농학박사학위논문

Studies on the *Streptococcus mutans* Sortase A Inhibitors from Medicinal Plants

약용식물로부터 *Streptococcus mutans*균의 Sortase A 저해제에 관한 연구

2018년 8월

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농생명공학부 응용생명화학전공
양 우 영

A Dissertation for the Degree of Doctor of Philosophy

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August 2018

Woo-Young Yang
Applied Life Chemistry Major
Department of Agricultural Biotechnology
Seoul National University

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지도교수 오 기 봉

이 논문을 농학박사학위논문으로 제출함
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Studies on the *Streptococcus mutans* Sortase A Inhibitors from Medicinal Plants

Advisor: Ki-Bong Oh

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of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

to the Faculty of
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at

SEOUL NATIONAL UNIVERSITY

by

Woo-Young Yang

Date Approved

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Abstract

Sortase A (SrtA) is a membrane-bound cysteine transpeptidase that is responsible for catalyzing the covalent anchoring of surface proteins to the Gram-positive bacterial cell wall. *Streptococcus mutans* is known to be the primary pathogen underlying dental caries and utilizes SrtA to anchor surface proteins to the cell wall peptidoglycan and forms a biofilm to facilitate its adhesion to the tooth surface. Studies have confirmed the important role of *S. mutans* SrtA in bacterial pathogenesis and its potential as a suitable target for pharmacological inhibition. In this study, the coding region of the *srtA* gene (620 bp) from *S. mutans* OMZ65 isolated from the human oral cavity was cloned, expressed in *Escherichia coli*, and purified by metal chelate affinity chromatography. To identify potential inhibitors of *S. mutans* SrtA, 100 commercially available medicinal plants were used for screening, and five specimens that had the highest scores were chosen for further molecular analysis. Among them, SrtA assay-guided separation of the crude extract of *Sophora japonica* flowers using various chromatographic techniques yielded a new maltol derivative along with six known maltol derivatives and flavonoids. Based on the results of combined spectroscopic methods, the structure of new compound was determined to be maltol-3-*O*-(4'-*O*-*cis*-*p*-coumaroyl-6'-*O*-(3-hydroxy-3-methylglutaroyl))-β-glucopyranoside. This compound exhibited potent inhibitory activity against SrtA, with an IC_{50} value of 34 μg/mL, and saliva-induced cell aggregation. In addition, several coumarin compounds including triphasiol were isolated from *Poncirus trifoliata* and evaluated for their bioactivities. Triphasiol showed strong inhibitory activity against *S. mutans* SrtA, with an IC_{50} value of 10 μg/mL. This compound also exhibited significant inhibition of *S. mutans* adherence to S-HAs (saliva-coated hydroxyapatite beads) and biofilm formation to the surface of resin teeth in a dose-dependent manner. These chemical and biological data revealed the potential of these compounds for the treatment of *S. mutans* infections

via inhibition of SrtA activity and prevention of dental plaque.

Key words : *Streptococcus mutans*, sortase A, medicinal plant, inhibitor, antibacterial, biofilm formation, aggregation, adhesion

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List of Abbreviations

HPLC : high performance liquid chromatography

NMR : nuclear magnetic resonance spectrometer

UV : ultra-violet

MS : mass spectrometer

MIC : minimum inhibitory concentrstion

IC_{50} : 50% inhibition concentration

E. coli : *Escherichia coli*

S. mutans: *Streptococcus mutans*

SrtA : sortase A

MeOH : methanol

EtOAc : ethyl acetate

Introduction

Since drug discovery, anti-microbial drugs have proved remarkably effective for the control of bacterial infections. However, it was soon evident that bacterial pathogens were unlikely to surrender unconditionally, because some pathogens rapidly became resistant to many of the first effective drugs. For example, the development of resistance to penicillin in *Staphylococcus aureus* by the production of a β-lactamase quickly decreased the usefulness of penicillin for serious staphylococcal infections, especially among hospitalized patients, in whom resistant strains are frequently found before they spread to the community. Also, all the antibiotics used clinically at the moment share a common mechanism of action, acting as inhibitors of the bacterial cell wall biosynthesis or affecting protein synthesis on ribosomes, resistance to these pharmacological agents represents a serious medical problem, which might be resolved by using new generation of antibiotics, possessing a different mechanism of action.

1. SrtA and mechanism of action

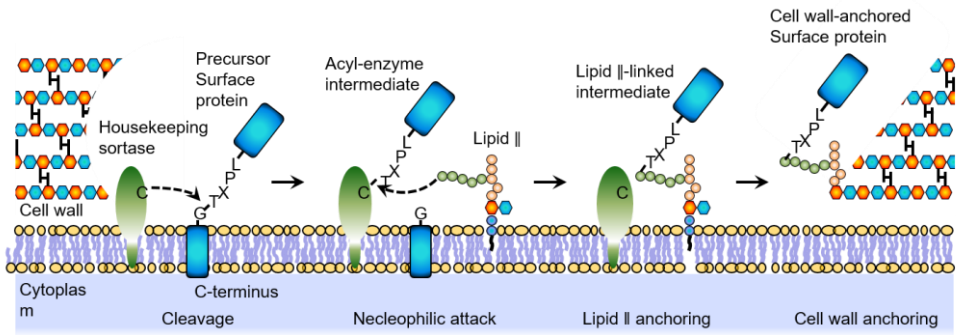
SrtA is a membrane-bound cysteine transpeptidase that is responsible for catalyzing the covalent anchoring of surface proteins to the Gram-positive bacterial cell wall. Since the discovery of *S. aureus* SrtA (Sa-SrtA) a little more than decade ago by Schneewind and colleagues (Mazmanian *et al*., 1999), over 800 genes encoding related proteins have been identified in ~260 distinct bacterial species (Finn *et al*., 2010). The vast majority of sortases are found in Gram-positive bacteria that contain a conventional cell wall (Pallen *et al*., 2001). Most bacterial species contain multiple sortase enzymes that have been named in an *ad hoc* manner (e.g. SrtA, SrtB, SrtC,

etc.) (Dramsi *et al*., 2005). Approximately 60% of all sortase proteins can be partitioned into six distinct families of enzymes that share related amino acid sequences. All sortase enzymes characterized to date function as cysteine transpeptidases that join proteins containing a cell wall sorting signal (CWSS) to an amino group located on the cell surface (Clancy *et al*., 2010).

The sorting reaction catalyzed by the SrtA protein from *S. aureus* is best understood and begins when a full length precursor protein containing an amino terminal leader peptide is exported from the cytoplasm through the secretory pathway (Fig. 1). The C-terminal CWSS is then processed by SrtA. The CWSS consists of a LPXTG motif (where X denotes any amino acid), followed by a segment of hydrophobic amino acids, and a tail composed primarily of positively charged residues. The C-terminal charged tail presumably retards export, positioning the protein for processing by the extracellular membrane associated SrtA enzyme. A highly conserved active site cysteine residue in SrtA then nucleophilically attacks the backbone carbonyl carbon of the threonine residue in the LPXTG motif, breaking the threonine and glycine peptide bond and creating a sortase-protein complex in which the components are linked via a thioacyl bond. The protein is then transferred by SrtA to the cell wall precursor lipid II, when the amino group in this molecule nucleophilically attacks the thioacyl linkage to create an isopeptide linked protein-lipid II product. Transglycosylation and transpeptidation reactions that synthesize the cell wall then incorporate this product into the peptidoglycan, where it is covalently linked to the cross-bridge peptide. Other sortases catalyse a similar transpeptidation reaction, but join remarkably different LPXTG motifs and amino groups. In a landmark study by Mazmanian *et al*., in 2000, gene knockout mutants of *srtA* in *S. aureus* were shown to result in defective surface expression of various LPXTG motif proteins and a defect in establishing renal abscesses and acute

Figure 1. Cell wall sorting mechanism of *S. aureus* SrtA

Surface proteins are synthesized in the cytoplasm as precursor proteins with a N-terminal signal sequence and C-terminal sorting signal. The C-terminal sorting signal consists of a positively charged tail, a hydrophobic region and a LPXTG motif. The active-site Cys of the sortase cleaves the amide bond between Thr and Gly of the LPXTG motif and generates an acyl-enzyme (thioester) intermediate. Nucleophilic attack by the amino group within the pentaglycine cross-bridge of lipid II links the C-terminal Thr of the surface protein to lipid II (Hendrickx *et al*., 2011)


Precursor
Surface
protein
Housekeeping
sortase
Cell wall
Cytoplas
m
C-terminus
Cleavage
Acyl-enzyme
intermediate
Lipid ‖
Necleophilic attack
Lipid ‖-linked
intermediate
Lipid ‖ anchoring
Cell wall-anchored
Surface protein
Cell wall anchoring

infection in mice. The reduced ability of an *S. aureus srtA* mutant to infect the host was also confirmed in several animal models of infection. Considering that bacterial adhesion is the crucial first step of Gram-positive pathogenesis and biofilm formation and that adhesion is promoted by several surface proteins at the cell wall, SrtA constitutes an ideal target for the development of new anti-virulence agents that will block a common enzyme responsible for linking many of these proteins to the cell wall rather than targeting a single surface protein involved in virulence (Chen and Wen, 2011).

2. *Streptococcus mutans* as cariogenic bacteria

Dental caries is a multifactorial, chronic bacterial disease, that causes demineralization and destruction of the hard tissues, usually by production of acid by bacterial fermentation of the food debris accumulated on the tooth surface (Taubman and Nash, 2006). Today, caries remains one of the most common diseases of people worldwide. Individuals are susceptible to this disease throughout their lifetime. About 700 different bacteria species have been identified from the human oral microbiome. In the pathogenesis of dental caries, an important role plays cariogenic bacteria, i.e. oral streptococci. Oral streptococcal species are major component in the pathogenesis of dental caries. especially It is believed that bacteria of the species *S. mutans* is the main factor that initiates caries and very important factor of enamel decay. *Lactobcillus* are important in further caries development, especially in the dentin. *S. mutans* is considered a major causative agent of human dental caries, one of the most common infectious diseases that affect humans (van Houte, 1994). This bacterium is also among the oral microorganisms that can cause infective endocarditis. Its main cariogenic virulence factors are its abilities to promote adhesion and accumulation on teeth, its acidogenicity, and its aciduricity

(Banas**,** 2004**;** Cvitkovitch *et al*., 2003). The adherence of bacteria to dental surfaces is the first step in the development of the complex biofilm community that constitutes dental plaque. Streptococci are recovered less frequently. *S. mutans* is a Gram-positive oral bacterium responsible for human dental caries (Hamada and Slade, 1980; Loesche, 1986). Formation of plaque biofilm on the tooth surface by *S. mutans* is an important step in the progression of dental caries, and many factors responsible for biofilm formation have been reported (Russell, 1992). Also, they metabolize sucrose to synthesize insoluble extracellular polysaccharides, which enhance their adherence to the tooth surface and encourage biofilm formation. *S. mutans* utilizes SrtA to anchor some surface proteins to the cell wall, thus easily coalescing into host teeth.

