A broad-spectrum bactericidal lipopeptide with anti-biofilm properties

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Previous studies of the oligoacyllysyl (OAK) series acyl-lysyl-lysyl-aminoacyl-lysine-amide, suggested their utility towards generating robust linear lipopeptide-like alternatives to antibiotics, although to date, none exhibited potent broad-spectrum bactericidal activity. To follow up on this premise, we produced a new analog (C₁₄KKc₁₂K) and investigated its properties in various media. Mechanistic studies suggest that C₁₄KKc₁₂K uses a non-specific membrane-disruptive mode of action for rapidly reducing viability of Gram-negative bacteria (GNB) similarly to polymyxin B (PMB), a cyclic lipopeptide used as last resort antibiotic. Indeed, C₁₄KKc₁₂K displayed similar affinity for lipopolysaccharides and induced cell permeabilization associated with rapid massive membrane depolarization. Unlike PMB however, C₁₄KKc₁₂K was also bactericidal to Gram-positive bacteria (GPB) at or near the minimal inhibitory concentration (MIC), as assessed against a multispecies panel of > 50 strains, displaying MIC₉₀ at 3 and 6 µM, respectively for GPB and GNB. C₁₄KKc₁₂K retained activity in human saliva, reducing the viability of cultivable oral microflora by >99% within two minutes of exposure, albeit at higher concentrations, which, nonetheless, were similar to the commercial gold standard, chlorhexidine. This equipotent bactericidal activity was also observed in pre-formed biofilms of Streptococcus mutans, a major periodontal pathogen. Such compounds therefore, may be useful for eradication of challenging poly-microbial infections.

Facing the global crisis of multidrug resistant bacteria¹-⁶, membrane active compounds (MACs) are earning a renewed attention for their potential to control infections⁷-¹⁰ by multiple mechanisms, including by affecting critical common bacterial processes such as communication¹¹,¹² and virulence¹³,¹⁴ at sub-inhibitory concentrations. Thus, although the molecular basis for these effects are relatively ill understood, various MACs are presently gaining interest for their potential to address antibiotic resistance challenges and promise to overcome infections while avoiding many of the known resistance mechanisms. Of particular interest are borderline-hydrophobic MACs which, at low micromolar concentrations instigate mild/transient membrane damages¹⁵-¹⁷, including partial loss of the transmembrane potential, believed to bare critical consequences on efflux function¹⁸ and expression of antibiotic resistance factors¹⁹. While borderline-hydrophobic MACs might exert a bacteriostatic mode of action at higher doses, outright-hydrophobic MACs tend to disrupt biological membranes structures abruptly²⁰-²², which often culminates in a rapid bactericidal outcome, at or near the minimal inhibitory concentration (MIC)²³,²⁴.

Many of the known antimicrobial peptides (AMPs) exert antibacterial activities over Gram-positive bacteria (GPB) and/or Gram-negative bacteria (GNB) through their MAC properties. Some, manage to breach the cytoplasmic membrane (CM) permeability barrier²⁰,²⁵,²⁶ while others selectively increase the outer membrane (OM) permeability in GNB by perturbing the organization and function of the lipopolysaccharides (LPS) layer²⁷-²⁹. Thus, owing to its hydrophilic attributes, the OM is often responsible for the low sensitivity of GNB to hydrophobic antimicrobials (that can be highly active on GPB), thereby further challenging the generation of broad-spectrum antibacterial compounds, particularly needed in poly-microbial infections, for instance.

A variety of strategies were proposed to generate chemical mimics of AMPs to alleviate complications associated with peptide drugs³⁰,³¹. AMP-mimics may also be beneficial in helping to improve understanding the complex molecular basis for AMPs function(s) owing to their relative molecular/structural simplicity. Namely, while charge and hydrophobicity represent most critical factors influencing AMP properties, it is often challenging to identify the respective optimal proportions for controlling potency or selectivity. For instance, excess
hydrophobicity might promote self-assembly in aqueous solutions and consequently might reduce potency\cite{32,33}. Charge considerations are similarly intricate, namely due to the fact that the relative proportion of anionic phospholipids in bacterial membranes can reach 20–30% in GNB and up to nearly 100% in GPB\cite{34,35}. In this respect, the peptidomimetic approach using oligo-acyl-lysines (OAKs) seems particularly suitable for engineering high affinity antibacterial MACs\cite{22,36,37,38} since OAKs composition consists exclusively of hydrophobic linear acyls and cationic lysine residues\cite{37}-\cite{39}, where the inherently simple and incremental nature of designed analogs provides a systematic tool for dissecting the relative importance of charge and/or hydrophobicity. In fact, OAK designs that concentrated on miniaturized sequences of the general formula: Acetyl-lysyl-lysyl-aminocyl-lysine-amide suggest its capacity to generate distinct MAC versions with rational antimicrobial behaviors. For instance, the inactive tetrapeptide H-KKc12K-NH2 became active upon conjugation of N-terminal acyls, gradually revealing increasing activity with increased acyl length. Thus, C6KKc12K or C8KKc12K remained essentially inactive\cite{40} unlike more hydrophobic analogs\cite{38,41}.

