Synthetic Simplification of Carolacton Enables Chemical Genetic Studies in *Streptococcus mutans*

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Abstract

Understanding the broader biological impact of carolacton, a macrolactone natural product, has been ongoing for the past decade. Multiple studies have shown connections to regulatory systems, acid tolerance mechanisms, biofilm formation, and recently folate dehydrogenase (FolD). Progress elucidating the cause of biofilm-specific activity in *Streptococcus mutans* has been limited due to low-throughput analyses of carolacton-treated cells. We disclose the discovery of a simplified carolacton-inspired analog that demonstrates inhibitory activity against *S. mutans* biofilm cells. This discovery permitted a proof of concept chemical genetic screen of *S. mutans* mutants identifying the carbon catabolite protein A signaling pathway as a putative target.

Graphical Abstract

Keywords

antibiotic; natural product; dental caries; diverted total synthesis; bacterial biofilm

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DEDICATION
This paper is dedicated to Prof. Christopher T. Walsh on the occasion of his 75th birthday. His guidance and mentorship to over 100 trainees has transformed the field of chemical biology, and the world is a better place for it.
Most chronic infections are caused by bacterial biofilms, a conglomeration of single- or multispecies cells encompassed by an extracellular matrix.\textsuperscript{1–4} These communities are challenging to eradicate due to changes in metabolic activity.\textsuperscript{5} Nearly all current antibiotics target cell-wall biosynthesis, protein synthesis, DNA replication, and folate biosynthesis, all of which are downregulated when bacteria form a biofilm, thereby decreasing efficacy.\textsuperscript{6} Increased efforts in developing biofilm-specific therapeutics are imperative to treat chronic biofilm infections.\textsuperscript{4} Streptococcus mutans is a model organism for studying biofilm formation in the oral cavity.\textsuperscript{7} S. mutans forms a robust biofilm and outcompetes neighboring bacteria through the production of lactic acid by glycolysis.\textsuperscript{8,9} This process lowers the pH of the environment leading to dental caries,\textsuperscript{10,11} a disease affecting over half of the population.\textsuperscript{12} S. mutans has the ability to survive these harsh conditions due to its acid tolerance response (ATR).\textsuperscript{13,14} Regulatory pathways in S. mutans such as equilibration of intracellular protons, rerouting of carbon to glucan production, and branched-chain amino acid synthesis maintain pH and thereby protect the cell.\textsuperscript{9,15} Management of these processes is controlled by the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) and a carbon catabolite control protein (CcpA).\textsuperscript{16} Along with other transcriptional regulators such as CodY, CcpA is able to activate or repress genes on the basis of carbon source availability.\textsuperscript{17} These processes contribute to the swift ATR of S. mutans in the oral cavity.

Carolacton (Figure 1, (−)-1) is a myxobacterial natural product whose activity has been shown to promote cell membrane defects in S. mutans during biofilm growth when environmental pH rapidly decreases.\textsuperscript{18–20} This specific and unique activity toward biofilm growth conditions has attracted a number of synthetic efforts, with three total syntheses and multiple analog campaigns disclosed.\textsuperscript{21–27} In addition, several studies investigating these specific biofilm perturbation effects at low nanomolar concentrations have been reported. Wagner-Döbler and co-workers have greatly contributed to understanding the comprehensive cellular activities of carolacton by initially showing that a ΔcomD strain was less sensitive to carolacton treatment.\textsuperscript{28} In a follow-up study, the activity of carolacton was connected to the master cellular regulator PknB.\textsuperscript{19} PknB is the only serine/threonine kinase present in S. mutans and has regulatory responsibilities that include genetic competence, cell wall metabolism, and bacterocin production.\textsuperscript{29} Transcriptional analysis has further established that carolacton greatly affects the regulatory network present in S. mutans biofilm, and Sudhakar et al. demonstrated that a CysR deletion strain greatly reduced carolacton susceptibility (~90%).\textsuperscript{30} More recently, it has been shown that carolacton inhibits folate dehydrogenase (FolD) in a TolC knockout of E. coli.\textsuperscript{31} These results were further supported by computational modeling and in vitro inhibition of FolD assays in S. pneumoniae, but an answer as to why carolacton specifically targets S. mutans biofilms was not discussed. Furthermore, there are outstanding questions, such as how PknB, CysR, and ComDE contribute to the biofilm response. Taken together, it is unclear if the binding interaction between carolacton and FolD causes the biofilm phenotype in S. mutans.

