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Article

Synthesis, Characterization, and Antimicrobial Investigation of a Novel Chlorhexidine Cyclamate Complex

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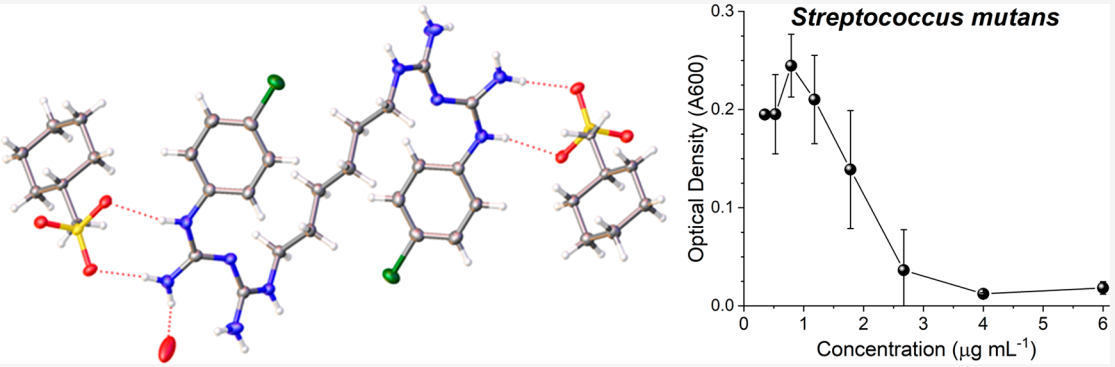

ABSTRACT: The synthesis, crystal structure, and antimicrobial efficacy are reported for a novel material comprising a 1:2 ratio of chlorhexidine (CHX) to *N*-cyclohexylsulfamate (i.e., artificial sweetener known as cyclamate). The chemical structure is unambiguously identified by incorporating a combination of single-crystal X-ray diffraction (SC-XRD), electrospray ionization mass spectrometry (ESI-MS), ^{1}H nuclear magnetic resonance (NMR) spectroscopy, correlation spectroscopy (COSY), and attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR). The new material: (1) is among only several reported structures identified to date incorporating the vital chlorhexidine antimicrobial drug; (2) exhibits broad spectrum antimicrobial activity at concentrations less than 15 μg/mL; and (3) provides a unique delivery method for the essential active pharmaceutical ingredient. Furthermore, substitution of inactive gluconate with bioactive cyclamate counterion potentially provides the additional benefit of improving the taste profile of chlorhexidine.

INTRODUCTION

Chlorhexidine (CHX) is a chemical disinfectant and antiseptic with broad antimicrobial activity against a variety of microorganisms including fungi and bacteria.[1] Since its introduction in the 1950s, it has become increasingly ubiquitous in cosmetic, healthcare, and pharmaceutical industries as a preservative, disinfectant, and antiseptic.[2−5] CHX is widely used in mouth rinses for the prevention of plaque formation and development of gingivitis.[6] In fact, CHX was included in the "World Health Organization (WHO) Model List of Essential Medicines" for antiseptic (i.e., 5% digluconate solution) and neonatal umbilical cord care (i.e., 7.1% digluconate solution or gel) applications.[7] Because the neutral chlorhexidine molecule exhibits low water solubility (i.e., less than 0.1 g/L),[8] it is typically delivered as an aqueous dication (H_2CHX) salt of an appropriate counterion (Figure 1). For example, water-soluble salts can be formed by protonating the guanidine groups with gluconic acid (i.e., chlorhexidine digluconate), acetic acid, or hydrochloric acid. Largely attributed to this low solubility and propensity to form micelles in solution, CHX does not typically crystallize, and only five crystal structures of CHX salts have been reported in the literature over the past 60 years despite its widespread use in global healthcare.[9]

In 2008, Dupont et al. reported the crystal structures of complexes between CHX and three anionic calix[4]arene derivatives.[10] Nearly a decade later, in 2016, Cattaneo et al. reported crystallographic characterization of the hydrated salts of CHX with SO_4^{2-} and CO_3^{2-}.[11] However, the effect of the anion on the antimicrobial activity of CHX was not investigated. Herein, we report the crystal structure of chlorhexidine dicyclamate as well as an investigation of the antimicrobial activity of dicyclamate (i.e., CHX-cyclamate or CHC) counter-