Here, we set out to verify the implied linear relationship (between potency and hydrophobicity) of this C-terminally amidated series by producing a previously untested intermediate analog, C13KKc12K (molecular structure depicted in Fig. 1a) and investigating its antibacterial activity, mode of action and potential application. Ultimately, the findings are discussed in the context of a series of analogs, CnKKc12K (n = 8, 10, 12, 14 and 16).

**Results**

As part of its biophysical characterization, the purified synthetic lipopeptide was first subjected to light scattering measurements in order to assess its potential for self-assembly in an aqueous environment, as compared with two analogs, C12KKc12K and C16KKc12K. Expectedly, the light scattered by these analogs at low concentrations and up to~10\( \mu \)M, displayed proportional amplitudes, where C12KKc12K was intermediate between C12KKc12K (lowest) and C16KKc12K (highest) (Fig. 1b) and started to deviate from each other as concentration range where both C12KKc12K and C16KKc12K were previously found to aggregate\cite{24,41}. At~20\( \mu \)M however, C14KKc12K revealed a sharp divergence since its light scattering pattern exhibited significantly higher amplitudes, likely to reflect its capacity to form supramolecular structures of larger sizes\cite{42}. We also assessed the aggregation tendency in a more complex medium such as the supernatant of centrifuged saliva and found it to display an overall similar trend to that in PBS, only starting at somewhat lower concentrations (i.e., the respective critical aggregation concentrations values were 8 ± 1 and 23 ± 3\( \mu \)M). Often, such self-assembly is deleterious to antibacterial potency of AMPs and OAKs\cite{24,32,33}. Though, in the case of C12KKc12K and C16KKc12K, we previously used their respective unsaturated N-terminal acyls to show that the less hydrophobic counterparts (i.e., C12,ωKKc12K and C16,ωKKc12K)\cite{24,43} displayed higher potencies, even as aggregates. The proposed explanation for this discrepancy was that the unsaturated analogs formed different structures where the self-assembled lipopeptides were not as tightly bound\cite{44,45}, thereby gaining the ability to dis-aggregate upon interaction with bacteria and the ability to exert their antibacterial activity, unlike the saturated counterparts. Consequently, we next verified the analogs propensities to disaggregate in presence of bacteria by monitoring their light scattering amplitudes. Figure 1c illustrates the rapidly reduced light scattered by C14KKc12K, indicating its greater tendency to disaggregate in presence of bacteria. While these findings raise interesting questions (such as whether higher bacterial concentrations and/or longer incubation periods will increase the OAK disassembly), the fact that C12KKc12K demonstrated a tendency for rapid disaggregation in presence of bacteria supports the rapid activity expected in certain applications (as exemplified later in this section). Collectively therefore, the data suggest that the OAK’s tendency for self-assembly in a biological milieu at high concentrations, might not interfere with its antibacterial activity.

Next, we characterized its antibacterial activity by determining the MIC against a multispecies panel of 54 representative bacteria, including various medically relevant strains. Table 1 summarizes the MIC values obtained with 30 GPB (five species) and 24 GNB (seven species). With one exception (one of the Acinetobacter baumannii clinical isolates), the data reveal that C12KKc12K was active on most tested bacteria, although GPB appear generally more sensitive (MIC\(<\) 3 and 6\( \mu \)M, respectively). Replacing LB with cation adjusted Mueller Hinton Broth resulted in essentially similar outcome. For instance, MICs of S. aureus 29213 or E. coli 25922 were 3 and 6\( \mu \)M, respectively. Also note that C12KKc12K displayed an unchanged MIC value (3\( \mu \)M) on both the wild type (AG100) and its efflux deletion-mutant (AG100A), unlike its analogs\cite{48} (this issue will be elaborated in the Discussion section).

To investigate the mode of action, we used standard methodologies for MAC characterization, including assessment of membrane damages and determination of time-kill kinetics over both Escherichia coli and Streptococcus mutans, respectively representing GNB and GPB. Figure 2a shows the OAK’s ability to affect viability of E. coli, reflecting a rapid bactericidal mode of action (e.g., >2-log-unit reduction within 2 hours exposure at \( \geq \)MIC).

