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Biohybrid Microswimmers for Antibiotic Drug Delivery Based on a Thiol-Sensitive Release Platform

Tetiana Studer[+],[a] Daria Morina[+],[a] Inga S. Shchelik,[a] and Karl Gademann*[a]

Abstract: This study reports on the design and engineering of biohybrid microswimmers exploiting a thiol-mediated self-immolative antibiotic release strategy. The design features a covalent attachment of a vancomycin conjugate via a disulfide-based linker to the surface of the microalgae *Chlamydomonas reinhardtii*. The antibiotic release from the surface of these biohybrids was triggered by the addition of a thiol-based reducing agent, and, subsequently, the inhibition of bacterial growth was observed for *Bacillus subtilis* and *Staphylococcus aureus*. These engineered microbots represent the first example of a microalgae-based drug delivery system with a thiol-mediated, reductive release of an antibiotic drug.

Introduction

Antimicrobial resistance constitutes a rising global health threat directly leading to the death of 1.27 million humans every year.[1–5] To overcome the resistance problem, different approaches such as the discovery of novel antibiotic scaffolds, improvement of existing drugs, and development of new drug delivery strategies are central in a broad panel of measures.[6–17] Among the latter, microswimmers are of great interest due to their potential to mask and carry active drugs and direct those towards the specific target site in a complex environment.[18,19] Both chemical and biological microswimmers have been explored for such tasks, with the latter being evolutionarily optimized to survive and perform in complex biological environments.[20–51] *Chlamydomonas reinhardtii* microalgae are generally recognized as safe (GRAS), biodegradable, and are not considered to trigger the immune response of the human body.[52] In addition, these biflagellate, 10 μm, single-cell microorganisms exhibit motility due to an optical sensor.[53–56] These unique qualities allowed microalgae to be used in numerous studies as micromotors for the manipulation and disruption of biological targets.[56–69] For instance, *C. reinhardtii* microalgae were reported to be used for the design of light navigated, antibiotic-loaded microswimmers, which were employed for the *Pseudomonas aeruginosa* bacterial growth inhibition inside of mice lungs and treatment of acute bacterial pneumonia.[57]

In order to use those microswimmers for drug delivery, both non-covalent and covalent attachment of the antibiotic to the cell offer viable strategies, which have been previously employed by our group.[13,66,69] We have designed and engineered microbots carrying vancomycin and ciprofloxacin via a photo-cleavable linker and demonstrated good antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* upon release by UV light.[13,69] As drawbacks, reduced penetration of light through the tissue limits the application of such systems potentially to skin-related infections and possible photodamage to healthy cells might further limit the application of such systems. A complementary approach utilizes endogenous stimuli such as the pH value or redox homeostasis.[15,16] In the latter, disulfide-containing linkers are frequently used in targeted drug delivery, which often involve "traceless" self-immolating linkers to be released by thiols in thiol exchange reactions.[15,16,70,71] In such a system, the linker will neither be remaining on the system nor on the cargo after release. In this study, we designed and engineered *C. reinhardtii* as a biohybrid microbot for the targeted delivery of vancomycin, based on a self-immolative, thiol-mediated release platform.

Our design features the following key elements as shown in Figure 1. The surface of the microalgae will be functionalized in a two-step strategy with the antibiotic (Figure 1A), where first the surface will be functionalized with the anchor and linker, and in a second step, antibiotics will be clicked to the surface. The drug-loaded microswimmers would therefore be composed of the following building blocks: the antibiotic vancomycin, a self-immolative disulfide linker, a click unit, and an acyl NHS surface anchor attached to the algae cells (Figure 1B). The drug-loaded microswimmers will then be exposed to living bacteria in microbiological assays, with the subsequent antibiotic release upon the addition of a thiol-based reductive agent (Figure 1C). This study presents the successful design, molecular engineering, and biological evaluation of such biohybrid, drug-loaded microbots to eliminate bacteria.