Although informative, traditional microbiological mutant screening with carolacton has major limitations. For example, CodY, which regulates the largest subnetwork of affected genes in their study, was not tested since ΔCodY is not able to form a robust biofilm. The
bioactivity of carolacton, which is monitored by LIVE/DEAD staining and CFU/mL counts, could not be measured against ΔCodY since there was no fixed biofilm samples and proceeded with limited throughput. These findings highlight the current limitations of using molecular genetic techniques to confirm the mechanism of action of carolacton and biofilm inhibitors in general. Over the past decade, chemical genetic approaches have been used in similar situations to identify convoluted mechanisms of natural products, specifically in signal transduction systems.\textsuperscript{32,33} Below, we describe how our diverted total synthesis (DTS) provided a new compound, (+)-2, that was successfully implemented in a proof of concept forward chemical genetic screen of S. mutans mutants.

Our interest in carolacton began in 2014 with our initial publication of a concise synthesis of the natural product in collaboration with the Phillips Lab.\textsuperscript{27} We quickly realized that further analysis of the activity of carolacton would be limited for the aforementioned reasons. Therefore, we focused on leveraging DTS for the purpose of two specific goals: (1) creating a simplified analog with quantifiable biofilm inhibitory activity and (2) developing a chemical probe that would enable further investigation of biofilm mechanisms. On the basis of previous synthetic discoveries, both from the Kirschning Lab and our own, we sought to apply DTS toward the construction of further simplified side-chain analogs.\textsuperscript{26}

In our first DTS effort, disclosed in 2017, we found that replacing the trisubstituted alkene with an aryl bioisostere provided analogs with unique biofilm phenotypes when observed with confocal microscopy (D4, Figure 1).\textsuperscript{34} Most importantly, we identified a simplified structure, C3, that inhibited 50% of biofilm formation at 63 μM. The discovery of this tool compound demonstrated the importance of the side-chain length and the terminal carboxylic acid in the bioactivity of carolacton. Therefore, we sought to design a second generation of analogs that would incorporate these factors and further simplify the synthesis (Figure 1).

To access these simplified analogs, we employed our previous synthetic route (Figure 2).\textsuperscript{27} Starting from the commercially available nonanediol, a TBS-mono protection followed by a Parikh-Doering oxidation and a Roush crotylation with (E)-crotylboronate furnished the alcohol side chain (+)-5. Esterification with the previously published carboxylic acid precursor followed by ring-closing metathesis provides macrocycle (−)-7. Deprotection of the TBS-silyl ether and subsequent oxidation affords (−)-8 and analog (−)-9, respectively. Finally, analog (+)-10 was accessed by removing the acetonide group with HF-pyridine.

To mimic the macrocyclic structure of carolacton, we sought the monohydrogenated versions of (−)-9 and (+)-10 analogous to our previous synthetic efforts. Unfortunately, all attempts to perform the selective hydrogenation were unsuccessful (Figure S1). We rationalized that the increased flexibility of the side chain significantly affected the selectivity of the hydrogenation. We postulated that the increased number of rotatable bonds brings the side chain into closer proximity and accordingly reduces the selectivity of the hydrogenation that was originally observed. With this knowledge and the oversaturated macrocycle material (+)-11 in hand, we questioned if maximal macrocycle flexibility would also correlate to unique bioactivity. For this reason, (+)-2 was accessed via a TEMPO oxidation from (+)-11 (Figure 2a) to yield the corresponding carboxylic acid.
We tested the biological activity of analogs (−)-9, (+)-10, and (+)-2 against S. mutans to explore the effect of our structural changes. First, compound-treated S. mutans was grown in planktonic conditions and compared to carolacton and C3, a first generation analog that has previously demonstrated biofilm inhibition. Interestingly, the fully saturated analog (+)-2 inhibited S. mutans with a minimum inhibitory concentration (MIC) of 250 μM (Figure 2b). It should be noted that this was the first observance of an MIC value for any compound structurally related to carolacton, including the natural product. In biofilm assays (0.1% sucrose supplement to promote biofilm formation), (−)-9, (+)-10, carolacton, and C3 all performed similarly (Figure 2c). In contrast, (+)-2 exhibited more potent inhibitory activity against biofilm growth, demonstrating an inhibition effect on S. mutans growth when biofilm formation is promoted. The difference between the biofilm and planktonic response suggests that the activity of (+)-2 trends with increased biofilm character and the likely increase in acidity that results.

