# Development of Bioactive Functions in Hydrangeae Dulcis Folium. III.[1) On the Antiallergic and Antimicrobial Principles of Hydrangeae Dulcis Folium. (1). Thunberginols A, B, and F

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**From the less polar fraction of Hydrangeae Dulcis Folium, the fermented and dried leaves of *Hydrangea macrophylla* SERINGE var. *thunbergii* MAKINO, eight antiallergic and antimicrobial principles were isolated together with several known compounds. Among the newly isolated bioactive constituents, the chemical structures of thunberginols A, B, and F have been determined on the basis of chemical and physicochemical evidence. Thunberginols A, B, and F were found to exhibit more potent antiallergic activity than phyllodulcin, hydrangenol, disodium cromoglycate (DSCG), and tranilast. In addition, these thunberginols showed antimicrobial activity against oral bacteria.**

**Keywords** thunberginol; antiallergic activity; oral bacteria; antimicrobial activity; Hydrangeae Dulcis Folium; *Hydrangea macrophylla* var. *thunbergii*

Hydrangeae Dulcis Folium (Amacha in Japanese), a crude drug indigenous to Japan, is listed in the Japanese Pharmacopeia XII as an oral refrigerant and a sweetening agent. This natural medicine is prepared from the leaves of *Hydrangea macrophylla* SERINGE var. *thunbergii* MAKINO (Saxifragaceae) *via* fermentation and drying. In regard to the bioactive constituents of this crude drug, three dihydroisocoumarins, phyllodulcin (**4**), hydrangenol (**5**), and phyllodulcin monomethyl ether (**4a**), have been isolated, and among them, **4** has been shown to be a sweet and antifungal principle.[2)]

As a part of our studies on bioactive constituents of natural medicines, we have reported that the less polar fraction obtained from Hydrangeae Dulcis Folium exhibited various biological activities, including potent antiulcer, antiallergic, and cholagogic activities.[1)] We have also developed a quantitative analysis method of dihydroisocoumarins such as **1**, **2** and their glucosides by means of HPLC. By use of this method, the chemical processing of this natural medicine was clarified together with the distribution in the plant and seasonal fluctuation of these dihydroisocoumarins.[3)] As a continuation of that work, we have isolated eight antiallergic and antimicrobial principles named thunberginols A (**1**),[4)] B (**2**),[4)] C,[5)] D,[5)] E,[5)] and F (**3**),[4)] and hydramacrophyllols A[6)] and B[6)] from the bioactive less polar fraction of Hydrangeae Dulcis Folium. Furthermore, we have developed an efficient chemical transformation from dihydroisocoumarin to benzylidenephthalide and isocoumarin. As an application of this synthetic method, thunberginols A (**1**) and F (**3**) were efficiently synthesized from phyllodulcin (**4**).[6)] In this paper, we present a full account of the structure elucidation of thunberginols A (**1**), B (**2**), and F (**3**) and evaluation of their antiallergic and antimicrobial activities.[4)]

Hydrangeae Dulcis Folium, cultivated and processed in Nagano Prefecture, was extracted with methanol and the extract was found to show strong antiallergic activity in the *in vivo* passive cutaneous anaphylaxis (PCA) test[7)] and the *in vitro* Schults–Dale test[8)] as described in the previous paper.[1)] The methanol extract was subjected to fractionation and purification as shown in Chart 1. Thus, the extract was partitioned into a mixture of ethyl acetate and water, and the ethyl acetate-soluble portion, which showed strong antiallergic activity in *in vivo* and *in vitro* tests,[1)] was subjected to column chromatography with ordinary-phase and reversed-phase silica gel and Sephadex LH-20. Eight new active principles, thunberginols A (**1**, 0.0086% from the crude drug),[9)] B (**2**, 0.0016%), C (0.0038%),[9)] D (0.0012%), E (0.0045%),[9)] and F (**3**, 0.0028%),[9)] and hydramacrophyllols A (0.00013%) and B (0.00047%) have so far been isolated from the ethyl acetate-soluble portion, together with phyllodulcin[2)] (**4**, 1.99%), hydrangenol[2)] (**5**, 2.35%), phyllodulcin monomethyl ether[2)] (**4a**, 0.0004%), umbelliferone[3)] (**6**, 1.41%), hydrangeic acid[10)] (**7**, 0.0024%), and dihydroresveratrol[11)] (**8**, 0.0008%). Isoarborinol (**9**, 0.0026%),[12)] and rubiarbonol B (**10**, 0.0022%)[13)] have been isolated from the chloroform-soluble portion.

Thunberginol A (**1**) was obtained as yellow prisms of mp 240 °C and the molecular formula, $C_{15}H_{10}O_5$, of **1** was confirmed by the molecular ion peak in the electron impact mass spectrum (EI-MS) and high resolution mass (HR-MS) measurement. The infrared (IR) spectrum of **1** showed absorption bands due to hydroxyl, chelated δ-lactone, and aromatic ring at 3436, 1669, 1611, 1526, and 1221 $cm^{-1}$. From the ultraviolet (UV) spectrum of **1**, it was presumed to possess the 3-phenylisocoumarin skeleton[14)] having a chelated 8-hydroxyl group [absorption maxima (log ε) in EtOH at 223 (4.0), 347 (4.1), and 365 (4.0) nm and in EtOH containing aluminium chloride at 224, 333, and 407 nm]. The proton nuclear magnetic resonance ($^1$H-NMR) spectrum of **1** showed the presence of two trisubstituted benzene rings [δ 7.69 (dd, $J$=8, 8 Hz, 6-H), 7.10 (br d, $J$=8 Hz, 5-H), 6.93 (br d, $J$=8 Hz, 7-H); 7.30 (d, $J$=2 Hz, 2′-H), 7.24 (dd, $J$=2, 8 Hz, 6′-H), 6.88 (d, $J$=8 Hz, 5′-H)], a conjugated olefin function [δ 7.23 (s, 4-H)] and a chelated hydroxyl group [δ 10.87 (br s)].

