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Full Length Research Paper

Antibacterial effects of *S*-(-)-tulipalin B isolated from *Spiraea thunbergii* Sieb. on *Escherichia coli*, a major food borne pathogenic microorganism

Jiyoung Lee[1], Jongsung Lee[1], Jihee Lim[1], Sanghee Sim[2] and Deokhoon Park[1*]

[1]Biospectrum Life Science Institute, SK Ventium 101-701, #522 Dangjung Dong, Gunpo City, Gyunggi Do, Rep. of Korea.

[2]School of Biotechnology, Yeungnam University, Gyeongsan City, Gyeongsangbuk Do, Republic of Korea.

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The crude ethanol extract of the leaves of *Spiraea thunbergii* Sieb. (Rosaceae) showed antibacterial activity against a food borne pathogen, *Escherichia coli*. Bioactivity-guided separation led to the isolation of a butyrolaton, 1(*S*-(-)-tulipalin B). The structure was determined from the interpretation of spectroscopic data (UV, MS, and NMR). The minimal inhibitory concentration (MIC) of compound 1 against *E. coli* was found to be 100/ml. In addition, we found the possibility that a methylene group may operate as a key factor in the antibacterial activities of compound 1 and the hydroxyl group may exert a synergistic effect with the methylene group.

Key words: *Spiraea thunbergii* Sieb., antibacterial activity, butyrolactones, S-(-)-tulipalin B, *Escherichia coli.*

INTRODUCTION

Food borne illness resulting from the consumption of food contaminated with pathogenic bacteria has been of vital concern to public health. Among the reported outbreaks in the United States during period of 1993–1997 for which the etiology was determined, bacterial pathogens caused the largest percentage of outbreaks (75%) and the largest percentage of cases (86%) (Olsen et al., 2000). Bacteria contaminating unwashed raw food, leaking packages, hands, and surfaces, which are introduced to domestic refrigerators, may directly contaminate other stored foods or attach to and persist on the internal surface of the refrigerator posing risks of indirect longer-term contamination during subsequent food preparation activities (Michaels et al., 2001) . Of these, *Escherichia coli* accounted for the largest number of outbreaks, cases, and deaths.

Herbs and spices with antibacterial activity have been widely used both traditionally and commercially to increase the shelf-life and safety of foods (Brul and Coote, 1999). With the recent upturn in consumer mistrust of synthetic additives, there has been a concomitant increase in the search for new natural compounds from plants to replace existing synthetic antimicrobials (Zink, 1997).

The *Spiraea thunbergii* Sieb. (Rosaceae) complex includes seven varieties of small foliose shrubs that are widespread in eastern Asia, and are used as ornamental trees. Previous chemical investigations of *S. thunbergii* Sieb. and its varieties have led to the reporting of seven new atisane-type diterpenoids (Nie and Hao, 1998) and 37 new diterpene alkaloids of the atisine- and hetisine-types (Hao et al., 1995; Nie and Hao, 1997; Wang et al., 2000; Hao et al., 2004; Hao et al., 2005).

In order to screen plant extracts with antibacterial activities against foodborne bacterial pathogens, includeing *E. coli,* from 171 plants, a disk diffusion assay was introduced. In this study, we found that, among 171 plant extracts, *S. thunbergii* Sieb. extract had a high antibacterial activity on *E. coli* (Table 1). Therefore, we attempted to isolate the effective compounds from *S. thunbergii* Sieb. and characterize their properties.

The present paper reports on the isolation of the constituents of the leaves of this plant and the antibacterial effects of the isolated compound. In addition, the present study of the antibacterial compound gives insight into the mode of action of this compound.

*Corresponding author. E-mail: pdh@biospectrum.com.

Table 1. Antibacterial activity of 171 plant extracts using disc diffusion assay (3,000 µg/disc).

