DOI: 10.1002/cbic.201402093

Specific Maltose Derivatives Modulate the Swarming Motility of Nonswarming Mutant and Inhibit Bacterial Adhesion and Biofilm Formation by *Pseudomonas aeruginosa*

Gauri S. Shetye,[a] Nischal Singh,[a] Changqing Jia,[a] Chan D. K. Nguyen,[a] Guirong Wang,[b] and Yan-Yeung Luk*[a]

We have demonstrated that specific synthetic maltose derivatives activate the swarming motility of a *Pseudomonas aeruginosa* nonswarming mutant (rhlA) at low concentration, but inhibit it at high concentration. Although these molecules are not microbicidal, active maltose derivatives with bulky hydrocarbon groups inhibited bacterial adhesion, and exhibited biofilm inhibition and dispersion (IC_{50} ~20 μM and DC_{50} ~30 μM, respectively). Because the swarming motility of the rhlA mutant is abolished by the lack natural rhamnolipids, the swarming activation suggests that maltose derivatives are analogues of rhamnolipids. Together, these results suggest a new approach of controlling multiple bacterial activities (bacterial adhesion, biofilm formation, and swarming motility) by a set of disaccharide-based molecules.

Introduction

The increase in antibiotic-resistant bacteria calls for fundamentally new approaches in treating a wide range of established and new infectious diseases.[1–3] Quorum sensing in bacterial biofilm formation[4–7] and the detrimental effects of biofilms has made the exploration of nonmicrobicidal anti-biofilm approaches an important area of research.[8–10] To this end, important and extensive work has explored the inhibition of the quorum sensing to reduce bacterial biofilm formation.[8,11–15] Another approach is based on the hypothesis that as bacterial adhesion is a major step in causing various diseases, inhibiting the adhesion of microbes or developing vaccines against microbial adhesins provides a potential therapeutic solution.[16–19] However, this anti-adhesion strategy has not yet reached an ultimate goal of drug development, probably because multiple adhesins are employed by the microbe for adhesion;[20] ; attachment of polymers secreted by the microbes might also facilitate the hosting of microbes that lead to the formation of biofilms. As many bacterial activities involve intertwined cell signaling processes,[21] we explored the impact of a class of nonmicrobicidal disaccharide hydrocarbons (Scheme 1) on multiple bacterial activities, including the activation of swarming motility of a nonswarming mutant of *Pseudomonas aeruginosa*, inhibition of bacterial adhesion, and inhibition and dispersion of biofilms.

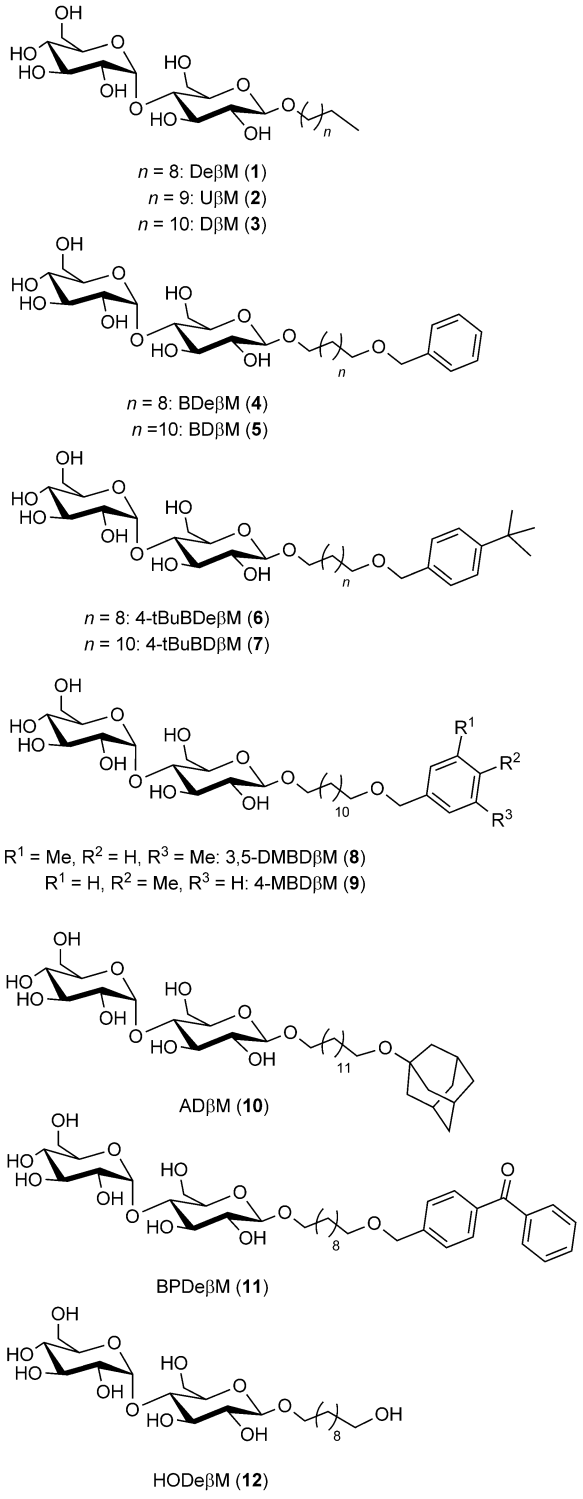
Microbial biofilm is one of the most prevalent sources for infectious diseases. Biofilm formation is gene regulated[5,22] and results in a dynamic surface-based multicellular organism.[4] Film-hosting microbes can exhibit 1000-fold higher resistance to antibiotics than planktonic microbes.[2,10] Complete eradication of biofilms has been a daunting challenge as these films exhibit resistance to many chemical agents. Interestingly, although the initial step of biofilm formation is believed to involve microbial adhesion on host surfaces (or on adsorbed polymer secreted by the microbes),[16,23] relatively few studies have explored the use of anti-adhesion agents to inhibit or disperse biofilm formation.[24]

In this work, we focused on nonmicrobicidal control of three activities of *P. aeruginosa*: swarming motility, bacterial adhesion, and biofilm formation. *Pseudomonas aeruginosa* is an opportunistic pathogen that causes severe infections in a wide range of immune-compromised situations.[25] Similarly to many other bioactivities, swarming motility of *P. aeruginosa* is controlled by quorum sensing.[26] A series of mutant studies has indicated that the production of rhamnolipid (a natural disaccharide derivative) by *P. aeruginosa* is indispensable.[27–30] This class of molecule is also produced by a few other bacterial species.[31] Rhamnolipids are involved in at least three different activities of *P. aeruginosa*. Firstly, it is necessary for making structured biofilms with channels and pores during the early stage of biofilm formation.[32] Secondly, when overproduced, it facilitates the dispersion of bacteria from biofilms.[32] Thirdly, its production is necessary for enabling swarming motility.[27,33,34] Deleting the *rhlA* gene (controls the synthesis of rhamnolipid) results in a non-swarming mutant of *P. aeruginosa*.[27,33,34] In spite

[a] G. S. Shetye, N. Singh, C. Jia, C. D. K. Nguyen, Dr. Y.-Y. Luk
Department of Chemistry, Syracuse University
Syracuse, New York 13244 (USA)
E-mail: yluk@syr.edu

[b] Dr. G. Wang
Department of Surgery, SUNY Upstate Medical University
Syracuse, New York 13210 (USA)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201402093.



Scheme 1. Library of maltose-derived hydrocarbons.