To assess potential membrane damages, we used an assay capable of differentiating permeability changes in bacterial membranes by testing the leakage of small organic molecules. The assay employs the engineered E. coli strain, ML-35p, which is constitutive for cytoplasmic \( \beta \)-galactosidase, lacks lactose permease, and expresses a plasmid-encoded periplasmic \( \beta \)-lactamase\cite{44}. The chromogenic \( \beta \)-galactosidase substrate ONPG is used to assess permeation of the CM, while OM permeability is assessed using nitrocefin, a chromogenic \( \beta \)-lactamase substrate. The data summarized in Fig. 2b suggest that both membranes were permeabilized at the MIC value (3\( \mu \)M) albeit the OM appears more susceptible. To validate the CM damages at low concentrations, bacteria were subjected to another permeability assay, this time monitoring cytoplasmic access to the DNA binder, ethidium bromide (Fig. 2c). The fact that ethidium bromide accumulated in E. coli provides confirming evidence for the CM permeabilization at the MIC value. The inset shows two representative kinetic curves illustrating ethidium bromide’s rapid accumulation in bacterial cytoplasm. Combined, these findings support the view that bacterial death has resulted from the OAK’s capacity to abruptly disrupt both membranes. A similar mode of action was attributed to the natural bacteria-derived 11-residue cyclic lipopeptide, polymyxin B (PMB)\cite{45}, an effect believed to stem from its high-affinity interaction with LPS. When compared, PMB and C12KKc12K exhibited a similar binding affinity to LPS originating from E. coli or Pseudomonas aeruginosa, as determined by their abilities to displace the binding
of dansyl-PMB (Fig. 3). Under similar conditions the analogs C8, C10 and C12 exhibited a binding affinity that was lower than that of C14KKc12K but increased with increasing hydrophobicity. This is further addressed below.

Figure 4a shows the OAK’s ability to affect viability of S. mutans, reflecting, again, the OAK’s rapid bactericidal mode of action at low micromolar concentrations (e.g., ~2 log units reduction within 2 hours exposure to 1.56 µM). Membrane damages were evident from the rapid and massive leakage of protons and cytoplasmic accumulation of ethidium bromide (Fig. 4b and c, respectively).

To determine the applicative potential of C14KKc12K, we next aimed to exploit apparent OAK advantageous properties such as protease stability and rapid bactericidal mode of action over a broad spectrum of bacteria. Hence, we performed a preliminary assessment for OAK’s ability to affect multiple bacterial species in saliva,
given that the list of susceptible strains (Materials & Methods section) included various bacterial species known to promote periodontal diseases47, 48 whose treatment with an orally active antimicrobial has been the standard clinical approach. For this purpose, we elected to use *S. mutans*, a prototypical oral pathogen. Unlike other salivary floating bacteria, *S. mutans* can adhere to the oral cavity surfaces (particularly to teeth) and promote biofilm formation, which disturbs the balanced oral microbiome by pH reduction, culminating in dental complications such as caries and periodontal inflammations49–51.

Table 1. MIC of C_{14}KK_{c12}K against a representative panel of bacteria. ^aMIC was determined by the microdilution method. Values represent the average of at least 2 independent experiments performed in duplicate.

| Species (number of strains tested) | MIC\(^a\) range (µM) |
|-----------------------------------|---------------------|
| **Gram-positive bacteria**        |                     |
| Streptococci (9)                 | 0.78–3.12           |
| Staphylococci (10)                | 1.56–3.12           |
| Enterococci (3)                   | 3.12–6.25           |
| Bacilli (2)                       | 6.25                |
| Listeria (6)                      | 3.12                |
| **Gram-negative bacteria**        |                     |
| Escherichia (9)                   | 3.12–6.25           |
| Pseudomonas (3)                   | 6.25–12.5           |
| Klebsiella (3)                    | 3.12–12.5           |
| Acinetobacter (5)                 | 3.12–>25            |
| Salmonella (1)                    | 3.12                |
| Fusobacterium (2)                 | 6.25                |
| Porphyromonas (1)                 | 3.12                |