3. Role of SrtA in *S. mutans*

In *S. mutans,* these surface proteins are anchored to the cell wall by SrtA, that recognize specific surface protein sorting signals. Since *srtA* knockout *S. mutans* variants showed a decreased ability to colonize the oral mucosa and teeth, reduced biofilm biomass, and consequently reduced caries formation (Lee and Boran, 2003). Specific cell-surface proteins, which aid in its primary attachment to the tooth surface. *S. mutans* possesses a single SrtA protein that belongs to the *srtA* subfamily (Comfort and Clubb, 2004). And six surface proteins containing sorting signal, namely, PAc (also known as P1 and surface protein antigen I/II), fructanase (FruA), wall-associated protein A (WapA), wall-associated protein E (WapE), glucan-binding protein C (GbpC), and dextranase (DexA) (Ajdić *et al*., 2002) (Fig. 2). This mechanism is very similar to *S. aureus* sorting signal mediated with SrtA. Therefore, SrtA has been regarded as a promising target for the development of efficient antibacterial agents.

4. Discovery of SrtA inhibitors

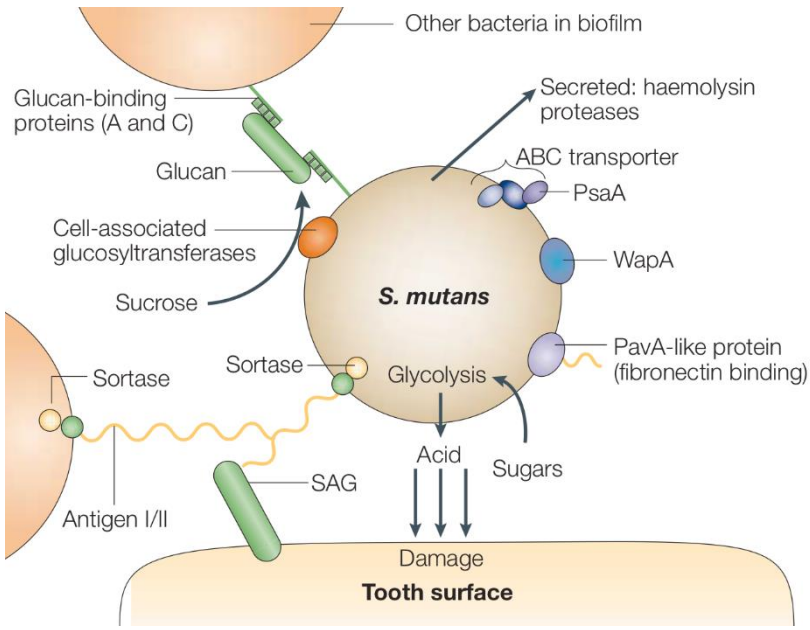
Because of the great interest in sortase as targets for anti-infective therapies, many studies toward finding a potent inhibitor have been conducted over the past decade (Clancy *et al*., 2010). Inhibitors development has mainly focused on SrtA from *S. aureus*. All reported *S. aureus* SrtA inhibitors are classified based on their type: non-specific, peptide-analogs, natural products, and synthetic small molecules. Natural products attract much attention since they can inhibit SrtA *in vitro* and *in vivo*, while they do not significantly affect bacterial viability. Although there is high amino acid sequence homology (43%) between the SrtA of *S. aureus* and *S. mutans*, there is no evidence that homologous proteins have the same inhibitors.

4.1. Natural products

After this discovery and the cloning of sortase, there have been several investigations for sortase inhibitors in compound libraries of natural products (Table 1). Early studies that screened large collections of medicinal plant extracts for sortase inhibitory activity, identified the ethyl acetate fraction from *Cocculus trilobus* as a potent SrtA inhibitor with an IC_{50} of 1.52 µg/mL (Kim *et al*. 2002). The first natural compound demonstrated as sortase inhibitory activity was described by Kim *et al*., in 2003 and was the glucosylsterol β-sitosterol-3-*O*-glucopyranoside extracted from isolated from the sponge *Spongosorites* sp. and evaluated for their inhibitory activity against *S. aureus* SrtA (Oh *et al*., 2005). Among 9 isolated compounds belonging to two distinct classes (topsentins and hamacanthins) deoxytopsentin showed the most the bulb of *Fritillaria verticillata*. This compound displayed an IC_{50} value of 18.3 µg/mL that two times lower than the positive control *p*HMB (IC_{50} = 40.6 µg/mL)

Figure 2. The molecular pathogenesis of dental caries associated with *S. mutans*

Initial attachment of *S. mutans* to tooth surfaces. This attachment is thought to be the first event in the formation of dental plaque. Accumulation of *S. mutans* on tooth surface in the presence of sucrose. Acid production by *S. mutans*. The metabolism of various saccharides (including glucose and fructose) by the accumulated bacterial biofilm (Mitchell, 2003).


Other bacteria in biofilm
Glucan-binding proteins (A and C)
Glucan
Cell-associated glucosyltransferases
Sucrose
Secreted: haemolysin proteases
ABC transporter
PsaA
WapA
S. mutans
PavA-like protein (fibronectin binding)
Sortase
Sortase
Glycolysis
Acid
Sugars
SAG
Antigen I/II
Damage
Tooth surface

and its Inhibitory potency was dependent upon the glucopyranoside side chain moiety, as the aglycone sitosterol was found to be inactive against the enzyme (Kim *et al*., 2003). Berberine chloride from rhizomes of *Coptis chinensis* were also found to be inhibitors of sortase (IC_{50} = 8.7 µg/mL) and exert antibiotic activity (Kim *et al*., 2004). The activity of *Curcuma longa* L. (turmeric) rhizome constituents against SrtA have also been extensively studied (Hu *et al*., 2013 a). The curcumin was shown to inhibit *S. aureus* SrtA with an IC_{50} value of 13.8 µg/mL, showing no growth inhibition even at MIC values greater than 200 µg/mL (Park *et al*., 2005)

Another promising group of natural compounds identified by these early screens of medicinal plant extracts is the flavonols. Natural flavonols of similar structure, inhibited for *S. aureus* SrtA (Kang *et al*., 2006). Morin, myricetin and quercetin acted as potent SrtA inhibitors with IC_{50} values of 11.29 µg/mL, 13.99 µg/mL and 15.91 µg/mL, respectively. All flavonols tested in this study exhibited no growth inhibition of *S. aureus* Newman strain at MIC values greater than 900 µg/mL, while they reduced bacterial clumping to fibrinogen in a dose-dependent manner. Recently, Oh *et al*. (2011) described a series of flavonoids isolated from the roots of *Sophora flavescens* that acted as *S. aureus* SrtA inhibitors. The most active compound was kurarinol with an IC_{50} value of 48.8 µg/mL and weak antibacterial activity against *S. aureus* (MIC = 99 µg/mL).

Marine invertebrates, such as sponges, are a prolific source of biologically active compounds with may interesting modes of action and have been widely used in the search of potent SrtA inhibitors. For example, bis (indole) alkaloids, consisting of two indole moieties connected to each other via heterocyclic units, have been potent SrtA inhibitory activity (IC_{50} = 15.67 µg/mL). A different study evaluated the SrtA inhibitory potential of four aaptamines, belonging to the family of 1H-benzo[de] [1,6]-naphthyridine alkaloids that were isolated from the *Aaptos aaptos* marine sponge (Jang *et al*., 2007). Increased anti-SrtA activity was observed for isoaaptamin

Table 1. *S. aureus* SrtA inhibitory activity of the natural products from medicinal plant and marine sponge.

Compound	Source	SrtA IC_{50} (μg/mL)	MIC (μg/mL)	References
β-Sitosterol-3-*O*-glucopyranoside	*F. verticillata*	18.3 (*S. aureus*)	200	Kim *et al*., 2003
Morin	Chinese herbs and fruit	11.3 (*S. aureus*)	>900	Kang *et al.*, 2006
		8.2 (*S. mutans*)		Huang *et al*., 2014
Myricetin	Chinese herbs and fruit	14.0(*S. aureus*)	>900	Kang *et al.*, 2006
Quercetin	*R. verniciflua*	15.9 (*S. aureus*)	>900	Kang *et al.*, 2006
Curcumin	*C. longa* L.	13.8 (*S. aureus*)	>200	Park *et al*., 2005
		3.6 (*S. mutans*)	61.7	Hu *et al*., 2013a, b
Berberine chloride	*C. chinensis*	8.7 (*S. aureus*)	100	Kim *et al*., 2004
Kurarinol	*S. flavescens*	48.8 (*S. aureus*)	99	Oh *et al*., 2011
Deoxytopsentin	*Spongosorites* sp.	15.6 (*S. aureus*)	6.25	Oh *et al*., 2005
Bromodeoxytopsentin	*Spongosorites* sp.	19.4 (*S. aureus*)	100	Oh *et al*., 2005
Isoaaptamin	*A. aaptos*	3.7 (*S. aureus*)	50	Jang *et al*., 2007
(-)-Discorhabdin *Z*	*Sceptrella* sp.	2.2 (*S. aureus*)	>100	Jeon *et al*., 2010
Halisulfate 1	*Coscinoderma* sp.	21.3 (*S. aureus*)	1.56-25	Bae *et al*., 2011

(IC_{50} = 3.7 µg/mL) (Table 1). An additional class of marine compounds displaying antibacterial and SrtA inhibitory activity are the pyrroloiminoquinone alkaloids found in the sponge *Sceptrella* sp. In particular, (–)-discorhabdin *Z*, one of the two new alkaloids characterized by an unusual hemiaminal group, inhibited *S. aureus* SrtA with an IC_{50} of 2.19 µg/mL and without defects on microbial viability (MIC > 100 µg/mL) (Jeon *et al*., 2010).

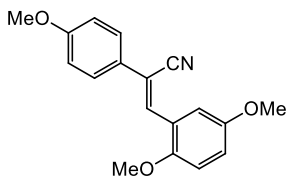
4.2. Synthetic small molecules.

The first important step in the synthesis of new derivatives of pharmacological interest is to identify a hit compound; this process can be facilitated by several different techniques. For SrtA in particular, High-Throughput Screening (HTS) of synthetic or natural compound libraries as well as virtual screening approaches have successfully used and have identified several inhibitor hits to date. Among the diarylacrylonitriles that were synthesized and tested for SrtA inhibitory activity (Z)-3-(2,5-dimethoxyphenyl)-2-(4-methoxyphenyl) acrylonitrile (DMMA) was the most active compound with an IC_{50} value of 9.2 µM. A subsequent study examined the inhibitory effects of DMMA in vivo providing the first data of SrtA inhibitor use in animals (Oh *et al*., 2010). A different class of promising SrtA inhibitor hits are the aryl(β-amino)ethyl ketones (AAEK) (Maresso *et al.*, 2007). Compounds AAEK1 and AAEK2 displayed IC_{50} values of 47 µM and 15 µM, respectively, for SrtA of *S. aureus*. The most recent HTS screen for potent SrtA inhibitors identified a further three promising classes of small molecules: rhodanines, pyridazinones and pyrazolethiones, which inhibited SrtA in a reversible way with IC_{50} values in the sub-micromolar range (Suree *et al*., 2009). The most potent compounds from each class were rhodanine, pyridazinone and pyrazolethione with IC_{50} values of 3.7 µM, 0.2 µM and 0.3 µM, respectively (Fig. 3).

