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Hydroxychalcone inhibitors of *Streptococcus mutans* glucosyl transferases and biofilms as potential anticaries agents

Bhavitavya Nijampatnam^a, Luke Casals^a, Ruowen Zheng^a, Hui Wu^{b,*}, and Sadanandan E. Velu^{a,*}

^aDepartment of Chemistry, University of Alabama at Birmingham, Birmingham, Alabama 35294, USA

^bDepartment of Pediatric Dentistry, UAB School of Dentistry, Birmingham, Alabama 35294, USA

Abstract

Streptococcus mutans has been implicated as the major etiological agent in the initiation and the development of dental caries due to its robust capacity to form tenacious biofilms. Ideal therapeutics for this disease will aim to selectively inhibit the biofilm formation process while preserving the natural bacterial flora of the mouth. Several studies have demonstrated the efficacies of flavonols on *S. mutans* biofilms and have suggested the mechanism of action through their effect on *S. mutans* glucosyltransferases (Gtfs). These enzymes metabolize sucrose into water insoluble and soluble glucans, which are an integral measure of the dental caries pathogenesis. Numerous studies have shown that flavonols and polyphenols can inhibit Gtf and biofilm formation at millimolar concentrations. We have screened a group of 14 hydroxychalcones, synthetic precursors of flavonols, in an *S. mutans* biofilm assay. Several of these compounds emerged to be biofilm inhibitors at low micro-molar concentrations. Chalcones that contained a 3-OH group on ring A exhibited selectivity for biofilm inhibition. Moreover, we synthesized 6 additional analogs of the lead compound and evaluated their potential activity and selectivity against *S. mutans* biofilms. The most active compound identified from these studies had an IC₅₀ value of 44 μM against biofilm and MIC₅₀ value of 468 μM against growth displaying >10 fold selectivity inhibition towards biofilm. The lead compound displayed a dose dependent inhibition of *S. mutans* Gtfs. The lead compound also did not affect the growth of two commensal species (*Streptococcus sanguinis* and *Streptococcus gordonii*) at least up to 200 μM, indicating that it can selectively inhibit cariogenic biofilms, while leaving commensal and/or beneficial microbes intact. Thus non-toxic compounds have the potential utility in public oral health regimes.

*Corresponding authors: Sadanandan Velu, Mailing address: Department of Chemistry, University of Alabama at Birmingham, 901, 14th Street S. Birmingham, AL 35294, USA, Phone: (205) 975-2475, Fax: (205) 934-2543, svelu@uab.edu. Hui Wu, Mailing address: Department of Pediatric Dentistry, University of Alabama at Birmingham, School of Dentistry, Birmingham, Alabama 35294, Phone: (205) 996-2392, Fax: (205) 975-4430, hwu@uab.edu.

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Supplementary data:

Docking protocols, synthetic procedures, assay protocols, and compound characterization data associated with this article can be found in the attached supplementary data.

Graphical Abstract

Keywords

Hydroxychalcone; inhibitors; glucosyltransferase (Gtfs); *Streptococcus mutans*; biofilms; dental caries

Dental caries is a multifactorial disease evident by localized destruction of susceptible dental surfaces. *S. mutans* is the primary etiological agent.¹ Through the function of its Gtfs, *S. mutans* metabolizes sucrose into water insoluble and soluble glucans.² Three types of Gtfs are reported: GtfB, GtfC, and GtfD. Both GtfB and GtfC predominantly synthesize water-insoluble glucans whereas GtfD synthesizes water-soluble glucans.³ These exopolymeric glucans are an integral measure of the cariogenic biofilms that provide an extracellular matrix, shielding the bacteria from the host immune responses, mechanical stresses, and antimicrobial agents.⁴ As *S. mutans* accumulate, the bacterium and other cariogenic species within the biofilm undergo active sugar metabolisms and produce copious amounts of their end-product, lactic acid, leading to the dissolution of the hydroxyapatite crystal lattice of the enamel.⁵

Current preventive approaches for dental caries include tooth-brushing and the use antimicrobial mouthwashes.⁶ Unfortunately, the removal of bacterial biofilms through brushing demands frequent repetition because the tooth surfaces are rapidly re-colonized by bacteria. Furthermore, the drawback of existing antimicrobial mouthwashes lies in their lack of selectivity, affecting pathogenic species as well as commensal beneficial species, which give rise to some undesired side effects.⁷ Therefore, emerging innovative approaches of dental caries prevention need to be selective and should strive to preserve the natural bacterial flora in the mouth.