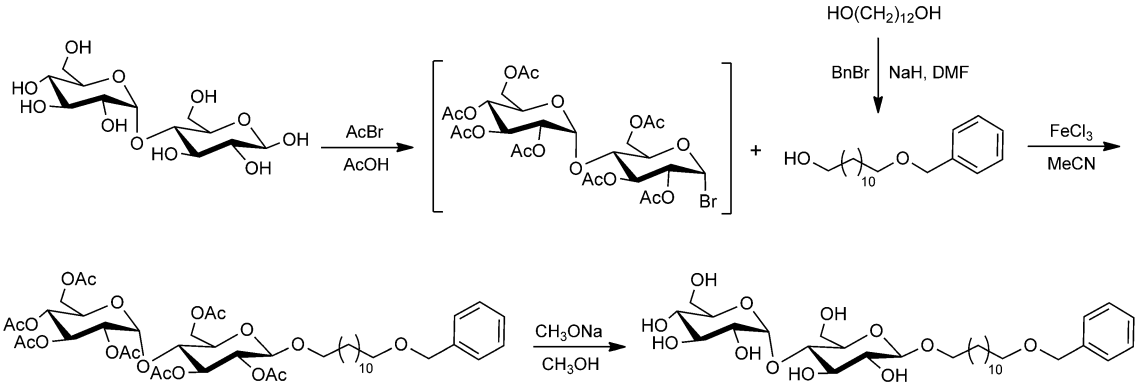
of all these biological activities, the protein receptor(s) for rhamnolipid has not yet been identified, although Kohler et al. demonstrated that type IV pili are important for swarming motility driven by rhamnolipids.[27]

P. aeruginosa is involved in many saccharide-based molecular recognition processes; the receptor proteins include lectins, PA-IL, PA-IIL, and flagellin.[35,36] The ligand for mediating adhesion of *P. aeruginosa* to epithelial cells in cystic fibrosis patients has been shown to be a disaccharide moiety, GalNAcβ(1→4)Galβ, from the asialo-GM1 glycolipid.[37] Synthetic molecules that tether different methylated GalNAcβ(1→4)Galβ moieties to different aliphatic chains have been shown to be potent anti-adhesion agents against *P. aeruginosa*,[38] and that the receptor for these ligands appears to be a protein of the pilus of the bacterium.[39,40] Structural variation and mimics of the disaccharide glucosamine (different stereochemistry and the presence of NAc group) have not been extensively evaluated for inhibiting the adhesion of *P. aeruginosa*.

Together, swarming motility and ligand-mediated adhesion suggest that disaccharide-based molecules can influence the signaling processes that control swarming motility, adhesion, biofilm formation, and biofilm dispersion. Prompted by previous studies,[41–44] we screened three different types of surfactants for biofilm inhibition and dispersion: anionic (SDS), cationic (dodecyl trimethyl ammonium chloride (DTAC)), and nonionic (tetra(ethylene glycol) monododecyl ether ($C_{12}EG_4OH$) and dodecyl β-maltoside (DβM)). Only DβM showed some activity. Preliminary screening of different surfactants led us to explore the effect of a series of maltose derivatives on swarming motility, bacterial adhesion, and biofilm formation of *P. aeruginosa*.

Results

Among *P. aeruginosa* mutants that show impaired swarming motility,[27,29,30,45] the *rhlA* mutant lacks the production of rhamnolipids.[33,46] Externally added rhamnolipids in agar gel activated the swarming motility of the *rhlA* mutant.[34] Rhamnolipid is a biosurfactant consisting of a disugar hydrophilic head group and two aliphatic chains. To evaluate the importance of the disugar head group and the surface activities separately, we synthesized a series of disaccharide hydrocarbons (maltose derivatives; Scheme 1) and screened these for their effect on the swarming motility of the *rhlA* mutant. The results were compared with those for commercially available generic surfactants: SDS, DTAC, $C_{12}EG_4OH$, decyl β-maltoside (DeβM, **1**), undecyl β-maltoside (UβM, **2**), and DβM (**3**). The disaccharide hydrocarbons have maltose stereochemistry (Glcα(1→4)Glcβ) bearing different hydrocarbon tails. To investigate the effect of aliphatic chain length, maltose derivatives with 10 (**1**), 11 (**2**), and 12 (**3**) carbons in the aliphatic chain were studied. To investigate the effect of terminal hydrocarbon bulkiness, benzyl decyl β-maltoside (BDeβM, **4**), benzyl dodecyl β-maltoside (BDβM, **5**), 4-tertiary butyl benzyl decyl β-maltoside (4-*t*BuDeβM, **6**), 4-tertiary butyl benzyl dodecyl β-maltoside (4-*t*BuBDβM, **7**), 3,5-dimethyl benzyl dodecyl β-maltoside (3,5-DMBDβM, **8**), 4-methyl benzyl dodecyl β-maltoside (4-MBDβM, **9**), and benzophenonyl decyl β-maltoside (BPDeβM, **11**) were synthesized (Scheme 2 and see the Supporting Information). To determine whether nonaromatic bulky substituents are effective, we synthesized adamantane dodecyl β-maltoside (ADβM, **10**). To examine the effect of polarity of the end group on the aliphatic chain, 12-hydroxy decyl β-maltose (HODeβM, **12**) was also synthesized.



Scheme 2. Synthesis of maltose derivative BDβM (**5**)

Specific maltose derivatives activated the swarming motility of the nonswarming mutant of *P. aeruginosa*

The swarming motility of *P. aeruginosa* is unusual in that it can form a pattern of tendrils that is not seen in the swarming behavior of many other bacteria.[26,33,34] When bacteria are inoculated at the center of a soft gel (0.5% agar), tendrils of PA14 strain of *P. aeruginosa* exhibit a floral pattern and occasionally resemble a fractal pattern.[47] When wild-type PAO1 strain of *P. aeruginosa* was inoculated on an M8 agar plate, the swarm pattern covered the entire agar surface over time without exhibiting prominent tendril formation. None of the three generic surfactants (SDS, DTAC, and $C_{12}EG_4OH$) promoted or activated swarming motility of a *P. aeruginosa rhlA* mutant (see Supporting Information and Figure 1). To our surprise, eight of the twelve maltose derivatives activated the swarming motility of the *rhlA* mutant to a great extent, but with different degrees and shapes of tendril formation (Figure 1). We categorized the maltose derivatives into three groups: swarming-activating without tendrils, swarming-activating with well-defined tendrils, and those that did not activate swarming (Figure 1). With 85 μM maltose derivative in the soft agar, the first group (**11**, **5**, **2**, and **4**) caused the *rhlA* mutant to exhibit swarming motility without well-defined tendrils but with small protrusions at the periphery of the swarming circle. Among this group, the swarming ring size for all but **4** was comparable to that for (wild-type) PAO1. The second group (**3**, **9**, **8**, and **10**) resulted in long, straight, well-defined tendrils and with a swarming circle size similar to that for PAO1. Among these, **10** resulted in narrow tendrils with frequent turns (similar to a fractal). The third group (**1**, **6**, **7**, and **12**) did not exhibit any significant activation of swarming of the mutant (Figure 1).

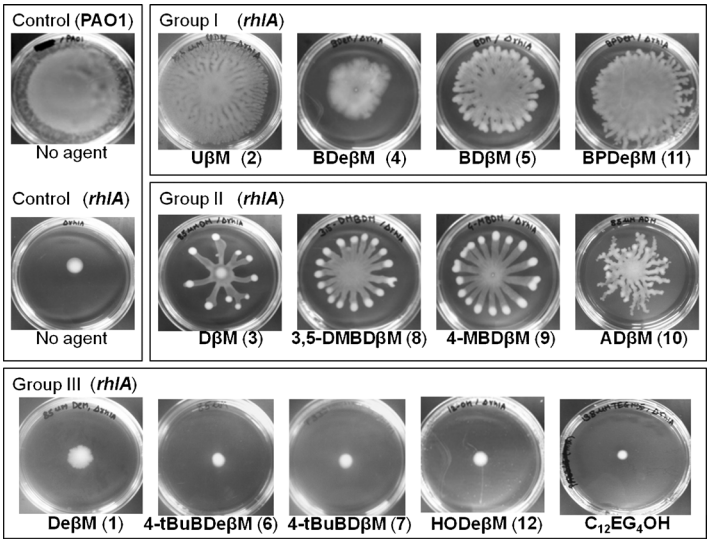


Figure 1. Nonswarming *P. aeruginosa* mutant *rhlA* inoculated on M8 agar plates with and without 85 μM maltose hydrocarbons or $C_{12}EG_4OH$ (generic surfactant). Pictures were taken 24 h after inoculation.