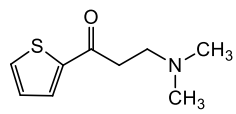
4.3. Peptides

Another important class of known SrtA inhibitors are the substrate-derived peptides. Peptidyl-diazomethane and peptidylchloromethane analogues Cbz (benzyloxycarbonyl)-Leu-Pro-Ala-Thr-CHN_2 and Cbz-Leu-Pro-Ala-Thr-CH_2Cl were both shown to act as time-dependent irreversible inhibitors of recombinant SrtA (Scott *et al*., 2002). Based on previous studies concerning the ability of the vinyl sulfone group to covalently modify the active site thiol in cysteine proteases. Connolly *et al*. (2003) synthesized a different irreversible inhibitor by replacing the scissile Thr-Gly in the substrate recognition motif of SrtA with a vinyl sulfone group ($C=C–SO_2Ph$). Subsequently, Jung *et al*. (2005), using the bisprotected L-threonine analogue (2R,3S)-3-amino-4-mercapto-2-butanol protected with silyl groups, synthesized two tetrapeptide analogues of the sorting sequence for SrtA and SrtB that were able to bind covalently to the tiol group of Cys184 in both enzymes.

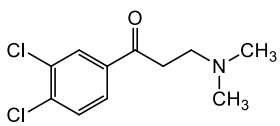
Figure 3. Structures of synthetic small molecules and *S. aureus* SrtA IC_{50} (μM).



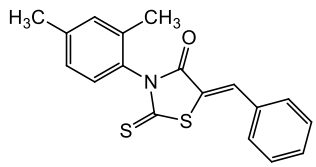
DMMA IC_{50} = 9.2 μM



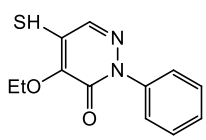
AAEK1 IC_{50} = 47 μM



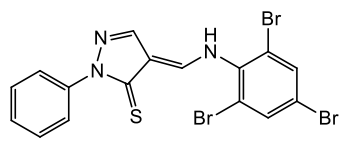
AAEK2 IC_{50} = 15 μM



Rhodanine IC_{50} = 3.7 μM



Pyridazinone IC_{50} = 0.2 μM



Pyrazolethione IC_{50} = 0.3 μM

5. *S. mutans* SrtA inhibitors

Currently, there have only been a few reports in the literature describing inhibitors of *S. mutans* SrtA. The inhibitory effect of morin on *S. mutans* UA159 SrtA was also evaluated and an IC_{50} of 8.21 µg/mL was reported. Morin did not have any effect on *S. mutans* viability but at a concentration of 9 µg/mL it significantly reduced biofilm formation by this strain (Huang *et al*., 2014). Ping Hu et al. studied the inhibition of *S. mutans* SrtA activity by curcumin *in vitro* (Hu *et al*., 2013a). Curcumin inhibited *S. mutans* SrtA with an IC_{50} value of 3.6 µg/mL and *S. aureus* SrtA with an IC_{50} value of 13.8 µg/mL; both values are far lower than the MIC (61.7 µg/mL) values identified, suggesting that *S. mutans* growth and viability are not compromised at the functional concentration of curcumin. A follow-up study by the same group investigated the effect of curcumin on *S. mutans* biofilm formation. The anti-biofilm action of curcumin was attributed to a reduction in the cell wall-anchored adhesion PAc that mediates attachment of *S. mutans* to the tooth surface (Jenkinson and Demuth, 1997). Eugenol (4-allyl-2-methoxyphenol) interacts with the cell wall and membrane first, inhibiting the biofilm formation and disrupting the cell-to-cell connections (Xu *et al*. 2016; Yadav *et al*. 2015). Since the SrtA is involved in the cell wall anchor, the eugenol may act through inhibition of this enzyme. *Trans*-chalcone, the precursor molecule of many flavonoids, would inhibit the *S. mutans* SrtA *in vitro* and possibly prevent bacterial biofilm growth. IC_{50} value of 5 µM was determined for *trans*-chalcone after incubating SrtA with varying concentrations of inhibitor overnight. Lignans and phenyl propanoids were isolated from the dried roots of *Pulsatilla koreana*, and the inhibitory activities toward SrtA from *S. mutans*. (7S,8S)-dihydrodehydrodiconiferyl alcohol (IC_{50} = 35.9 µM), (-)-rosmarinic acid (IC_{50} = 20.1 µM), and caffeic acid (IC_{50} = 20.2 µM) as potent SrtA inhibitors (Table 2).

Table 2. *S. mutans* SrtA inhibitors and IC_{50}.

Compound	Source	IC_{50}	Reference
Curcumin	*Curcuma. longa* L.	91.5μM	Hu *et al*.,2013a,b
(7S,8S)-dihydrodehydro diconiferyl alcohol	*Pulsatilla koreana*	9.2 μM	Lee *et al*., 2014
Morin	Chinese herbs and fruit	37.39 μM	Huang *et al*., 2014
Rosmarinic acid	*Pulsatilla koreana*	20.1 μM	Lee *et al*., 2014
Caffeic acid	*Pulsatilla koreana*	20.2 μM	Lee *et al*., 2014
Trans-chalcone	*Arabidopsis thaliana*	5 μM	Wallock-Richards *et al*., 2015

6. Purpose of this study

Mainstream medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants, as traditional antibiotics become ineffective and as new disease remain intractable to this kind of drug. Another factor in the renewed interest in plant antimicrobials in the past 30 years has been the rapid rate of plant species extinction. From this point of view, medicinal plants are resources for antibacterial drug discovery. As part of ongoing efforts to identify potent SrtA inhibitors from medicinal plants, one hundred commercially available specimens were screened and found that the ethyl acetate fractions from *Pueraria thunbergiana*, *Spatholobus suberectus, Rhus verniciflua*, *Poncirus trifoliata,* and *Sophora japonica* significantly inhibited SrtA derived from *S. mutans* OMZ65 isolated from the human oral cavity. Bioassay-guided separation of the crude extract of *S. japonica* flowers using various chromatographic techniques yielded two maltol derivatives along with five flavonol glycosides. Several SrtA active coumarin derivatives including ponciol and triphasiol were isolated from *P. trifoliata.* The effects of these compounds on *S. mutans* SrtA activity in vitro and subsequent biofilm formation were investigated.

Materials and Methods

1. General experimental procedures

The optical rotations were measured on a JASCO P-1020 polarimeter using a 1 cm cell. UV spectra were recorded on a HITACHI U-3010 spectrophotometer. NMR spectra were recorded in MeOH-d_4 containing Me4Si as an internal standard on Varian 300, Bruker Avance 400 and 600 spectrometers. Proton NMR spectra were measured at 600 MHz and carbon NMR spectra were measured at 150 MHz. Mass spectrometric data were obtained at the Korea Basic Science Institute (Daegu, Korea) and were acquired using a JEOL JMS 700 mass spectrometer with meta-nitrobenzyl alcohol (NBA) as a matrix for the FABMS. HPLC was performed on a SpectraSystem p2000 equipped with a SpectraSystem RI-150 refractive index detector. All solvents were spectroscopic grade or were distilled from glass prior to use.

2. Plant materials

Medicinal plants were purchased from Kyungdong Oriental Herbal Market (Kyung Dong Pharm. Co. Ltd), Seoul, Korea. Each plant was extracted with methanol at room temperature and concentrated in vacuum evaporation. Each extract residue was suspended in water and sequentially fractioned with *n*-hexane and ethyl acetate. The inhibitory activity of each fraction on the *S. mutans* SrtA was estimated.

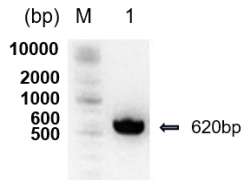
3. Cloning and expression of SrtA

The coding region of the *srtA* gene (620 bp) was PCR-amplified from the genomic DNA of *S. mutans* OMZ65, isolated from the human oral cavity (College of Dentistry, Seoul National University), using the forward (5′-GAAGGATCCGCTTGGAATACCAATAGA-3′, *Bam*HI) and reverse (5′-GAACTCGAGAAATGATATTTGATTATAGGACTG-3′, *Xho*I) primers. Amplification was carried out in the following conditions on a thermal cycler (Astec, Tokyo, Japan), hot start at 98 ℃ for 5 min, 30 cycles of denaturation (98 ℃, 30 sec), annealing (58 ℃, 30 sec), extension (72 ℃, 40 sec), and a final extension at 72 ℃ for 5 min. The PCR product was cloned into the *Bam*HI and *Xho*I restricted pET-21a(+) and confirmed by agarose gel electrophoresis (Fig. 4). The resulting plasmid, pET-*srtA*, was maintained in *E. coli* DH5α. The cloned DNA fragment was sequenced with T7 promoter and T7 terminator primers. The positive transformant was grown in an LB medium supplemented with 50 μg/mL of ampicillin at 37 ℃ for 16 h. The pre-incubated cells were inoculated into 5 mL of LB broth containing ampicillin and incubated with shaking at 37 ℃ until OD_{600} reached approximately 0.5. At this point, IPTG was added to the medium to a final concentration of 0.2mM and incubation was continued for an additional 4 h to induce the fused SrtA expression. Cells were harvested by centrifugation at 12,000 ×g for 1 min, resuspended by lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl) and lysed by sonication. Clear extracts containing fused protein were purified on a Ni-NTA affinity column (Qiagen). The column was washed twice with wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). The bound proteins were eluted with elution buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 250 mM imidazole, pH 6.0). Purified protein was dialyzed by using Snakeskin Dialysis Tubing (ThermoScientific, Rockford, IL, U.S.A.) (Kim *et al*., 2002).

Figure 4. *S. mutans* SrtA gene cloning.

(A) The coding region of the *srtA* gene (620 bp) was PCR-amplified from the genomic DNA of *S. mutans* OMZ65. (B) The PCR product was cloned into the *Bam*HI and *Xho*I restricted pET-21a(+) and confirmed by agarose gel electrophoresis.