Plant	Activity	Plant	Activity
Phryma leptostachya var. asiatica		*Isodon inflexus* (Thunb.) Kudo	
Diospyros kaki Thunb	++	*Vicia angustifolia var. segetilis*	
Forsythia koreana (flower)		*Pyrrosia lingua* (root)	
Hypochoeris radicata L		*Hedera japonica Tobler*	
Pteridium aquilinum var.latiusculum	+	*Houttuynia cordata Thunb*	
Staphylea bumalda (leave)		*Persicaria hydropiper* (L.)	
Psidium guajava LINN	++	*Akebia quinata* (Thunb.) Decne	
Capsella bursa-pastoris		*Geranium thunbergii Siebold & Zucc*	+
Cinnamomum camphora Sieb		*Leonurus japonicus Houtt*	
Allium monanthum MAX	+	*Ardisia japonica* (Thunb.) Blume	
Sedum sarmentosum Bunge		*Corydalis incisa* (Thunb.) Pers	
Oenanthe javanica		*Ligustrum obtusifolium*	
Aralia elata Seem		*Cassia mimosoides var. nomame*	
Aralia elata Seem (bud)		*Arisaema ringens* (root)	
Paeonia suffruticosa		*Arisaema ringens* (leave)	
Sargassum fulvellum		*Arisaema ringens* (stem)	
Magnolia kobus		*Isodon inflexus*	
Mentha arvensis		*Sigesbeckia pubescens Makino*	
Plantago asiatica L.		*Scrophularia buergeriana Miq*	
Duchesnea chrysantha	+++	*Coniogramme japonica*	
Centella asiatica (L.) Urbain		*Ostericum praetericum*	
Selaginella involvens (sw) Spring.		*Angelica japonica*	
Allium odorum		*Peucedanum japonicum*	
Brassica oleracea	+	*Lysimachia mauritiana*	
Amaranthus magostanus		*Lathirus japonicus*	
Eriobotrya japonica		*Sophora flavenscens*	+
Morus alba LINNAEUS (root)		*Sida spinosa*	
Morus alba LINNAEUS (leave)		*Dryopteris crassirhizoma*	
Dioscorea batatas DECNE.		*Angelica dahurica*	
Rhododendron yedoense var. Poukhanense (branch)		*Angelica dahurica*	
Rhododendron yedoense var. Poukhanense (flower)		*Angelica dahurica*	
Cryptomeria japonica		*Melampylum roseum*	
Saururus Chinensis (Lour) Baill		*Polistichum poliblepharum*	
Cirsium japonicum var. ussuriense		*Kadsula japonica*	
pine needle		*Pimpinella komarorii*	+
Magnolia kobus	+	*Dioscorea quinqueloba*	
Artemisia princceps var. ORIENTARIS		*Sedum sarmentosum*	
Platycodon grandiflorum		*Korth alsella japonica*	
Artemisia asiatica		*Perilla frutescens var. japonica*	
Houttuynia cordata Thunb		*Patrinia vilosa*	
Rhododendron lateritium Planch		*Helianthus tuberosus*	
Acanthopanax koreanum Nakai		*Sedum bulbiferum Makino*	
Zea mays L.		*Ficus nipponic.*	
Pisum sativum L		*Messerschmidia sibirica*	
Prunus yedoensis (flower, branch)		*Ficus carica* (fruit)	
Achyranthes japonica N.		*Ficus carica* (branch)	

Table 1. contd.