Figure 2. Mode of action investigated against *E. coli* ML-35p as GNB representative. (a) Bactericidal kinetics upon exposure to C_{14}KK_{c12}K 0, 1, 2 and 4 MIC multiples (squares, circles, triangles and inverted triangles, respectively); dashed line, limit of detection (500 CFU/ml); asterisks denote lack of detected CFUs. (b,c) Membrane permeabilization, expressed as percentage of the positive control dermaseptin S4 (1–15) at 6.25 µM. Symbols: triangles, outer membrane; inverted triangles, cytoplasmic membrane. (c) Cytoplasmic membrane permeation to ethidium bromide. The inset shows representative permeation kinetics by the OAK at the MIC (triangles) and the positive control (inverted triangles); Min, minutes; F.U., fluorescence units (excitation: 535 nm, emission: 590 nm). Results are from at least two independent experiments performed in duplicate. Error bars represent the standard deviation.
In the literature, CHX MIC against *S. mutans* varies between 0.3 and 4 µM \( \mu \text{M} \) (0.3 µM, in our hands, representing a nearly 3-fold higher potency than \( \text{C}_{14}\text{KKc}_{12}\text{K} \)). However, when comparing their bactericidal kinetics (Figs 4a and 5c) CHX was bactericidal only at 40 multiples of its MIC (11.2 µM) i.e., at ~10 times the OAK’s bactericidal concentration (1.56 µM). While we are unable to explain this discrepancy, it is possible that these potency manifestations (i.e., the measure of inhibitory concentration versus time-kill kinetics) reflect an aptitude to compensate one for the other (likely related to mechanistic differences).

Moreover, noteworthy is the fact that, when compared at high concentrations (e.g., \( \geq 0.5 \text{mM} \)) \( \text{C}_{14}\text{KKc}_{12}\text{K} \) and CHX were (again) equipotent in their ability to affect viability of *S. mutans* in a preformed biofilm, killing >99% of the initial massive inoculum (10^9 CFU/ml) within one hour of exposure (Fig. 5d).

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**Figure 3.** Dansyl-polymyxin binding assay. Interaction with LPS was assessed by incubation (1.5 hr) of \( \text{C}_{14}\text{KKc}_{12}\text{K} \) and polymyxin B with 2 µM pure monodansyl-polymyxin and 3 µg/ml LPS from *E. coli* (a) or *P. aeruginosa* (b). Symbols: triangles, \( \text{C}_{14}\text{KKc}_{12}\text{K} \); inverted triangles, Polymyxin B; The Y axis represents fluorescence measurements (excitation: 340 nm, emission: 485 nm). Results are from two independent experiments performed in duplicate. Error bars represent the standard deviation.

**Figure 4.** Mechanistic studies (GPB). \( \text{C}_{14}\text{KKc}_{12}\text{K} \) mode of action was investigated against *S. mutans* ATCC 35668 as GPB representative. (a) Bactericidal kinetics upon exposure to 0, 1, 2 and 4 MIC multiples (squares, circles, triangles and inverted triangles, respectively); dashed line, limit of detection (500 CFU/ml); asterisks denote lack of detected CFUs. (b,c) Membrane damages instigated by \( \text{C}_{14}\text{KKc}_{12}\text{K} \), expressed as percentage of the positive control dermaseptin S4(1–15) at 6.25 µM. Membrane depolarization (b) assessed by displacement of DiSC3(5), and membrane permeation (c) assessed by accumulation of EtBr. The insets in (b and c) show representative depolarization and permeation kinetics, of the OAK (triangles) and the positive control (inverted triangles) at 0.78 and 6.25 µM, respectively; Min, minutes; F.U., fluorescence units (excitation: 620 nm, emission: 680 nm in panel b; excitation: 535 nm, emission: 590 nm in panel c). Results are from at least two independent experiments performed in duplicate. Error bars represent the standard deviation.
Antibacterial membrane-active lipopeptides are currently gaining extensive interest for their potential to affect critical bacterial processes ranging from communication to antibiotic functions, in mono- and combination-therapy. For example, PMB and daptomycin are two naturally occurring bactericidal cyclic lipopeptides, clinically used against GNB and GPB, respectively, with MIC values ranging between 0.12 and 8 µg/ml (PMB) or 0.015 and 32 µg/ml (daptomycin). In both cases, the acyl moiety plays a critical role.

Acyl conjugation was also shown to enhance the antimicrobial properties of various AMPs and synthetic mimics alike. For instance, acylated derivatives of dermaseptin, a broad spectrum amphibian AMP, have drastically influenced its activity spectrum, enabling its conversion to specific activity on either GPB or GNB, depending on the acyl selected.
expands and reinforces previous findings that illustrated the potential of N-terminal acyl conjugation to the core sharp premature deviancy from linearity (as evidenced by high intensity) suggests that C_{14}KKc_{12}K forms drastic hydrophobicity, did not increase correspondingly, as evidenced by light scattering measurements (Fig. 1). Its HPLC using a hydrophobic column, C_{14}KKc_{12}K possesses an intermediate hydrophobicity value compared with hydrophobicity and self-assembly of this particular sequence. As expected from their respective elution time in sent a quite remarkable yet ill-understood counter-intuitive outcome regarding the relationships between the outright-hydrophobic OAKs (e.g., C_{14}KKc_{12}K) are more likely to escape extrusion due to their tighter/deeper efflux pumps. Consequently, borderline-hydrophobic OAKs would be more susceptible to extrusion, whereas this hypothesis. Combined, our findings hint to a scenario implicating a simultaneous/competitive attraction required for antimicrobial properties.