(A)



1. 98℃ 5min
2. 98 ℃ 30sec
   58 ℃ 30sec
   72 ℃ 40sec
   — 30 cycles
3. 72 ℃ 5min
4. 4 ℃ ∞

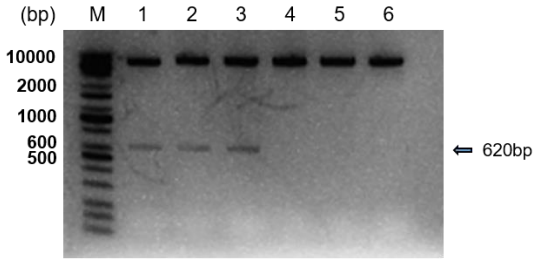
BamH I

SM SrtA F: 5'- gaa gga tcc gct tgg aat acc aat aga

Xho I

SM SrtA R: 5'- gaa ctc gag aaa tga tat ttg att ata gga ctg

(B)



Lane 1-6 ; pET-SrtA-Δ40 BamH I/Xho I Digestion

4. SrtA activity assay

The SrtA enzyme inhibitory activity of the isolated compounds was determined by quantifying increased fluorescence intensity upon cleavage of synthetic peptide substrate according to a previously documented procedure (Ton-That *et al*., 1999). For the reactions, 300 μL of reaction mixture (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.75 μg synthetic peptide substrate, Dabcyl-QALPETGEE-Edans, and 55 μg of recombinant SrtA) was added to each well of a 96-well microtiter plate; each well, contained the prescribed concentration of test sample (final 0.5% DMSO). The appropriate blanks contained all of the above, with the exception of the test sample. After incubation at 37 ℃ for 1 h, the increase in the fluorescence intensity was recorded by a fluorescence spectrophotometer (FLx 800, BioTek Instruments Inc., VT, USA) using excitation and emission wavelengths of 350 and 495 nm, respectively. Curcumin and *p*-hydroxymecuribenzoic acid (*p*HMB), known SrtA inhibitors, were used as positive controls.

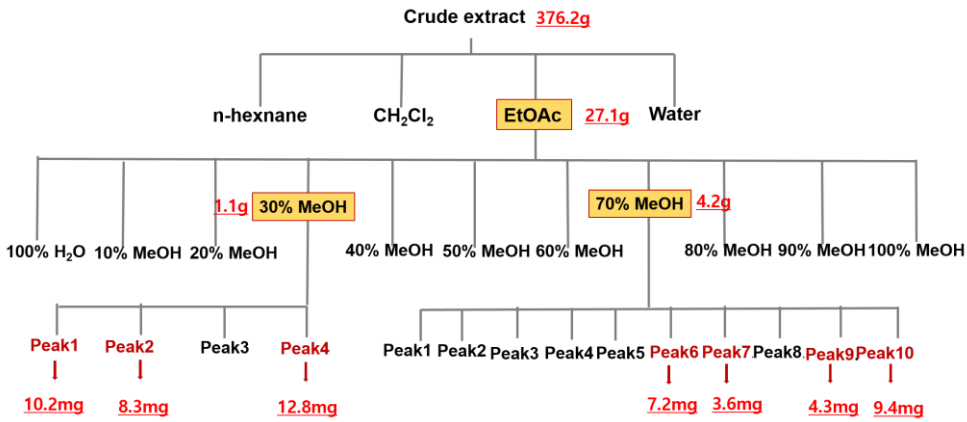
5. Antibacterial activity assay

The minimum inhibitory concentrations (MICs) of test compounds were determined according to a published protocol (Frankel *et al*. 2004). A. culture of *S. mutans* OMZ65 (5 mL) in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) was grown to saturation at 37 °C and diluted to an OD_{600} of 0.01. The culture was incubated for an additional 2 h and diluted to an OD_{600} of 0.005. In each well of a 96-well plate, 180 μL of cells were mixed with 20 μL of a concentrated test compound solution in 10% DMSO (final 1% DMSO). Culture plates were incubated overnight at 37 °C, and OD_{600} was measured using spectrophotometer. MIC values were determined as the lowest concentration of test compounds that inhibited cell growth. Ampicillin was used as a positive control.

6. Isolation of SrtA inhibitors from the flowers of *Sophora japonica* L.

The dried flowers of *S. japonica* (3.2 kg) were macerated and were repeatedly extracted with MeOH (10 L × 3). The combined crude extracts (376.2 g) were successively partitioned between H_2O and organic solvents such as *n*-hexane, CH_2Cl_2, and EtOAc (Fig. 5). Due to its potent inhibitory activity against SrtA, the EtOAc fraction (27.1 g) was separated by C_{18} reversed-phase vacuum flash chromatography using a sequential mixture of MeOH and H_2O as eluents (eleven fractions in gradient, H_2O–MeOH, from 100:0 to 0:100), acetone, and finally EtOAc. Based on the results of SrtA inhibitory activity test, the two fractions eluted with H_2O-MeOH (30:70) (4.2 g) and H_2O-MeOH (30:70) were chosen for separation. One-half of H_2O-MeOH (30:70) fraction was separated by semi-preparative reversed-phase HPLC (YMC-ODS column, 10 mm × 250 mm; H_2O–MeOH, 50:50) to yield, in order of elution, compounds **1**, **2**, **3**, and **4** as amorphous solids. These metabolites were then purified by reversed-phase HPLC in the same condition as described above. The purified metabolites were isolated in the following amounts: 13.2, 8.4, 8.6 and 17.8 mg of **1**–**4**, respectively. The fraction eluted with H_2O–MeOH (70:30) (1.1 g) was separated by reversed-phase HPLC (Zorbax 5 μm Eclipse-XDB-C_{18} column, 250 × 4.6 mm; H_2O–MeOH, 50:50). The purified metabolites were isolated in the following amounts: 10.2, 8.3, and 12.8 mg of **5**–**7**, respectively (Fig. 5).

Figure 5. Fractionation scheme of SrtA active extracts from *S. japonica*.


Crude extract 376.2g
n-hexnane
CH_2Cl_2
EtOAc 27.1g
Water
100% H_2O
10% MeOH
20% MeOH
1.1g 30% MeOH
40% MeOH
50% MeOH
60% MeOH
70% MeOH 4.2g
80% MeOH
90% MeOH
100% MeOH
Peak1
Peak2
Peak3
Peak4
10.2mg
8.3mg
12.8mg
Peak1 Peak2 Peak3 Peak4 Peak5 Peak6 Peak7 Peak8 Peak9 Peak10
7.2mg
3.6mg
4.3mg
9.4mg

7. SrtA inhibitors from the dried fruits of *Poncirus trifoliata*

S. mutans SrtA inhibitory metabolites form the dried fruits of *P. trifoliata* were kindly provided by Prof. J. Shin (College of Pharmacy, Seoul National University) and used in this study.

8. Saliva-induced aggregation assay

Saliva used was freshly prepared clarified unstimulated whole saliva. *S. mutans* NG8 (wild-type), *srtA*-defective mutant, and *srtA*-complemented mutant cells (Lee and Boran, 2003) grown for 16 h in Todd-Hewitt broth at 37 ℃ were washed twice with KPBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 6.5 mM Na_2HPO_4, pH 7.2) and resuspended to an OD_{700} of approximately 1. Assay mixtures using whole saliva contained 800 μL of cells and 200 μL of fresh clarified saliva. The aggregation assay was performed with *S. mutans* NG8 in the presence and absence of test compounds. The mixtures were mixed by inversions at time zero and incubated at room temperature without further mixing. The OD_{700} of the mixtures was measured with a spectrophotometer (model UVmini-1240, Shimadzu, Kyoto, Japan) at time intervals as 20 min (Lee *et al*., 1989).

9. Biofilm formation assay

S. mutans OMZ65 was cultured in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) under aerobic condition for 24 h at 37 °C. Biofilm formation was measured by staining with safranin (Petersen *et al*., 2004). Briefly, various concentration of triphasiol were added to 0.1 % sucrose containing BHI broth in 24-well plates (Nunc, Copenhagen, Denmark) or 24-well plates containing resin teeth (Endura, Shofu Inc., Kyoto, Japan). Then, the culture was created in the allotted

broths by inoculating them with seed cultures of *S. mutans* (5×10^5 CFU/mL) and incubated for 24 h at 37 °C. After incubation, the supernatants were removed and the culture wells or resin teeth were rinsed with distilled water. Biofilm formation was stained with 0.1 % safranin and photographed. The bound safranin was released from the stained cells with 30 % acetic acid, and the absorbance of the solution was measured at 530 nm. The biofilm that formed on the surface of the resin teeth was also stained with 0.1 % safranin and photographed.

10. Bacterial adherence

The bacterial adherence assay was based on a previously described method (Liljemark *et al*. 1981). Briefly, *S. mutans* was grown in BHI for 24 h at 37 °C. The cells were then diluted in BHI to approximately 10^8 CFU/mL. Thirty micrograms of hydroxyapatite beads (diameter of 80 μm, Bio-Rad, Hercules, CA, USA) were coated with clarified human saliva for 1 hour at room temperature. The saliva-coated hydroxyapatite beads (S-HAs) were washed 3 times with 0.01 M potassium phosphate buffer (KPB; pH 7.0) and immersed in the bacterial suspension (1×10^7 CFU/mL) with various concentration of triphasiol (Hay *et al*., 1971) To allow bacteria to be adherent, the mixture was gently agitated for 90 min at 3 °C. Following this, S-HAs were rinsed to remove nonadherent bacteria and were transferred to a new tube that contained KPB buffer. The adherent *S. mutans* onto the S-HAs were dispersed using a sonicator (Sonics Inc., Newtown, CT) at 50 W for 30 secs and the supernatants were spread on bacitracin (3.2 mg/mL) contained mitis-salivarius agar (MSA) plate (Difco, Detroit, MI, USA). After 48 h of cultivation, the numbers of colonies were counted. 0.1% NaF was used as a positive control (Jeng *et al*., 1998).

Results

1. Cloning and expression of *S. mutans* OMZ65 SrtA

The genomic DNA of *S. mutans* OMZ65 was extracted according to the previously methods (Lee *et al*. 2002). The coding region of the *srtA* gene (620 bp) was PCR-amplified from the genomic DNA. The PCR product was cloned into the *Bam*HI and *Xho*I restricted pET-21a (+) and confirmed by *srtA* sequence analysis (Fig. 6). The resulting plasmid, pET-*srtA*, was maintained in *E. coli* DH5α. Recombinant SrtA was expressed in *E. coli* and purified by using metal chelate-affinity chromatography. The SDS-PAGE analysis of expressed and purified SrtA is shown in Fig. 7. Compared with the periplasmic fractions from induced *E. coli* DH5α containing pET-21a and *E. coli* DH5α containing pET21-*srtA* without induction (lanes 1 and 2), a new band appeared in the induced *E. coli* DH5α containing pET21-*srtA* sample (lane 4). The estimated molecular weight of this new band was approximately 22.4 kDa, which was consistent with the theoretical molecular weight of the purified proteins. SrtA was purified from the soluble phase by His-tag affinity chromatography using a nickel-charged column. The purity of the eluate was dialyzed and confirmed by SDS-PAGE.

2. Screening of *S. mutans* SrtA inhibitory activity of medicinal plants

A total of 100 medicinal plant extracts were sequentially fractioned with n-hexane, and ethyl acetate, and water, and the inhibitory activity of each fraction on the SrtA activity was estimated. Among the 300 medicinal plant fractions tested, the ethyl acetate fractions of *Pueraria thunbergiana*, *Spatholobus suberectus, Rhus verniciflua*, *Poncirus trifoliata*, and *Sophora japonica* showed potent inhibitory

activity (Table 3). In this study, the active constituents of *S. japonica* were investigated further.