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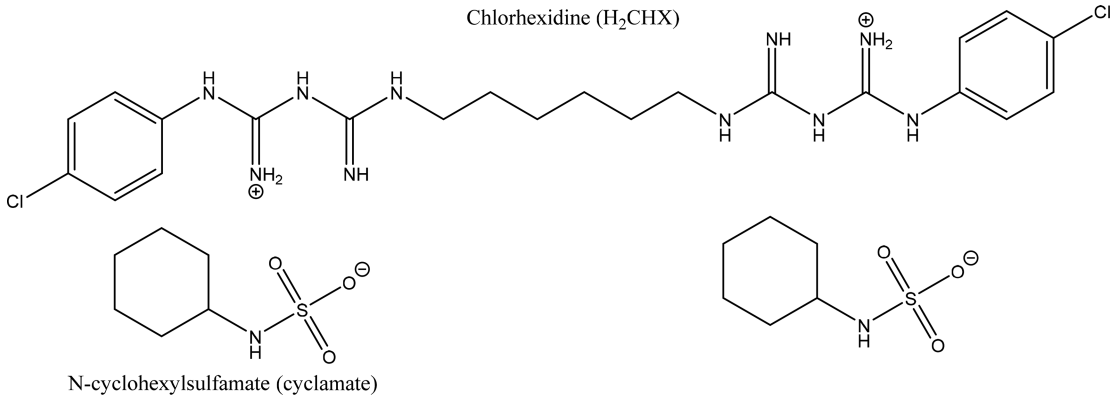

Figure 1. Molecular structure of chlorhexidine-cyclamate salt.

ion as compared to digluconate (i.e., CHX-gluconate or CHG) and dihydrochloride (i.e., CHX-HCl) counterparts.

In some cases of chlorhexidine (digluconate) oral treatments, patients often report an initial unpleasant bitter taste, while prolonged use often produces taste disturbances which may last for several hours.[12,13] The presence of these side effects may lead to reduced patient compliance and incomplete antimicrobial effect causing a reduction in overall treatment efficacy. In the current work, an artificial sweetener was utilized as a counterion for chlorhexidine in attempts to mitigate chlorhexidine side effects and thus enhance its oral treatment compliance. Hence, the biologically inactive gluconate or acetate counterions are replaced by the bioactive and functional cyclamate anion.

Sodium cyclamate is a relatively stable and inexpensive artificial sweetener produced by the sulfonation of cyclohexylamine.[14,15] Besides its potential capability to mask the bitter taste of CHX, it is known and well-studied that the combination of molecules can have an enhancing or synergistic effect on chemical and physical properties (e.g., antimicrobial activity).[16] In fact, Cavicchioli et al. reported how the complexation of cyclamate with Ag(I) caused more than a 4-fold reduction in minimum inhibitory concentration (MIC) against *Mycobacterium tuberculosis* as compared to $AgNO_3$.[17]

Because of the critical role that CHX plays in human health, a considerable amount of research has been devoted to understanding its antibacterial mechanism.[18] Isotopic labeling studies demonstrated that the uptake of CHX by bacteria occurs rapidly, reaching maximum binding at ca. 20 s, and is concentration-dependent.[19] At low concentrations, CHX affects the intracellular cytoplasmic membrane integrity, while at high concentrations it causes congealing of cytoplasm.[1] On the other hand, CHX has little effect on the germination of bacterial spores, exhibits low activity against many viruses, and its effect against mycobacteria is bacteriostatic. It is therefore highly desirable to modulate the chemistry of CHX (e.g., conjugation or complexation with other molecules) to discover synergistic effects.

MATERIALS AND METHODS

Synthesis. Synthesis of CHX-cyclamate was carried out in both methanol and water environments. Chlorhexidine digluconate (20 wt %), chlorhexidine dihydrochloride, and sodium *N*-cyclohexylsulfamate (referred to as sodium cyclamate herein) were supplied by Sigma-Aldrich (St. Louis, MO). All materials were used as received by the manufacturer without further purification.

The aqueous synthesis entailed dropwise addition of an aqueous 1 wt % sodium cyclamate solution to a 20 wt % CHG solution, to achieve a 2:1 molar ratio in accordance with charge balance considerations, yielding a precipitate. The heterogeneous mixture was filtered, washed with copious amounts of water, and dried in a 40 °C vacuum. Synthesis in methanol was conducted by combining dilute solutions of chlorhexidine dihydrochloride (0.2 wt %) and sodium cyclamate (0.14 wt %) to achieve a final solution comprising 0.1 wt % chlorhexidine dihydrochloride and (chlorhexidine)(cyclamate)$_2$ stoichiometry. Slow evaporation of solvent yielded crystal formation. Although the syntheses in water and methanol yielded the same product (Figure 7), the crystals from the aqueous synthesis were used for subsequent analyses.

Characterization. X-ray diffraction data were collected using a Bruker D8 Venture PHOTON 100 CMOS system equipped with a Cu Kα INCOATEC ImuS microfocus source (λ = 1.54178 Å). The data were collected at 100 K. Indexing was performed using *APEX3* (Difference Vectors method).[20] Data integration and reduction were performed using SaintPlus 6.01.[21] Absorption correction was performed by multiscan method implemented in SADABS.[22] Space group was determined using XPREP implemented in APEX3.[20] The structure was solved using SHELXT (direct methods) and was refined using SHELXL-2017[23−25] (full-matrix least-squares on F^2) through OLEX2 interface program.[26] All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed in geometrically calculated positions and were included in the refinement process using riding model.

Infrared spectra were collected using a Bruker Vertex 70 FTIR spectrometer (Bruker Optics, Billerica, MA) equipped with a GladiATR diamond ATR accessory (Pike Technologies, Madison, WI). The spectral range was 80−4000 cm^{-1} with a resolution of 4 cm^{-1}. All measurements were carried out at room temperature on as-prepared samples.