Ulmus macrocarpa Hance		*Ficus carica* (leaf)	
Brassica napus L. var. oleifera	+	*Orostachys iwarenge (Mak.) Hara*	+
Brassica napus L. var. oleifera (root)		*Dicranopteris pedatum*	
Brassica napus L. var. oleifera (flower)	+	*Castanea crenata* (leaf, branch)	+++
Lepisorus thunbergianus		*Firmiana simplex*	
Albiza julibrissin	+	*Quercus acuta*	
Zanthoxylum piperitum		*Quercus acuta* (Pericarp)	
Viscum album var. coloratum		*Eriobotrya japonica*	
Sasa quelpaertensis Nakai		*Eriobotrya japonica* (Pericarp)	
Spiraea thunbergii Sieb	+++	*Eriobotrya japonica* (Fruit)	
Rhododendron Schlippenbachii		*Eriobotrya japonica* (leaf)	
Rhododendron Schlippenbachii (branch)		*Achiyranthes Japonica*	
*Codonopsis lanceolata (*S.et Z)Trautv		*Circaea cordata*	++
Hizikia fusiforme		*Damnacanthus major*	
Taraxacum Platycarpum H. dahlst		*Angelica keiskei*	
Cynanchum wilfordii Hemsley		*Angelica keiskei*	
Polygonum cuspidatum S. et Z.		*Asparagus officinalis*	
Magnolia obovata Thunb		*Euphorbia jolkini Boissier*	+
Althaea rosea		*Cirsium Japonicum var. ussuriense*	
Solanum migrum L.		*zanthoxylum coreanum*	
Aster ageratoides TURCZ		*Hyloceresus undatus*	
Elsholtzia splendens Nakai		*Aleurites fordii*	
Corydalis ochotensis		*Clematis mandshurica*	
Cocculus trilobus (Thunb.) DC.	+++	*Siphonostegia chinensis*	
Gynostemma pentaphyllum		*Quercus glauca Thunb.*	
Mosla punctulata (J.F.Gmelin) Nakai		*Siegesbeckia glabrescens*	
Gnaphalium affine D.Don		*Rosa multiflora Thunb.*	+++
Solidago serotina Aiton		*Quercus salicina Blume*	+++
Phytolacca americana L		*Lillium lancifolium*	
Solidago virg-aurea var asiatica		*Miscanthus sinensis*	
Veratrum patulum		*Angelica cartilaginomarginata*	
Veratrum patulum (root)		*Arisaema amurense var. serratum*	
Adonis amurensis Regel & Radde	+	*Asparagus cochichinensis*	
Phacelurus latifolius (Steud.) Ohwi		*Smilax chiana*	
Cassia tora L.		*zanthoxylum piperium*	
Microlepia strigosa (thunb.) Presl		*Desmodium oldhami*	
Arachniodes aristata (Forst.) Tindale		*Geum aleppicum*	+++
Cytromium fotunei J.		*Lythrum salicaria*	++
Orixa japonica Thunb.		*Sorbus alnifolia*	
Dryopteris erythrosora			

MATERIALS AND METHODS

General

The HPLC (High Performance Liquid Chromatography) system used comprised a multi- solvent delivery 600 E controller, a dual 600 pump, and a photo diode array detector (model 996). Ultra-violet (UV) spectra were obtained on a PDA (Photodiode array detector, Waters 996) instrument. The HPLC-grade organic sol-vents and bulk organic solvents were purchased from the Duksan and Oriental Chemical companies.

^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance-500 spectrometer (500 MHz) using acetone-d_6 as solvent and tetramethylsilane (TMS) as an internal standard, and the chemical shifts were reported in (ppm) units relative to the TMS signal and coupling constants (*J*) in Hz. A complete attribution was performed on the basis of the 2 D-experiment (heteronuclear multiple bond correlation, HMBC) . High resolution-mass spectrometry (electro-spray ionization) or HRMS (ESI) data were measured on a JEOL HX 110A Tandem HR mass spectrophotometer at the Korea Basic

Science Institute.

Analytical thin-layer chromatography (TLC) was performed on a precoated silica gel plastic plate (0.25 mm, 60 F_{254}, E. Merck). TLC spots were visualized under a UV lamp at 254 and 365 nm, and by spraying with diluted H_2SO_4 and p-anisaldehyde with methanol reagent, followed by heating at 200 for 1 min.

Plant material

The fresh leaves of *S. thunbergii* Sieb. were collected from Jeju Island, Republic of Korea in 2005. A voucher sample has been deposited at the Jeju Bio Diversity Research Institute of the Jeju Hi-Tech Industry Development Institute.