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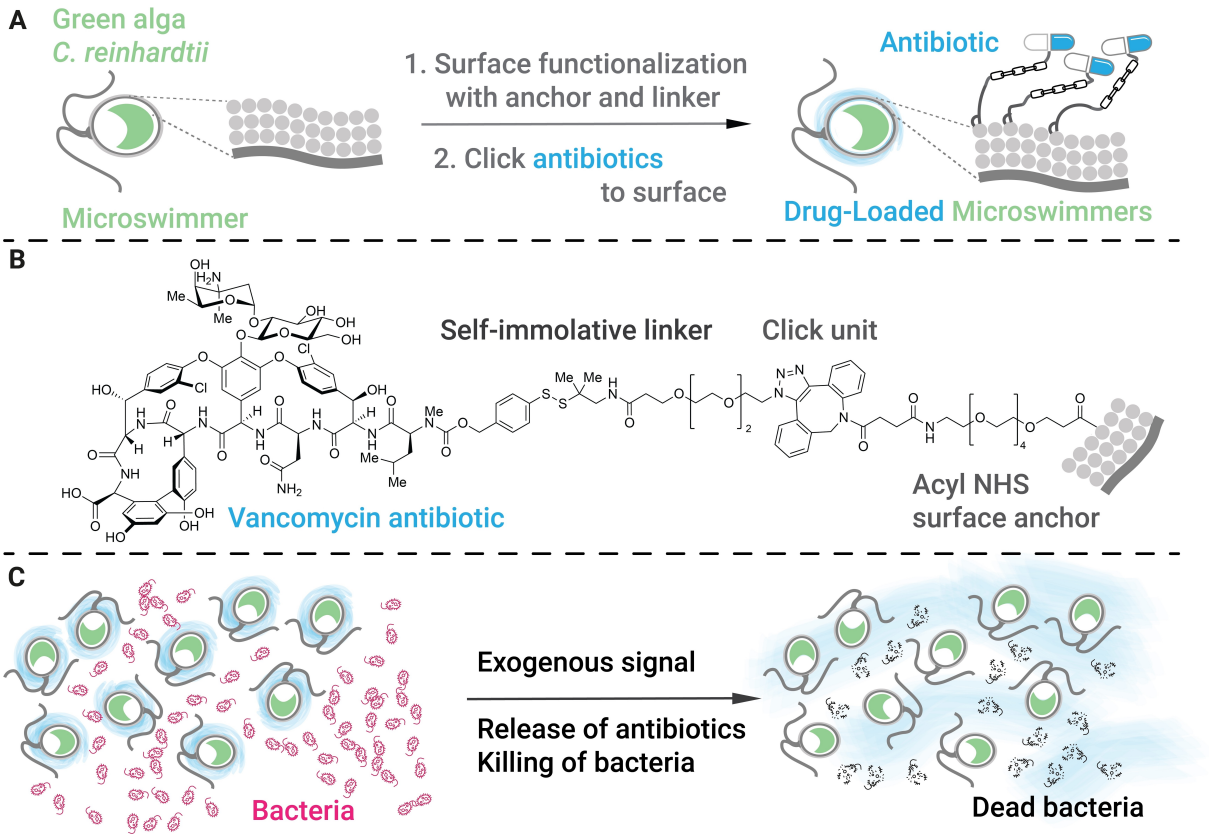


Figure 1. Design of a drug delivery system based on *C. reinhardtii* for release of vancomycin via reduction of a self-immolative linker.