Concentration-dependent study revealed an "activity-reversal" that modulates the swarming motility of *rhlA* mutants

For the last group (**1**, **6**, **7**, and **12**), which did not activate swarming motility, there are two possible explanations for the inactivity. One is that these maltose derivatives are not recognized by a receptor, and thus do not trigger cell signaling. Alternatively, they might bind to the receptor but function as inhibitors (antagonists of the receptor ligand, rather than agonists), thus preventing signaling for swarming. To explore which mechanism is more likely, we performed two experiments.

Firstly, we investigated the effects of 5–170 μM **6** and **7** (third group) on mutant swarming (Figure 2). Surprisingly, both **6** and **7** activated swarming motility at low concentrations (5–10 μM), but appeared not to be active at and above 40 μM (Figure 2). There was a significant difference in activation ability between the two: **6** gave a maximum swarming area of ~39.6 cm^2 at 10 μM, whereas for **7** it was ~5.5 cm^2 at 5 μM (Figure 3; control (no agent): 1.4 cm^2). Although this observation of "activity reversal" appeared to be unusual, the classical cell signaling quorum sensing molecule N-acyl homoserine lactone (AHL) of *Photobacterium fischeri* also demonstrated "activity reversal": photoluminescence decreased at high concen-

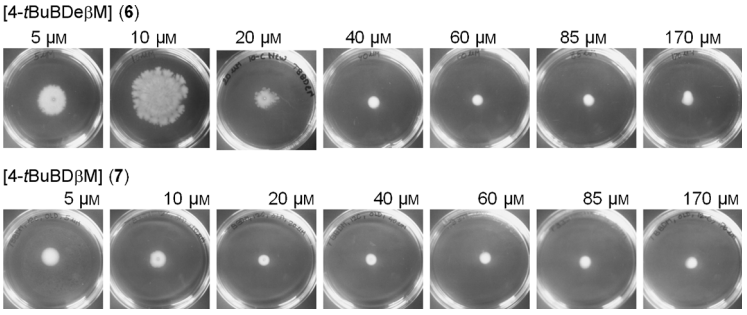


Figure 2. Effect of 4-*t*BuBDeβM (**6**) and 4-*t*BuBDβM (**7**) on swarming of *rhlA* mutant.

trations of AHLs.[48] This effect, however, has not been studied extensively since the initial report.

Second, we mixed a swarming-activating maltose hydrocarbon (**3**) with three of the swarm-nonactivators (**7, 6**, or **12**) in the same swarming plate (soft agar) to examine if swarming activation by **3** was inhibited the other agent. Interestingly, both **7** and **6** inhibited the activity of **3**, whereas **12** had no effect (Figure 4). At 20 μM **3**, *rhlA* swarmed to its maximum extent after 24 h (as for PAO1). In the additional presence of **6** or **7**, swarming was inhibited: 40 μM **6** completely inhibited swarming motility, 20 μM **7** caused complete inhibition (indistinguishable from the control: no agent added to *rhlA* mutants) (Figures 4 and 5). In contrast, 80 μM **12** with 20 μM **3** did not cause any effect on swarming motility. These results are consistent with the possibility that both **7** and **6** compete with and displace **3** at the receptor, and thus inhibit cell signaling. In contrast, **12** might not bind strongly to the receptor.

The mechanism of tendril formation in swarming motility is a challenging topic, and has been studied by several groups.[30,31,33,45] Déziel and co-workers have proposed a mechanism in which the di-rhamnolipid is an attractant, whereas mono-rhamnolipid and 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAA) might function as wetting agents and repellents, respectively, during swarming of *P. aeruginosa*.[34] We believe that these results and our findings are consistent with the existence of a receptor for rhamnolipids. Furthermore, as externally added rhamnolipids in the soft gel also activated the swarming motilities of the *rhlA* mutant,[34] swarm-activating maltose derivatives might be ligands that share receptor(s) with the rhamnolipids.

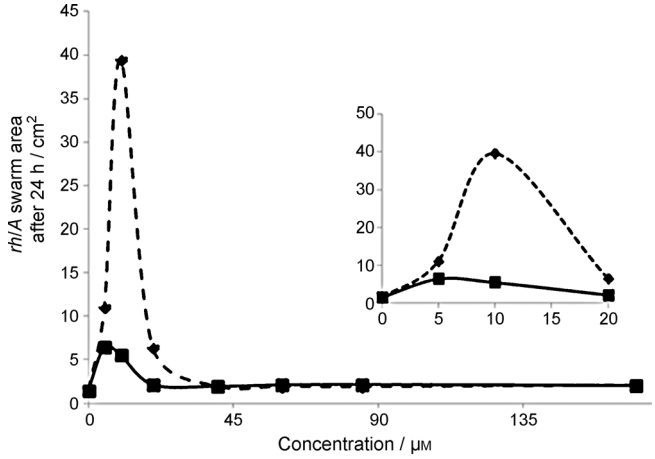


Figure 3. Swarming area of *rhlA* mutant inoculated for 24 h plotted against concentration of 4-*t*BuBDeβM (**6**; ◆) and 4-*t*BuBDβM (**7**; ■) in soft agar gel.

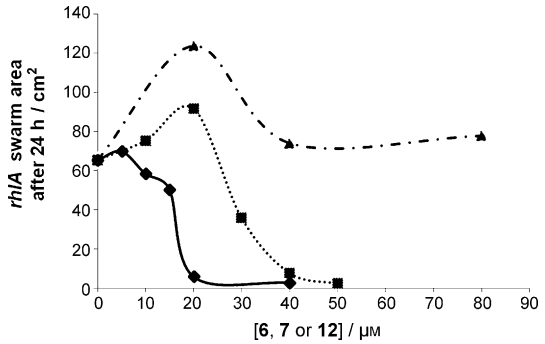


Figure 5. Swarm area of *rhlA* mutant 24 h after inoculation onto soft gel containing 20 μM DβM (**3**) with different concentrations of HODeβM (**12**; ▲), 4-*t*BuBDeβM (**6**; ■), or *t*BuBDβM (**7**; ◆).

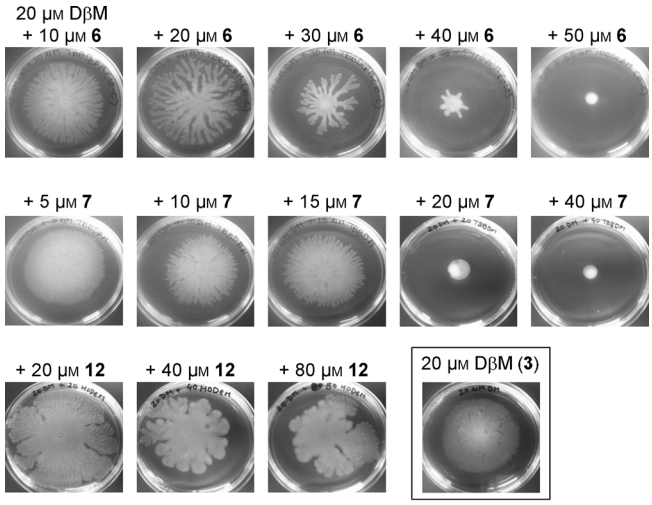


Figure 4. Nonswarming *P. aeruginosa* mutant *rhlA* inoculated on M8 soft agar (0.5% agar) plates containing mixtures of 20 μM DβM (**3**) with different concentrations of 4-*t*BuBDeβM (**6**), 4-*t*BuBDβM (**7**), or HODeβM (**12**).

Specific maltose derivatives exhibited nonmicrobicidal anti-biofilm and anti-adhesion activities

Although rhamnolipids has been demonstrated to be necessary for swarming,[27,33,34] rhamnolipids also play a critical role in forming structured biofilms with channels at early stages.[32] Interestingly, biofilm formation and swarming motility appear to be inversely regulated,[49] and at the late stage of biofilm formation rhamnolipids appear to assist in dispersing bacteria from biofilms.[32,50] These findings prompted us to examine the effect of the disaccharide derivatives on biofilm formation and dispersion, as well as to examine if there is correlation be-

tween activation of swarming motility and biofilm control (dispersion and inhibition).