Several studies have established the importance of Gtfs in the *S. mutans* biofilm formation.^{8–11} Mutants defective in genes *gtfB* and *gtfC*, which express GtfB and GtfC respectively, exhibited markedly reduced levels of smooth surface caries lesion compared to the parental *S. mutans*.⁸ Thus, *S. mutans* Gtfs are valid targets for the discovery of inhibitors of cariogenic biofilms, which will have potential applications in the treatment of dental caries.

Efforts have been placed in search for *S. mutans* biofilm inhibitors by several research groups.^{12–15} Since diet is one of the key factors that define oral health, research has mainly

been focused on widely consumed food products. Numerous studies have been conducted comparing the efficacies of flavonols and low-molecular-weight polyphenols found in cranberries.¹⁶ Flavones and flavonols were inhibitors of Gtfs; of these, a compound called apigenin (4',5,7-trihydroxyflavone, Fig.1) was the most effective inhibitor of Gtfs.¹⁷ Flavonols and their glycosides such as myricetin and myricetin 3-rhamnoside exhibited significant but moderate effects (Fig. 1).^{18, 19} In addition, scaffolds found in tea (*Camellia sinensis*) that inhibit *S. mutans* biofilms attract attention due to their unique polyphenol compositions and their prevalence in the human diet.²⁰ Early studies were carried out on extracts containing multiple constituents however recent reports focus on effects of single defined component *in vitro* and *in vivo*.²¹ Furthermore, these natural product compounds inhibited *S. mutans* biofilms at high micromolar and even millimolar concentrations.

Undoubtedly, flavonols are a promising class of scaffolds for the development of anticaries therapeutics as they may selectively inhibit the formation of cariogenic biofilms. By targeting *S. mutans*' virulence instead of its viability, the agents developed will be non-bactericidal, preserving the natural bacterial flora of the mouth, and will also be less likely to develop resistance to therapy. Encouraged by reported findings on flavonols, we were interested to examine the effect of hydroxychalcones (Fig. 1), precursors of flavonols and isoflavonols, for their effect on *S. mutans* biofilm and Gtfs. Chalcones were originally isolated from natural sources and are abundant in edible plants. Structurally, they can be defined as open-chain flavonoids in which two aromatic rings are joined by a three carbon α,β -unsaturated carbonyl system.²² Being a minor subgroup of the flavonoid family, like other members, chalcones have been reported responsible for a variety of biological activities, including antiviral, anticancer, antimicrobial, anti-inflammatory, antioxidative, antimalarial, anti-leishmania, antinociceptive, and antiproliferative activities.^{23, 24} Hence, chalcones are considered to be a class of compounds with important therapeutic potential. To our knowledge, there are no studies that report the antibiofilm activity of chalcones against *S. mutans*. We therefore investigated this class of compounds to identify novel selective antibiofilm agents that can have potential applications in dental caries prevention.

Although the inhibition of both GtfB and GtfC are required for the maximum reduction in biofilm formation, only the structural information of GtfC's active site is currently known. However, genes encoding these two Gtfs have a very similar amino acid composition (76% homology), and are subject to the same regulatory processes.²⁵ Thus, we used GtfC as a target for the drug discovery efforts. We initially performed docking analysis using FlexX/LeadIT package against GtfC active site (resolution: 3.11 Å, PDB code: 3AIC) on a polyphenol database which included various scaffolds such as flavonols, chalcones, stilbenes, and other complex structures. Of these compounds, we focused on chalcones due to their properties discussed above. A total of 14 chalcones with various hydroxyl substitution patterns were obtained from National Cancer Institute (NCI) compound library and screened for their effect on *S. mutans* growth and its biofilm. Table 1 summarizes the results of the preliminary screen conducted at 200 μ M.