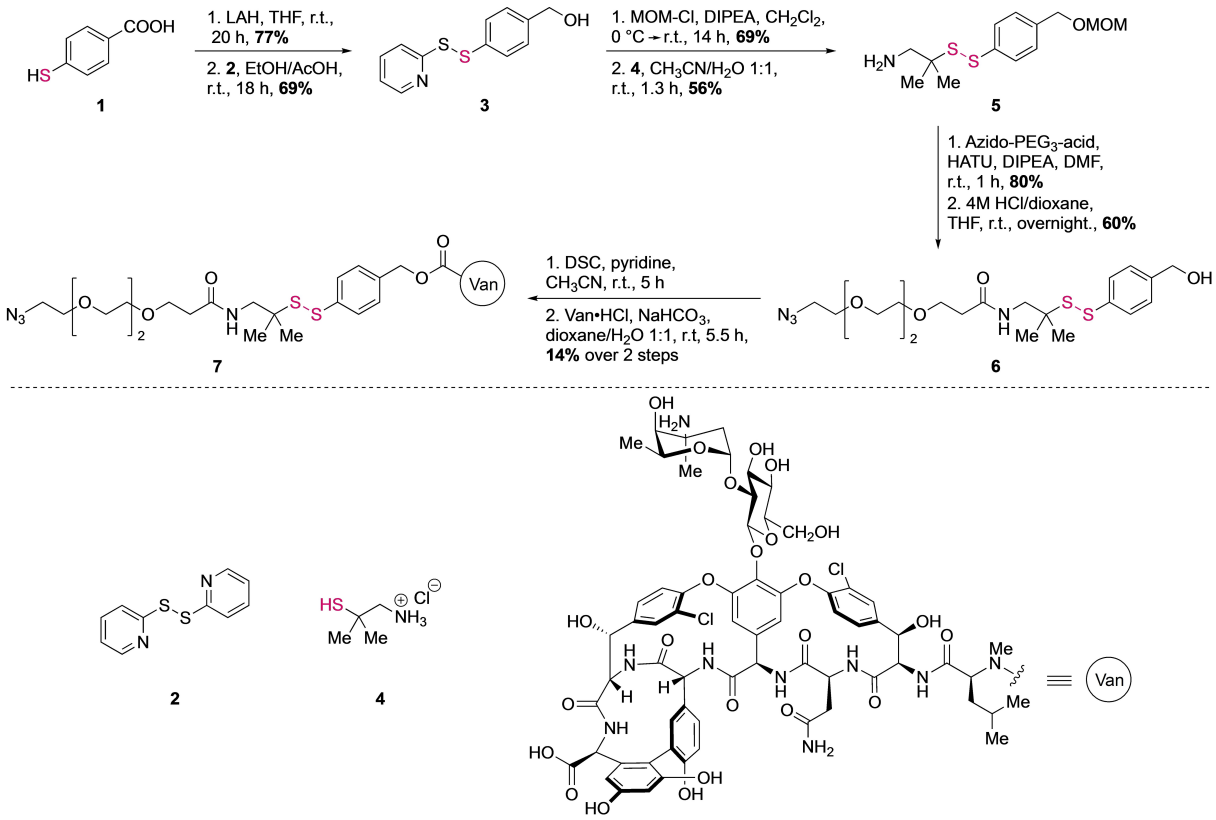
Results and Discussion

Synthesis of the vancomycin linker

First, the synthetic route to the vancomycin self-immolative linker was developed (Scheme 1). The synthesis of the targeted vancomycin derivative started with the reduction of 4-mercaptobenzoic acid (**1**), followed by a disulfide exchange reaction with 2,2′-dithiodipyridine (**2**), resulting in the formation of mixed disulfide **3**.[72,73] The primary hydroxy group of compound **3** was MOM protected, and subsequent disulfide exchange of the 2-thiopyridyl group with dimethyl cysteamine **4** resulted in the desired disulfide amine **5**. The azide derivative **6** was then obtained through the amide coupling between the azido-PEG_3-acid and compound **5**, followed by the MOM group deprotection reaction. In the final step, the disulfide-containing benzyl alcohol **6** was reacted with *N,N′*-disuccinimidylcarbonate allowing the formation of the activated N-succinimidyl-carbonate, which was subsequently coupled to vancomycin. The target compound **7** was obtained after reversed phase HPLC purification. The identity and purity of target vancomycin azide **7** were confirmed by ESI-MS-, ^{1}H NMR-, and analytical RP-HPLC-analyses.

Optimization of reductive release

Next, several reducing agents were tested to identify the optimal conditions for the disulfide-reduction mediated release of vancomycin from the linker **7**. A solution of compound **7** in PBS buffer (10 μM) was treated with solutions of dithiothreitol (DTT) or glutathione (GSH) at 2 mM concentrations, respectively. Aliquots of the reaction mixture were taken at different time points, followed by the quenching with iodoacetamide, and analyses of concentrations of linker **7** and vancomycin by UHPLC-MS measurements (single ion monitoring for vancomycin and compound **7** and compared to authentic samples via calibration curves, see Figure S1 in Supporting Information for details). Approximately 80% of vancomycin were released after 6 h in the presence of DTT (2 mM) at r.t. (Figure 2A, magenta curve). At an elevated temperature of 37 °C, faster release of vancomycin was observed, already reaching a maximum of 80% after 1 h (Figure 2B). In comparison to the vancomycin release with DTT, the reductive cleavage of **7** with GSH was significantly slower and only 23% and 76% of vancomycin was observed after 6 h at r.t and 37 °C, respectively (Figure 2C and Figure 2D). Therefore, DTT was selected as a reducing agent in further experiments for in vitro cleavage of vancomycin.



Scheme 1. Synthesis of disulfide containing vancomycin derivative **7**.

Antibacterial potency of the synthesized linker

The antibiotic activity of the chemical construct was studied next. As a negative control, the minimum inhibitory concentration (MIC) values of the newly synthesized vancomycin derivative **7** were determined against *B. subtilis* 6633 and *S. aureus* 33591 (MRSA), and compared to vancomycin.[72,73] The disulfide containing vancomycin derivative **7** displayed no significant activity against both *B. subtilis* and *S. aureus* with MIC values of 16–32 μg/mL and 32 μg/mL, respectively, compared to MIC values of vancomycin of 0.25 μg/mL and 1–2 μg/mL, respectively.