P. aeruginosa lectins PA-IL and PA-IIL are known to recognize carbohydrate moieties on human cell surfaces, with higher specificities for galactose and fucose, respectively.[35,36] Additionally, in a series of studies, Irvin and co-workers showed that the pili are likely responsible for adhesion of *P. aeruginosa* on biotic (mammalian cell surface) and abiotic surfaces (polystyrene).[51] By using solid-phase binding assays, the disaccharide (GalNAcβ(1→4)Galβ) hydrocarbons were shown to bind to pili of *P. aeruginosa*.[52] These results support the proposal that GalNAcβ(1→4)Galβ hydrocarbons are potent inhibitors of *P. aeruginosa* adhesion.[38] Although the maltose derivatives and GalNAcβ(1→4)Galβ are completely different disaccharides, we examined the anti-adhesion and anti-biofilm activities of the maltose derivatives, because molecular recognition and binding involving individual saccharides are often weak. Thus, the hydrocarbon derivatives might contribute to recognition and cause tolerance to variation of saccharide stereochemistry.

To study anti-adhesion and anti-biofilm activities, we first examined the toxicity of maltose derivatives and generic surfactants (see the Supporting Information) on the growth of planktonic bacteria. Microbicidal activity of the agents would invalidate anti-adhesion and anti-biofilm results. Furthermore, agents that control microbial behavior without killing the microbes have the potential for developing therapeutic agents that do not invoke drug resistance. At 170 μM these agents did not exhibit any noticeable inhibition of the growth of *P. aeruginosa* (PAO1); in fact, most maltose derivatives promoted planktonic growth (Figure 6). Cationic surfactant DTAC (170 μM) showed some inhibition. (This concentration was higher than that used in the anti-adhesion and anti-biofilm assays.)

We used an assay based on crystal violet (CV) dye to measure the amount of PAO1 biofilm formed 24 h after bacterial inoculation, with and without maltose derivatives. Five of the twelve maltose derivatives (**11**, **5**, **6**, **3**, and **9**) inhibited (> 80%) PAO1 biofilm; six (**10**, **8**, **7**, **4**, **2**, and **1**) inhibited (40–60%) PAO1 biofilm; **12** showed insignificant inhibition (~30%; Figure 7). This indicates that the anti-biofilm activity is highly sensitive to the structure of the maltose derivative. Increasing the methylene units in the aliphatic chain length (10 (**1**) to 11 (**2**) to 12 (**3**)) increased inhibition (50–70%). Incorporating a benzophenone group in a maltose derivative with a ten-carbon aliphatic chain increased the inhibition from 50% (**4**) to 90% (**5**). In general, adding bulky groups to the aliphatic chain (**11**, **5**, and **6**) appeared to increase the inhibition of PAO1 biofilm formation. These anti-biofilm activities of the active maltose derivatives were also verified by fluorescence static biofilm assays. *Pseudomonas aeruginosa* strain PAO1-EGFP (constitutively expressing enhanced green fluorescent protein, EGFP) was allowed to form biofilm on sterile steel coupons. Biofilms treated with maltose derivatives showed significant reduction in fluorescence signal in comparison with the control (untreated coupons, see the Supporting Information). These results indicate a reduction in biofilm on the steel coupons, consistent with the results of the crystal violet assays.

To measure the anti-adhesion activity of the maltose derivatives, we measured EGFP fluorescence of PAO1-EGFP on polystyrene (black 96-well plates) 2 h after bacterial inoculation to

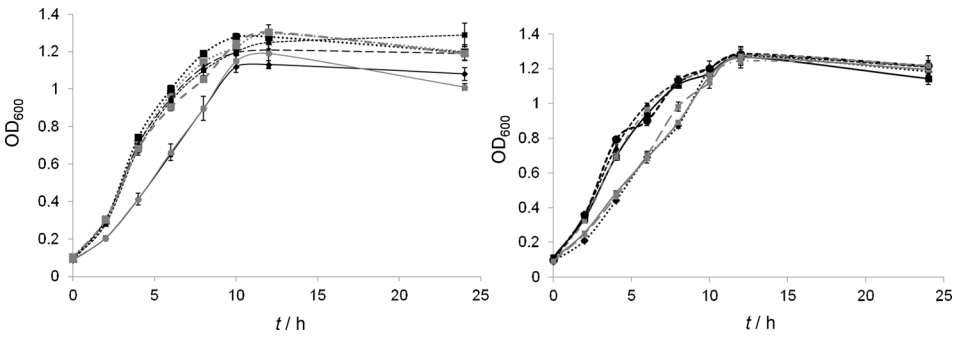


Figure 6. *P. aeruginosa* growth–response curve in absence and presence of 170 μM of maltose derivatives DeβM (**1**; -----▲-----), UDβM (**2**; ···■···), DβM (**3**; ···■···), BDeβM (**4**; ——●——), BDβM (**5**; ···■···), and BDeβM (**6**; –·–■–·–), 4-*t*BuBDβM (**7**; ——■——), 3,5-DMBDβM (**8**; ——●——), 4-MBDβM (**9**; ——▲——), ADeβM (**10**; ——■——), BPDeβM (**11**; -----■-----), and HODeβM (**12**; ——■——); ◆: control (no agent). Error bar is standard error of the mean from six replicates.

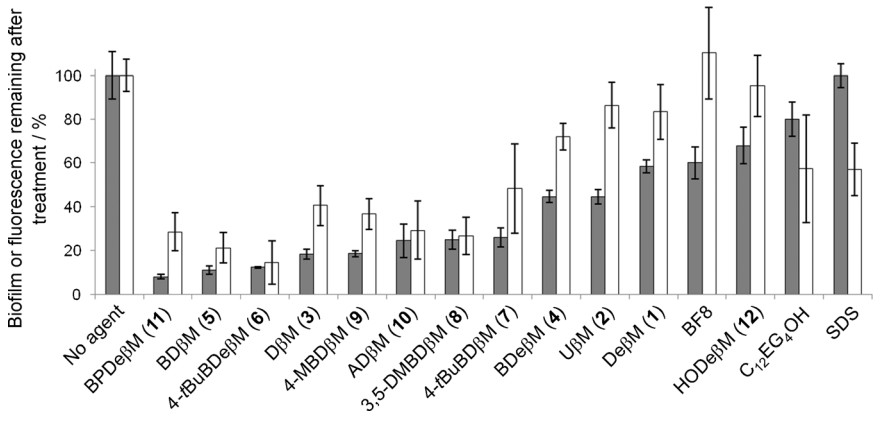


Figure 7. Inhibition of biofilm and adhesion by maltose derivatives, generic surfactants (SDS, $C_{12}EG_4OH$), and the known anti-biofilm agent BF8. Anti-biofilm (■) and anti-adhesion (□) assays were performed at 110 and 85 μM, respectively, except for BF8 anti-adhesion (100 μM). Data from CV dye and fluorescence assays, respectively. Errors bar are standard error of the mean from six replicates.

quantify adhered bacteria. Relative to the amount of adhered bacteria without agents, 85 μM **6** and **5** inhibited (>80%) PAO1-EGFP adhesion. Four derivatives (**11**, **3**, **7**, and **4**) also inhibited PAO1-EGFP adhesion (30–70%). These anti-adhesion activities show a trend similar to that for anti-biofilm activities, in terms of structure, but without exact correlation (Figure 7). Derivatives **11**, **5**, and **6** exhibited high activities for both adhesion inhibition and biofilm formation; **11** was the most active anti-biofilm agent; **6** was the most active anti-adhesion agent; **12** showed no significant inhibition of either biofilm formation or adhesion (Figure 7). We also tested the known biofilm inhibitor, brominated furanone (BF8),[53,54] and two generic surfactants, SDS and $C_{12}EG_4OH$. BF8 showed no inhibition of PAO1 adhesion but ~35% inhibition of biofilm formation. SDS and $C_{12}EG_4OH$ showed no biofilm inhibition, but ~40% inhibition of PAO1 adhesion (Figure 7).