^{1}H NMR measurements were performed on 1 wt % samples in deuterated dimethyl sulfoxide (DMSO) solution. All NMR spectra were acquired on a Bruker Avance spectrometer (Bruker−Biospin, Billerica, MA, USA) with a 5 mm BBI probe operating at 500.0 MHz for ^{1}H at 25 °C. The ^{1}H NMR resonance of the compounds were further assigned using the homonuclear shift correlation 2D NMR (COSY) method.

Antimicrobial Assays. *Salmonella enterica* serovar Typhimurium LT2,[27] *Staphylococcus aureus* USA300 LAC,[28] and *Streptococcus mutans* Clark (ATCC) were used to study the effect of chlorhexidine compounds on survival. *S. enterica* and *S. aureus* were cultured in Muller Hinton media (Sigma-Aldrich) and *S. mutans* was cultured in Reinforced Clostridial Media (Oxoid). Stock solutions for chlorhexidine·2HCl (CHX-HCl; 2.2 mg mL^{-1}) and chlorhexidine cyclamate (CHC; 2 mg mL^{-1}) were prepared by dissolving the compounds in DMSO prior to use. Chlorhexidine gluconate (CHG; 2 mg mL^{-1}) was provided as a 19% w/v solution and further diluted in deionized water.

Growth analyses were conducted as previously described with slight alterations.[29] Single bacterial colonies were inoculated into 2 mL of medium in 10 mL capacity culture tubes. Inoculated cultures of *S. aureus* and *S. enterica* were grown aerobically at 37 °C with shaking at

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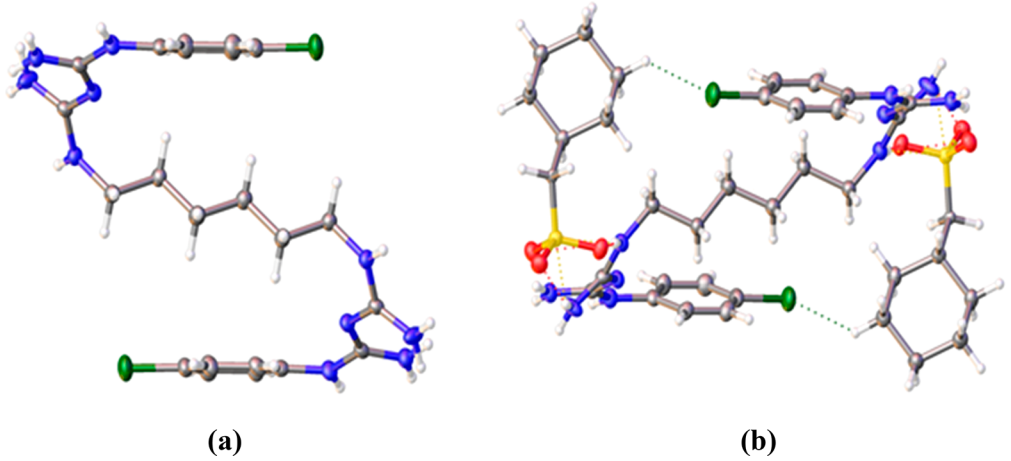
Figure 2. View of the CHX unit with two cyclamate units.



(a) **(b)**

Figure 3. (a) The S-shaped CHX coils and (b) depiction of the C−H−Cl hydrogen bonding interactions.

200 rpm for 24 h. *S. mutans* was cultured statically for 48 h. End-point minimum inhibitory concentrations (MICs) were determined for CHX-HCl, CHC, and CHG using the broth microdilution method from Clinical and Laboratory Standards Institute.[30] Overnight cultures were standardized, in triplicates, to 0.5 McFarland standards (OD_{600} = 0.1). MICs were determined in cultures grown in 96-well microtiter plates. 100 μL of the standardized culture was subcultured into wells containing 100 μL of medium containing the antimicrobial compound. Control wells containing 200 μL of media only or media with antimicrobial compound were used to standardize the data. The microtiter plates were aerobically incubated statically at 37 °C. The *S. aureus* and *S. enterica* cultures were analyzed after 20 h, and *S. mutans* was analyzed after 48 h. Culture optical densities (A_{600}) were determined using a Biotek EPOCH 2 microplate reader.

RESULTS AND DISCUSSION

Synthesis of CHX-cyclamate was carried out in aqueous and organic (i.e., methanol) solvent using commercially available precursors. Initial observation of CHX-cyclamate salt formation occurred upon mixing the cationic chlorhexidine precursors with sodium cyclamate, which yielded precipitation.

The single crystal X-ray diffraction (SC-XRD) analysis, carried out at 100 K, shows that CHX-cyclamate crystallizes in the monoclinic $P2_1/c$ space group. The asymmetric unit consists of half a molecule of protonated chlorhexidine (CHX) cation and one molecule of cyclamate anion. A disordered solvent is also present which was modeled as a water molecule (atom O1) with an occupancy of ∼0.5. The hydrogen atoms of the water could not be modeled accurately. The unit cell parameters and crystallographic details are listed in Table S1.