3. Structure identification of metabolites (**1**–**7**) isolated from *S. japonica*

The EtOAc fraction of the dried flowers of *S. japonica* was separated by C_{18} reversed-phase vacuum flash chromatography using a sequential mixture of MeOH and H_2O as eluents (eleven fractions in gradient, H_2O–MeOH, from 100:0 to 0:100), acetone, and finally EtOAc. Based on the results of SrtA inhibitory activity test, the two fractions eluted with H_2O-MeOH (30:70) (4.2 g) and H_2O-MeOH (30:70) were chosen for separation. H_2O-MeOH (30:70) fraction was separated by semi-preparative reversed-phase HPLC to yield compounds **1**–**4**. These metabolites were then purified by reversed-phase HPLC in the same condition as described above. The fraction eluted with H_2O–MeOH (70:30) was separated by reversed-phase HPLC to yield compounds **5**–**7**.

Compounds **1**, **3–7** were identified to be the maltol 3-*O*-(4′-*O*-*p*-coumaroyl-6′-*O*-(3-hydroxy-3-methylglutaroyl))-β-glucopyranoside (**1**) (Kite *et al*., 2007), kaemferol-3-rutinoside (**3**) (Sang *et al*., 2002), isorhamnetin 3-*O*-β-D-rutinoside (**4**) (Sang *et al*., 2002), rutin (**5**) (Colombo *et al*., 2014; Phan *et al*., 2011), quercetin-3'-*O*-methyl-3-*O*-α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside (**6**) (Markham and Hammett, 1994; Xu *et al*., 2011), and quercetin (**7**) (Markham and Hammett, 1994; Xu *et al*., 2011), respectively, by combined spectroscopic analyses. The spectroscopic data for these compounds were in good agreement with those in

Figure 6. *srtA* sequence analysis of *S. mutans* OMZ65.

Range 1: 121 to 742 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1149 bits(622)	0.0	622/622(100%)	0/622(0%)	Plus/Plus