Examining the dose dependence of maltose-derivative inhibition of PAO1 biofilm formation revealed that the active agents exhibited IC_{50} values of around 25 μM (**11** and **6**: 23 μM; **5**: 28 μM; **9**: 29 μM; **3**: 48 μM; Figure 8 and Table 1). The IC_{50} structure–activity trend was similar to that obtained from activity screening at 110 μM (Figure 7). The anti-biofilm activities (crystal violet assays) of the maltose derivatives appeared to be lower in LB media than in M9+ media. There was no significant inhibition in medium without sodium chloride (see the Supporting Information).

Nonmicrobicidal dispersion of biofilm by maltose derivatives

Chemical dispersion of formed biofilm is often more relevant to medical applications, and more challenging than inhibition of biofilm formation. We screened the maltose derivatives (110 μM) for their ability to disperse 24 h *P. aeruginosa* biofilm. For all twelve derivatives, the trend was the same as for the anti-biofilm activity, with **11** and **5** being the most potent

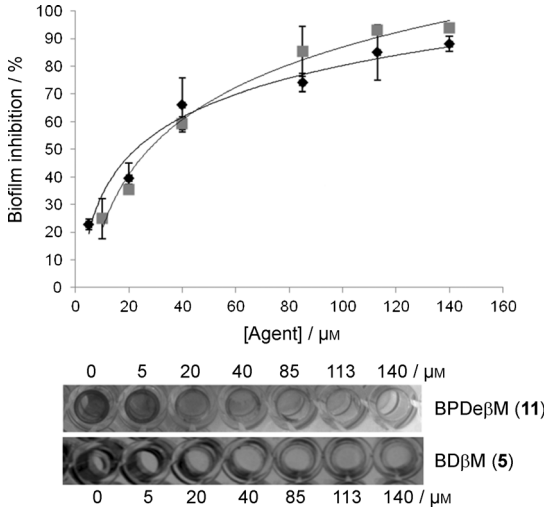


Figure 8. Dose–response curves and biofilm inhibition for BPDeβM (**11**; ◆) and BDβM (**5**; ■) from the biofilm inhibition assay (CV dye). Error bars are standard error of the mean from six replicates.

Table 1. IC_{50} and DC_{50} values of selected maltose derivatives.

Disugar hydrocarbons	IC_{50} [μM]	DC_{50} [μM]
BPDeβM (**11**)	23	31
4-*t*BuBDeβM (**6**)	23	43
BDβM (**5**)	28	32
4-MBDβM (**9**)	29	77
DβM (**3**)	48	66

(Figure 9). For **11** and **5**, biofilm dispersion dose dependence revealed half-maximal dispersion (DC_{50}) values of 31 μM and 32 μM, respectively (**7**: 43 μM; **3**: 66 μM; **9**: 77 μM; Figure 10, Table 1 and see the Supporting Information). These DC_{50} values are comparable to those for quorum-sensing-based small molecule biofilm inhibitors,[11,12] but we believe that the mechanism of the anti-biofilm activities of these maltose derivatives is likely not directly due to disruption of quorum sensing (see the Supporting Information). Together with the adhesion inhibition data, the dual action (anti-adhesion and anti-biofilm) activities of these non-microbicidal disaccharide hydrocarbons offers potential for development as therapeutic agents.

Discussion

The bioactivities (anti-biofilm, anti-adhesion, and swarm-activating) of maltose hydrocarbons are highly dependent on the particular structures of the molecules. Most notably, the bulky maltose derivatives, BDβM (**5**) and 4-*t*BuBDβM (**7**), differs only in *tert*-butyl substitution on the benzene ring; but BDβM at 85 μM activated the swarming motility of *rhlA* to the full extent, whereas 4-*t*BuBDβM (**7**) showed activation at 10 μM (Figure 1) but inhibition at higher concentrations. Compounds **6** and **7** differ by two methylene units in the aliphatic chain, yet exhibited different levels of swarming activation and different concentrations for transition from activating to inhibiting the swarming of *rhlA* mutants. For biofilm inhibition and dispersion, both the length of the aliphatic chains and the structure of the bulky substituents play important roles in maintaining high anti-biofilm activities. A general trend appears to be that with the specific aliphatic chain lengths, bulky substituents increase anti-biofilm activity. For example, pairs of maltose derivatives (**4** and **5**, **6** and **7**) have the same bulky benzyl and *tert*-butyl benzyl groups, respectively, but with ten and twelve methylene units within each pair of molecules. Because of their tert-butyl groups, **5** and **6** are similar in size of hydrocarbon chain, whereas **4** is small and **7** is large, in comparison to **5** and **6**. Examining the activities of these four structures indicated that **5** and **6** are significantly more active than **4** and **7**, thus suggesting a specific size with bulky substituents would be optimal for anti-biofilm activities. The most active maltose derivative, BPDeβM (**11**), has a relative polar benzophenone group and a 10-carbon aliphatic chain. IC_{50} and DC_{50} data also support this structure–activity relationship, as **11** and **6** had higher anti-biofilm activities than **9**. This level of structural sensitivity, we believe, is consistent with the maltose derivatives having different agonist/antagonist effects on cell-signaling

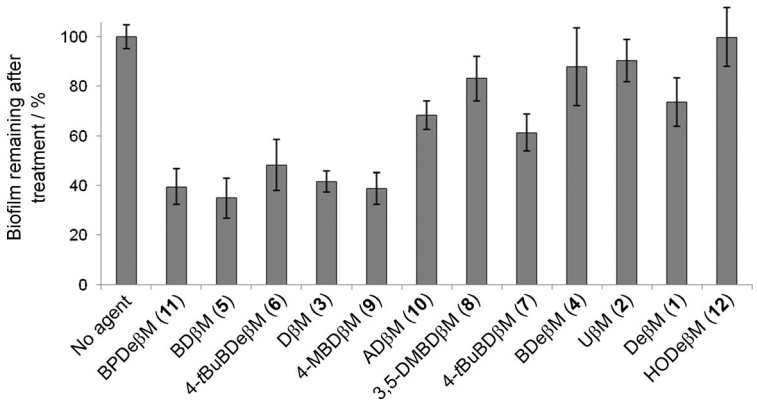


Figure 9. Dispersion of PAO1 biofilm by adding maltose derivatives (110 μM) to 24 h biofilms followed by CV dye assay. Error bars are standard error of the mean from six replicates.

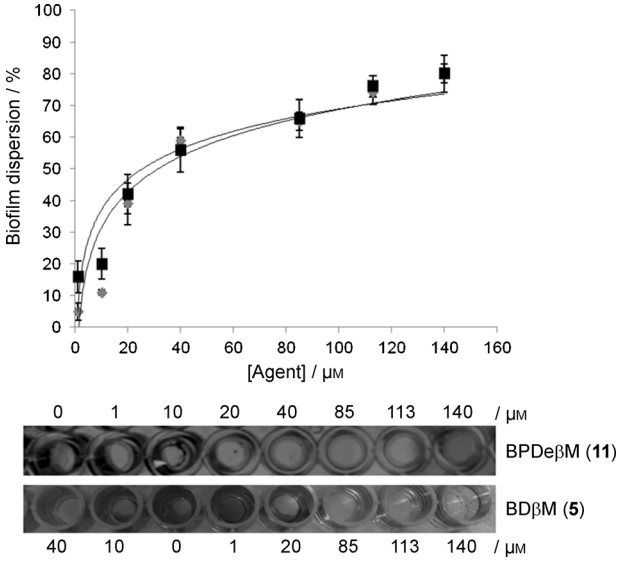


Figure 10. Dose–response curve and for BPDeβM (**11**; ◆; DC_{50} = 31 μM) and BDβM (**5**; ■; DC_{50} = 32 μM) from a CV biofilm inhibition assay. Error bars are standard error of the mean from six replicates.

events. The effect of disaccharide stereochemistry and further bulky derivative structures on biofilm and swarming motility is an on-going subject of our study. Although both swarming activation and anti-biofilm activity are highly sensitive to the structure of the maltose derivatives, there is no strict correlation between these two biofunctions. For example, of the four maltose derivatives that did not activate swarming of *rhlA* mutant at 85 μM, two were effective biofilm inhibitors: **6** and **7** gave 87 and 74% biofilm inhibition at 110 μM, respectively, whereas two (**12** and **1**) were sluggish (32 and 41% inhibition, respectively).