Other interesting studies reported synthetic ultra-short lipopeptides (3–5 residues) with potent activities against plant-related pathogenic fungi\(^{[45]}\) and bacteria\(^{[46]}\), though at higher doses\(^{[47]}\). The present study provides evidence for the capacity of equivalent lipopeptides (generated via the OAK approach) to yield small molecules susceptible to be useful as simple investigation tools to help clarifying debated mechanistic aspects and potentially useful in biomedical applications. Namely, the findings reported herein, establish C_{14}KKc_{12}K as the shortest broad-spectrum antibacterial OAK known hitherto. The data also represent a quite remarkable yet ill-understood counter-intuitive outcome regarding the relationships between the hydrophobicity and self-assembly of this particular sequence. As expected from their respective elution time in HPLC using a hydrophobic column, C_{14}KKc_{12}K possesses an intermediate hydrophobicity value compared with C_{12}KKc_{12}K and C_{16}KKc_{12}K (Table 2). However, their tendencies for self-assembly, which presumably also depend on hydrophobicity, did not increase correspondingly, as evidenced by light scattering measurements (Fig. 1). Its sharp premature deviancy from linearity (as evidenced by high intensity) suggests that C_{14}KKc_{12}K forms drastically different aggregates in terms of three-dimensional organization. In contrast, our findings point to a dramatic increase in antibiotic efficiency since C_{14}KKc_{12}K achieved not only the lowest MIC value for this analog series (e.g., C_{14}KKc_{12}K MIC over S. mutans is 0.78 versus 1.56 μM for C_{12}KKc_{12}K or C_{16}KKc_{12}K) but has also upgraded its mode of action from bacteriostatic to bactericidal, including on GNB (Table 2). In this respect, the study expands and reinforces previous findings that illustrated the potential of N-terminal acyl conjugation to the core structure of AMPs\(^{[45,46,49]}\) or OAKs as a potent and versatile strategy for optimizing the hydrophobic/cationic balance required for antimicrobial properties.

The data also hint to a relationship between antibacterial potency and bacterial efflux function. Although so far, OAK properties were often rationalized strictly in terms of interactions with bacterial membranes, it is now clear that efflux\(^{[45,71]}\) represents another decisive factor whose contribution to the mode of action must be accounted for. Previously, we proposed that related but borderline-hydrophobic analogs (e.g., C_{13}XKKc_{12}K\(^{[18]}\) or C_{10}KKc_{12}K\(^{[40]}\) are substrates of the AcrAB-TolC system, the resistance nodulation division (RND) efflux pump present in E. coli and various enteric bacteria. The fact that C_{14}KKc_{12}K was potently active on isogenic mutant strains (whose efflux-pump components were deleted) supports the view that inactivity on normal GNB stems from the OAK's rapid extrusion by these pumps\(^{[48]}\). Additional support for this view comes from the fact that bactericidal OAKs are also prone to deeper insertion within the CM\(^{[37]}\). In this respect, the fact that C_{14}KKc_{12}K is equally potent on both the wild type (AG100) and its deletion-mutant strain (AG100A) reinforces this hypothesis. Combined, our findings hint to a scenario implicating a simultaneous/competitive attraction of the OAK molecules in the periplasm, to the CM anionic phospholipids and the membrane-embedded efflux pumps. Consequently, borderline-hydrophobic OAKs would be more susceptible to extrusion, whereas outright-hydrophobic OAKs (e.g., C_{14}KKc_{12}K) are more likely to escape extrusion due to their tighter/deeper anchoring within the CM.