Acetylation of **1** with acetic anhydride in pyridine

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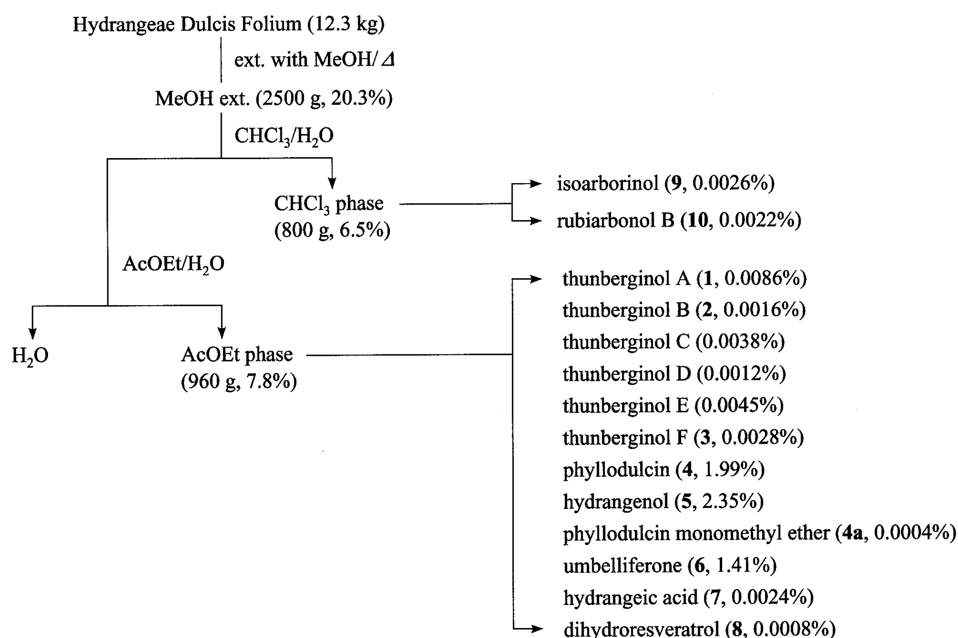


Chart 1

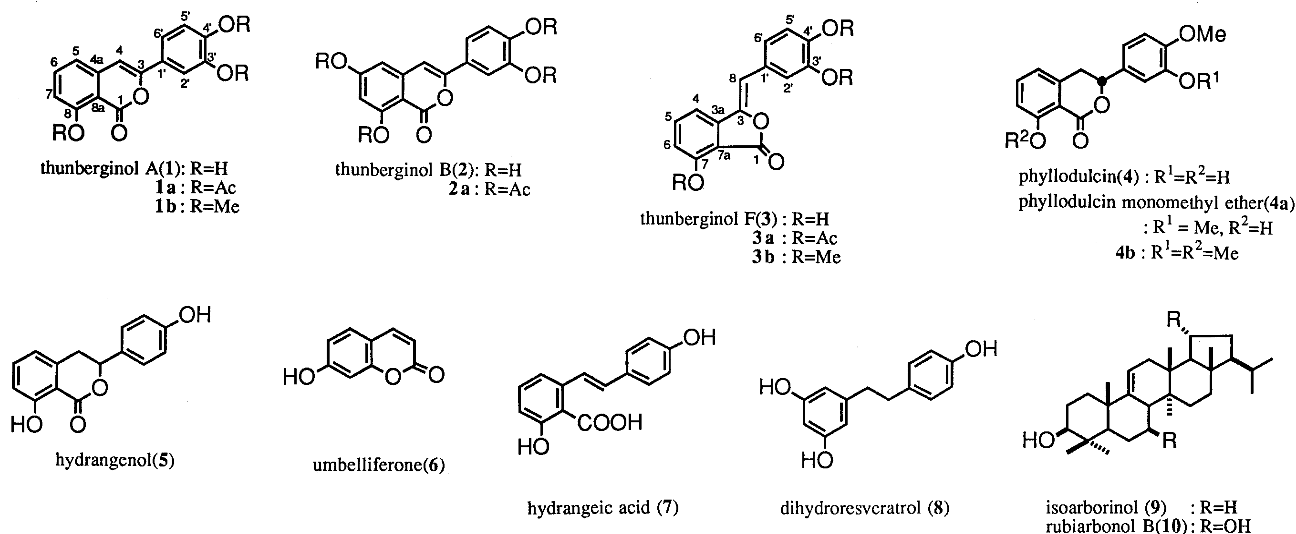


Chart 2

furnished the triacetate (**1a**), while methylation of **1** with diazomethane in a mixture of methanol and ether yielded the trimethyl ether (**1b**). The IR spectra of **1a** and **1b** showed the presence of δ-lactone (**1a**: $1732\,cm^{-1}$, **1b**: $1719\,cm^{-1}$). Furthermore, examination of the $^{13}C$-NMR data (Table II) of **1** and **1a** led us to presume the structure of thunberginol A as 3-(3,4-dihydroxyphenyl)-8-hydroxyisocoumarin (**1**). Finally, the chemical structure of **1** was determined by chemical correlation with phyllodulcin (**4**). Thus, diazomethane methylation of **4** and subsequent 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) oxidation in benzene provided thunberginol A trimethyl ether (**1b**) in 70% yield.