A time-resolved bacterial growth experiment was performed in a 96-well setup to study the dynamic influence of compound **7** on bacterial growth in the presence and absence of DTT. First, complete inhibition of bacterial growth of *B. subtilis* ATCC 6633 was achieved at concentrations above 4 μg/mL of compound **7** in the presence of DTT (Figure 3A and 3 C). Control experiments such as (1) only addition of DTT (2 mM) without linker **7** resulted in normal bacterial growth or (2) only addition of compound **7** without DTT resulted in bacterial growth in line with the MIC values discussed above.

For *S. aureus* ATCC 33591, we were pleased to observe the desired inhibition of bacterial growth after the release of vancomycin at a concentration of target **7** of 8 μg/mL and higher (Figures 3B and 3D). Control experiments in the presence of only DTT with no target **7**, or only target **7** without the reducing agent gave the expected results of normal bacterial growth (Figure S2 in Supporting Information). Taken together, these data support the potential of thiol-mediated release of construct **7** against different bacterial strains.

Antibacterial activity of the algae drug delivery loaded with the antibiotic

Next, we moved to the covalent functionalization of the microalgae surface with the linker **7** and subsequent thiol-mediated vancomycin release. Compound **7** was attached to the surface of *C. reinhardtii* in a two-step procedure that includes modification of the microalgae surface with an NHS-dibenzocyclooctyne anchor (DBCO) in the first step, and biorthogonal click-reaction with the vancomycin derivative **7** in the second step.[13,69] Previous studies in our group using fluorescently labelled vancomycin demonstrated the suitability of this approach for surface functionalization.[13,69] After repeated centrifugation and washing, the functionalized biohybrid microbots were treated with 2 mM solution of DTT in PBS during 3 h. Next, the cells were centrifuged down and the collected supernatant solution was analyzed by UHPLC-MS in SIM mode (for the detection of vancomycin). After 3 h incubation, the release efficiency of vancomycin from the algae surface reached 18% (Figure 4A). The change of the medium to TAP for algae surface functionalization led to the decrease of