Isolation of anti-microbial compound

The fresh leaves of the plant material (420 g) were cut into small pieces and extracted three times with EtOH at room temperature for 7 days, and filtered. The original EtOH (63.42 g) extract was evaporated to dryness *in vacuo,* and was then suspended in approximately 100 ml of water. The water suspension was partitioned three times with hexane (approximately 200 ml x 2). The residual water fraction was then partitioned three times with EtOAc (approximately 400 ml x 2). The EtOAc layer (20.62 g) was concentrated and chromatographed on a silica gel column (70 × 400 mm) with an $CHCl_3$-MeOH step gradient system with increasing polarity; from 2 to 7%, 10, and 15% MeOH to give nine fractions (Fraction No.1 - No. 9).

Fraction No. 7 (8.22 g) was column-chromatographed (30 × 310 mm) on silica gel with a gradient elution from 0% MeOH to 15% MeOH in $CHCl_3$ to yield eight fractions; Frs. A (78.4 mg), B (478.4 mg), C (1603.6 mg), D (661.0 mg), E (1497.6 mg), F (2456.3 mg), G (1177.7 mg), H (267.0 mg). The fifth fraction (7E) was further fractionated using C-18 MPLC (adsorbent; 200 g, Analytichem BONDESIL C18, 40 um, preparative grade, Varian, glass column; 10 mm i.d., 140 mm length) eluting with 500 ml of H_2O, then 1000 ml of 20% and 40% MeOH in H_2O and MeOH (1000 ml), respectively. The bioactive 0 and 20% MeOH in H_2O-eluted fractions were again chromatographed on a C18 HPLC column eluted with 6% ACN in H_2O (0-36 min) to yield compound 1 (25 mg).

Compound 1

Oil; R_f 0.41 (Et_2 O); UV max (H_2 O) nm (log) 204 (3.96), MS, [ ]D, and ^1H NMR in accordance with lit. values (Tschesche et al., 1968; Tschesche et al., 1969; Hutchinson, 1974); ^{13}C NMR (75 MHz, $CDCl_3$): 67.7 (t, C-5), 73.1 (d, C-4), 126.7 (t, C-3), 137.8 (s, C-2), 169.1 (s, C-1).

Chemicals

Purified -methylene-gamma-butyrolactone (tulipalin A) (2), gamma-butyrolactone (3), *S*-(-)- -hydroxy-gamma-butyrolactone (4), *R*-(+)-alpha-hydroxy-gamma- butyrolactone (5), and *S*-(-)- -hydroxy-gamma-butyrolactone (6) were purchased from Sigma (St. Louis, Mo.). Stock solutions (1 M) were made in dimethylsulfoxide (DMSO). The final DMSO concentration in the experiments was always kept below 2% (vol/vol). This concentration of DMSO did not affect the growth of bacteria.

Preparation of bacterial strains

E. coli KCTC 1039 samples used in this study were obtained from the Korean Collection for Type Cultures (KCTC). Luria-Bertani (LB) agar and LB broth were used as culture media.

Antibacterial activity assay

The antibacterial activity was determined using both agar diffusion and broth dilution techniques as described previously by Cheesbrough (1984) and Gatsing et al. (2006).

Agar diffusion susceptibility testing was performed using the disc method. A disc of blotting paper was impregnated with 50 l of a 60 mg/ml (for crude extract) or 10 mg/ml (for pure compounds) solution of each sample dissolved in DMSO. Thus, the disc potencies were 3 mg and 500 g for crude extract and pure compounds, respectively. Erythromycin (Sigma) was used as the standard drug. After drying, the disc was placed on a plate of sensitivity testing agar inoculated with the test organism. Petri dishes were left at room temperature for about 45 min to allow the extract or the compounds to diffuse from the disc into the medium, and were then incubated at 37°C for 24 ~ 48 h, after which the zones showing no growth were noted and their diameters were recorded as the zones of inhibition.