One possible mechanism of anti-biofilm and anti-adhesion activity can attributed to the surfactant properties of the molecules (a simple washing effect). However, in addition to the strong dependency of bioactivity on the structural details of the agents, two observations suggest that the activities were not simply the result of washing. Firstly, the effective concentrations for biofilm inhibition and dispersion (IC_{50} and DC_{50}) were significantly lower than the critical micelle concentration (CMC) of a typical maltoside (CMC of DβM (**3**): 170 μM).[55] Secondly, other generic surfactants examined in this study, including SDS and $C_{12}EG_4OH$, did not show any anti-biofilm activity. Neither did any of the generic surfactants activate swarming motility *rhlA* mutant—$C_{12}EG_4OH$ actually showed a higher surface activity than disaccharide derivative **3**.[55] For these reasons, the activation of the swarming motility of *rhlA* mutant by disaccharide derivatives is not likely a result of just lowering the surface tension of the soft gel. We also note that inhibiting the bacterial adhesion is likely not the sole cause of anti-biofilm activity, because preformed biofilm was also dispersed by the same maltose derivatives.

Many receptors for saccharide derivatives exist on bacterial surfaces.[16,38,40] Among these, several inter-related systems appear to be associated with the anti-biofilm activities of the maltose derivatives. Firstly, pili (particularly type IV) are important for swarming motility,[27] thus suggesting that a specific moiety on the pilus is likely involved in rhamnolipid-activated swarming motility. Secondly, the broad biological activity spectrum of rhamnolipids (building of porous biofilm, bacterial dispersion from mature biofilm, and enabling swarming motility) suggests the existence of at least one receptor; its identity has not been discovered but it might be strongly associated with the protein SadB (surface attachment defective).[33] Thirdly, when *P. aeruginosa* swarms on soft agar gel, it appears to differentiate into two phenotypes: hyperactive swarming at the tip of the swarming tendril, and less-mobile bacteria at the center of the swarming ring and on the stem of tendrils.[29,56] Among the genes screened by Déziel, *gltK* is up-regulated in bacteria at the tendril tip but not in bacteria at the center of the swarming pattern.[29] In *P. aeruginosa* this gene encodes an inner-membrane component of the ATP-binding cassette (ABC) transporter system (glucose transport).[57] Interestingly, gltK is a member of the MalK family of proteins, which transport maltose in *E. coli*.[58,59] Fourthly, Irvin and co-workers reported that 90% of *P. aeruginosa* adhesion is likely caused by pili proteins,[39] and that these are responsible for binding to the GalNAcβ(1→4)Galβ moieties on human cells.[40,52] Additionally, pili are important for adhesion of *P. aeruginosa* to polystyrene surfaces.[51] Together with our results (disaccharide hydrocarbons inhibited adhesion of *P. aeruginosa* on polystyrene and also modulated swarming motility), the maltose derivatives might target the pilus protein that recognizes GalNAcβ(1→4)Galβ on mammalian cells.[40] As the maltose derivatives activated swarming motility of the nonswarming mutant (a biological function also exhibited by rhamnolipid),[33] these disaccharide derivatives might also target the rhamnolipid receptor(s). It is not clear whether GalNAcβ(1→4)Galβ on mammalian cells and rhamnolipids share a bacterial receptor, but this would suggest that rhamnolipids are ligands for pilus proteins.

Whether or not this is the case, we believe that maltose derivatives could be promiscuous (binding to multiple bacterial proteins).

We explored the potential of maltose derivatives to activate *P. aeruginosa* quorum sensing circuits (*las* and/or *rhl* systems) by using a previously established gene reporter strains (PAO1/plasI-LVAgfp and PAO1/prhlI-LVAgfp) for identifying small molecule quorum sensing inhibitor.[8] These reporter strains produce natural AHL signals, and binding of these signal molecules to the Lux-type receptor proteins (LasR and RhlR) activated the expression of plasmid-encoded GFP. Our results indicate that maltose derivatives did not compete with the natural signaling molecules to cause a decrease in fluorescent signal (see the Supporting Information). Furthermore, the ability of the maltose derivatives to activate quorum sensing in *P. aeruginosa* in the absence of natural AHLs was studied with double knockout strains PAO-JP2 (plasI-LVAgfp) and PAO-JP2 (prhlI-LVAgfP), which do not produce AHLs.[9] The maltose derivatives did not show any significant increase in the fluorescent signals (see the Supporting Information), indicating that they did not agonize the quorum sensing receptors.

Conclusions

This class of maltose derivative, with a wide range of aliphatic chain structures, exhibited three versatile biological functions: activation of swarming motility, inhibition of bacterial adhesion, and inhibition of biofilm formation. Natural rhamnolipids activated the swarming motility of a nonswarming mutant of *P. aeruginosa*, *rhlA*; this series of maltose derivatives represent the first class of synthetic molecules to activate the swarming motility of this nonswarming mutant. Because the rhlA mutant does not produce natural rhamnolipids, our results suggest that the active maltose derivatives function as analogues of rhamnolipids. As the bioactivities were highly sensitive to the structural details of the agents, and because of the cross-inhibition activities between the agents, this suggests that one or more protein receptors exist for maltose derivatives as well as rhamnolipids. As biofilm formation and swarming motilities are common for a wide range of microbes, and as other bacteria also produce rhamnolipids-like molecules,[31] these and other disaccharide derivatives have potential anti-biofilm activity for other microbial species. Because multiple biological activities (adhesion, biofilm formation, and swarming) are affected by a common set of molecular structures without killing the bacteria, this class of molecules might form the basis of an effective approach to control *P. aeruginosa* biofilm-related disease.

Experimental Section

Organic synthesis of maltose derivatives. The maltose derivatives were synthesized by a general organic synthetic route (Scheme 2). As an example, we describe here the synthesis of benzyl dodecyl β-maltoside, BDβM (**5**). Condensation of benzyl bromide with 1,12-dodecanediol in presence of NaH yielded dodecanoyl benzyl ether. Glycosidation of dodecanoyl benzyl alcohol with an acetobromo maltose in the presence of an acid catalyst ($FeCl_3$) gave the acetyl protected disaccharide alkyl conjugate. Deprotection of acetyl groups with sodium methoxide followed by neutralization (to pH ~ 6.5) with Amberlite H^+ resins (Zemplen deacetylation) yielded **5**. Synthesis of other maltose derivatives was conducted similarly (see the Supporting Information).

Bacterial strains and growth media. Wild type *P. aeruginosa* PAO1 and PAO1-EGFP strains were obtained from Dr. Guirong Wang (Upstate Medical University, Syracuse). The non-swarming *P. aeruginosa* mutant, *rhlA* (PW6886, *rhlA*-E08::ISphoA/hah) was obtained from the PA two-allele library (PAO1 transposon mutant library, Manoil Lab, University of Washington Genome Sciences).[60] Strains PAO-JP2 (plasI-LVAgfp) and PAO-JP2 (prhlI-LVAgfp) were obtained from Dr. Helen E. Blackwell (University of Wisconsin-Madison). Plasmids plasI-LVAgfp and prhlI-LVAgfp were obtained from Dr. Hiroaki Suga (University of Tokyo). All bacterial strains were grown in lysogeny broth (LB: tryptone ($10\,g\,L^{-1}$), yeast extract ($5\,g\,L^{-1}$), and NaCl ($10\,g\,L^{-1}$)) at 37 °C. For biofilm inhibition and dispersion assays 95% M9+ medium with 5% LB was used unless otherwise stated. M9+ medium contained NH_4Cl (18.7 mM), KH_2PO_4 (21.7 mM), Na_2HPO_4 (47.7 mM), NaCl (8.6 mM), $CaCl_2$ (0.1 mM), $MgSO_4$ (1 mM), anhydrous α-D(+)-glucose (0.2%), L-Arg (0.4%), citric acid monohydrate (0.2%), casamino acids (0.5%), sodium succinate dibasic hexahydrate (0.2%), and L-glutamic acid monopotassium salt monohydrate (0.2%).[11]

Stock solutions of generic surfactants and maltose derivatives. Stock solution of all the agents (11.5 mM) were prepared in autoclaved water, sterilized by filtering through a 0.2 μM syringe filter, and stored at −20 °C in sealed vials. Appropriate amounts of sterile water were added to controls in all assays to eliminate solvent effect.