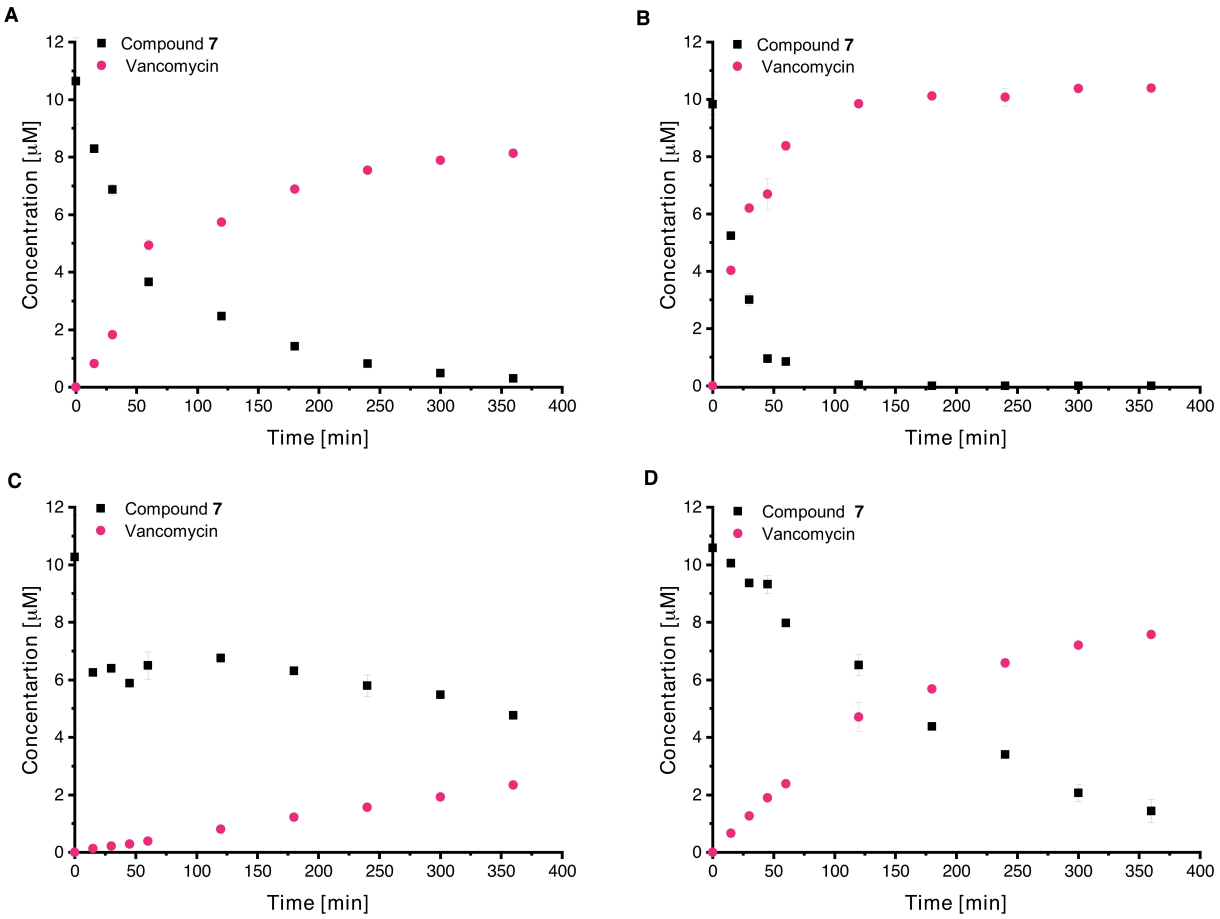


Figure 2. Vancomycin release efficiency from vancomycin derivative **7** (c = 10 μM) at different time points. Panels **A** and **B**: the decay of compound **7** (■) and formation of vancomycin (●) in the presence of DTT (2 mM) at r.t. and 37 °C, respectively. Panels **C** and **D**: the decay of compound **7** (■) and formation of vancomycin (●) in the presence of GSH (2 mM) at r.t. and 37 °C, respectively. The data points represent mean values ± SD (n = 3).

vancomycin release after addition of DTT to 7% after 3 h of incubation (Figure 4B). Therefore, PBS buffer was chosen for the following antimicrobial experiments.

The toxicity effect of DTT on microalgae was studied as a control experiment. The non-functionalized algae were treated with solutions of DTT at different concentrations (two-fold dilution ranging from 100 mM to 6.25 mM), incubated at 25 °C for 4 days, and monitored by OD_{750} measurements over time. Only at a concentration of DTT above 25 mM, significant impact on algal growth was observed (Figure S8 in Supporting Information). Importantly, the presence of DTT at a chosen concentration of 2 mM did not significantly affect the microalgae viability. Therefore, it was concluded to use this concentration of reducing agent for the further bacterial experiments.

After successful reduction-mediated release of vancomycin from the surface of modified microalgae, the antimicrobial activity of the system was investigated in the presence of *B. subtilis* and *S. aureus* (MRSA). The biohybrid microbots were added to the bacterial suspensions (*B. subtilis* and *S. aureus*) in PBS, followed by the addition of DTT. Antimicrobial activity was assessed by OD_{600} measurements over up to 14 h for *B. subtilis* and *S. aureus*, respectively. Complete inhibition of *B. subtilis* growth was observed in the samples containing DTT after 14 h incubation at 37 °C (Figure 5A). The CFU experiments were in agreement with the live growth measurements and demonstrated an overall 10^7 cells density difference between the experiments (see Figure S4 in Supporting Information). The inhibition of *S. aureus* was also observed in bacteria growth experiments (Figure 5B), which again was supported by CFU measurements (10^3 cells density difference, see Figure S5 in Supporting Information).

The following control experiments were carried out. First, we wanted to evaluate the role of the algal cells on the growth of bacteria. Consequently, only the supernatant after thiol-mediated release of vancomycin was mixed with the suspensions of *B. subtilis* and *S. aureus*. The samples were incubated at 37 °C for 10 h and 14 h for *B. subtilis* and *S. aureus*, respectively. The OD_{600} measurements revealed the complete growth inhibition for both *B. subtilis* and *S. aureus* (Figure 5C and 5D, respectively) with the corresponding 10^5 and 10^4 cell density difference for *B. subtilis* and *S. aureus*, respectively, according to the CFU experiments (see Figure S6 and Figure S7 in Supporting Information).