Thunberginol B (**2**), obtained as pale yellow needles of mp 244 °C, was more polar than thunberginol A (**1**) in thin layer chromatographic (TLC) examination and its molecular formula was $C_{15}H_{10}O_6$ as determined by high MS measurement. The UV and IR spectra of **2** indicated a close similarity of its structure to that of **1**. The $^1H$-NMR spectrum of **2** showed the presence of one trisubstituted benzene ring [δ 7.27 (d, $J$=2 Hz, 2'-H), 7.22 (dd, $J$=2, 8 Hz, 6'-H), 6.85 (d, $J$=8 Hz, 5'-H)], one tetrasubstituted benzene ring [δ 6.48 (d, $J$=2 Hz, 7-H), 6.31 (d, $J$=2 Hz, 5-H)], and a conjugated olefin proton [δ 7.11 (s, 4-H)]. Acetylation of **2** with acetic anhydride in pyridine furnished the tetraacetate (**2a**). Comparison of the $^{13}C$-NMR data (Table II) for **2** and **2a** with those for **1** have led us to formulate the structure of thunberginol B as 3-(3,4-dihydroxyphenyl)-6,8-dihydroxyisocoumarin (**2**).

Thunberginol F (**3**) was obtained as yellow needles of mp 242—243 °C. The MS and high MS measurement of **3** revealed its molecular formula to be $C_{15}H_{10}O_5$. The UV spectrum of **3** suggested the benzylidenephthalide structure[15] [absorption maxima (log ε) at 225 (4.1), 290

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Table I. The $^{1}$H-NMR Data for **1**, **1a**, **1b**, **2**, **2a**, **3**, **3a**, and **3b**

| | **1**$^{a)}$ | **1a**$^{b)}$ | **1b**$^{b)}$ | **2**$^{a)}$ | **2a**$^{b)}$ | | **3**$^{a)}$ | **3a**$^{b)}$ | **3b**$^{b)}$ |
|---|---|---|---|---|---|---|---|---|---|
| 4-H | 7.23 s | 6.91 s | 6.75 s | 7.11 s | 6.86 s | 4-H | 7.41 br d (8) | 7.61 br d (8) | 7.29 br d (8) |
| 5-H | 7.10 br d (8) | 7.37 br d (8) | 7.01 br d (8) | 6.48 d (2) | 7.20 d (2) | 5-H | 7.58 dd (8, 8) | 7.73 dd (8, 8) | 7.63 dd (8, 8) |
| 6-H | 7.69 dd (8, 8) | 7.71 dd (8, 8) | 7.60 dd (8, 8) | | | 6-H | 6.91 br d (8) | 7.20 br d (8) | 6.91 br d (8) |
| 7-H | 6.93 br d (8) | 7.14 br d (8) | 6.91 br d (8) | 6.31 d (2) | 6.96 d (2) | 7-OH | 9.27 br s | | |
| 8-OH | 10.87 br s | | | 10.94 br s | | 8-H | 6.61 s | 6.39 s | 6.34 s |
| 2′-H | 7.30 d (2) | 7.69 d (2) | 7.36 d (2) | 7.27 d (2) | 7.68 d (2) | 2′-H | 7.38 d (2) | 7.73 d (2) | 7.46 d (2) |
| 5′-H | 6.88 d (8) | 7.30 d (8) | 6.92 d (8) | 6.85 d (8) | 7.31 d (9) | 5′-H | 6.79 d (8) | 7.24 d (8) | 6.89 d (9) |
| 6′-H | 7.24 dd (2, 8) | 7.73 dd (2, 8) | 7.47 dd (2, 8) | 7.22 dd (2, 8) | 7.72 dd (2, 9) | 6′-H | 7.04 dd (2, 8) | 7.64 dd (2, 8) | 7.39 dd (2, 9) |
| OMe | | | 3.94 | | | OMe | | | 3.93 |
| | | | 4.00 | | | | | | 3,97 |
| | | | 4.02 | | | | | | 4.03 |
| OAc | | 2.32 | | | 2.32 | OAc | | 2.31 | |
| | | 2.33 | | | 2.33 | | | 2.34 | |
| | | 2.45 | | | 2.35 | | | 2.45 | |
| | | | | | 2.44 | | | | |

Coupling constants ($J$ values in Hz) are given in parentheses. *a*, *b*) The $^{1}$H-NMR spectra were measured in *a*) DMSO-$d_6$ or *b*) $CDCl_3$.

Table II. The $^{13}$C-NMR Data for **1**, **1a**, **2**, **2a**, **3**, **3a**, and **3b**$^{a)}$