Swarming assay. Swarm agar plates were made with M8 medium (Na_2HPO_4 (50 mM), KH_2PO_4 (25 mM), NaCl (4 mM)), supplemented with glucose (0.2%), casamino acid (0.5%), $MgSO_4$ (1 mM), and solidified with Bacto agar (0.5%),[33] and inoculated with bacterial culture (3 μL, $OD_{600} = 0.4–0.6$). Swarm agar plates were incubated at 37 °C for 12 h and then incubated for an additional 12 h at room temperature. For each set of experiment all the swarm plates were poured from same batch of agar and allowed to dry for 1 h before inoculation. Each swarming experiment waas repeated at least three times.

Effect of maltose derivatives on the growth of *P. aeruginosa*. Optical density was measured with an ELx800 TM absorbance microplate reader (BioTek Instruments, Inc., Winooski, VT) with Gen5TM data analysis software. OD_{600} values were taken in sterile conditions at 0, 2, 4, 6, 8, 10, 12, and 24 h after inoculation in 96-well polystyrene plates, with or without agents in LB broth.

Crystal violet biofilm inhibition assay. The inhibitory effect of maltose hydrocarbons on *P. aeruginosa* biofilm formation was determined by crystal violet-dye-based biofilm inhibition assays. Overnight culture of wild-type *P. aeruginosa* (PAO1) was subcultured (initial $OD_{600} = 0.01$) in M9+/LB (95:5) or LB. Aliquots (200 μL, $OD_{600} = 0.1$) of the subculture were placed in wells of a 96-well polystyrene microtiter plate. Test compounds (concentrations as indicated) were then added to the wells, and the plates were wrapped in Press'n'Seal (GLAD, Oakland, CA) followed by incubation. After (24 h, 37 °C), the medium was discarded, and the plates were washed with water and dried (1 h, 37 °C). The plates were stained with aqueous crystal violet (CV; 200 μL, 0.1%), followed by incubation (RT, 20 min). The CV stain was then removed, and the plates were washed with water. Remaining biofilm-adhered stain was resolubilized in acetic acid (200 μL, 30%). After the stain was dis-

solved (15 min), solubilized CV dye (100 μL) was transferred from each well into the corresponding wells of a new polystyrene microtiter dish. Biofilm inhibition was quantified by measuring OD_{600} (negative control (no biofilm formation; background) was subtracted). The percent inhibition was calculated by the comparison of the OD_{600} for biofilm grown in the absence of compound (control) versus biofilm grown in the presence of compound under identical conditions. Assays were repeated at least three times and the biofilm inhibition values reported are the average of 6 replicate wells from one experiment.

Anti-adhesion assay. An overnight culture of PAO1-EGFP was subcultured (initial $OD_{600}=0.01$) in M9+/LB (95:5) containing carbenicillin (300 $\mu g\,mL^{-1}$, to maintain the plasmid of PAO1-EGFP) at 37 °C in a rotary shaker (250 rpm). After reaching $OD_{600}=0.1$, aliquots (200 μL) were transferred to the wells of black polystyrene microtiter plate with and without (control) maltose derivatives. This black 96-well plate was saran wrapped and incubated (37 °C, 2 h). Then, bacterial cultures were discarded, and fresh M9+/LB (95:5) medium was added to the wells. The fluorescence of surface-adhered bacteria was measured by a Synergy 2 microplate reader ($\lambda_{ex}=500$ nm, $\lambda_{em}=540$ nm) with Gen5 data analysis software. Background signal (M9+/LB (95:5) was subtracted from all the samples. Assays were repeated at least three times; inhibition values are the averages of six replicate wells from one experiment.

Dispersion assay for preformed biofilm. The plate for the biofilm dispersion assay was prepared as for the crystal violet assay but without adding any maltose derivative at the time of inoculation with bacteria. PAO1 was allowed to grow for 24 h at 37 °C. After 24 h, bacterial culture was pipetted out and replaced with 200 μL of fresh medium containing maltose derivative (110 μM). After 24 h incubation, biofilms were fixed and quantified by using crystal violet dye as described above. The amount of dispersed biofilm was determined by comparing (normalizing) the amount of biofilm at 48 h with and without maltose hydrocarbons. Assays were repeated at least three times; biofilm dispersion values are the averages of six replicates from one experiment.

Dose-dependence assays for biofilm inhibition and dispersion. Maltose derivatives with the five highest activities for biofilm inhibition and dispersion were selected for dose-response analysis. Maltose derivatives (1, 5, 10, 20, 40, 85, 113, and 140 μM) were added to bacterial cultures (200 μL) in a 96-well plate. Quantification of biofilm inhibition and dispersion was as for the respective crystal violet assays. Assays were repeated at least three times; inhibition values are the averages of six replicates from one experiment.

Acknowledgements

We thank NSF-CMMI (CAREER #0845686), NSF-11-560 (#1242505), and NSF-EFRI (#1137186, coPI, Y.Y.L) for partial financial support. We thank Dr. Helen E. Blackwell (University of Wisconsin–Madison) for the generous donation of strains PAO-JP2 (plasI-LVAgfp) and PAO-JP2 (prhlI-LVAgfp), and Dr. Hiroaki Suga (The University of Tokyo) for the generous donation of plasmids plasI-LVAgfp and prhlI-LVAgfp. We also thank Prof. James Hougland and Ivan Korendovych (Syracuse University) for the instrumentation.

Keywords: biofilm inhibition · disaccharide hydrocarbons · inhibitors · *Pseudomonas aeruginosa* · swarming motility