To probe this hypothesis further, we used confocal imaging to visualize the effect of (+)-2 on biofilm formation. Compound-treated S. mutans biofilm was grown in glass bottom plates to further promote biofilm formation and to allow for direct visualization of biofilm phenotypes (Figure 3a). In our confocal conditions (THB media, sucrose, and glass bottom plate), the biofilm growth inhibition was improved with an IC\textsubscript{50} of 44 μM, further demonstrating its preference for biofilm mechanisms (Figure 3b). It should be noted that carolacton does not have an IC\textsubscript{50} under these conditions. Visualized with LIVE/DEAD stain, high densities of nonviable cells were observed at 125 and 63 μM, and percent viabilities were calculated from the fluorescence measurements. According to these calculations, analog (+)-2 caused 76% and 59% of the biofilm cells to become nonviable at 125 and 63 μM, respectively. These results in conjunction with crystal violet staining experiments (see SI) confirmed that the observed increase in biofilm mass corresponded to the accumulation of cells with cell membrane defects (shown in red). To further validate the observation that our compound was causing viability issues to S. mutans in this biofilm assay, we conducted CFU/mL (colony forming units) counts to determine the biofilm activity of (+)-2 (Figure 3c). From the CFU/mL calculations, it was found that (+)-2 decreased biofilm cell viability by 96% at 125 μM, 94% at 63 μM, and 58% at 32 μM compared to the DMSO control. More interestingly, it was discovered that (+)-2 decreased biofilm cell viability between 75% and 93% at low concentrations (between 62 nM and 2 μM) (Figure 3d). At the same concentrations, carolacton decreased biofilm cell viability between 30% and 78%. Therefore, compared to carolacton, our simplified analog has a more potent effect on S. mutans biofilm.

After confirmation of the biofilm specific activity, we sought to investigate the mechanism by which (+)-2 acts. Carolacton’s activity is dependent on the drastic drop in pH that is observed during biofilm formation.\textsuperscript{19} With structural and biological similarities between (+)-2 and carolacton, we deemed it appropriate to investigate if (+)-2 acted via a similar mechanism. To test this, we measured the susceptibility of pre-acidified planktonic cultures to (+)-2. This experiment had been previously used to connect the activity of carolacton to decreases in environmental pH. We found that (+)-2 inhibits planktonic cells with an IC\textsubscript{50} of approximately 10 μM when the media was pre-acidified (Figure 4a, red). This data supports our hypothesis that (+)-2 acts, at least in part, via an acid-mediated mechanism.
We were next prompted to further characterize the mechanism by which (+)-2 targets the ATR of *S. mutans*. Taking advantage of the inhibitory activity of our compound, we utilized a proof of principle forward chemical genetic approach. Recently, the Quivey lab reported an extensive library of *S. mutans* mutants with complete phenotypic profiles. After comparison of these biofilm phenotypes with the previous reports highlighted above, we selected 17 mutants that we hypothesized to be associated with the ATR. This group contained genes responsible for *S. mutans* acid tolerance mechanisms, two component systems, cell division, cellular regulation, and glucan synthesis (list of mutants; Figure S4).