For the broth dilution susceptibility testing, the solutions (maximum concentration) of the active compounds (i.e. the compounds that induced zones of inhibition) were prepared in DMSO and serially diluted (2-fold), then, 0.5 ml of each dilution was introduced into a test tube containing 4.4 ml of Selenite broth; 0.1 ml of bacterial suspension (5×10^5 cfu/ml) was subsequently added, and the mixture was then homogenized. The total volume of the mixture was 3 ml, with the test-compound concentrations in the tube ranging from 400 to 6.25 g/ml and those of the standard compounds, i.e. erythromycin ranging from 100 to 3.125 µg/ml, respectively. After 24 h of incubation at 37°C, the minimum inhibitory concentration (MIC) was reported as the lowest concentration of antimicrobial that prevented visible growth.

RESULTS AND DISCUSSION

Separation of the active compound was performed by a series of silica gel column chromatographic steps, with final purification performed by HPLC to yield an active compound, referred to as compound 1. When the purified compound 1 was applied to HPLC, the HPLC chromatograms again showed one peak at R_t = 33.2 min.

Compound 1 had an optical rotation value of -0.4^o (c 0.47, H_2O) . Chemical structure of compound 1 was determined to be *S*-(-)-tulipalin B (Figure 1) on the basis of analyses of the MS, IR, ^1H, and ^{13}C NMR spectroscopic data, including HMQC, HMBE, and ^1H-^1H COSY experiments. Although previous reports showed that compound 1 exists in several plants, including *Botrytis* species (Bergman and Beijerxbergen, 1968; Schönbeck and Schroeder, 1972), the existence of compound 1 in *S. thunbergii* Sieb. was reported for the first time in this study.

The absolute C-4 configuration of this compound 1 was anticipated to be *S*, because compound 1 that possesses the *S*- configuration exhibits a negative specific rotation value. Structurally similar types of bioactive compounds have been reported from *S. thunbergii* (Hiradate et al., 2004; Kim et al., 1998), *Tulips* (Bergman, 1966; Bergman et al., 1967; Bergman and Beijersbergen, 1968; Beijers

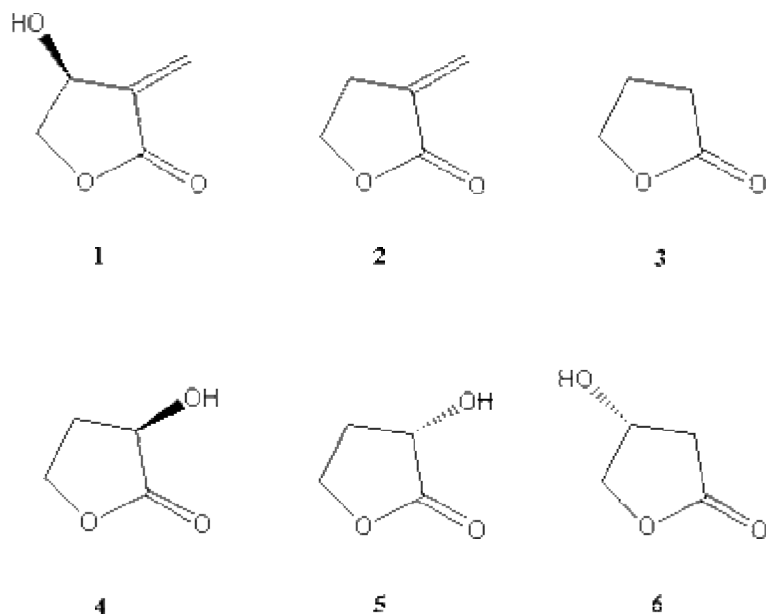

Figure 1. Chemical structures of compound 1 and other homologous compounds, 2-6.