[1] L. Hall-Stoodley, J. W. Costerton, P. Stoodley, *Nat. Rev. Microbiol.* **2004**, *2*, 95 – 108.
[2] D. Davies, *Nat. Rev. Drug Discovery* **2003**, *2*, 114 – 122.
[3] R. Bax, N. Mullan, J. Verhoef, *Int. J. Antimicrob. Agents* **2000**, *16*, 51 – 59.
[4] D. G. Davies, M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, E. P. Greenberg, *Science* **1998**, *280*, 295 – 298.
[5] M. E. Taga, B. L. Bassler, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14549 – 14554.
[6] C. A. Lowery, T. J. Dickerson, K. D. Janda, *Chem. Soc. Rev.* **2008**, *37*, 1337 – 1346.
[7] J. Engebrecht, M. Silverman, *Nucleic Acids Res.* **1987**, *15*, 10455 – 10467.
[8] K. M. Smith, Y. Bu, H. Suga, *Chem. Biol.* **2003**, *10*, 563 – 571.
[9] G. D. Geske, R. J. Wezeman, A. P. Siegel, H. E. Blackwell, *J. Am. Chem. Soc.* **2005**, *127*, 12762 – 12763.
[10] J. J. Richards, C. Melander, *Anti-Infect. Agents Med. Chem.* **2009**, *8*, 295 – 314.
[11] R. Frei, A. S. Breitbach, H. E. Blackwell, *Angew. Chem. Int. Ed.* **2012**, *51*, 5226 – 5229; *Angew. Chem.* **2012**, *124*, 5316 – 5319.
[12] C. A. Bunders, M. J. Minvielle, R. J. Worthington, M. Ortiz, J. Cavanagh, C. Melander, *J. Am. Chem. Soc.* **2011**, *133*, 20160 – 20163.
[13] T. Zang, B. W. K. Lee, L. M. Cannon, K. A. Ritter, S. Dai, D. Ren, T. K. Wood, Z. S. Zhou, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6200 – 6204.
[14] M. Hentzer, L. Eberl, J. Nielsen, M. Givskov, *BioDrugs* **2003**, *17*, 241 – 250.
[15] D. Ren, J. J. Sims, T. K. Wood, *Environ. Microbiol.* **2001**, *3*, 731 – 736.
[16] R. Autar, A. S. Khan, M. Schad, J. Hacker, R. M. J. Liskamp, R. J. Pieters, *ChemBioChem* **2003**, *4*, 1317 – 1325.
[17] N. Firon, S. Ashkenazi, D. Mirelman, I. Ofek, N. Sharon, *Infect. Immun.* **1987**, *55*, 472 – 476.
[18] R. J. Pieters, *Med. Res. Rev.* **2007**, *27*, 796 – 816.
[19] M. Hartmann, H. Papavlassopoulos, V. Chandrasekaran, C. Grabosch, F. Beiroth, T. K. Lindhorst, C. Röhl, *FEBS Lett.* **2012**, *586*, 1459 – 1465.
[20] I. Ofek, D. L. Hasty, N. Sharon, *FEMS Immunol. Med. Microbiol.* **2003**, *38*, 181 – 191.
[21] J. W. Hastings, K. H. Nealson, *Annu. Rev. Microbiol.* **1977**, *31*, 549 – 595.
[22] K. Sauer, A. K. Camper, G. D. Ehrlich, J. W. Costerton, D. G. Davies, *J. Bacteriol.* **2002**, *184*, 1140 – 1154.
[23] I. Ofek, D. L. Hasty, R. J. Doyle, *Bacterial Adhesion to Animal Cells and Tissues*, New Edition, ASM, Washington DC, **2003**.
[24] E. M. V. Johansson, S. A. Crusz, E. Kolomiets, L. Buts, R. U. Kadam, M. Cacciarini, K.-M. Bartels, S. P. Diggle, M. Cámara, P. Williams, R. Loris, C. Nativi, F. Rosenau, K.-E. Jaeger, T. Darbre, J.-L. Reymond, *Chem. Biol.* **2008**, *15*, 1249 – 1257.
[25] V. E. Wagner, B. H. Iglewski, *Clin. Rev. Allergy Immunol.* **2008**, *35*, 124 – 134.
[26] D. B. Kearns, *Nat. Rev. Microbiol.* **2010**, *8*, 634 – 644.
[27] T. Köhler, L. K. Curty, F. Barja, D. C. Van, J.-C. Pechère, *J. Bacteriol.* **2000**, *182*, 5990 – 5996.
[28] Y. Lequette, E. P. Greenberg, *J. Bacteriol.* **2005**, *187*, 37 – 44.
[29] J. Tremblay, E. Déziel, *BMC Genomics* **2010**, *11*, 587.
[30] J. Overhage, S. Lewenza, A. K. Marr, R. E. W. Hancock, *J. Bacteriol.* **2007**, *189*, 2164 – 2169.
[31] A. M. Abdel-Mawgoud, F. Lépine, E. Déziel, *Appl. Microbiol. Biotechnol.* **2010**, *86*, 1323 – 1336.
[32] M. E. Davey, N. C. Caiazza, G. A. O'Toole, *J. Bacteriol.* **2003**, *185*, 1027 – 1036.
[33] N. C. Caiazza, R. M. Q. Shanks, G. A. O'Toole, *J. Bacteriol.* **2005**, *187*, 7351 – 7361.
[34] J. Tremblay, A.-P. Richardson, F. Lépine, E. Déziel, *Environ. Microbiol.* **2007**, *9*, 2622 – 2630.
[35] A. Imberty, M. Wimmerová, E. P. Mitchell, N. Gilboa-Garber, *Microbes Infect.* **2004**, *6*, 221 – 228.
[36] M. Andreini, M. Anderluh, A. Audfray, A. Bernardi, A. Imberty, *Carbohydr. Res.* **2010**, *345*, 1400 – 1407.
[37] L. Saiman, A. Prince, *J. Clin. Invest.* **1993**, *92*, 1875 – 1880.
[38] F. Schweizer, H. Jiao, O. Hindsgaul, W. Y. Wong, R. T. Irvin, *Can. J. Microbiol.* **1998**, *44*, 307 – 311.
[39] R. T. Irvin in *Pseudomonas: Model Organism, Pathogen, Cell Factory* (Ed.: B. H. A. Rehm), Wiley-VCH, Weinheim, **2008**, pp. 45 – 83.
[40] H. B. Sheth, K. K. Lee, W. Y. Wong, G. Srivastava, O. Hindsgaul, R. S. Hodges, W. Paranchych, R. T. Irvin, *Mol. Microbiol.* **1994**, *11*, 715 – 723.

[41] J. R. Mireles II, A. Toguchi, R. M. Harshey, *J. Bacteriol.* **2001**, *183*, 5848 – 5854.
[42] C. M. Toutain-Kidd, S. C. Kadivar, C. T. Bramante, S. A. Bobin, M. E. Zegans, *Antimicrob. Agents Chemother.* **2009**, *53*, 136 – 145.
[43] A. Houari, P. Di Martino, *Lett. Appl. Microbiol.* **2007**, *45*, 652 – 656.
[44] D. H. Dusane, J. K. Rajput, A. R. Kumar, Y. V. Nancharaiah, V. P. Venugopalan, S. S. Zinjarde, *Lett. Appl. Microbiol.* **2008**, *47*, 374 – 379.
[45] A. T. Y. Yeung, E. C. W. Torfs, F. Jamshidi, M. Bains, I. Wiegand, R. E. W. Hancock, J. Overhage, *J. Bacteriol.* **2009**, *191*, 5592 – 5602.
[46] E. Déziel, F. Lépine, S. Milot, R. Villemur, *Microbiology* **2003**, *149*, 2005 – 2013.
[47] T. Matsuyama, M. Matsushita, *Crit. Rev. Microbiol.* **1993**, *19*, 117 – 135.
[48] A. Eberhard, A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson, N. J. Oppenheimer, *Biochemistry* **1981**, *20*, 2444 – 2449.
[49] N. C. Caiazza, J. H. Merritt, K. M. Brothers, G. A. O'Toole, *J. Bacteriol.* **2007**, *189*, 3603 – 3612.
[50] S. R. Schooling, U. K. Charaf, D. G. Allison, P. Gilbert, *Biofilms* **2004**, *1*, 91 – 99.
[51] C. L. Giltner, E. J. Van Schaik, G. F. Audette, D. Kao, R. S. Hodges, D. J. Hassett, R. T. Irvin, *Mol. Microbiol.* **2006**, *59*, 1083 – 1096.
[52] H. B. Sheth, K. K. Lee, W. Y. Wong, G. Srivastava, O. Hindsgaul, R. S. Hodges, W. Paranchych, R. T. Irvin, *Mol. Microbiol.* **1994**, *11*, 715-723..
[53] G. S. Shetye, N. Singh, X. Gao, D. Bandyopadhyay, Y.-Y. Luk, *Med. Chem. Commun.* **2013**, *4*, 1079 – 1084.
[54] J. Pan, D. Ren, *Bioorg. Med. Chem. Lett.* **2013**, *23*, 6559 – 6562.
[55] R. Zhang, L. Zhang, P. Somasundaran, *J. Colloid Interface Sci.* **2004**, *278*, 453 – 460.
[56] E. Déziel, Y. Comeau, R. Villemur, *J. Bacteriol.* **2001**, *183*, 1195 – 1204.
[57] L. O. Adewoye, E. A. Worobec, *Gene* **2000**, *253*, 323 – 330.
[58] T. Ferenci, *J. Bacteriol.* **1980**, *144*, 7 – 11.
[59] M. Reyes, N. A. Treptow, H. A. Shuman, *J. Bacteriol.* **1986**, *165*, 918 – 922.
[60] M. A. Jacobs, A. Alwood, I. Thaipisuttikul, D. Spencer, E. Haugen, S. Ernst, O. Will, R. Kaul, C. Raymond, R. Levy, L. Chung-Rong, D. Guenthner, D. Bovee, M. V. Olson, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14339 – 14344.

Received: March 13, 2014
Published online on June 18, 2014