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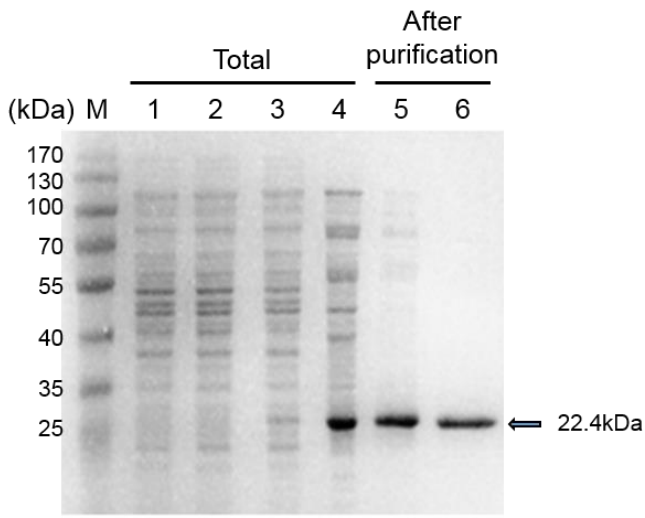
```

Figure 7. SDS-PAGE of the SrtA expressed in *E. coli*.

The SrtA was purified by Ni-chelate affinity column chromatography. The column was washed twice with wash buffer. The bound proteins were eluted with elution buffer. Purified protein was dialyzed by using Snakeskin Dialysis Tubing (ThermoScientific).



Lane 1 : pET-21a(+)
Lane 2 : pET-SrtA
Lane 3 : Induced pET-21a(+)
Lane 4 : Induced pET-SrtA
Lane 5 : Eluted pET-SrtA
Lane 6 : After dialysis

the literature (Fig. 8). The exception was compound **2**, whose configuration was unreported. The molecular formula of compound **2** was deduced to be $C_{27}H_{30}O_{14}$ by HRFABMS analysis. The ^{1}H and ^{13}C NMR spectra of this compound were very similar to those of compound **1,** revealing the presence of coumaric acid, *β*-glucopyranose, 3-hydroxybutyric acid, and maltol (Table 4). Based upon the results of combined COSY, HSQC and HMBC analyses, these moieties as well as the assembly among them were confirmed to be the same with **1**. The noticeable differences in the NMR data between these compounds were those of coumaric acid in which the *Z*-configuration was assigned for the benzylic double bond on the basis of vicinal coupling constant between the olefinic protons ($J_{\alpha,\beta}$ = 12.8 Hz). Thus compound **2** was determined to be the maltol-3-*O*-(4′-*O*-*cis*-*p*-coumaroyl-6′-*O*-(3-hydroxy-3-methylglutaroyl))-β-glucopyranoside, an isomer of **1**.

4. *S. mutans* SrtA inhibitory activity of isolated compounds (1–7)

The inhibitory potencies, expressed as IC_{50} values, of the isolated compounds toward *S. mutans* SrtA are given in Table 5 and are compared to that of known SrtA inhibitors, curcumin (IC_{50} = 91.5 μM) and *p*HMB (IC_{50} = 112.5 μM). Notably, compounds **2**–**4** were more potent than the positive control compounds. Compound **2** exhibited the most potent inhibitory activity against *S. mutans* SrtA, with an IC_{50} value of 58.6 μM, and approximately 1.6 times more potent than compound **1**. These results revealed that the *Z*-configuration of *p*-coumaric acid at the b position of compound **2** (Fig. 8) is important for the exhibition of strong SrtA-inhibitory activity. Compounds **3** and **4** were also found to be strong inhibitors, with IC_{50} values of 60.7

Table 3. SrtA inhibitory activity of medicinal plant extracts (ethyl acetate fraction).

Medicinal name	Botanical name	SrtA inhibiton ratio at 100 μg/mL (%)
갈근	*Pueraria thunbergiana*	76.1
갈화	*Pueraria thunbergiana*	28.1
감국	*Chrysanthemum sinese*	47.6
맥문동	*Liriope platyphylla*	40.8
공사인	*Amomum villosum*	47.4
괄루인	*Trichosanthes kirilowii*	-
과체	*Cucumis melo*	-
관중	*Dryopteris crassirhizoma*	-
계혈등	*Spatholobus suberectus*	85.2
연교	*Forsythiae fructus*	-
구척	*Cibotium barometz*	10.8
내복자	*Raphanus sativus*	-
승마	*Cimicifuga heracleifolia*	26.3
운방풍	*Seseli mairei*	-
당귀	*Angelica gigas*	-
대두황권	*Glycine max*	-
대풍자	*Hydnocarpus anthelmintica*	2.7
동과자	*Benicasa cerifera*	11.0
동규자	*Malva verticillata*	37.6
제니	*Adenophora trachelioides*	32.7
목방기	*Cocculus triolbus*	54.6
오미자	*Schizandra chinensis*	33.7
삼능	*Sparganium stoloniferum*	-
진피	*Citrus unshiu*	-
적작약	*Paeonia albiflora*	58.4
치자	*Gardenia jasminoides*	42.6
백단향	*Santalum album*	11.3
백두구	*Amomum cardamomum*	-
백두옹	*Pulsatilla chinesnsis*	54.0
백부자	*Aconitum koreanum*	23.4
천문동	*Asparagus cochinchinensis*	4.1
보두	*Strychnos ingnatii*	-
복분자	*Rubus coreanus*	31.8
부소맥	*Triticum aestivum*	8.8
부평초	*Spirodelae Herba*	27.7
비자	*Torreya nucifera*	-
사상자	*Torilis japonica*	12.9

산사	*Crataegus pinnatifida*	54.2
상심자	*Fructus mori*	41.7
상표초	*Mantidis Ootheca*	10.8
생지황	*Rehmania glutinosa*	29.3
소엽	*Perilla sikokiana*	5.2
노봉방	*Polistes mandarinus*	11.9
치커리	*Cichorium intybus*	22.7
소자	*Perilla sikokiana*	-
속단	*Phlomis umbrosa*	63.2
숙지황	*Rehmania glutinosa*	40.8
천초	*Zanthoxylum bungeanum*	41.3
애엽	*Artemisia argyi*	26.9
여정실	*Lingustrum japonicum*	2.2
연자육	*Nelumbo nucifera*	13.0
영능향	*Lysimachia foenum*	50.8
오가피	*Acanthopanax senticosus*	35.7
오수유	*Evodia officinalis*	29.8
택사	*Alisma orientale*	19.3
와송	*Orostachys japonicas*	40.7
우슬	*Achyrnthes bidentate*	20.1
욱리인	*Prunus humilis*	5.5
패모	*Fritillaria verticillata*	-
유향	*Boswellia carterii*	0.4
자단향	*Pterocarpus santalinus*	46.5
진범	*Acohitum pseud-leave*	38.7
잠사	*Bombyx mori*	20.0
저실자	*Cuscuta Australia*	15.1
정력자	*Lepidium apetalum*	31.2
복신	*Pachyma hoelen*	27.4
길경	*Platycodon grandiflorum*	10.7
조협	*Gleditschia japonica*	29.8
지부자	*Kochia scoparia*	1.3
지실	*Poncirus trifoliata*	70.1
창이자	*Xanthium strumarium*	26.5
창출	*Atractylodes japonica*	48.2
천마	*Gastrodia elata*	31.7
초오	*Acontium kusnezoffii*	16.9
칠피	*Rhus verniciflua*	68.9
토복령	*Smilax china*	30.0
파고지	*Psoralea corylifolia*	67.3
산작약	*Paeonia obovate*	30.5
수산약	*Dioscorea batatas*	5.0
식고본	*Angelica tenuissima*	19.8
호도인	*Juglans rejia*	48.7
향부자	*Cyperus rotundus*	-

황련	*Coptis chinensis*	23.0
황율,건율	*Castanea crenata*	15.2
흑축	*Pharbitis nil*	12.1
희첨	*Siegeseckia pubescens*	13.2
감초	*Glycyrrhiza uralensis*	42.8
강활	*Nothoptergium forbessi*	58.9
건강	*Zingiber officinale*	6.4
건지황	*Rehmania ghitinosa*	45.3
결명자	*Cassia tora*	62.8
고본	*Angelica tenuissima*	48.6
고삼	*Sophora flavescens*	29.1
과루인	*Trichosanthes kirilowii*	13.9
괴화	*Sophora japonica*	68.2
구맥	*Dianthus chinensis*	38.4
구기자	*Lycium chinense*	22.4
구절초	*Chrysanthemum sibiricum*	41.9
남성	*Arisaema amurense*	-
낭탕근	*Scopolia japonica*	-
*p*HMB (positive control)		94.7

and 62.1 μM, respectively. In addition, methoxy group at the C-3′ position did not affect the SrtA inhibitory activity because the inhibitory activities of compounds **3** and **4** were similar.

Sortase is essential for the functional assembly of surface proteins and for the pathogenesis of Gram-positive bacteria. It has been reported that sortase inhibitors should act as anti-infective agents and disrupt the pathogenesis of bacterial infections without affecting microbial viability. Therefore, to rule out effects of test compounds on *S. mutans* cell aggregation due to inhibition of cell growth, the minimum inhibitory concentration (MIC) values of isolated compounds were determined. As shown in Table 4, compounds **1**–**7** exhibited no growth-inhibitory activity against *S. mutans* OMZ65 (MIC >200 μg/mL).

5. Inhibitory activity of compound 2 on saliva-induced aggregation of *S. mutans* NG8

SrtA is responsible for controlling *S. mutans* adherence, aggregation ability and biofilm formation, and it has been verified as one of the virulence factors of caries. It was expected that inhibitors of SrtA would block the SrtA-mediated protein anchoring and prevent adherence and aggregation ability of *S. mutans* cells. Based on these findings, we next investigated the effect of compound **2** on the saliva-induced aggregation of *S. mutans* strain NG8 (wild-type) as well as its isogenic knockout mutants (Lee and Boran, 2003) Aggregation was assessed as a reduction in OD at 700 nm of reactive solutions (Magnusson, 1976). The strain NG8 was used in this test as the SrtA sequence alignment of this strain (GenBank accession number AF542085) showed 100% identity with that of *S. mutans* OMZ65 over its entire length (data not shown). In saliva-induced aggregation (Fig. 9A), NG8 and the *srtA*-complemented mutant aggregated upon incubation with saliva, but the *srtA*-deletion

mutant failed to aggregate. As expected, treatment of NG8 with compound **2** significantly reduced the aggregation ability of the bacterial cells in a dose dependent manner (1 ×, 2 ×, 4 × the SrtA IC_{50}) (Fig. 9B). These assay data suggest that SrtA inhibitor concentrations above the IC_{50} value are important for the inhibition of *S. mutans* aggregation via sortase activity. It is important to note that the onset and of inhibition of aggregation in *S. mutans* NG8 treated with compound **2** (4 × IC_{50}) (Fig. 9B) are comparable to the behavior of untreated *srtA*-deletion mutant (Fig. 9A). This result supported the observation that compound **2** was an effective inhibitor of SrtA activity.

6. *S. mutans* SrtA inhibitory activity of compounds from *P. trifoliata*

The inhibitory potencies (IC_{50} values) of four coumarin compounds isolated from *P. trifoliata* (Fig. 10, Table 6) toward *S. mutans* SrtA are also given in Table 6 and are compared to that of known SrtA inhibitors. Notably, ponciol and triphasiol exhibited potent inhibitory activities against *S. mutans* SrtA, with IC_{50} values of 14.9 μg/mL and 10.1 μg/mL, respectively. The MIC values of these compounds were also determined. As shown in Table 6, ponciol and triphasiol exhibited no growth-inhibitory activity against *S. mutans* OMZ65 (MIC >128 μg/mL).