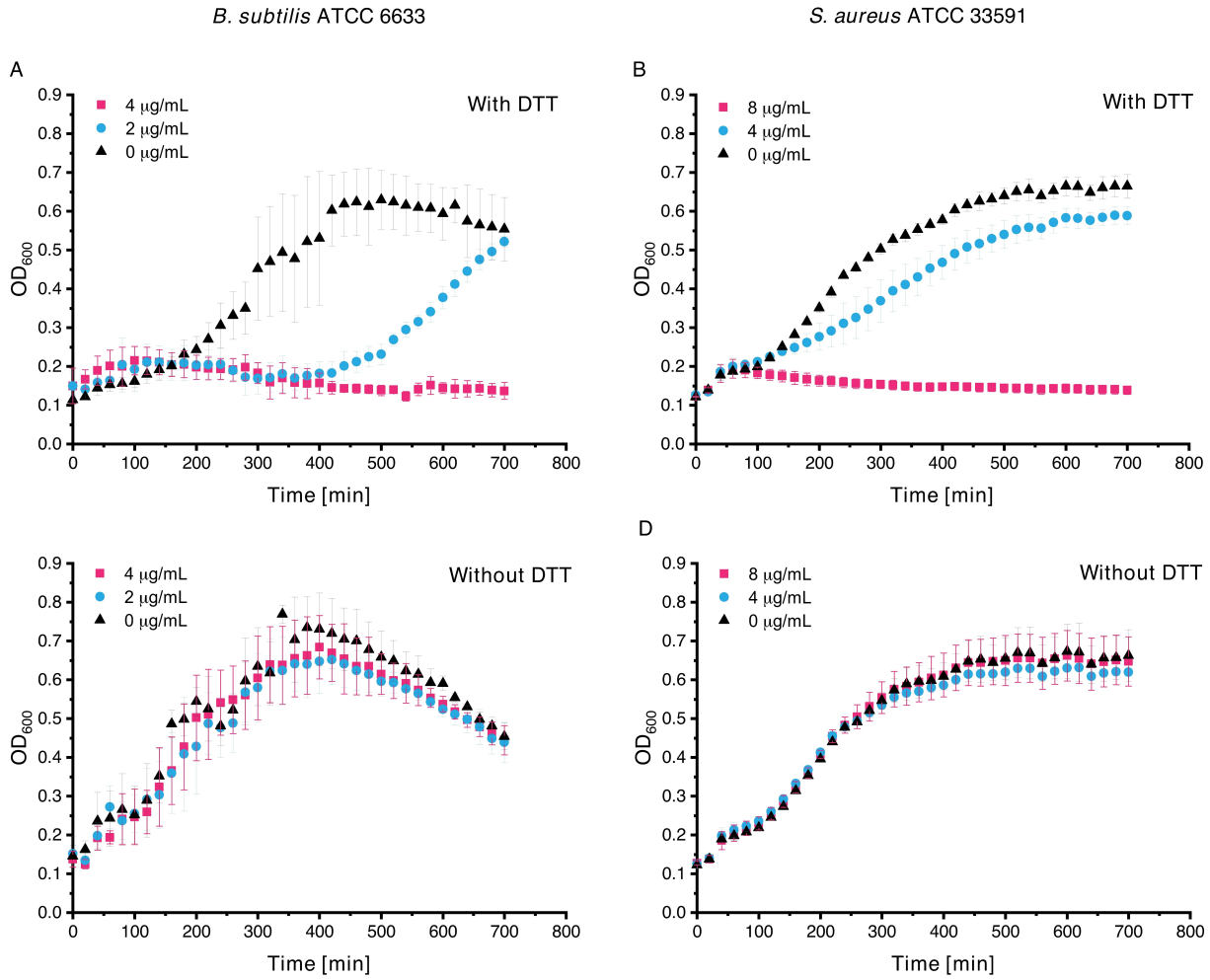


Figure 3. Bacterial growth curves in the presence of compound **7**. **A**: *B. subtilis* ATCC 6633 after addition of DTT. **B**: *S. aureus* ATCC 33591 after addition of DTT. **C**: *B. subtilis* ATCC 6633 without addition of DTT **D**: *S. aureus* ATCC 33591 without addition of DTT. The data points represent mean values ± SD (n = 3).

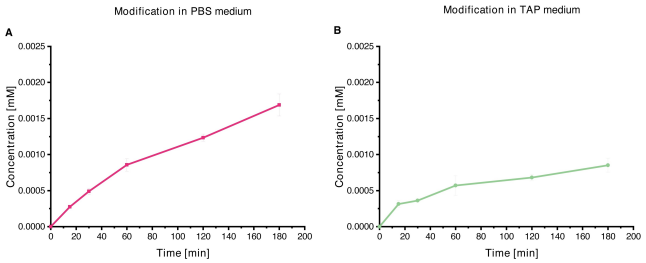


Figure 4. Release of vancomycin from the surface of functionalized algae at different time points at 37 °C. **A**: The cells were modified in PBS medium and the release was mediated by DTT (2 mM). **B**: The cells were modified in TAP medium and the release was mediated by DTT (5 mM). The data points represent mean values ± SD (n = 3).