The overall structure consists of a symmetrically diprotonated CHX molecule surrounded by two cyclamate units (Figure 2), and the structural formula can be described as $[C_{22}H_{32}N_{10}Cl_2]\cdot[C_7H_{13}O_3S]_2$. The CHX molecules adopt a spiral conformation as observed in previous reported structures with the simple CO_3^{2-} and SO_4^{2-} anions, but instead of the U-shaped coils,[11] they arrange into S-shaped coils (Figure 3a). The two Cl ends of the CHX are antiparallel to each other and show weak C−H−Cl hydrogen bonding interactions (3.168 Å)[31] with the hydrogens of the cyclamate ring (Figure 3b).

The SO_3^- anions from the cyclamate ring form strong to moderate hydrogen bonds (2.059−2.706 Å) with the −NH/NH_2 groups (2.064/2.153 Å, respectively) of three adjacent chlorhexidine cations all arranged in a left-handed conformation. Each CHX unit interacts with four cyclamate units, and each of the cyclamate units shows interaction with two cyclamate units. Interestingly, the cyclamate molecules also show strong hydrogen bonding among each other whereby the oxygens of the SO_3^- of one cyclamate interacts with the hydrogens from the $-CH_2$ of the other cyclamate, forming dimers extending along

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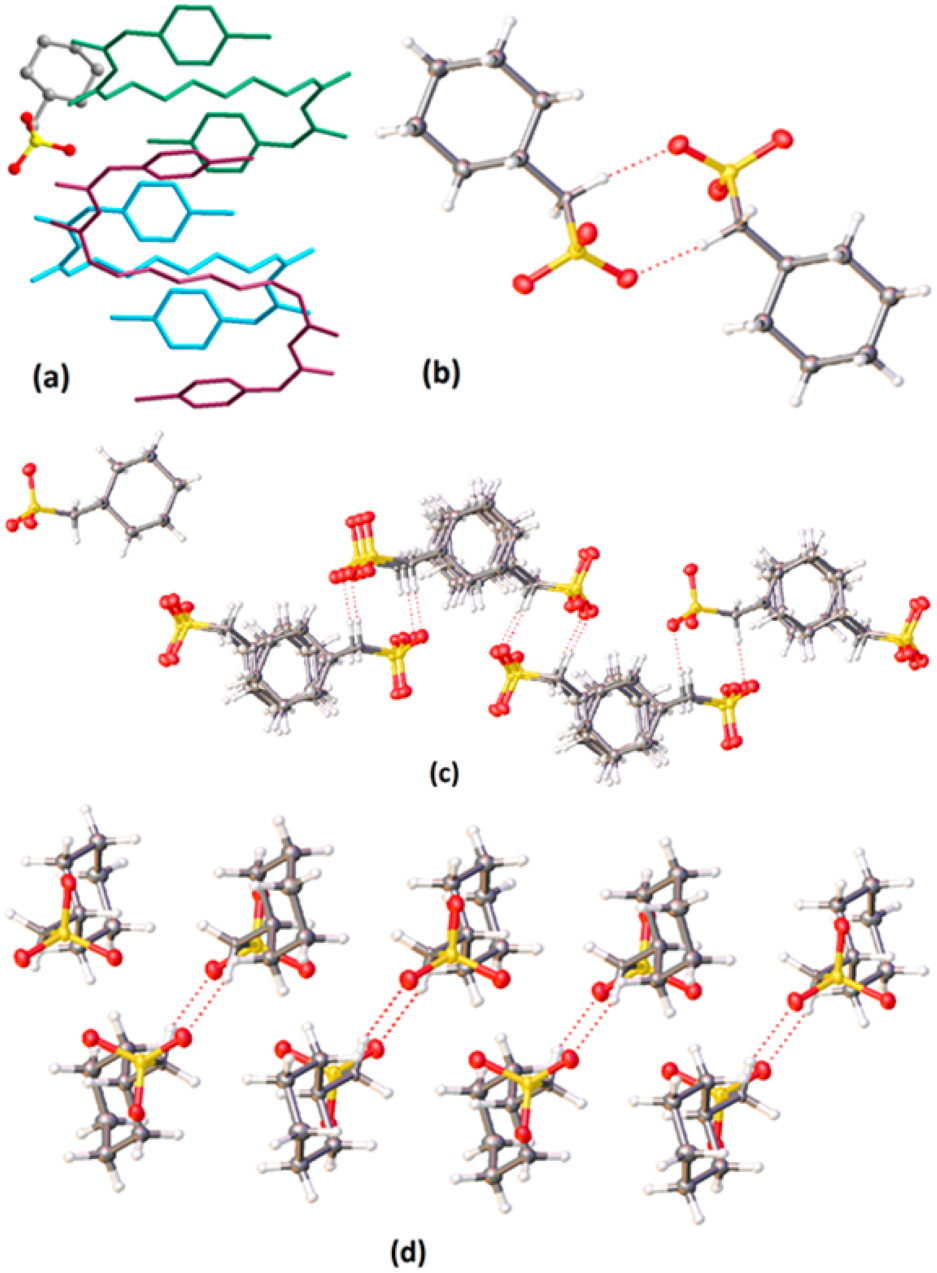