| | **1** | **1a** | **2** | **2a** | | **3** | **3a** | **3b** |
|---|---|---|---|---|---|---|---|---|
| 1 | 165.4 | 158.4 | 165.8 | 157.8 | 1 | 164.7 | 168.6 | 165.7 |
| 3 | 153.0 | 152.4 | 152.8 | 153.0 | 3 | 141.8 | 142.1$^{b)}$ | 143.0$^{b)}$ |
| 4 | 100.9 | 101.1 | 100.7 | 101.9 | 3a | 142.5 | 142.2$^{b)}$ | 143.2$^{b)}$ |
| 4a | 138.7 | 139.6 | 140.2 | 140.5 | 4 | 110.5 | 123.7$^{c)}$ | 111.1$^{c)}$ |
| 5 | 116.8 | 122.8 | 103.3 | 116.1 | 5 | 136.7 | 136.1 | 136.4 |
| 6 | 137.8 | 135.7 | 165.0 | 155.7 | 6 | 115.9 | 124.8 | 111.3$^{c)}$ |
| 7 | 114.3 | 124.0$^{b)}$ | 101.5 | 116.8 | 7 | 157.1 | 148.7 | 158.6 |
| 8 | 160.7 | 152.1 | 162.7 | 153.3 | 7a | 108.1 | 117.4 | 107.1 |
| 8a | 105.4 | 113.4 | 98.0 | 110.8 | 8 | 106.8 | 106.4 | 107.1 |
| 1′ | 122.5 | 130.3 | 122.5 | 130.0 | 1′ | 125.0 | 131.7 | 126.3 |
| 2′ | 112.4 | 120.6 | 112.3 | 120.8 | 2′ | 116.7 | 117.4 | 110.7 |
| 3′ | 145.9 | 142.5 | 145.7 | 142.5 | 3′ | 145.5 | 142.3$^{b)}$ | 149.0 |
| 4′ | 148.0 | 143.6 | 147.8 | 143.8 | 4′ | 146.4 | 144.3 | 149.5 |
| 5′ | 116.2 | 124.1$^{b)}$ | 116.1 | 124.1 | 5′ | 115.9 | 128.5 | 112.7 |
| 6′ | 117.2 | 123.4 | 117.1 | 123.5 | 6′ | 122.5 | 123.3$^{c)}$ | 123.7 |

*a*) Compounds **1**, **2**, and **3** were measured in DMSO-$d_6$, and compounds **1a**, **2a**, **3a**, and **3b** in $CDCl_3$. *b*, *c*) Assignments may be interchangeable within the same column.

(4.4), and 381 (4.0) nm], while its IR spectrum showed absorption bands due to hydroxyl, chelated $\gamma$-lactone, and aromatic ring moieties (3272, 1738, 1605 $cm^{-1}$). The $^{1}$H- (Table I) and $^{13}$C-NMR (Table II) data for **3** showed the presence of two trisubstituted benzene rings [$\delta$ 7.58 (dd, $J$=8, 8 Hz, 5-H), 7.41 (br d, $J$=8 Hz, 4-H), 6.91 (br d, $J$=8 Hz, 6-H); 7.38 (d, $J$=2 Hz, 2′-H), 7.04 (dd, $J$=2, 8 Hz, 6′-H), 6.79 (d, $J$=8 Hz, 5′-H)], a conjugated olefin [$\delta$ 6.61 (s, 8-H)], and a weakly chelated hydroxyl group [$\delta$ 9.27 (br s)]. The $^{1}$H- and $^{13}$C-NMR signals of **3** could be analyzed completely on the basis of $^{1}$H–$^{1}$H correlation spectroscopy COSY, $^{1}$H–$^{13}$C COSY, and long-range $^{1}$H–$^{13}$C COSY ($J_{C-H}$=5, 10 Hz) experiments. Ordinary acetylation of **3** provided the triacetate (**3a**), while the trimethyl ether (**3b**) was obtained by diazomethane methylation. Comparisons of spectral data for **3**, **3a**, and **3b** with those for various phthalide derivatives[15] showed the structure of **3** to be 7,3′,4′-trihydroxybenzylidenephthalide. The chemical structure, including the geometry of the benzylidene side chain in **3**, was confirmed by NOE experiments on **3b**. Namely, nuclear Overhauser effect (NOE) enhancements were observed in the following pairs of protons of **3b** [4-H, 8-H; 8-H, 2′-H; 8-H 6′-H; 6-H, 7-OMe ($\delta$ 3.93); 2′-H, 3′-OMe ($\delta$ 3.97); 4′-OMe ($\delta$ 4.03), 5′-H] in the nuclear Overhauser effect spectroscopy (NOESY) and difference NOE spectra. Consequently, the structure of thunberginol F was shown to be ($Z$)-3-(3,4-dihydroxybenzylidene)-7-hydroxyphthalide (**3**).

Table III. Inhibitory Effects of Thunberginols A (**1**), B (**2**), and F (**3**), Phyllodulcin (**4**), and Hydrangenol (**5**) on the Schults-Dale (S.D.) Reaction in Sensitized Guinea Pig Tracheal Chain and the Histamine Release from Rat Mast Cells Induced by Compound 48/80 and Calcium Ionophore A-23187

| | $IC_{50}$ (M) | | |
|---|---|---|---|
| | S.D. | Compound 48/80 | A-23187 |
| Thunberginol A (**1**) | $9.8\times10^{-6}$ | $2.2\times10^{-5}$ (99.0) | $9.4\times10^{-6}$ (120.3) |
| Thunberginol B (**2**) | $1.0\times10^{-5}$ | $7.6\times10^{-5}$ (110.6) | $9.1\times10^{-5}$ (77.1) |
| Thunberginol F (**3**) | $9.3\times10^{-6}$ | $9.8\times10^{-6}$ (83.9) | $2.0\times10^{-5}$ (94.7) |
| Phyllodulcin (**4**) | $3.2\times10^{-5}$ | $>10^{-4}$ | $>10^{-4}$ (35.0) |
| Hydrangenol (**5**) | $3.2\times10^{-5}$ | $>10^{-4}$ (22.6) | $>10^{-4}$ |
| DSCG | $>10^{-4}$ | $>10^{-4}$ | $>10^{-4}$ |
| Tranilast | $4.7\times10^{-5}$ | $>10^{-4}$ (25.7) | $>10^{-4}$ |

The values in parentheses denote the inhibition percentage of histamine release at $10^{-4}$ M.