Furthermore, the influence of DTT itself on bacterial growth was investigated. The presence of DTT at a concentration 2 mM showed no significant effect on the growth of both *B. subtilis* and *S. aureus* (Figures S10 and S11 in Supporting Information, respectively). On the other hand, a moderate inhibition of *B. subtilis* and *S. aureus* growth was observed when the bacteria were grown in the presence of non-functionalized microalgae and DTT at the same concentration in a MHB/PBS 1:1 mixture (Figure S11 in Supporting Information). The reason for the decreased bacterial growth could be in nutrient deficiency in the presence of microalgae. Indeed, a significant increase in *B. subtilis* and *S. aureus* growth was observed when nutrients were provided in doubled concentration in the Mueller-Hinton broth to the bacteria (Figures S12 and S13 in Supporting Information).

Motility of functionalized algae cells

In the next step, the locomotion abilities of the biohybrid microswimmers were investigated. Microalgae were functionalized according to the established two-step procedure with the linker **7** in either PBS or TAP medium. After the second incubation, the microalgae were washed three times with PBS or TAP medium, respectively, placed in water for 1 h, and directly used for microscope studies. No motility for the cells functionalized in PBS media could be observed (Movie S1 in Supporting Information). In contrast, the motility was main-

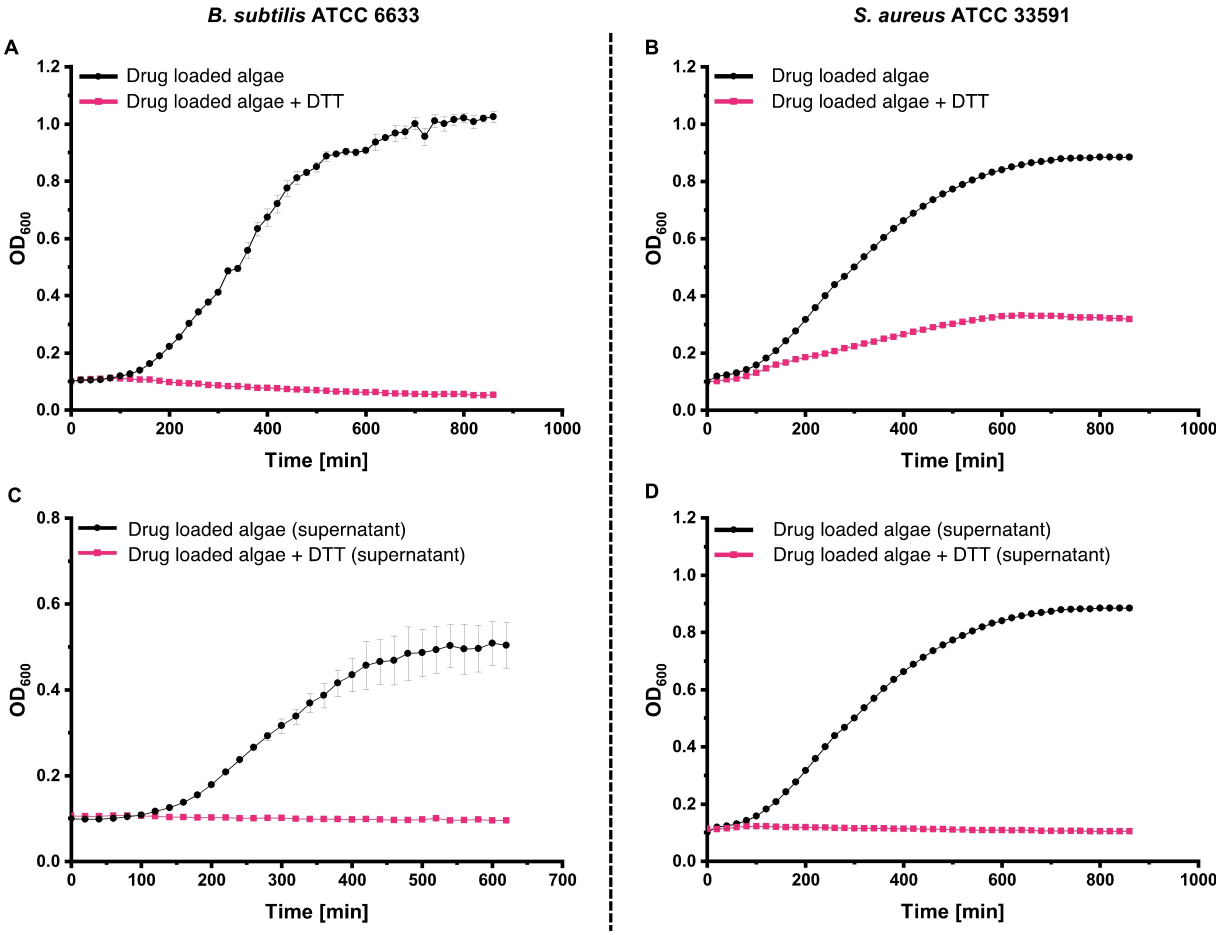


Figure 5. A: Growth curves of *B. subtilis* in the presence of drug-loaded algae with vancomycin derivative **7** and DTT (■, 2 mM) and without DTT (●). **B**: Growth curves of *S. aureus* in the presence of drug-loaded algae with vancomycin derivative **7** (30 μM) in the presence of DTT (■, 2 mM) and without DTT(●). **C**: Growth curves of *B. subtilis* in the presence of supernatant collected from the functionalized microalgae treated with DTT (■, 2 mM) for 1 h and not treated with DTT (●). **D**: Growth curves of *S. aureus* in the presence of supernatant collected from the functionalized microalgae treated with DTT (■, 2 mM) for 1 h and not treated with DTT (●).

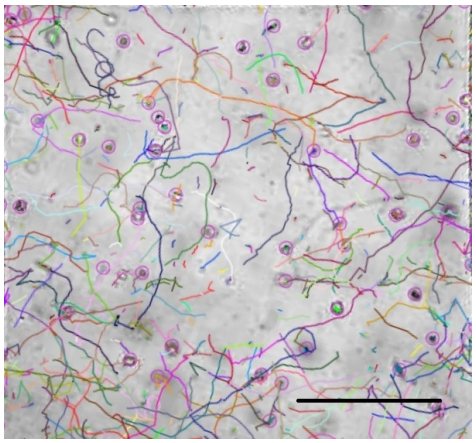


Figure 6. Swimming trajectories of functionalized microswimmers in TAP medium, as observed by bright field microscopy, and analyzed by the TrackMate[74] plugin in FIJI. Scale bar represents 100 μm.