Figure 4. (a) The cyclamate showing interaction with three CHX units; (b) the cyclamate dimer; (c) extension of dimers along the *c*-axis; and (d) alternating cyclamate molecules along the *b*-axis.

the *c*-axis. Adjacent cyclamate molecules alternate with the sulfonate groups pointing at opposing ends giving rise to the dimer network along the *b*-axis. The oxygen atom in the water molecule also shows hydrogen bonding with the hydrogens from the unprotonated $-NH_2$ of the CHX. All these different kinds of hydrogen bonding interactions[32] are represented in Figure 4. The resulting structure with alternating CHX coils and cyclamate dimers gives rise to a three-dimensional network with extensive hydrogen bonding. The structural arrangement is significantly different from the analogous sulfonate-derivatized calixarenes that are arranged into bilayers and form inclusion complexes with CHX.[10] The packing arrangement is depicted in Figures 5 and 6.

No significant $\pi-\pi$ interactions were observed in the structure. The C−N bond lengths within the biguanidine units of CHX showed some delocalization of single and double bonds (1.319−1.363 Å) indicating resonance between the protonated forms. The selected bond lengths and angles are summarized in Tables S2 and S3, respectively.

Figure 7 compares the infrared absorption spectra of CHX-cyclamate samples prepared in two different solvents (methanol and water) to the sodium *N*-cyclohexylsulfamate, chlorhexidine dihydrochloride, and lyophilized chlorhexidine digluconate raw materials. The presence of both components (i.e., chlorhexidine and cyclamate) is immediately apparent in the spectra of CHX-cyclamate samples. As an example, chlorhexidine bands corresponding to ν(C═C), ν(C═N), and $\delta(NH_2)$ vibrations are clearly evident in the region above 1480 cm^{-1} where sodium cyclamate has no infrared absorption.[33−35] Similarly, N−H stretching vibrations of -NH, ═NH, and NH_2 functional groups of chlorhexidine can be identified in the 3000−3500 cm^{-1} high frequency range. The cyclamate component is manifested, for

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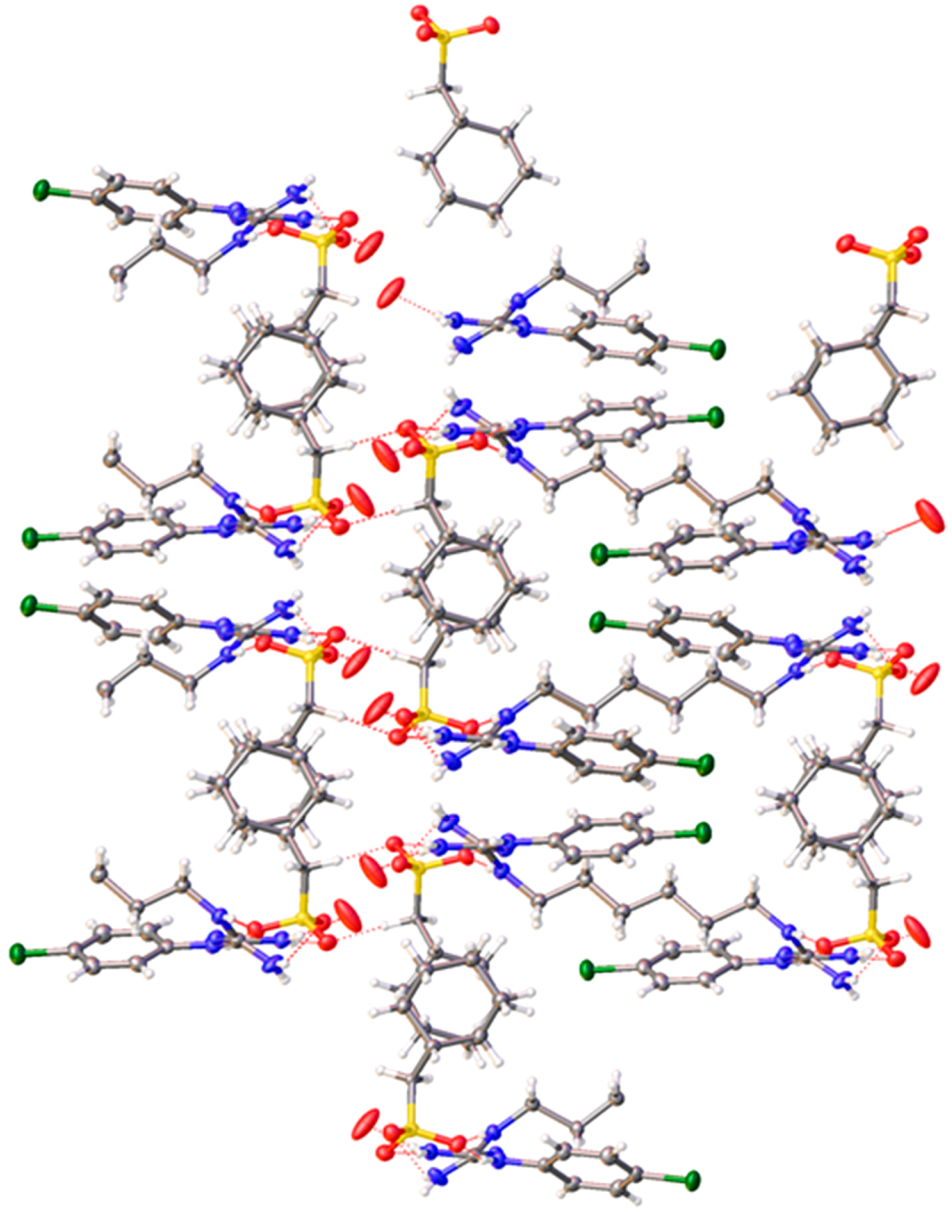