The initial screen highlighted chalcone activities ranging from almost complete inhibition of both *S. mutans* growth and biofilm (Compounds **2–4**, **8**, **11**), selective inhibition of *S. mutans* biofilm (Compound **9**), to no effect on either *S. mutans* growth or biofilm

(Compounds **5–7**, **10**, **12**, **14**). Active compounds inhibited biofilm in the range of 27–95% at 200 μ M concentration. However, several of these compounds also proportionally affected the growth of *S. mutans*, indicating that these small molecules may not be selective biofilm inhibitors. Nonetheless, four compounds that showed promise by exhibiting greater than 80% biofilm inhibition were pursued further into serial dilutions in order to determine their bactericidal and anti-biofilm IC₅₀ values, and to observe if any selectivity for biofilm inhibition over growth inhibition arises at lower doses. Both the growth and biofilm IC₅₀ values remained in the low micromolar ranging from 32 – 64 μ M. While compounds **2**, **4**, and **11** failed to demonstrate biofilm-selective inhibition, compound **9** did elicit selectivity with biofilm IC₅₀ value 32 μ M, without affecting the growth of *S. mutans* at 200 μ M concentration. Through this study, we observed several trends in the elicited biofilm and bactericidal effects in relation to chalcone structures.

Chalcones that did not possess any bactericidal or anti-biofilm properties also did not have any OH groups on the ring B of the chalcone skeleton (Compound **5**). Despite containing methyl protected hydroxyl groups on the ring B, compounds **10** and **12** also failed to produce any biofilm inhibition. The only analogs that do not contain free hydroxyl groups on the ring A are compounds **13** and **14**, which contain methoxy and methylenedioxy groups in the 4th and 5th positions. While compound **13** was marginally active, reducing biofilm and growth by 30% at 200 μ M, compound **14** exhibited no anti-biofilm effects. These results suggest that the presence of hydroxyl groups on either ring of the chalcone scaffold is crucial for the inhibition of *S. mutans* biofilm.

Compounds **5** and **6** differ only in the 2' position of OH group in ring A, where the latter contains an OH group in place of hydrogen. Interestingly, both compounds are inactive, suggesting the OH in 2' position may not contribute to the studied bioactivities. While compound **7** also follows this pattern of activity, this effect is not observed in the case of compound **11**.

Furthermore, analysis of compounds **1–4** which contain an OH group at the 2nd position of ring A seemed to exhibit potent anti-biofilm and bactericidal activities. Contrary to these compounds, chalcones that bare a hydroxyl group at the 3rd position of ring A (Compounds **7–9**) seemed to exhibit a range of potent and selective anti-biofilm activities. With the exception of the inactive compound **7**, other compounds exhibited greater than 81% inhibition of biofilm at 200 μ M and presented some selectivity over growth inhibition. Strikingly, when tested at lower concentrations, the best compound that emerged from this study (**9**) almost exclusively inhibited *S. mutans* biofilm, suggesting the importance of OH group in the 3rd position in the ring A.

There was only one compound that contained a mono-hydroxyl group at the 4th position of the ring A (compound **11**). This compound was inactive against both *S. mutans* growth and biofilm. Compounds **5–6**, **10–14** contain OH group at the 4th position and majority of these compounds also fared poorly in the preliminary screen besides from compound **11** which was toxic to *S. mutans* at low micromolar concentrations. The importance of OH group at this position remains unclear and warrants further investigation.

Since we are in search of compounds that selectively target *S. mutans* biofilm without affecting its growth, the best candidate to pursue further was compound **9**, which has a biofilm IC₅₀ value 32 μ M. No previous biological activities have been reported with regard to this synthetic polyhydroxy analog. This compound did not affect the bacterial growth at least up to a concentration of 200 μ M, showing that it is a selective biofilm inhibitor. While compound **11** exhibited comparable biofilm inhibition to compound **9** (IC₅₀ value of 34 μ M, and 32 μ M respectively,) compound **11** also inhibited *S. mutans* growth at this concentration.

Thus, compound **9** was selected as the lead scaffold for the further development of the selective antibiofilm agents. In order to further establish its potency, the biofilms produced by *S. mutans* treated with this compound at various concentrations were imaged as shown in Fig. 2A. Fig. 2B depicts the dose-dependent inhibition of *S. mutans* growth and biofilm at various concentrations. Considerable selectivity can be seen, especially at higher concentrations. Finally, the effect of it on the survival rates of *S. mutans*, and two commensal species (*S. sanguinis*, *S. gordonii*) were used to assess the toxicity of this scaffold. At higher concentrations (200 μ M), approximately 10–18% inhibition is seen with all strains when compared to DMSO (Fig. 2C). It is important to note that at the biofilm IC₅₀ value of 32 μ M, no significant bactericidal activity is observed. Thus, we concluded that the compound **9** is a non-toxic and a biofilm-selective inhibitor.