Considering the OAK properties in the context of a series of analogs (as outlined in Table 2), the resulting perspective provides an overall rational picture describing a bell-shaped continuum of effects that exacerbate (possibly through accumulation) with increasing hydrophobicity. Thus, the least hydrophobic among the tested lipopeptides (C_{10}KKc_{12}K) was clearly devoid of growth inhibitory activity against GNB but managed to induce mild damages, mostly to the OM\(^{[40]}\) without affecting bacterial proliferation. While these effects are readilyparable, they may cause some delay in initial bacterial doubling time upon minor hydrophobicity increase (as observed with C_{10}KKc_{12}K) where they start affecting the CM as well, expressed by partial depolarization\(^{[48]}\). Additional increase in hydrophobicity (to yield C_{14}KKc_{12}K) further intensifies these effects to cause a variety of non-reparable damages to both GNB and GPB as expressed by a full-fledged growth inhibitory activity (i.e., MIC). These damages are nonetheless not severe enough to jeopardize bacterial viability, hence only leading to a bacteriostatic mode of action, as observed previously\(^{[40]}\) and in this study. Owing to its optimal hydrophobicity level, C_{14}KKc_{12}K was able to induce the most severe damages that culminated in rapid bactericidal outcomes. In contrast, excess hydrophobicity (e.g., in C_{16}KKc_{12}K\(^{[24]}\)) leads to the formation of tight peptide aggregates, which in turn, inverse the MAC potency-trend by limiting OAK’s availability for optimal interactions with bacterial targets.

| RX | H (%) | MIC (μM) | S.m. | E.c. | Effect observed at low concentrations (<3μM) |
|----|------|----------|------|------|---------------------------------------------|
| HX | 27   | >50<sup>26</sup> | >50<sup>26</sup> | Not observed (normal growth) |
| C_{14}X | 41 | 50 | >50<sup>26</sup> | Weak perturbations of GNB outer membrane (normal growth) |
| C_{10}X | 46 | 6.25 | >50<sup>26</sup> | Weak growth inhibition of some GPB and transient membrane damages including partial depolarization of CM (slight delay in GNB growth) |
| C_{12}X | 51 | 1.56 | 16<sup>40</sup> | Impairing membrane damages leading to a bacteriostatic mode of action in GNB: OM permeabilization & delayed growth in GNB |
| C_{14}X | 54 | 0.78 | 3.12 | High efficacy in membranes disruption leading to a bactericidal mode of action in both GPB & GNB |
| C_{16}X | 62 | 1.56 | 25<sup>44</sup> | Trend reversal (reduced potency) due to excess hydrophobicity as self-assembly approaches the critical aggregation concentration |

Table 2. Modulating biophysical properties of a core sequence by conjugating an N-terminal acyl.

*R* = N-terminal acyl, X = KKc_{12}K; \(^{24}\)Hydrophobicity, defined as % acetonitrile eluent in C_{18} HPLC column; \(^{26}\)Minimal inhibitory concentration as determined by the microdilution method over S. mutans and E. coli representing GPB and GNB, respectively.
In conclusion, our results support the view that N-terminal acyl-manipulations of the core sequence KKc12K, exhibit straightforward structure-activity relationships. Thus, simply by switching the N-terminal acyl, the OAK properties became tunable, gradually evolving from lack of “visible” antibiotic activity on to exerting bacteriostatic activity over GPB only, and ultimately, exercising broad-spectrum bactericidal activity. The fact that C8KKc12K was equally potent on both wild type and efflux mutant strains suggests that stronger anchoring within the CM enables hydrophobic MACs to escape extrusion by RND pumps, thereby providing a rational for the observed increased potency.

Besides their potential role as investigation tools, such compounds may be useful in treating infections involving multiple microbial populations, such as oral mucositis. Our findings may have relevance to various biofilm-associated micro-environmental niches that hamper drug efficacy in infections or industry related issues. Future studies might clarify this issue. Interestingly, a 35-residue-long MAC currently in phase 2 clinical trials, C16G272, 73 displayed specific antistreptococcal bactericidal properties in saliva. In this respect, the OAK platform might present advantages in the capacity to generate superior anti-biofilm candidates, including in terms of biological robustness, simplicity and production costs.

Materials and Methods
Peptide synthesis. OAKs were synthesized in-house (433 A Peptide Synthesizer; Applied Biosystems, Foster City, CA, USA) by the solid-phase method using 9-fluorenylmethylxycarbonyl (Fmoc) active-ester chemistry on 4-methylbenzhydrylamine (MBHA) resin. OAKs were then deprotected and cleaved from the resin using trifluoroacetic acid:H2O (95:5) and purified to >95% chromatographic homogeneity by reverse phase high performance liquid chromatography (RP-HPLC) using C18 column (Vydac), a flow rate of 2 ml/min and a linear acetonitrile gradient of 1% /min (Alliance; Waters, Milford, MA, USA). Peaks identity was verified by mass-spectrometry (Xevo G2 ToF; Waters, Milford, MA, USA). Purified OAKs were then lyophilized and kept as dry powder at −20°C.