The antiallergic activities of thunberginols A (**1**), B (**2**), and F (**3**) were examined by utilizing the Schults–Dale reaction on sensitized guinea pig tracheal muscle and a bioassay to test the inhibitory activity against histamine release from rat mast cells induced by compound 48/80 or calcium ionophore A-23187. The inhibitory activities ($IC_{50}$ values) of **1**, **2**, and **3** on the Schults–Dale reaction and on histamine release from mast cells are summarized in Table III, together with those of phyllodulcin (**4**), hydrangenol (**5**), and two commercial antiallergic agents, disodium cromoglycate (DSCG) and tranilast. Thunberginols (**1**, **2**, **3**) showed more potent inhibitory activities than **4**, **5**, DSCG, and tranilast in those *in vitro* bioassays. Thunberginol F (**3**) was the most effective in the experiment where compound 48/80 was used as an inducer, while thunberginol A (**1**) was significantly effective against calcium ionophore A-23187-induced histamine release.

Thunberginols (**1**, **2** **3**) exhibited a little inhibition of histamine-induced contraction of tracheal chain isolated

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TABLE IV. Inhibitory Effects of Thunberginols A (1), B (2), and F (3), Phyllodulcin (4), and Hydrangenol (5) on the Histamine-Induced Contraction in Isolated Guinea Pig Tracheal Chain

| Compound | Concentration (M) | Inhibition (%) |
|---|---|---|
| Thunberginol A (**1**) | $10^{-5}$ | 14.6 ± 11.7 |
| | $3 \times 10^{-5}$ | 24.1 ± 2.7[a] |
| | $10^{-4}$ | 93.4 ± 4.6[b] |
| Thunberginol B (**2**) | $10^{-5}$ | 16.5 ± 5.9 |
| | $3 \times 10^{-5}$ | 13.7 ± 7.4 |
| | $10^{-4}$ | 65.2 ± 6.9[b] |
| Thunberginol F (**3**) | $10^{-5}$ | 12.1 ± 4.0 |
| | $3 \times 10^{-5}$ | 12.0 ± 4.7 |
| | $10^{-4}$ | 43.2 ± 2.6[b] |
| Phyllodulcin (**4**) | $10^{-5}$ | 0.0 |
| | $3 \times 10^{-5}$ | 4.2 ± 2.6 |
| | $10^{-4}$ | 38.2 ± 5.0[b] |
| Hydrangenol (**5**) | $10^{-5}$ | 0.0 |
| | $3 \times 10^{-5}$ | 0.0 |
| | $10^{-4}$ | 52.3 ± 0.5[b] |
| Diphenhydramine | $10^{-5}$ | 76.9 ± 2.5[b] |

Each value represents the mean with standard error of 3—8 experiments (*a*) $p < 0.05$, *b*) $p < 0.01$).

TABLE V. Antimicrobial Activities of Thunberginols A (1), B (2), and F (3), Phyllodulcin (4), and Hydrangenol (5) against Oral Bacteria (MIC, ppm)

| Compound | *B. melaninogenicus* | *F. nucleatum* |
|---|---|---|
| Thunberginol A (**1**) | 5 | 10 |
| Thunberginol B (**2**) | 10 | 10 |
| Thunberginol F (**3**) | 10 | 10 |
| Phyllodulcin (**4**) | 100 | 100 |
| Hydrangenol (**5**) | 10 | 5 |

from sensitized guinea pigs, as shown in Table IV. This finding suggested that the antiallergic activity of thunberginols involves a mechanism other than competition with histamine. Furthermore, thunberginols A (**1**) and F (**3**) were also found to exhibit potent antiallergic activity in the *in vivo* PCA test.[7] Details of the various *in vivo* antiallergic bioassays including the PCA test will be reported elsewhere.[16]

On the other hand, we have also observed antimicrobial activity of **1**, **2**, and **3** against two oral bacteria, *Bacteroides melaninogenicus* and *Fusobacterium nucleatum*, causing peridontal disease and halitosis (Table V).

## Experimental

The following instruments were used to obtain physical data: melting points, Yanagimoto micro-melting point apparatus (values are uncorrected); specific rotations, Horiba SEPA-200 digital polarimeter (l=5 cm); UV spectra, Shimadzu UV-1200 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; MS and HR-MS, Hitachi M-80 mass spectrometer; $^{1}$H-NMR spectra, JEOL EX-270 (270 MHz) spectrometer; $^{13}$C-NMR spectra, JEOL EX-270 (68 MHz) spectrometer with tetramethylsilane as an internal standard.

The following experimental conditions were used for chromatography: ordinary-phase column chromatography; Silica gel 60 (Merck, 70—230 mesh), reversed-phase column chromatography; Silica gel 60 silanized (Merck, 70—230 mesh), gel filtration chromatography; Sephadex LH-20 (Pharmacia); TLC, pre-coated TLC plates with Silica gel 60 $F_{254}$ (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 60 $F_{254}$(Merck, 0.25 mm) (reversed phase); HPTLC, pre-coated TLC plates with Silica gel RP-18 60 $WF_{254S}$ (Merck, 0.25 mm) (reversed phase). Detection was done by spraying 1% $Ce(SO_4)_2$–10% aqueous $H_2SO_4$, followed by heating.