As compound **9** was obtained from the NCI library in a very small quantity, we were not able to ascertain its structure with NMR spectroscopic analysis. In addition, the stereochemistry of the double bond (E or Z) present in this molecule was not reported in the NCI database. So, we synthesized both E and Z isomers of the compound **9** and characterized them. The preparation of the E isomer of **9** (**9a**) was achieved by the Claisen-Schmidt aldol condensation²⁸ of the desired benzaldehyde (**15a**) and acetophenone (**16a**) in EtOH using aqueous NaOH as the base in 93% yield. Demethylation of the intermediate methoxy substituted compound **17a** was accomplished by the treatment with BBr₃ in CH₂Cl₂ under N₂ atmosphere led to the formation of compound **9a** (E isomer) in 87% yield (Scheme 1).

The stereospecific synthesis of the Z isomer of compound **9b** was performed using a reported procedure of acid mediated aldol condensation²⁸ as shown in Scheme 2. BF₃.Et₂O mediated condensation of 2,4-dihydroxybenzaldehyde (**19**) with m-hydroxyl acetophenone (**20a**) resulted in the Z-isomer **9b** in contrast with the results obtained when using NaOH. This method was tolerant of pre-existing hydroxyl groups and thus required no protecting groups.

Taking compound **9** as our lead, we prepared a small collection of both E and Z isomers of a few hydroxychalcone derivatives (**18b–d** and **21b**) with different substitutions in rings A, B using the synthetic procedures described above. Selected examples and their activities are shown in Table 2.

We have included the E and Z isomers of the lead compound, E and Z isomers of a synthetic derivative and two other compounds that further explore the hydroxyl regiochemistry of chalcones. Due to the possibility of photo-isomerization of chalcones, which has been

previously demonstrated to be correlated with the substituents in the aromatic rings and pH dependent,²⁶ all samples were tested immediately upon making them. The biofilm and growth inhibition profiles were screened using the assay protocols as described earlier. All compounds besides compound **18c** demonstrated considerable selectivity in biofilm inhibition. Compound **18c** was the most toxic to planktonic cells with a selectivity index of 1.5; a result that is consistent to the observation reported earlier. Comparing the activities of the E and Z pairs (**9a** and **9b** and **18b** and **21b**), the Z isomers demonstrate slightly better inhibitions of both biofilm and growth. The most active compound (**9**) that emerged from the NCI screen had a biofilm IC₅₀ value of 32 μ M, while the synthesized cis isomer of this compound (**9b**) exhibited a slight decrease in activity with an IC₅₀ value of 44 μ M. Corresponding E isomer (**9a**) also had a slightly decreased activity with an IC₅₀ value of 68 μ M. Both **9a** and **9b** demonstrated a selectivity index of about 10 towards biofilm inhibition. The Z isomer (**21b**) fared slightly better at being selective compared to its E isomer when comparing compounds with a hydroxyl in the 4th position of ring A also. Compound **18d** (mixture of E/Z) produced biofilm activity with moderate selectivity. Thus, the demonstrated selectivity of these compounds makes them amenable for the development of non-toxic biofilm inhibitors.

In order to establish the relationship between the biofilm inhibition and the effect on Gtfs, we performed a zymogram assay on six of our active compounds and qualitatively observed the inhibition of Gtfs on an SDS-page gel through the intensity of the glucan bands produced. Data from selected compounds is presented in Fig. 3A. Each of these compounds was added to overnight cultures at 50 μ M concentrations, and the Gtfs were collected from supernatants. One of the duplicate SDS-PAGE gel is Coomassie blue stained while the other is subjected to the zymogram assay, where the glucan bands produced represent Gtfs enzyme activities. GtfB and GtfD remain unresolved above the 150 kD reference ladder while a clear separation can be seen with the single band of GtfC.

With regards to effects on protein level, compounds **9b**, **18b**, **18c** and **21b** showed an inhibitory effect on the production of Gtfs. Compound **18c** shows the largest inhibition and this result is logical given the bactericidal effects of the compound. Mechanism by which these compounds affect the Gtf expression is unknown and warrants further investigation. With regards to Gtf enzyme inhibitory activity, several of the polyhydroxy chalcones exhibited a prominent effect. Comparing the activities of the E and Z pairs (**9a**, **9b** and **18b**, **21b**), the Z isomers (**9b** and **21b**) exhibit the best inhibitory effects, consistent with the biofilm inhibition. A decrease is observed in the bottom GtfC band, but a decrease in the upper band GtfBD is also observed, suggesting that the compound inhibited Gtfs. This is expected as GtfC and GtfB share 76% homology. Surprisingly, the E isomers **9a** and **18b** did not exhibit a drastic effect, suggesting that perhaps these isomers use a different pathway to illicit biofilm inhibition. Compound **18d** demonstrates some weak inhibition of the GtfC band. A dose-dependent zymogram experiment of compound **9b** was performed to determine if any selectivity would emerge (Fig. 3B). All Gtfs were inhibited by compound **9b** dose-dependently. Thus, we conclude this compound affects the production and activity of all Gtfs.