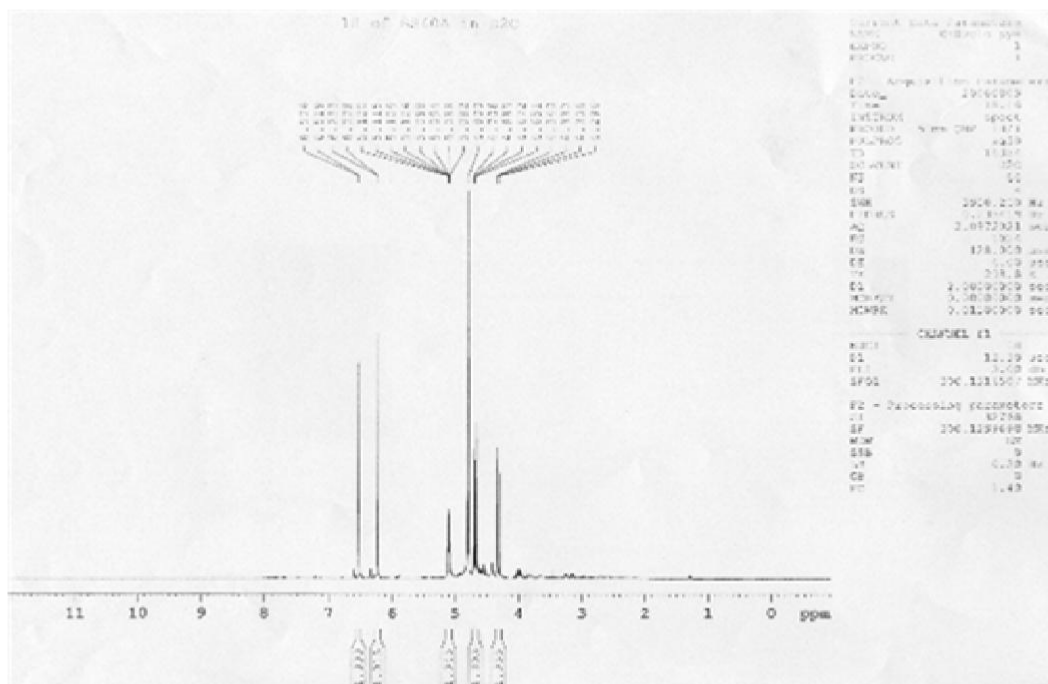

Figure 1S. ^{1}H NMR spectrum of compound 1 (D2O, 300 MHz).

bergen and Lemmers, 1971; Beijersbergen and Lemmers, 1972), *Artabotrys hexapetalus* (Wong and Brown, 2002), and *Alstroemeria* (Hausen et al., 1983; Christensen and Kristiansen, 1995a; Christensen and Kristiansen, 1995b; Christensen and Kristiansen, 1995c; Christensen, 1999). However, they were not reported to have antibacterial activities. Therefore, our report is the first to indicate that compound 1 (*S*-(-)-tulipalin B) exists in *S. thunbergii* Sieb. and has antibacterial activity against *E. coli*, a major of food borne pathogenic microorganisms.

