 0.1 or 2.0 %, followed by exposing to the termites. The consumptions of the discs, termite mortalities and boron contents in the termite bodies were measured. The S-Water significantly accelerated the consumptions of the discs at the retentions of less than 0.1 % boric acid ($p < 0.01$). However, regardless of the addition of the S-Water, the termite mortalities were almost same after the 21-day exposure. The results of boron analyses showed that boron was incorporated even into soldier bodies. Such transferability of boron from workers to soldiers indicated that boron could be spread among other termites which take no food directly, and accumulated in their bodies. When the paper discs permeated with extracts at 2.0 % retention of boric acid were exposed to workers and soldiers, boron contents in workers were lower than those of the same experiment without soldiers. Workers might take more food in order to feed soldiers which cannot eat by themselves. A part of boron ingested by workers were thought to be incorporated into soldiers through such trophallaxis. The determined boron contents of workers fed with 2.0 % boric acid plus the S-Water tended to be larger than those of the termites fed without the S-Water. These results suggested that the S-Water inhibited the excretion of boron from the termite bodies or increased the accumulation of it in the termite bodies. Thus, the S-Water will be available for a bait-additive in the future baiting system.

CONCLUSION

A major termite control methods nowadays is chemical treatment of wood and/or soil. Such treatment needs a large amount of pesticide, so that bad influences upon our environments sometimes happened. It is not too much to say that our excessive consumption of synthetic chemicals have caused today's serious environmental problems. In order to solve these problems, the utilization of safer natural compounds are taken an increasing interest in various purposes. In this dissertation, various wood components affecting termite behavior (semiochemicals) were investigated. Based on the results, the preliminary attempts to the application of these components for termite controls were conducted.

We have acquired the knowledge of the chemical characteristics of woods. A lots of antitermitics have been isolated and identified from resistant woods, nevertheless, the investigation of their structure-antitermitic activity relationships are still insufficient. PART I dealt with the wood components, saponins and flavonoids, which were known to have the antitermitic activity and discussed the structure-activity relationships.

Monodesmosidic saponins with methyl oleanolate as aglycone were synthesized and they were subjected to antitermitic bioassays. Methyl oleanolate glycosides showed the higher termiticidal activities than their aglycon, methyl oleanolate, although no clear correlation was found between the structures and the termiticidal activities against *R. speratus*. The feeding behavior of *R. speratus* was most inhibited by both methyl oleanolate-3-yl β -D-glucoside and methyl oleanolate-3-yl β -D-cellobioside, and that of *C. formosanus* was most inhibited by methyl oleanolate-3-yl β -D-cellobioside. The activity decreased with lengthening of the chain of the sugar moiety. From these results, it is assumed that the adequate polarity is necessary to reveal the antitermitic activities of triterpenoid saponins,

because the molecular hydrophilicity increases with the increasing amounts of sugar residues.

On the structures of flavonoids and their antifeedant activities, it was found that compounds containing two hydroxyl groups at C-5 and C-7 in A-rings showed high antifeedant activity. Further, the presence of carbonyl group at C-4 in pyran rings of the compounds was necessary for the occurrence of the high activity. Also, 3-hydroxyflavones and 3-hydroxyflavanones with 3',4'-dihydroxylated B-rings exhibited the higher activity than those with 4'-hydroxylated.

These informations on the structure-activity relationships obtained might be useful for further syntheses of the more active compounds.

In PART II, two cases on the termite-wood interaction were investigated in order to find new termite control agents.

First case is the feeding-preference of steamed larch wood. By the steam treatment, Japanese larch heartwood lost its some extent of resistance and turned to be seriously attacked by termites. The larch heartwood has several kinds of flavonoids. They showed the high antifeedant activity against *C. formosanus* contributing to its higher durability. However, these flavonoids were not detected in the hot-water extracts of the steamed heartwood, indicating that flavonoids underwent some degradation during steaming. On the other hand, arabinogalactan, which is contained in the non-steamed larch heartwood about 5%, showed the feeding-preference activity. According to the sugar analyses, the main sugars in the hot-water extracts of the non-steamed larch heartwood were arabinose and galactose, which suggests that arabinogalactan is a main carbohydrate of the hot-water extracts. In the steamed heartwood, the ratio of arabinose and galactose decreased, suggesting that arabinogalactan was degraded and converted to other substances through steaming. The water soluble fraction obtained from the hot-water extracts of the steamed heartwood after successive extraction by *n*-hexane,

diethyl ether and ethyl acetate had the high feeding-preference activity. As the fraction contained mainly mono- or low molecular saccharides and a little amounts of the colored substances, it was supposed that the saccharides affected the feeding behavior of termite. However, the feeding-preference activity was found in both the first fraction of the water eluates and 50% MeOH eluate after passing through the column of a Superpak-2 resin. This suggests that the several feeding-preference constituents are existed in the steamed larch. Further study is needed to know the precise structures of the constituents.

Another case of termite-wood interaction is the preference for decayed woods. Wood decayed by brown-rot fungi, *Gloeophyllum trabeum* and *Serpula lacrymans*, are known to attract *C. formosanus* as well as other rhinotermitids. Attractants produced by *S. lacrymans* have not been elucidated yet, whereas attractant by *G. trabeum* is known due to the substance analogous to a trail-following pheromone of *C. formosanus*, (Z,Z,E)-3,6,8-dodecatrien-1-ol (DTE-OH). As the both *n*-hexane extracts from *S. lacrymans*-decayed wood and *G. trabeum* mycelia showed trail-following activity, the author tried to isolate and identify the trail-following active substances in combination with bioassay and chemical analyses. The trail-following activity of *G. trabeum* mycelia was 105 times as high as that of *S. lacrymans*-decayed wood. GC-MS-SIM analysis showed that *G. trabeum* mycelia has the peaks corresponded with DTE-OH but the trail-following active compound in the *S. lacrymans* decayed wood was not DTE-OH. It is possibly a type of alcohol, although further confirmation is needed.

In PART III, wood components which affect the survival and feeding of *C. formosanus* were tried to apply for termite control agents. Their modes-of action were also discussed.

To utilize polyphenols, which are abundant in woody plants, for termite control agents, they were mixed with heavy metals and evaluated their termiticidal activity. The improved activities were different with the difference of mixed metals.

Addition of Ni to polyphenols tended to increase the ingestion and enhanced the termiticidal activity, whereas, mixture of catechin and Ni showed no protein-precipitation capacity. It was suggested that the termiticidal activity was not due to the increase of the astringent property, but due to the increase of ingestion of Ni. Furthermore, impregnation of catechin on zeolite gravel enhanced the prevention of penetration by the workers of *C. formosanus*. Impregnation of other flavonoids tested in Chapter 2 may also improve the preventive effect of zeolite gravel on the penetration by the termite.

For bait-toxicant application, the water-soluble fraction prepared from the hot-water extracts of the steamed larch heartwood with high feeding-preference activity was used incorporating boric acid as toxicant. Addition of the water-soluble fraction obtained from the hot-water extracts gave the higher uptake of boric acid by *C. formosanus*. The lower retention of boric acid was useful for the toxicant because of the delayed effectiveness.

Although antitermitic activities of wood components tested are not high as the conventional synthetic termiticides, these components can be converted to more active ones by simple methods as described in this dissertation. New termite control agents are formed by the biological or chemical transformation of wood components by fungi or steaming. Incorporation of these substances derived from wood components and commercial termiticides may result in reducing the dosage of the termiticides. The author hopes that this dissertation will be an exemplary model of the utilization of the wood components for the environmentally acceptable termite control agents.

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