Organization in solution. To assess the OAK’s self-assembly in solution, serial two-fold dilutions of the OAK (initial concentration of 200 µM) were prepared in phosphate buffered saline (PBS; 10 mM Na2HPO4, 154 mM NaCl, pH 7.4) and incubated for 2 hr at room temperature (RT). Light scattering at a 90° angle was measured through a 1 nm slit while holding both excitation and emission at 400 nm (Spectrophotometer Fluorolog-3 FL3–22; Horiba Jobin Yvon, Edison, NJ, USA).

To evaluate the disassembly of these aggregates upon bacterial exposure, an OAK solution (200 µM) was incubated (2 hr in PBS, at RT) after which, bacteria were added (10⁵ CFU/ml) and the light scattering evolution of these suspensions was monitored as described above.

Bacteria. Gram-positive bacteria tested were: American Type Culture Collection (ATCC) strains Staphylococcus aureus 25923, 29213, MRSA 39592, 43300, BAA-43 (HSJ 216), BAA-1720 (252), S. epidermidis 12228, S. xylosox 29971, Enterococccus faecalis 29212, E. faecium 35667, Bacillus subtilis 33677, B. cereus 11778, Listeria grayi 19120, L. innocua 33090, L ivanovii 19119, L. monocyrogenes 19115, L. seeligeri 35967, L. welshimeri 35897, Streptococcus agalactiae 13813, 27596, S. bovis 9809, S. mutans 35668, 700610 (UA159), S. pneumoniae 49619, 6303, S. pyogenes 19615, S. sobrinus 27352 (6715) and clinical isolates MRS A10017 (USA300), 15903, VRE Nu28.

Gram-negative bacteria tested were: ATCC strains Escherichia coli 25922, 43894, Pseudomonas aerugiosna 27853, 9027, Acinetobacter baumannii 19606, A. caloaceticus 31299, Salmonella Typhimurium 14028, Fusobacterium nucleatum 23726, Porphyromonas gingivalis 53977, clinical isolates E. coli 14182, 13484, U-16327, U-16329, P. aeruginosa 1278, Klebsiella pneumoniae 1287, K2–224, C2, A. baumannii 1279, 1280, 1281, F. nucleatum PK 1594, the engineered E. coli ML–35p and the isogenic K-12 pair AG100, AG100a (∆acrAB). Dental related bacteria (generous gift of Prof. Doron Steinberg and Gilad Bachrach from the Hebrew University of Jerusalem) appear in bold characters.

Culture conditions. Staphylococci, Bacilli, Escherichia, Pseudomonas, Acinetobacter, Salmonella and Klebsiella species were grown in Luria Bertani broth (LB; 5 g/1 NaCl, 5 g/1 yeast extract, 10 g/1 tryptone). Enterococci and E. coli ML–35p were grown in Tryptic Soy Broth (TSB). Listeria and Streptococci were grown in Brain Heart Infusion (BHI). All bacteria were grown over-night at 37 °C with shaking. S. mutans 35668 plated on BHI agar for enumeration was grown for 48 hr. S. mitans UA159 was grown in 3% CO2 enriched atmosphere. Fusobacterium and Porphyromonas were grown in Wilkins-Chalgren growth medium and an anaerobic atmosphere.

Minimal inhibitory concentration (MIC) was determined using the microdilution assay. Mid-log-phase bacteria at 5 x 10⁸ CFU/ml were incubated in a 96-well plate with serial two-fold dilutions of the tested compound for 18–24 hr at 37 °C (final volume of 200 µl). O.D. at 620 nm was measured (Synergy HT, BioTek Instruments, Winooski, VT, USA), and the MIC was determined as the lowest concentration for which no increase in O.D. was detected.

Bactericidal kinetics was assessed by incubating 5 x 10⁷ CFU/ml of mid-log-phase bacteria with the OAK for 3 hr at 37 °C with shaking. Aliquots were taken at t = 0, 0.5, 1, 2 and 3 hr, subjected to serial 10-fold dilutions in saline (NaCl = 0.85%) and plated for enumeration after 24–48 hr incubation at 37 °C.

Cytosplasmic membrane permeation to etidium bromide (EtBr) was evaluated as follows: Mid-log-phase bacteria at 1 x 10⁸ CFU/ml were centrifuged for 5 min at 15,000g. Pellet was washed twice with PBS containing 0.5% glucose (pH = 7.4), suspended in the same buffer and incubated for 10 min at 37 °C with shaking. 180 µl of the bacterial suspension were mixed in a 96-well plate with 25 µl of the tested compound and EtBr (EtBr final concentration = 1 µg/ml) and fluorescence was recorded immediately (excitation: 535 nm, emission: 590 nm) for up to 30 min at 37 °C with shaking (Synergy HT, BioTek Instruments, Winooski, VT, USA).