**Extraction and Isolation** The air-dried leaves (12.3 kg) of Hydrangeae Dulcis Folium, cultivated and processed in Nagano Prefecture in 1990, were extracted with MeOH under reflux (100 l, 2 h × 2) and the solvent was evaporated from the extract under reduced pressure to give the MeOH extract (2500 g, 20.3% from the leaves). The MeOH extract (2490 g) was suspended in water and the suspension was extracted successively with $CHCl_3$, AcOEt, and 1-BuOH, to give the $CHCl_3$-soluble portion (800 g), AcOEt-soluble portion (960 g), 1-BuOH-soluble portion (510 g), and $H_2O$-soluble portion (220 g).

The $CHCl_3$-soluble portion (225 g) was subjected to column chromatography ($SiO_2$ 4 kg, eluted with $CHCl_3$–MeOH) to give 4 fractions [fr. 1 (38.5 g), fr. 2 (78.0 g), fr. 3 (63.6 g), fr. 4 (42.5 g)]. fr. 3 (10.6 g) was further purified by column chromatography (500 g, $CHCl_3$ : MeOH = 50 : 1) to afford isoarborinol (**9**, 15 mg, 0.0026%). Fr. 4 (16.2 g) was also purified by $SiO_2$ column chromatography (1 kg, $CHCl_3$ : MeOH = 30 : 1) followed by reversed-phase $SiO_2$ column chromatography (100 g, 90% MeOH) to give rubiarbonol B (**10**, 29 mg, 0.0022%).

The AcOEt-soluble portion (427 g) was subjected to column chromatography ($SiO_2$ 4 kg, eluted with $CHCl_3$ : MeOH : $H_2O$ = 50 : 1 : 0.1) to give 11 fractions [fr. 1 (21.4 g), fr. 2 (12.9 g), fr. 3 (27.7 g), fr. 4 (329 g), fr. 5 (12.2 g), fr. 6 (6.4 g), fr. 7 (1.3 g), fr. 8 (0.8 g), fr. 9 (5.4 g), fr. 10 (5.4 g), fr. 11 (4.1 g)]. Fr. 4 (329 g) was applied to a Sephadex LH-20 (800 g) column eluted with MeOH to afford phyllodulcin (**4**, 108 g, 1.99% from the natural medicine), hydrangenol (**5**, 128 g, 2.35%), phyllodulcin monomethyl ether (**4a**, 22 mg, 0.0004%), thunberginols A (**1**, 472 mg, 0.0086%) and E (245 mg, 0.0045%), and umbelliferone (**6**, 77 g, 1.41%). Fr. 5 (12.2 g) was also purified on a Sephadex LH-20 (800 g, MeOH) column and then subjected to repeated separation by $SiO_2$ column chromatography (*n*-hexane–AcOEt, $CHCl_3$–MeOH) to give thunberginol C (210 mg, 0.0038%). Fr. 6 (6.4 g) was also purified on a Sephadex LH-20 (800 g, MeOH) column and then subjected to $SiO_2$ column chromatography ($CHCl_3$–MeOH) to give thunberginol F (**3**, 153 mg, 0.0028%) and dihydroresveratrol (**8**, 45 mg, 0.00082%). Fr. 7 (1.3 g) was also purified on a Sephadex LH-20 (800 g, MeOH) column and then subjected to $SiO_2$ column chromatography ($CHCl_3$–MeOH) followed by reversed-phase $SiO_2$ column chromatography and HPLC (Chromatorex ODS 250 × 10 mm i.d., 30% MeOH) to give hydramacrophyllols A (7.6 mg, 0.00013%) and B (25.8 mg, 0.00047%). Fr. 8 (0.8 g) was also purified on a Sephadex LH-20 (800 g, MeOH) column and then subjected to reversed-phase $SiO_2$ column chromatography (10 g, 60% MeOH) to give thunberginol B (**2**, 87 mg, 0.0016%). Fr. 9 (5.4 g) was also purified on a Sephadex LH-20 (800 g, MeOH) column and then subjected to reversed-phase $SiO_2$ column chromatography (10 g, 40% MeOH) to give thunberginol D (65 mg, 0.0012%) and hydrangeic acid (**7**, 132 mg, 0.0024%). Known compounds (**4**, **4a**, **5**, **6**, **7**, **8**, **9**, **10**) were identified by comparison of their physical data with reported values.[2,3,10,11,12,13]

Thunberginol A (**1**): Yellow prisms, mp 240 °C (MeOH–$H_2O$). High-resolution (HR)-EI-MS : Calcd for $C_{15}H_{10}O_5$ ($M^+$) 270.0527, Found 270.0539. UV [EtOH, nm (log ε)]: 223 (4.0), 347 (4.1), 365 (4.0), UV [EtOH + $AlCl_3$, nm]: 224, 333, 407. IR (KBr, $cm^{-1}$): 3436, 1669, 1611, 1526, 1221. $^{1}$H-NMR: given in Table I. $^{13}$C-NMR: given in Table II. EI-MS (*m/z* %): 270 ($M^+$, 100), 242 (45).

Thunberginol B (**2**): Pale yellow needles, mp 244 °C (MeOH–$H_2O$). HR-EI-MS: Calcd for $C_{15}H_{10}O_6$ ($M^+$) 286.0477, Found 286.0496. UV [EtOH, nm (log ε)]: 235 (4.1), 268 (4.1), 324 (4.3), UV [EtOH + $AlCl_3$, nm]: 235, 275, 332, 380. IR (KBr, $cm^{-1}$): 3374, 3171, 1676, 1620, 1528, 1250. $^{1}$H-NMR: given in Table I. $^{13}$C-NMR: given in Table II. EI-MS (*m/z* %): 286($M^+$, 100), 258 (53).