Figure 5. Packing arrangement showing the network formation with CHX coils and cyclamate dimers.

instance, by the two prominent sets of bands near the 1030 and 1170 cm^{-1} region, associated with as/sym (SO_2) vibrations.[36,37] Evidence of both precursors in the prepared samples along with the fact that the vibrational bands are significantly different in their shape and positions from the initial raw materials suggests the formation of the salt between chlorhexidine and cyclamate ions. Finally, comparison of the spectrum of CHX-cyclamate synthesized in methanol versus water reveals that the two samples display overall similar vibrational profiles with some variations in their relative bands' intensities likely originating from the small differences in the purity and local structure of the two samples.

^{1}H NMR spectroscopy (Figure 8) and COSY (Figure S6) confirmed that both chlorhexidine and cyclamate exist in the crystal dissolved in DMSO. The ^{1}H NMR chemical shifts corresponding to specific protons of chlorhexidine and cyclamate are indicated in Figure 8. Specifically, the ^{1}H NMR spectrum showed the presence of signals for the benzene rings of CHX, resonating at 7.34 and 7.38 ppm, as well as characteristic signals of methylene protons of CHX at 1.04, 1.46, and 3.06 ppm. In addition, the methylene protons of the cyclohexane ring of cyclamate were identified at 1.16, 1.61, 1.88, 2.87 ppm. Because the line broadening is greater than the proton-carbon scalar coupling, the proton peaks directly coupled with carbon were not well observed. COSY was further performed to confirm the peak assignment of the NMR spectrum. According to peak integrals in Figure 8, the stoichiometric ratio between chlorhexidine and cyclamate is 1:2.

The ability of CHX-HCl, CHC, and CHG to inhibit the growth of the bacterial pathogens *Staphylococcus aureus* LAC, *Streptococcus mutans*, and *Salmonella enterica* serovar Typhimurium was examined. *S. aureus* LAC is a Gram-positive community-associated methicillin-resistant CA-MRSA strain and a representative strain of the USA300 clone, which is a leading cause of skin and soft tissue infections in North America.[38] *S. mutans* is also Gram-positive and the leading causes of dental caries.[39] *Salmonella enterica* serovar Typhimu-

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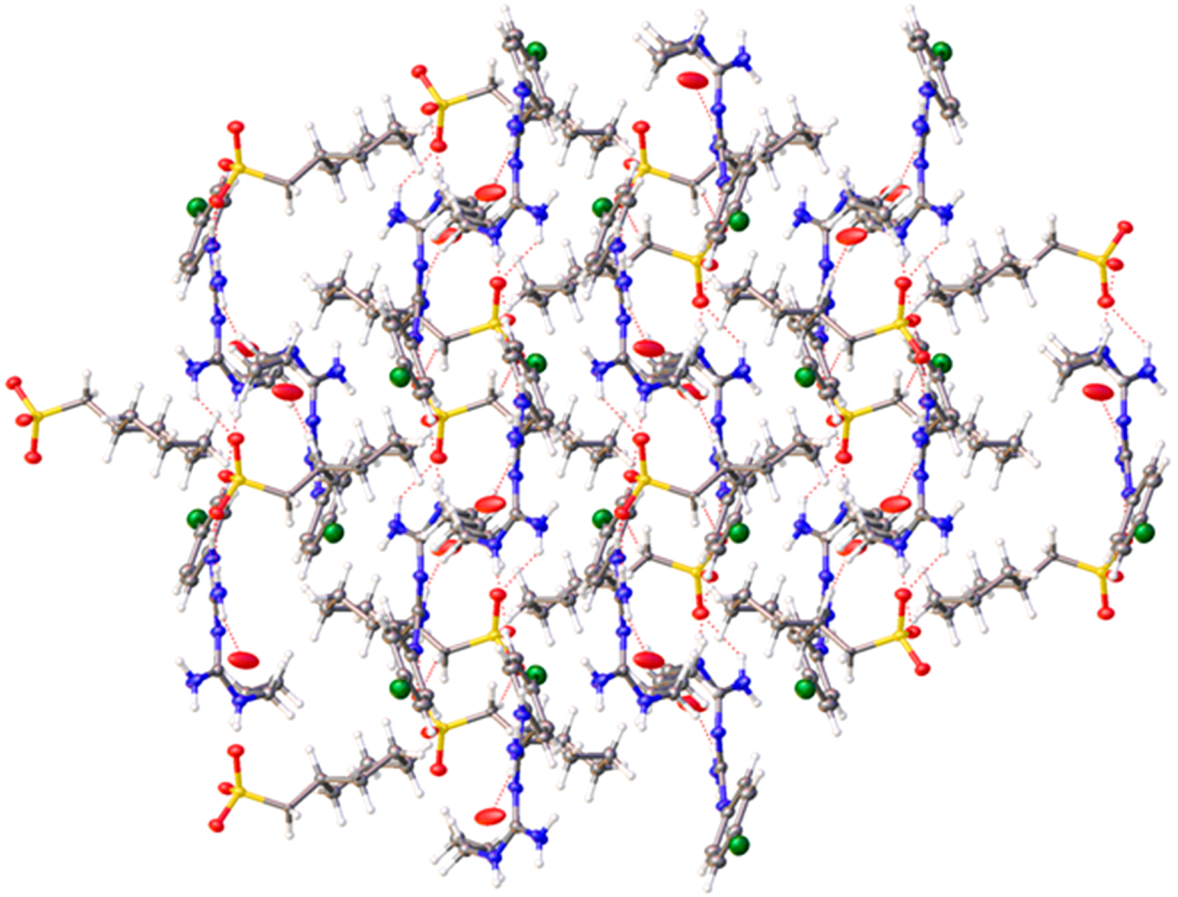