Outer and cytoplasmic membrane permeation in Gram-negative bacteria was assessed using the engineered E. coli ML–35p by monitoring the chromogenic hydrolysis of two indicators: ortho-nitrophenyl-β-galactoside.
compound for 1.5 hr at RT and fluorescence (excitation: 340 nm, emission: 485 nm) was measured immediately. Saliva was centrifuged for 5 min at 15,000 g and 4 °C, and the supernatant was spiked with 1 µl S. mutans (Synergy HT, BioTek Instruments, Winooski, VT, USA). Biofilms were scraped from the bottom of the plates and biofilms were washed three times with milliQ water. Solutions of the tested compounds were placed on the plates and incubated for up to 3 hr at 37 °C without shaking. Plates were decanted in 0.5 hr intervals and biofilms were washed three times with milliQ water. Solutions of the tested compounds were placed on the plates and incubated for up to 3 hr at 37 °C without shaking. Plates were decanted in 0.5 hr intervals and biofilms were washed three times with milliQ water to remove any compound residues. Biofilms were scraped from the bottom of the wells, suspended in BHI and sonicated for 5 min in a sonication bath, subjected to serial 10-fold dilutions in saline and plated on BHI agar for enumeration.

Cytoplasmic membrane depolarization was assessed by monitoring the displacement of the membrane binding fluorescent dye DiSC3(5) (3,3′-dipropylthiadicarbocyanine iodide) as follows. Mid-log-phase bacteria at 5 × 10^8 CFU/ml were centrifuged for 5 min at 15,000 g and the supernatant was removed. Pellet was washed three times with sodium phosphate buffer (SPB; 10 mM NaH2PO4, pH = 7) and suspended in the same buffer (O.D. at 620 nm = 1). Bacteria were then 10-fold diluted into SPB containing 3% TSB. 100 µl of bacterial suspension were placed in a 96-well plate with 100 µl of the tested compound and 20 µl of either ONPG (final concentration = 2.5 µM) or nitrocefin (final concentration = 25 µM). Hydrolysis of ONPG and nitrocefin was monitored immediately by recording the absorbance at 420 nm and 486 nm respectively, for 30 min at 37 °C with shaking (Synergy HT, BioTek Instruments, Winooski, VT, USA).

Dansyl-polymyxin displacement assay was assessed by displacement of dansyl-polymyxin B bound to lipopolysaccharide (LPS) as follows. Polymyxin B sulfate was covalently attached to dansyl chloride and mono-dansyl Polymyxin B (DPMB) was purified by RP-HPLC. 180 µl of 5 mM HEPES containing 3 µg/ml LPS (from E. coli or P. aeruginosa) and 2 µM DPMB were incubated in a 96-well plate with 20 µl of the tested compound for 1.5 hr at RT and fluorescence (excitation: 340 nm, emission: 485 nm) was measured immediately (Synergy HT, BioTek Instruments, Winooski, VT, USA).

Bactericidal kinetics in saliva was assessed as follows. Whole saliva was pooled from two-three healthy volunteers at a time, after obtaining their informed consent. To test for specific activity against S. mutans, saliva was centrifuged for 5 min at 15,000 g and 4 °C, and the supernatant was spiked with 1 × 10^8 CFU/ml of mid-log-phase S. mutans 35668. Otherwise, activity against natural oral microflora was also performed on whole saliva. Saliva was mixed with the tested compound, incubated at 37 °C with shaking, and aliquots were taken at t = 0, 2, 5 and 10 min. Aliquots were subjected to serial 10-fold dilutions in saline and plated on BHI agar for enumeration.

Anti-biofilm activity was evaluated against established biofilms in 96-well plates as follows. 200 µl of mid-log-phase S. mutans 35668 at 5 × 10^8 CFU/ml in BHI containing 2% sucrose were placed in each well, and plates were incubated for 24 hr at 37 °C without shaking. Unattached cells were removed by decanting the plates and biofilms were washed three times with milliQ water. Solutions of the tested compounds were placed on the biofilms for up to 3 hr at 37 °C without shaking. Plates were decanted in 0.5 hr intervals and biofilms were washed three times with milliQ water to remove any compound residues. Biofilms were scraped from the bottom of the wells, suspended in BHI and sonicated for 5 min in a sonication bath, subjected to serial 10-fold dilutions in saline and plated on BHI agar for enumeration.

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**Author Contributions**

O.M. synthesized reagents, performed research, analyzed data, wrote the paper; F.Z. synthesized reagents (mono-dansyl PMB); U.C. and A.M. designed the experiments, analyzed data, wrote the paper.

**Additional Information**

**Competing Interests:** The authors declare that they have no competing interests.

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