As mentioned above, a significant benefit of (+)-2 is that it elicits an inhibitory effect that allowed for the expedited screen of this library with the goal of identifying a nonsusceptible mutant strain. Each mutant was dosed with 125 μM of (+)-2, and viability was measured (Figure 4b). Fourteen of the mutant strains were as susceptible as the WT strain, UA159, but two mutants, SMU_484 (ΔpknB) and SMU_1276c (ΔezrA), were found to be more susceptible. It is interesting that the master regulator PknB, which has been previously implicated as the target of carolacton, is a slightly more susceptible mutant to our tool compound (+)-2 (p = 0.131). The activity of (+)-2 was significantly reduced against the SMU_1591 mutant when compared to the parent UA159 strain, signifying that this transcriptional regulator is partially responsible for the observed inhibition patterns described above. SMU_1591 is deficient in the gene that codes for carbon catabolite protein A (CcpA). This transcriptional regulator is responsible for the control of carbon usage within the cell and regulates a number of downstream pathways associated with the ATR mechanism of *S. mutans* (Figure 4c). Specifically, CcpA has been shown to regulate EPS formation (ftf, gtBC), cell attachment (fruA), acetate metabolism (pta, ack), branched-chain amino acid synthesis (ilvCE), oxidative stress tolerance (cid, lrg), and other virulence mechanisms.

This work details the successful implementation of DTS to develop a tool compound, (+)-2, that demonstrates biofilm specificity and is suitable for chemical genetic screening. Analog (+)-2 demonstrates three distinct advantages over the natural product: (1) the synthesis is greatly expedited due to the structural simplification, (2) it enabled a preliminary screen of *S. mutans* mutants that resulted in identification of the CcpA signaling pathway, and (3) it is more potent than the natural product eliciting an IC₅₀ and an MIC and causing higher levels of cell death in biofilm samples. Further characterization of the activity of analog (+)-2 and its connection to its parent structure, carolacton, is necessary and currently underway in our laboratory.

Taken together, our proof of concept chemical genetic screen has identified the CcpA signaling pathway as the one that likely harbors the target of (+)-2. It should be noted that CcpA regulates a large number of targets downstream in *S. mutans* and was also implicated in the activity of carolacton via transcriptomic studies by Sudhakar et al. Intriguingly, that study did not show a connection between CcpA and FolD, the proposed target of carolacton. These findings can be rationalized in one of three ways: (1) carolacton and (+)-2 have two distinct targets, (2) the target of carolacton in *S. mutans* is still elusive, or (3) CcpA regulates FolD by a currently unknown mechanism. Previous studies have also postulated that the CcpA signaling pathway is an ideal target for the oral microbiome as multispecies
communities devoid of CcpA allowed for commensal bacteria to outcompete S. mutans.\textsuperscript{40,41} 
Future studies will utilize (+)-2 as a chemical probe to better understand these biofilm mechanisms and apply them to multispecies communities. The work disclosed herein further illustrates the power of natural product total synthesis and chemical genetic approaches to identify biological targets and better understand novel mechanisms of action.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**REFERENCES**


Figure 1. 
First and second generation of carolacton-inspired analogs.
Figure 2.
(a) Synthesis of the second generation of analogs. Inhibition of S. mutans planktonic cells grown in (b) THB media to determine MIC and (c) THB media supplemented with 0.1% sucrose (w/v) to promote biofilm growth. Data shown represents biological triplicates from three individual experiments.
Figure 3.
(a) S. mutans biofilm response to treatment of analog (+)-2. Biofilm mass calculated via crystal violet analysis relative to control. Confocal images were taken with a 100× objective, and LIVE/DEAD stain was used to visualize cell viability. Image zooms contain a 5 μm scale bar. (b) Dose response of analog (+)-2 when S. mutans is grown with 0.1% sucrose supplement and in a 96-well glass bottom plate. (c) Biofilm CFU/mL analysis at 125, 63, and 32 μM in the same conditions as (a). (d) Biofilm CFU/mL represented from 0.0625 to 2 μM.
Figure 4.
(a) Analog (+)-2 kills S. mutans planktonic cells when grown in pre-acidified THB media.
(b) The S. mutans mutant screen identifies SMU_1446 as a putative target of analog (+)-2. The student’s t test was performed to calculate statistical significance of each mutant to the WT strain UA159. “*” refers to a p value < 0.05. “ns” denotes a difference that is not statistically significant. “% growth” represents [compound treated] / [vehicle control] × 100.
(c) A map of genes that have been shown to be regulated by CcpA.