7. Inhibitory activity of triphasiol on saliva-induced aggregation of *S. mutans* NG8

The effect of triphasiol on the saliva-induced aggregation of *S. mutans* strain NG8 (wild-type) as well as its isogenic knockout mutants was also investigated according to the methods as described above (Fig. 11). As expected, treatment of NG8 with triphasiol significantly reduced the aggregation ability of the bacterial cells in a dose

dependent manner (0.5 ×, 1 ×, 2 × the SrtA IC_{50}) (Fig. 11B). It is important to note that the onset and magnitude of inhibition of aggregation in *S. mutans* NG8 treated with triphasiol are comparable to the behavior of untreated *srtA*-deletion mutant. This result supported the observation that triphasiol was an effective inhibitor of SrtA activity.

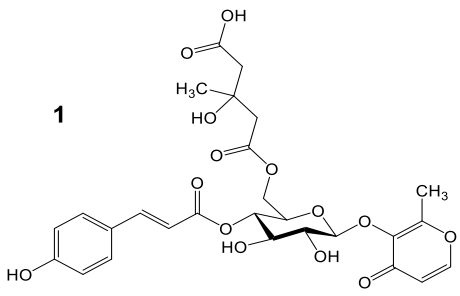
8. Inhibition of bacterial adherence and *S. mutans* biofilm formation

The inhibitory effect of triphasiol on the adhesion of *S. mutans* OMZ65 to saliva-coated hydroxyapatite beads (S-HAs) was investigated. The adherence of S. *mutans* cultured in the presence of 5 μg/mL of triphasiol was not significantly different from that of the control group. However, when the bacteria were cultured in the presence of 10 to 40 μg/mL of the triphasiol, adherence was significantly repressed (Fig. 12) in a dose-dependent manner.

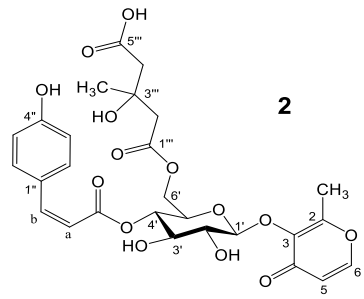
To determine whether the triphasiol inhibits the formation of *S. mutans* biofilm, cells were treated with the compound, and then biofilm formation was observed by safranin staining. As shown in Fig. 13, this compound inhibited the formation of *S. mutans* biofilm. Furthermore, the inhibitory effect of the compound at 40 μg/mL was similar to that of the positive control (0.1% NaF). *S. mutans* attached to and aggregated on the surface of polystyrene dishes, and visibly formed biofilm in the control group; however, biofilm formation was decreased in the presence of triphasiol at concentrations higher than 10 μg/mL. Biofilm formation was also decreased in the presence of the positive control. Also, triphasiol reduced the adherence of *S. mutans* to S-HAs at concentrations higher than 10 μg/mL compared to the negative and positive control groups.

Figure 8. Structures of compounds isolated from *S. japonica*.

Maltol 3-*O*-(4′-*O*-*p*-coumaroyl-6′-*O*-(3-hydroxy-3-methylglutaroyl))-β-glucopyranoside (**1**), maltol-3-*O*-(4′-*O*-*cis*-*p*-coumaroyl-6′-*O*-(3-hydroxy-3-methylglutaroyl))-β-glucopyranoside (**2**), kaemferol-3-rutinoside (**3**), isorhamnetin 3-*O*-β-D-rutinoside (**4**), rutin (**5**), quercetin-3'-*O*-methyl-3-*O*-α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside (**6**), quercetin (**7**).



1



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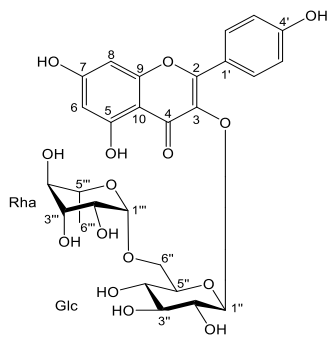

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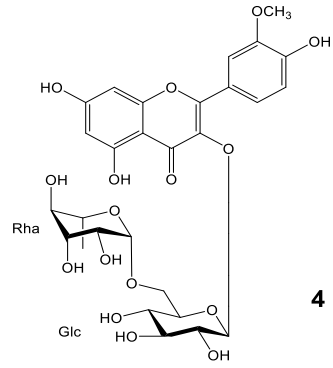

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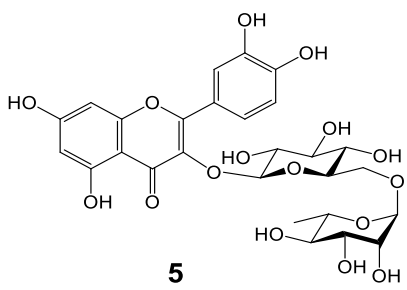

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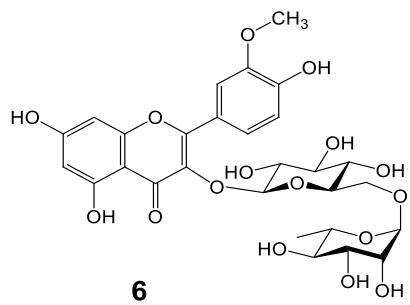

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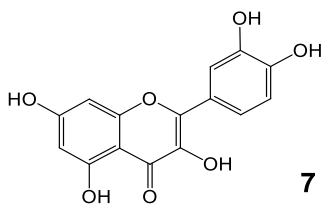


7

Table 4. NMR spectroscopic data for compound 2[a].

Position	**2**	
	δ_C[b]	δ_H[c]
2	164.7, C	
3	143.4, C	
4	177.1, C	
5	117.5, CH	6.44, d (5.6)
6	157.3, CH	7.99, d (5.6)
1′	104.8, CH	4.92, d (8.0)
2′	75.6, CH	3.50, dd (9.1, 8.0)
3′	75.7, CH	3.65, t (9.2)
4′	72.7, CH	4.91, t (9.6)
5′	73.8, CH	3.67, m
6′	63.8, CH_2	4.16, dd (12.2, 2.5) 4.12, dd (12.2, 5.5)
1″	127.7, C	
2″	134.0, CH	7.66, d (8.7)
3″	116.0, CH	6.74, d (8.7)
4″	160.3, C	
5″	116.0, CH	6.74, d (8.7)
6″	134.0, CH	7.66, d (8.7)
a	116.1, CH	5.79, d (12.8)
b	146.4, CH	6.91, d (12.8)
C=O	167.2	
1‴	172.2, C	
2‴	46.5, CH_2	2.69, d (14.6) 2.63, d (14.6)
3‴	70.8, C	
4‴	46.1, CH_2	2.61, d (15.3) 2.58, d (15.3)
5‴	175.2, C	
3‴-CH_3	27.9, CH_3	1.32, s
2-CH_3	16.0, CH_3	2.42, s

[a] Data were obtained in MeOH-d_4 solution.

[b] Data were measured at 150 MHz.

[c] Data were measured at 600 MHz.

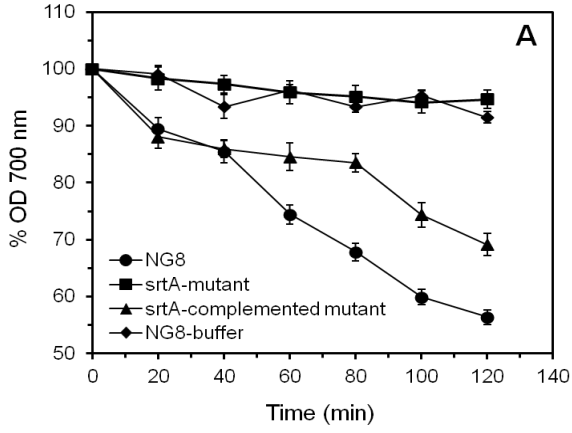
Table 5. Inhibitory effects of compounds 1–7 on the activity of SrtA enzyme and bacterial growth of *S. mutans* OMZ65.

Compound	SrtA IC_{50} μM (μg/mL)	MIC μM (μg/mL)
1	94.3 ± 1.9 (54.5 ± 1.1)	>345.9 (200)
2	58.6 ± 1.6 (33.9 ± 0.9)	>345.9 (200)
3	60.7 ± 1.2 (36.1 ± 0.7)	>336.4 (200)
4	62.1 ± 1.9 (38.8 ± 1.2)	>320.2 (200)
5	134.1 ± 1.6 (113.5 ± 0.98)	>327.9 (200)
6	185.9 ± 3.4 (116.1 ± 2.1)	>324.2 (200)
7	210.8 ± 2.9 (139.5 ± 1.9)	>661.7 (200)
Curcumin	91.5 ± 1.1 (33.7 ± 0.4)	>542.9 (200)
*p*HMB	112.5 ± 1.7 (33.1 ± 0.5)	NT

Curcumin and *p*HMB (*para*-hydroxymecuribenzoic acid) were used as reference inhibitors of SrtA. NT: not tested. IC_{50} values are means ± SD (n = 3).

Figure 9. Saliva-induced aggregation of *S. mutans* NG8 and *srtA* mutants without (A) or with compound 2 (B).

NG8-buffer refers to the aggregation assay performed with *S. mutans* NG8 in the absence of saliva. C1, C2, and C3 refer to the saliva-induced aggregation of *S. mutans* NG8 in the presence of 58.6 (1 × IC_{50}), 117.2 (2 × IC_{50}), and 234.4 μM (4 × IC_{50}) compound **2**, respectively.


A
% OD 700 nm
Time (min)
NG8
srtA-mutant
srtA-complemented mutant
NG8-buffer

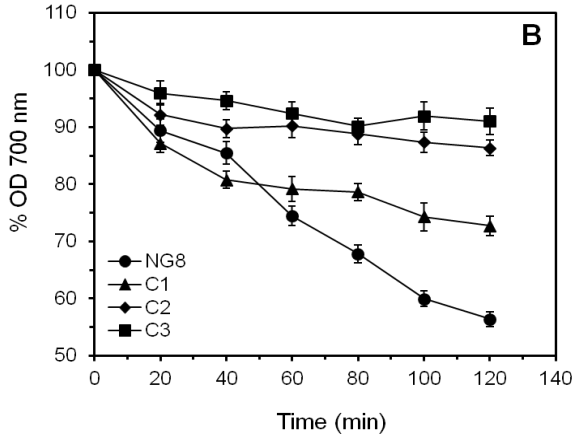
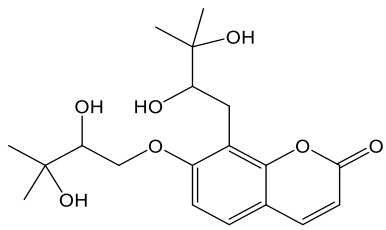
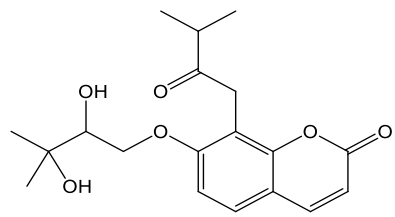

B
% OD 700 nm
Time (min)
NG8
C1
C2
C3

Figure 10. Structures of ponciol and triphasiol from *P. trifoliata*.



Ponciol



Triphasiol

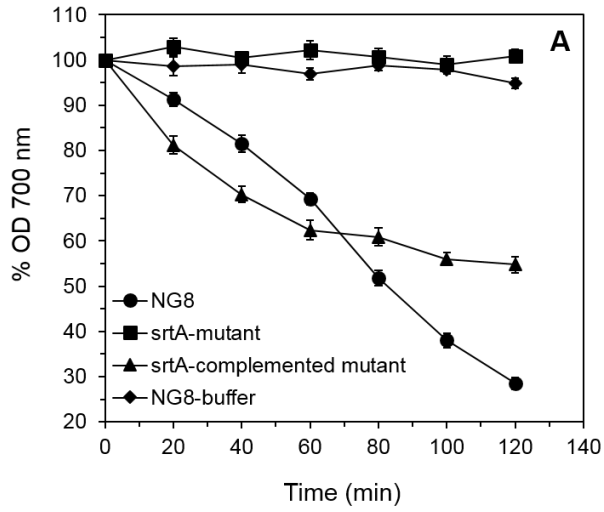
Table 6. Inhibitory effects of compounds from *P. trifoliata* on the activity of SrtA and bacterial growth of *S. mutans* OMZ65.

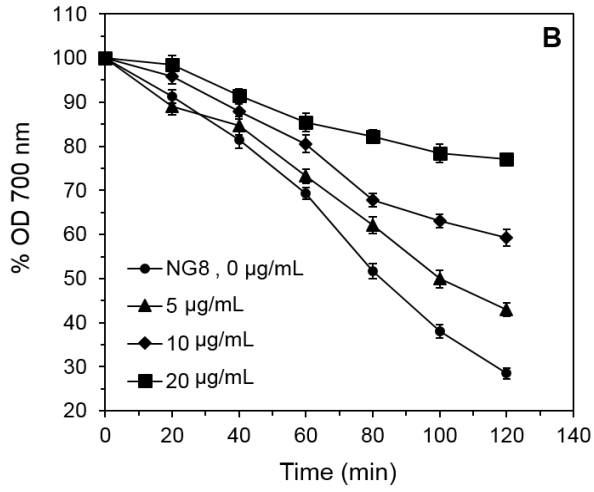
Compound	SrtA IC_{50} (μg/mL)	MIC (μg/mL)
Coumaric acid	>128	>128
Poncirin	>128	>128
Ponciol	14.9	>128
Triphasiol	10.1	>128
Curcumin	91.5	>128
*p*HMB	112.5	NT

Curcumin and *p*HMB (*para*-hydroxymecuribenzoic acid) were used as reference inhibitors of SrtA. NT: not tested.

Figure 11. Saliva-induced aggregation of *S. mutans* NG8 and *srtA* mutants without (A) or with triphasiol (B).

NG8-buffer refers to the aggregation assay performed with *S. mutans* NG8 in the absence of saliva. Saliva-induced aggregation of *S. mutans* NG8 in the presence of triphasiol (0, 5, 10 and 20 μg/mL, respectively).


A
% OD 700 nm
Time (min)
NG8
srtA-mutant
srtA-complemented mutant
NG8-buffer


B
% OD 700 nm
Time (min)
NG8 , 0 μg/mL
5 μg/mL
10 μg/mL
20 μg/mL

9. Inhibition of *S. mutans* biofilm formation on the surface of resin teeth

Adhesion of *S. mutans* to pellicle-coated tooth surfaces is the first step in the formation of dental plaque, a type of biofilm on teeth. Biofilm formation increases the bacterial resistance to both the host defense system and antimicrobials. Dental caries and periodontitis are caused by dental biofilm. Biofilm formation on the surface of resin teeth was observed (Fig. 14). Triphasiol (10 to 40 μg/mL) inhibited the formation of *S. mutans* biofilm on the surface of resin teeth. The positive control also showed an inhibitory effect on *S. mutans* biofilm formation. In addition, these data were similar to the safranin stain data as shown above. The results suggest that triphasiol may be considered useful for the prevention of dental plaque.

Figure 12. Effect of triphasiol on adherence to saliva coated hydroxyapatite bead (S-HAs) of *S. mutans* OMZ65.

The colony-forming units (CFU) of *S. mutans* that adhered to 30 mg of saliva-coated hydroxyapatite beads incubated at various concentrations of triphasiol. When cultured in the presence of 0 to 40 μg/mL of triphasiol, adherence ability of *S. mutans* was significantly repressed.

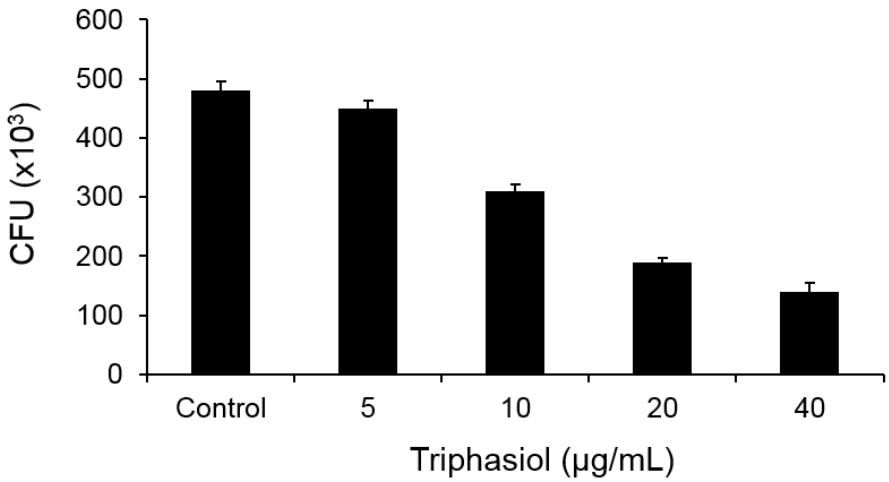

600
500
400
300
200
100
0
CFU (x10³)
Control
5
10
20
40
Triphasiol (μg/mL)

Figure 13. Effect of triphasiol on *S. mutans* biofilm formation.

S. mutans OMZ65 was inoculated into BHI broth with various concentrations of triphasiol and incubated for 48 h at 37°C. The biofilm that formed on the dish surface was measured by staining with 0.1 % safranin. The bound safranin was released from the stained cells with 30 % acetic acid, and the absorbance of the solution was measured at 530 nm. Triphasiol at 10, 20 and 40 μg/mL inhibited the formation of *S. mutans* OMZ65 biofilm.

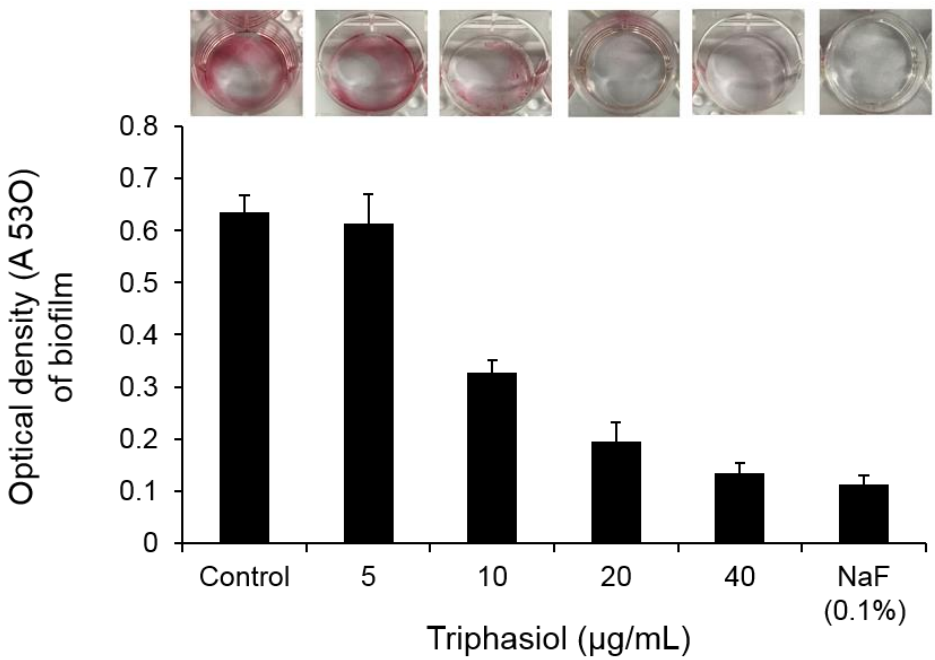
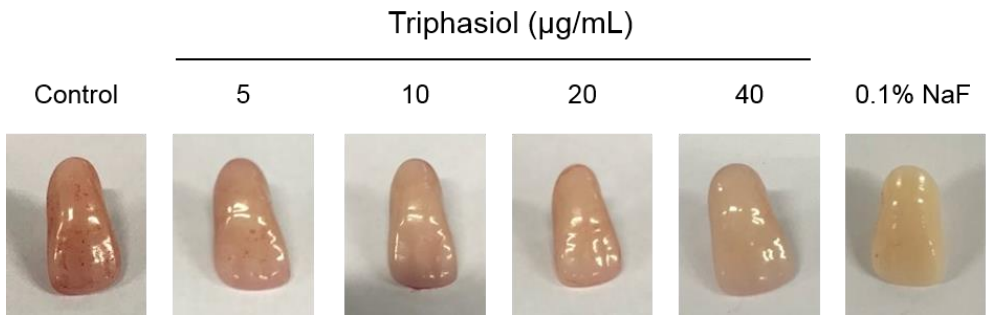

Optical density (A 53O) of biofilm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
5
10
20
40
NaF (0.1%)
Triphasiol (μg/mL)

Figure 14. Effect of triphasiol on biofilm formation on resin teeth surface.