Docking analysis of the two different isomers **9a** and **9b** within the GtfC active site revealed slightly different binding modes (Fig. 3C), which may explain the differences observed between the inhibitions seen in the zymograms and biofilm activity. A previous report indicated that binding of acarbose to Glu515 compromised the acid/base catalyst function, while interactions with Asp477 and Asp588 blocked the acceptor glycosyl moiety, explaining the inhibitory properties shown by acarbose when bound to GtfC²⁷. While the docking analysis showed that these three residues were within proximity to the both isomers, the Z isomer, **9b** showed the hydroxy groups positioned appropriately and closer to the three key residues when compared to the E isomer, **9a**.

We used a sucrose-dependent *Drosophila* colonization model¹³ to examine the effect of both compounds **9a** and **9b** on *S. mutans* colonization *in vivo*. Briefly, Gfp tagged *S. mutans* bacteria were infected into flies along with the treatment appropriate compound at 100 μ M in a feeding-assay. DMSO was used as a negative control (Fig. 3D) and *gtfB* mutant, a known biofilm defective strain, was used as a positive control (Fig. 3D.a). The fluorescence intensity was observed in guts of the flies fed with the Gfp tagged bacteria after 7 days of infection. A marked reduction in fluorescence was observed by both compounds **9a** and **9b** (Figs. 3D.d and 3D.c), with **9b** eliciting a more potent effect, producing an effect similar to that observed in the *gtfB* mutant (Fig. 3D.b). This data is consistent with the zymogram and the biofilm inhibition observed previously.

Thus, we have demonstrated the *S. mutans* antibiofilm activities of polyhydroxychalcones, and have observed some trends in activities with respect to the stereochemistry of the double bond and the regiochemistry and numbers of hydroxyl groups present in the molecules. Several low micromolar biofilm inhibitors emerged. Chalcones which contained a hydroxyl group in the third position of ring A exhibited higher selectivity for biofilm over growth. The lead compounds were further evaluated for their effect on *S. mutans* Gtfs and their effect on commensal bacteria and found that these scaffolds have an effect of Gtf expression and activity. The relationship between the biofilm activity and Gtf inhibition was established using a zymogram assay. These results were further validated by conducting a docking analysis of Z and E isomers of one of the lead compounds. We have also demonstrated the effect of two lead compounds on *S. mutans* colonization *in vivo*. Further studies to optimize the activity and selectivity of the lead compounds and establish polyhydroxychalcones as selective antibiofilm agents are currently in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DMSO	dimethylsulfoxide
E	trans isomer
Gfp	green fluo-rescent protein
Gtfs	glucosyltransferases
kD	kilodalton
SAR	structure activity relationship
SDS-PAGE	sodium do-decyl sulfate polyacrylamide gel electrophoresis
μM	micromolar
Z	cis isomer

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Highlights

- Low micromolar inhibitors of *S. mutans* biofilm and Gtf are identified.
- The lead compounds are E and Z isomers of hydroxychalcones.
- The Z isomers of chalcones produced more potent anti-biofilm activities.
- Chalcones with a 3-OH group on ring A showed better selectivity for biofilm inhibition.
- These compounds do not affect bacterial growth.

Figure 1.
Known inhibitors of *S. mutans* biofilm and *S. mutans* Gtfs and general structures of flavone and hydroxychalcone

Figure 2.

A) Microscopic evaluation of *S. mutans* biofilms inhibited by compound 9. *S. mutans* UA159 wild type was co-incubated with compound 9 at various concentrations and its subsequent biofilms were stained with Syto9. B) *S. mutans* growth and biofilm affected by compound 9. *S. mutans* UA159 was co-incubated with compound 9 at various concentrations and their growth was measured at OD₄₇₀. Using the crystal violet protocol, their biofilm was measured at OD₅₆₂. C) Effect of compound 9 on commensal streptococci. *S. mutans* UA159, *S. sanguinis*, *S. gordonii* were co-incubated with compound 9 at various concentrations and their growth was measured at OD₄₇₀.