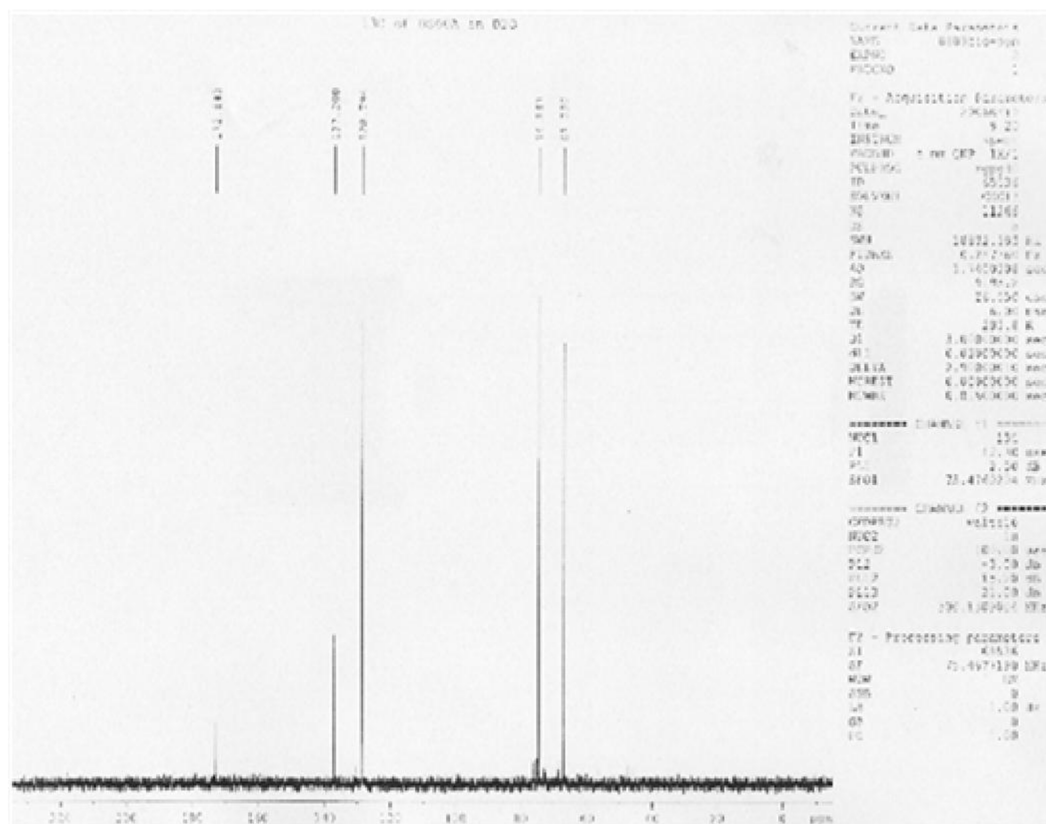

Figure 2S. ^{13}C NMR spectrum of compound 1 (D2O, 300 MHz).

Table 2. Minimum Inhibitory Concentration (MIC) of the S-(-)-tulipalin B (1) and other homologous compounds, 2-6 against *Escherichia coli*.

Bacteria strains	**MIC (ug/ml)**					
	1	**2**	**3**	**4**	**5**	**6**
E. coli	100	400	N.A.	N.A.	N.A.	N.A.

Key. 1: S-(-)-tulipalin B, 2: alpa-methylene-gamma-butyrolactone (tulipalin A), 3: gama-butyrolactone, 4: S-(-)-alpha-hydroxy-gamma-butyrolactone, 5: R-(+)-alpha-hydroxy-gamma-butyrolactone, 6: S-(-)-beta-hydroxy-gamma-butyrolactone, N.A.: No active

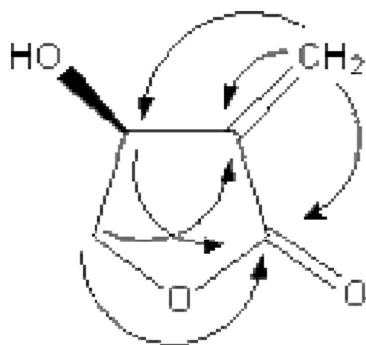

Figure 3S. Partial structures of compound 1, assembled with the aid of HMBC correlations.

The MIC (minimum inhibitory concentration) of compound 1 was determined, and is shown in Table 2.

In our study, compound 1 (*S*-(-)- tulipalin B) exhibited antibacterial activity against *E. coli*. It also showed more activity than the compound 2 (tulipalin A). However, structurally similar types of bioactive compounds - 3, 4, 5, and 6 did not show antibacterial activity against the food borne pathogenic bacterial strains tested, even at high concentrations (Table 2).

In this assay, we found that compound 1 and 2, both of which contain a methylene group, exerted antibacterial activities against *E. coli*, while other compounds that did not contain a methylene group did not. This suggests the possibility that a methylene group may operate as a key

factor in the antibacterial activities of compound 1. In addition, the hydroxyl group of *S*-(-)-tulipalin B seems to exert a synergistic effect with the methylene group, because *S*-(-)- tulipalin B showed antibacterial activity four times higher than that of tulipalin A against *E. coli*.

In conclusion, in this study, we demonstrated that compound 1, which was isolated from *S. thunbergii* Sieb., has antibacterial activities. That is, compound 1 exhibited a strong activity against *E. coli*, one of major food borne pathogenic microorganisms. To the best of our knowledge, its antibacterial function is reported here for the first time.

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