S. mutans biofilm on resin tooth surfaces were incubated triphasiol. Triphasiol (0 to 40 μg/mL) inhibited the formation of *S. mutans* biofilm on the surface of resin teeth. 0.1% NaF was used as positive control.


Triphasiol (μg/mL)
Control
5
10
20
40
0.1% NaF

Discussion

S. mutans is known to be the primary pathogen underlying dental caries and is part of the normal flora of the oral cavity. Therefore, the control of *S. mutans* should take into account the survival characteristics the normal flora in order to minimize any influence on the growth of the oral flora. SrtA is responsible for controlling *S. mutans* adherence ability and biofilm formation, and it has been verified as one of the virulence factors of caries (Lee *et al*., 1989; Russell, 1992). Moreover, studies have shown that the srtA mutant of *S. mutans* does not influence the growth yield (Lévesque *et al.*, 2005). Several studies have confirmed the important role of *S. mutans* SrtA in bacterial pathogenesis and its potential as a suitable target for pharmacological inhibition (Suree *et al*., 2007).

Medicinal plants have been widely recognized as a prolific sources of structurally unique and biologically active secondary metabolites. Many studies have been reported that bioactive compounds derived from medicinal plants show diverse biological activities such as cytotoxic, antiviral, antimicrobial, and enzyme inhibitory for therapeutic uses. The purpose of this study is to investigate natural products from medicinal plants and determine their inhibitory activities to *S. mutans* SrtA. In the course of searching for bioactive metabolites from Korean medicinal plants *S. japonica* and *P. trifoliata* were selected for chemical investigation based on chemical analysis and bioactivity screening.

Dried flowers and buds of *S. japonica* are a medicinal herb used in China, Japan and Korea to treat hemorrhoids and hematemesis. Previous phytochemical studies indicated that flavonoids were the main chemical constituents of *S. japonica*, which have been isolated from its seeds, fruits, stem barks, woods, and leaves (Kim and Yun-Choi, 2008). In this study, SrtA assay-guided separation of the crude

extract of *S. japonica* flowers using various chromatographic techniques yielded a new maltol derivative along with six known maltol derivatives and flavonoids (Fig. 8). Based on the results of combined spectroscopic methods, the structure of new compound was determined to be maltol-3-*O*-(4′-*O*-*cis*-*p*-coumaroyl-6′-*O*-(3-hydroxy-3-methylglutaroyl))-β-glucopyranoside. Among the isolated compounds, this compound exhibited the most potent inhibitory activity against *S. mutans* SrtA, with an IC_{50} value of 58.6 μM. The preliminary structure-activity data revealed that the *Z*-configuration of *p*-coumaric acid at the b position of this compound (Fig.8) is important for the exhibition of strong SrtA-inhibitory activity. In addition, as shown in the previous paper (Lee *et al*. 2014), SrtA inhibitory activity of the phenyl propanoids such as caffeic acid and ferulic acid revealed that the presence of a hydroxyl group at the C-3 position is important. The replacement of the C-3 hydroxyl group of caffeic acid (IC_{50} = 20.2 μM) with a methoxy group (ferulic acid) led to a total loss of activity (IC_{50} > 100 μM). The saliva-induced aggregation activity data revealed the potential of this compound for the treatment of *S. mutans* infections via inhibition of SrtA activity. The inhibition of cell aggregation in *S. mutans* treated with the compound are comparable to the behavior of untreated *srtA*-deletion mutant (Fig. 9).

P. trifoliata (Rutaceae), also known as trifoliate orange, is a close relative to the *Citrus* trees. Traditionally, trifoliata oranges (*P. trifoliata*) have been widely used in folk medicine as a remedy for gastritis, dysentery, inflammation, digestive ulcers, etc (Lee *et al*., 2005). A scientific investigation of trifoliate orange fruit has revealed its anti-inflammatory, antibacterial and anti-anaphylactic activities (Kim *et al*., 1999). Several compounds such as poncirin, coumarins, auraptine, hesperidin and naringin have been identified from poncirus fruits (Avula *et al*., 2005). In this study, the

inhibitory potencies of four coumarin derivatives isolated from *P. trifoliata* against *S. mutans* SrtA were investigated and compared to that of known SrtA inhibitors. Notably, ponciol and triphasiol exhibited potent SrtA inhibitory activities, with IC_{50} values of 14.9 μg/mL and 10.1 μg/mL, respectively, without affecting bacterial viability. The inhibitory effects of triphasiol on the adhesion and biofilm formation of *S. mutans* to saliva-coated hydroxyapatite beads (S-HAs) were investigated further. This compound exhibited significant inhibition of *S. mutans* adherence to S-HAs and biofilm formation at concentrations higher than 10 μg/mL. Dental caries and periodontitis are caused by dental biofilm. Therefore, the inhibitory effect of triphasiol on the biofilm formation of *S. mutans* to the surface of resin teeth was also evaluated. Triphasiol inhibited the formation of *S. mutans* biofilm on the surface of resin teeth in a dose-dependent manner. These results suggest that triphasiol may be considered useful for the prevention of dental plaque.

Because of the great interest in sortase as targets for anti-infective therapies, many studies toward finding a potent inhibitor have been conducted over the past decade (Clancy *et al*., 2010). Inhibitors development has mainly focused on SrtA from *S. aureus* and many studies regarding the identification of SrtA inhibitors from natural products have been reported (Cascioferro *et al*., 2014). Some plant natural products, especially several flavonoids, are effective inhibitors of SrtA. However, given the limited number of inhibitors and the development of drug resistance, the discovery of new inhibitors is urgent. Although there is high amino acid sequence homology (43%) between the SrtA of *S. aureus* and *S. mutans*, there is no evidence that homologous proteins have the same inhibitors. Moreover, compared with *S. mutans*, the major pathogen of dental caries, *S. aureus* is a leading cause of hospital- and community-acquired infections ranging from minor skin infections to osteomyelitis, meningitis, endocarditis, septicaemia and toxic shock syndrome (Luo

et al., 2017). Therefore, targeting *S. mutans* SrtA may be more effective than targeting *S. aureus* SrtA. Recently, flavonoids are polyphenolic plant natural products and two in particular, morin and curcumin, display good inhibition against *S. mutans* SrtA, although the exact molecular basis of their activity has not been determined.

The efficiency of the most promising SrtA inhibitors needs to be comprehensively evaluated in *in vivo* models of infection, prior to supporting an anti-virulence role for each inhibitor that is worthy of further development into a novel therapeutic agent. At the minimum, *in vitro* evaluation of compounds on live bacteria cells, such as inhibition of biofilm formation or bacterial adhesion, should constitute a minimum requirement for the initial assessment of anti-virulence activity of SrtA inhibitors following demonstration of SrtA inhibition in biochemical assays. The future of anti-virulence therapies hold great promise and excitement as many of the SrtA inhibitors described to date are worthy candidates for further investigation.

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초 록

서울대학교 대학원

농생명공학부 응용생명화학전공

양우영

치아 우식증으로 알려져 있는 충치 질환은 세계적으로 가장 널리, 많은 사람들이 앓고 있거나 앓아 본 경험이 있는 쉽게 접할 수 있는 질병이며, 이 질환 유발에 있어서 가장 중요한 원인균은 그람 양성 세균인 *Streptococcus mutans* 로 알려져 있다. 그람 양성 세균의 세포 표면 단백질은 숙주 세포에 부착 및 감염 등 병원성 유발에 매우 중요하다. 세포 표면 단백질이 합성되어 세포벽 표면에 부착되는 과정에서 transpeptidase 의 일종인 sortase A (SrtA)가 표면단백질의 LPXTG 모티프를 인식하여 T 와 G 사이를 절단하며 절단된 -COOH 말단은 펩티도글라이칸을 구성하는 펜타글라이신 가교 (cross-bridge) 의 아미노 그룹으로 세균의 세포벽에 연결된다. 따라서 SrtA 는 새로운 항생제 개발에 있어 유용한 분자표적으로 주목 받고 있다. 특히 현재까지의 SrtA 저해제에 관한 연구는 항생제 내성균인 *Staphylococcus aureus* 를 중심으로 이루어져 왔으나 최근 *S. mutans* 의 SrtA 저해제에 대한 연구가 증가 추세에 있다. 본 연구에서는 *S. mutans* 의 *srtA* 유전자 클로닝을 통해 재조합 SrtA 를 만들고 활성평가계를 구축하였으며, 약용식물 100 여가지의 에틸아세테이트 추출물을 제조 후 활성 검색을 수행하여 우수활성을 보이는 약용식물을 도출하였다. 이들 중 *Sophora*

japonica 에서 SrtA 저해활성을 나타내는 물질을 분리하고 구조 규명하였으며 또한 *Poncirus trifoliata* 유래의 우수활성 물질과 함께 이들 물질의 유용성을 평가하였다. 또한 우수 저해활성 물질에 대해서는 *S. mutans* 세포의 응집 억제능, 인공치아 표면에서의 치태 형성억제, 그리고 치아 에나멜과 구성성분이 같은 Hydroxyl-Apatite 비즈에 타액을 도말 후 비즈 표면에 접착 억제활성 실험을 수행하였다. 또한 세포 성장 억제 활성을 알기 위해 MIC 평가를 실시한 결과 이 화합물들은 *S. mutans* 균의 SrtA 작용을 효과적으로 억제하며 약한 항균 활성을 가지는 것을 확인하였다. 이러한 실험결과들은 향후 보다 우수한 SrtA 저해제 개발연구에 중요한 생물학적 지견을 제공할 것이라 생각된다.

주요어 : 연쇄상구균, 치아우식증, 약용식물, 효소저해제,
천연물분리, 세포표면단백질, Sortase A

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