Thunberginol F (**3**): Yellow needles, mp 242—243 °C (MeOH). HR-EI-MS: Calcd for $C_{15}H_{10}O_5$ ($M^+$) 270.0527, Found: 270.0497. UV [EtOH, nm (log ε)]: 225 (4.1), 290 (4.4), 381 (4.0). IR (KBr, $cm^{-1}$): 3272, 1738, 1605, 1468. $^{1}$H-NMR: given in Table I. $^{13}$C-NMR: given in Table II. MS (*m/z* %): 270 ($M^+$, 100).

**Acetylation of Thunberginol A (1)** A solution of **1** (10 mg) in pyridine (1.0 ml) was treated with $Ac_2O$ (0.5 ml) and the reaction mixture was stirred at room temperature (20 °C) under an $N_2$ atmosphere for 2 h. The reaction mixture was poured into ice-water and the whole was extracted with AcOEt. The AcOEt extract was washed successively with 5% aqueous HCl, saturated aqueous $NaHCO_3$ and brine, and then dried over $Na_2SO_4$, and filtered. After removal of the solvent from the filtrate under reduced pressure, the residue was chromatographed on an $SiO_2$ column (1 g, *n*-hexane : AcOEt = 1 : 1) to yield thunberginol A triacetate

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(**1a**, 9 mg, 61%), a white powder. HR-EI-MS: Calcd for $C_{21}H_{16}O_8$ ($M^+$) 396.0884, Found 396.0867. UV [EtOH, nm (log ε)]: 237 (4.4), 299 (4.4), 346 (4.6). IR (KBr, $cm^{-1}$): 1773, 1732, 1642, 1505, 1196. $^1$H-NMR: given in Table I. $^{13}$C-NMR: given in Table II. EI-MS (*m/z* %): 396 ($M^+$, 11), 354 (35), 312 (42), 270 (100).

**Methylation of Thunberginol A (1)** A solution of **1** (10 mg) in MeOH (3.0 ml) was treated with 10% trimethylsilyldiazomethane in *n*-hexane (3.0 ml) and the reaction mixture was left standing at room temperature (20 °C) for 4 h. The reaction mixture was concentrated to dryness under reduced pressure. The residue was chromatographed on an $SiO_2$ column (1 g, *n*-hexane : AcOEt = 1 : 1) to yield thunberginol A trimethyl ether (**1b**, 8 mg), a white powder. HR-EI-MS: Calcd for $C_{18}H_{16}O_5$ ($M^+$) 312.0998, Found 312.0998. UV [EtOH, nm (log ε)]: 236 (4.5), 319 (4.3), 363 (4.4). IR (KBr, $cm^{-1}$): 1719, 1518, 1257. $^1$H-NMR: given in Table I. EI-MS (*m/z* %): 312 ($M^+$, 100), 284 (37).

**Methylation of Phyllodulcin (4)** A solution of **4** (100 mg) in MeOH (15 ml) was treated with $CH_2N_2$–$Et_2O$ and the reaction mixture was left standing for 5 h at room temperature, then concentrated to dryness under reduced pressure. The residue was purified on an $SiO_2$ column (10 g, benzene : acetone = 10 : 1) to afford phyllodulcin monomethyl ether (**4a**, 12.8 mg, 12.3%) and dimethyl ether (**4b**, 143 mg, 86.7%). Compounds **4a** and **4b** were identified by comparison of their physical data with reported values.[2)]

**DDQ Oxidation of 4b to Give 1b** A solution of **4b** (20 mg) in dry benzene (1.0 ml) was treated with DDQ (28.9 mg, 2.0 eq) and the reaction mixture was stirred at 100 °C for 4 h. The reaction mixture was filtered to remove the precipitate and the filtrate was concentrated under reduced pressure. The crude product (32.6 mg) was purified on an $SiO_2$ column (2.5 g, benzene : AcOEt = 2 : 1) to give thunberginol A trimethyl ether (**1b**, 14 mg, 70.4%), which was shown to be identical with authentic **1b**, derived from natural thunberginol A (**1**), by $^1$H-NMR and TLC comparisons.

**Acetylation of Thunberginol B (2)** A solution of **2** (10 mg) in pyridine (1.0 ml) was treated with $Ac_2O$ (0.5 ml) and the reaction mixture was stirred at room temperature (20 °C) under an $N_2$ atmosphere for 2 h. The reaction mixture was poured into ice-water and the whole was extracted with AcOEt. The AcOEt extract was washed successively with 5% aqueous HCl, saturated aqueous $NaHCO_3$ and brine, and then dried over $Na_2SO_4$, and filtered. The solvent was evaporated under reduced pressure. The residue was chromatographed on an $SiO_2$ column (1 g, *n*-hexane : AcOEt = 1 : 1) to yield thunberginol B tetraacetate (**2a**, 11 mg), a white powder. HR-EI-MS: Calcd for $C_{23}H_{18}O_{10}$ ($M^+$) 454.0897, Found 454.0897. UV [EtOH, nm (log ε)]: 251 (4.5), 300 (4.5), 342 (4.7). IR (KBr, $cm^{-1}$): 1775, 1736, 1612, 1507, 1202. $^1$H-NMR: given in Table I. $^{13}$C-NMR: given in Table II. EI-MS (*m/z* %): 454 ($M^+$, 10), 412 (28), 328 (100), 286 (52).