Figure 6. View of packing along the (100) plane showing alternating cyclamate units.

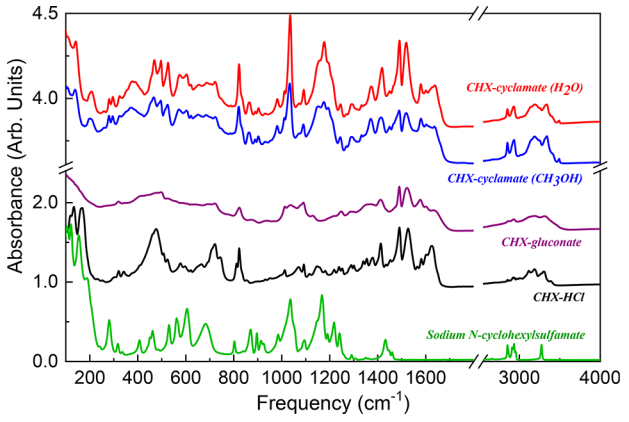

Figure 7. FTIR spectra of sodium *N*-cyclohexylsulfamate, CHX-HCl, CHX-gluconate, and CHX-cyclamate crystals prepared from methanol and water. Spectra are offset for clarity.

rium is a Gram-negative and a primary enteric pathogen affecting humans.[40]

The MICs of CHX-cyclamate, CHX-gluconate, and CHX-HCl were determined in a liquid culture after static growth. All of the three bacteria displayed typical dose−responses to the compounds utilized (Figure 9a,b and Figure S8). The MICs for CHX-cyclamate, CHX-gluconate, and CHX-HCl are reported in Table 1 and Table S4 for *S. mutans*, *S. aureus*, and *S. enterica*. CHX-HCl demonstrated the lowest MIC values for all tested bacteria, potentially due to the additional cellular toxicity provided by the coordinated strong acid. The gluconate and cyclamate counterparts exhibited parity efficacy versus the oral strain *S. mutans*. CHX-cyclamate was slightly less effective against *S. aureus* and more so against *S. enterica*. Nevertheless, all of the three tested compounds were on the same order of efficacy with efficient Gram-positive and Gram-negative bacteria inhibition at ppm concentration levels. On the basis of this data, we posit that the cyclamate counterion does not deactivate chlorhexidine's antimicrobial mode of action. The MIC values for CHX-gluconate are in good agreement with those reported in the literature.[8]

CONCLUSION

A novel analogue of an essential antimicrobial drug chlorhexidine digluconate was synthesized, characterized, and evaluated for its antibacterial properties. Substitution of the biologically inert gluconate anion with the bioactive *N*-cyclohexylsulfamate, an artificial sweetener known as cyclamate, counterpart yielded a material that can potentially enhance the taste profile while maintaining parity antimicrobial efficacy against *S. mutans*, which is known to cause dental caries. The novel material is an important progression to the solid state understanding of an indispensable biocide that does not easily crystallize under normal conditions.[41] Moreover, the newly developed technology furthers our understanding of CHX and paves the way for further research to improve the current leading antimicrobial treatment in a worldwide campaign to promote oral and overall healthcare.