Figure 3.

A) Gtf level and activity determined by SDS-PAGE analysis and zymographic assay. *S. mutans* UA159 wild type were co-incubated with compounds of interest at 50 μ M. Gtfs were concentrated from *S. mutans* UA159 wild type and then ran through duplicate SDS-PAGE. I) The upper panel is Coomassie blue staining of proteins analyzed by SDS-PAGE. II) The bottom panel shows glucan bands produced in the zymographic assay. The intensity of the bands reflects Gtf enzyme activity. **B)** Effects of compound **9b** at different concentrations. *S. mutans* UA159 wild type bacteria were co-incubated with compound **9b** various concentrations. Protein profiling (I) and activity (II) of Gtfs were evaluated as described in (A). **C)** Docking poses of compounds **9a** in blue and **9b** in green and the key residues interactions of GtfC active site. **D)** Fluorescent microscopy images of *S. mutans* colonization in *Drosophila*. a) Treatment with DMSO, b) *gtfB* mutant strain, c) Treatment with 50 μ M **9b** and d) Treatment with 50 μ M **9a**

Scheme 1.
Synthesis of the E chalcones

Scheme 2.
Synthesis of the Z chalcones

Table 1
Biofilm and growth inhibition profiles of chalcone derivatives obtained from the NCI library

Compd No	NSC code	R ¹	R ²	Biofilm inhibition 200 μ M (%) ^a	Growth inhibition 200 μ M (%) ^a	<i>S. mutans</i> Biofilm IC ₅₀ (μ M) ^a	<i>S. mutans</i> Growth IC ₅₀ (μ M) ^a
1	73255	2-OH	4'-OH	27 $\pm$ 5	48 $\pm$ 8	NA	NA
2	73257	2-OH	3'-OH	84 $\pm$ 3	86 $\pm$ 0.5	64 $\pm$ 4	32 $\pm$ 0.3
3	46672	2,5-di-OH	4'-OMe	87 $\pm$ 8	76 $\pm$ 0.9	NA	NA
4	94615	2-OH	4'-OH, 3'-OMe	86 $\pm$ 2	85 $\pm$ 0.6	61 $\pm$ 2	44 $\pm$ 3
5	401492	2,4-di-OH	H	NI	NI	NA	NA
6	636790	2,4-di-OH	2'-OH	NI	NI	NA	NA
7	636810	3-OH	2'-OH	NI	NI	NA	NA
8	640543	3-OH	2'-OH, 3',5'-di-Cl	81 $\pm$ 2	82 $\pm$ 2	NA	NA
9	17051	3-OH	2,4'-di-OH	93 $\pm$ 1	NI	32 $\pm$ 5	NI
10	90932	4-OH	3',4',5'-tri-OMe	NI	NI	NA	NA
11	640536	4-OH, 5-OMe	2'-OH	85 $\pm$ 3	71 $\pm$ 5	34 $\pm$ 2	40 $\pm$ 5
12	677786	4-OH, 3-NMe ₂ , 5-NMe ₂	4'-OMe	NI	NI	NA	NA
13	196539	4-OMe, 5-OMe,	4'-OMe, 3'-OH	27 $\pm$ 4	30 $\pm$ 0.7	NA	NA
14	196540	4,5-OCH ₂ O	4'-OMe, 3'-OH	NI	NI	NA	NA

^a Average of at least 5 measurements; NI_{No} inhibition; NA_{NI}Not available.

Table 2

Biofilm and growth inhibition profiles of synthesized analogs of compound **9**

No	E/Z	R ¹	R ²	Biofilm IC ₅₀ (μM) ^a	Growth IC ₅₀ (μM) ^a	SI ^b
9a	E	3-OH	2,4'-di-OH	68 ± 3	639 ± 25	9.39
9b	Z	3-OH	2,4'-di-OH	44 ± 5	468 ± 16	10.6
18b	E	4-OH	2,4'-di-OH	162 ± 21	1063 ± 118	6.6
21b	Z	4-OH	2,4'-di-OH	77 ± 8	782 ± 96	10.1
18c	E	2-OH	2',4',5'-tri-OH	224 ± 23	323 ± 36	1.5
18d	E/Z	4-OH	2',4',5'-tri-OH	76 ± 6	376 ± 6	4.9

^a Average of at least 5 measurements.

^b Selectivity index calculated by dividing growth IC₅₀ value by biofilm IC₅₀ value.