**Acetylation of Thunberginol F (3)** A solution of **3** (10 mg) in pyridine (1.0 ml) was treated with $Ac_2O$ (0.5 ml) and the reaction mixture was stirred at room temperature (20 °C) under an $N_2$ atmosphere for 2 h. The reaction mixture was poured into ice-water and the whole was extracted with AcOEt. The AcOEt extract was washed successively with 5% aqueous HCl, saturated aqueous $NaHCO_3$ and brine, then dried over $Na_2SO_4$, and filtered. The solvent was evaporated under reduced pressure. The residue was chromatographed on an $SiO_2$ column (1 g, *n*-hexane : AcOEt = 1 : 1) to yield thunberginol F triacetate (**3a**, 10 mg), a white powder. HR-EI-MS: Calcd for $C_{21}H_{16}O_8$ ($M^+$) 396.0844, Found 396.0866. UV [EtOH, nm (log ε)]: 274 (4.2), 345 (4.5). IR (KBr, $cm^{-1}$): 1780, 1608, 1506, 1211. $^1$H-NMR: given in Table I. $^{13}$C-NMR: given in Table II. EI-MS (*m/z* %): 396 ($M^+$, 9), 354 (28), 312 (35), 270 (100).

**Methylation of Thunberginol F (3)** A solution of **3** (11 mg) in MeOH (3.0 ml) was treated with 10% trimethylsilyldiazomethane in *n*-hexane (3.0 ml) and the reaction mixture was left at room temperature (20 °C) for 4 h then concentrated to dryness under reduced pressure. The residue was chromatographed on an $SiO_2$ column (1 g, *n*-hexane–AcOEt) to yield thunberginol F trimethyl ether (**3b**, 8 mg), a white powder. HR-EI-MS: Calcd for $C_{18}H_{16}O_5$ ($M^+$) 312.0998, Found 312.0971. UV [EtOH, nm (log ε)]: 227 (4.4), 273 (4.7), 357 (4.4). IR (KBr, $cm^{-1}$): 1773, 1601, 1518, 1256. $^1$H-NMR: given in Table I. $^{13}$C-NMR: given in Table II. EI-MS (*m/z* %): 312 ($M^+$, 100), 297 (25).

**Bioassay Tests for the Antiallergic Activity of Thunberginols A (1), B (2), F (3)** The methods of bioassay testing are basically the same as those for phyllodulcin (**4**), hydrangenol (**5**), and the extracts from Hydrangeae Dulcis Folium described in the previous report.[1)]

**i) Effect on the Schults–Dale Reaction** Sensitized male Hartley guinea pigs (Kiwa Laboratory Animals Ltd., Wakayama) weighing about 300 g were used. A guinea pig was killed by a blow on the back and exsanguinated by severing both carotid arteries. The trachea was removed and freed from the surrounding fat and connective tissue. The cleaned trachea was cut into rings about 2 mm wide and three rings were chained with silk thread. The preparation was mounted in a Magnus bath containing a Krebs–Henseleit medium consisting of NaCl (118 mM), KCl (4.7 mM), $KH_2PO_4$ (1.2 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (2.5 mM), $NaHCO_3$ (25 mM), and glucose (10 mM) at 37 °C and gassed continuously with a mixture of 95% $O_2$–5% $CO_2$. The initial loading tension was set at 1 g. The contractile response was recorded isometrically. Each test compound was added to the preparation 10 min before the treatment with egg albumin (0.1 μg/ml). The contraction was expressed as a percentage of the maximal response to histamine at $1 \times 10^{-5}$ M and the results ($IC_{50}$ value) are shown in Table III.

**ii) Histamine Release from Rat Peritoneal Mast Cells** Male Wistar rats (Kiwa Laboratory Animals Ltd.) weighing 300—400 g were exsanguinated and injected intraperitoneally with 10 ml of physiological solution consisting of NaCl (154 mM), KCl (2.7 mM), $CaCl_2$ (0.9 mM), glucose (5.6 mM), and HEPES (Dotite, 5 mM, pH 7.4). The abdominal region was gently massaged for 2 min and then the peritoneal exudate was collected. Mast cells were obtained from the peritoneal exudate according to the method of Nemeth and Rohlich.[17)] The cell suspension from the exudate was mixed with various concentrations of test compounds and the whole mixture was preincubated at 37 °C for 15 min. Then compound 48/80 or calcium ionophore A-23187 was added and the mixture was incubated at 37 °C for 10 min. After cooling, the amount of histamine released in the reaction mixture was measured by the quantitative fluorescence method[18)] and the results are summarized in Table III.

**iii) Effect on the Contraction Induced by Histamine** Isolated tracheal chain of guinea pig was prepared and suspended in a Magnus bath in the same manner as described in i. Each test compound was added to the preparation 10 min before the treatment with histamine ($10^{-5}$ M) and the results are given in Table IV.

**Test Organisms Used for Antimicrobial Activity Assay and Determination of Minimal Inhibitory Concentration (MIC)** Microorganisms used in this experiment were obtained from the Institute for Fermentation (Osaka, Japan). The following strains were used: *Bacteroides melaninogenicus* IID891 and *Fusobacterium nucleatum* GIFU4637. Bacteria were anaerobically cultured in the standard agar medium (Nissui). The agar dilution method was used to assess the antimicrobial activity of **1**, **2**, and **3**. Test compounds were dissolved in a minimum amount of dimethyl sulfoxide (DMSO) and the culture medium was incubated at 37 °C for 24 h. The MIC (ppm) was the lowest concentration giving complete inhibition of visible growth after incubation.

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