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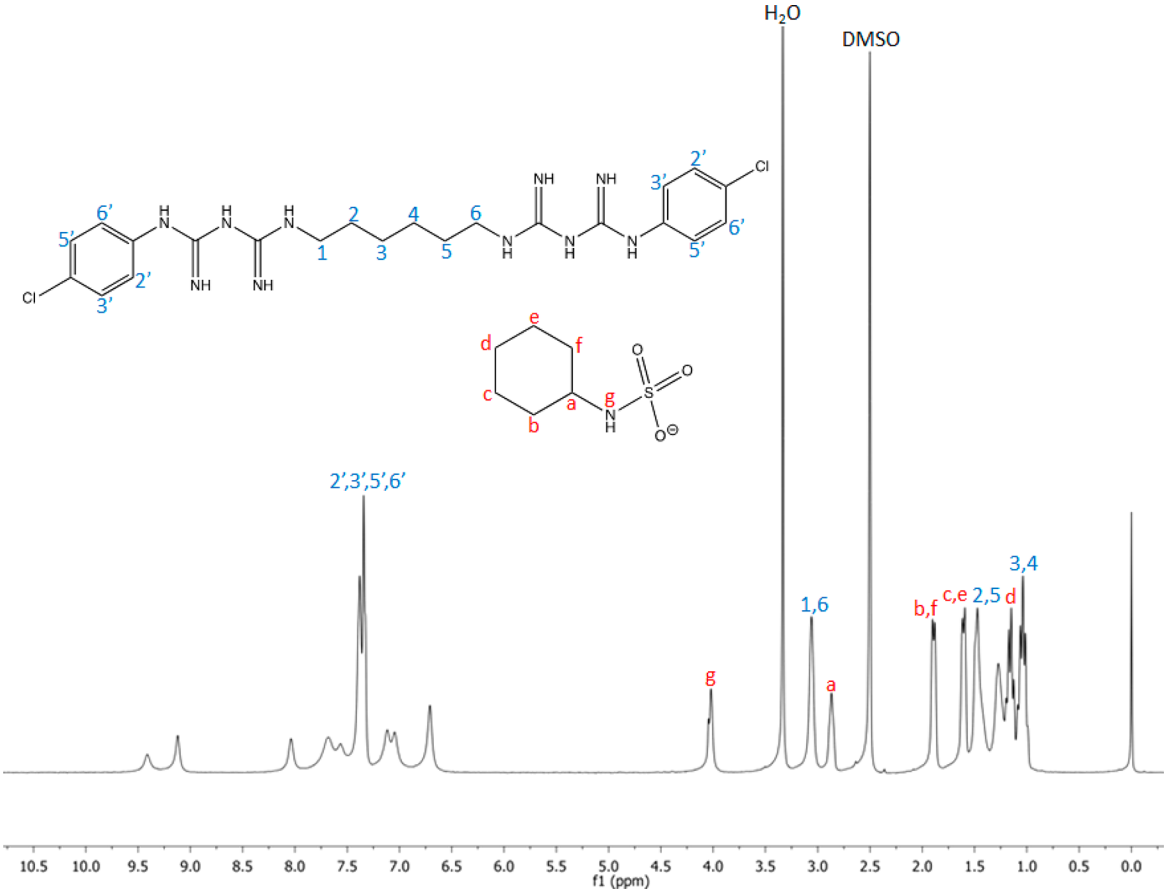

Figure 8. ^{1}H NMR spectroscopy of crystal dissolved in deuterated DMSO.

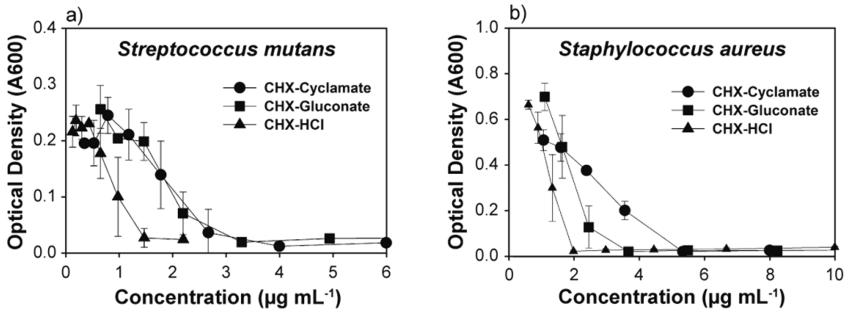

Figure 9. MIC assays for CHX-cyclamate, CHX-gluconate, and CHX-HCl with (a) *S. mutans* and (b) *S. aureus*.

Table 1. MIC Values for CHX-Cyclamate, CHX-Gluconate, and CHX-HCl

	minimum inhibitory concentration (MIC)	
compound	*S. mutans* (μg mL^{-1})	*S. aureus* (μg mL^{-1})
CHX-HCl	1.5	2.0
CHX-gluconate	2.5	3.5
CHX-cyclamate	2.5	5.0

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.cgd.0c00107.

Materials and methods; characterization of CHX-cyclamate using liquid chromatography-mass spectrometry, correlation spectroscopy (COSY) ^{1}H nuclear magnetic resonance, X-ray diffraction, PXRD, selected bond lengths and angles; antimicrobial assays (PDF)

Accession Codes

CCDC 1979952 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CHX, chlorhexidine; CHX-cyclamate, chlorhexidine dicyclamate; CHX-gluconate, chlorhexidine digluconate; CHX-HCl, chlorhexidine dihydrochloride

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