tained for the functionalized microswimmers in TAP medium (Movie S2 in Supporting Information). The swimming trajectories of the microalgae modified in TAP were analyzed with the software Fiji and the plugin TrackMate (Figure 6).[74] This experiment therefore provides strong evidence for the swimming abilities of the functionalized, antibiotic-loaded microbots.

Conclusion

In conclusion, we designed and engineered biohybrid microbots as antibiotic delivery agents, by cell surface functionalization with vancomycin based on a self-immolative linker platform. Vancomycin conjugate **7** was synthesized and covalently attached to the surface of green microalgae *C. reinhardtii* in a two-step procedure. Upon addition of a thiol reductive agent, the antibiotic was released from the biohybrid algae microswimmers and demonstrated strong inhibition of bacterial growth for two different strains of *Bacillus* and *Staphylococcus*. Control experiments provided evidence that (1) the vancomycin derivative **7** itself did not feature any significant antibacterial effect, (2) thiol-mediated release is necessary for activity, (3)

components such as the unfunctionalized algae, DTT, or the masked linker added separately did not influence bacterial growth, and (4) the functionalized microbots retain their swimming ability. When compared to vancomycin itself, the antibiotic-loaded microswimmers do not display antibiotic activity on their own, which could thus limit the development of potential antimicrobial resistance. Only upon addition of reductant, and thus with full control of space and time, antibiotic activity is unleashed. Potential applications might involve skin and soft tissue related infections, among others.[57,59] Overall, this study reports on the first antibiotic-loaded microbots based on a thiol-mediated release platform.

Experimental Section

Synthetic procedures

4-(2-Pyridinyldithio)benzenemethanol (**3**): A solution of 4-mercaprobenzoic acid (**1**) (1.29 g, 8.34 mmol) in dry THF (6.25 mL) was added to a solution of LAH (25.0 mL, 25.0 mmol) in dry THF under inert atmosphere at 0 °C, and the resulting mixture was stirred for 20 h at r.t. Afterwards, H_2O (10 mL) was added dropwise over 1 h, followed by the addition of aq. HCl solution (1 N, until pH = 2, 50 mL) at 0 °C with the subsequent stirring for 10 min. The mixture was extracted with Et_2O (3×80 mL), and the organic phase was washed with H_2O (3×50 mL), and brine (3×20 mL), dried over anhydrous $MgSO_4$, filtered, and then concentrated at reduced pressure. The crude residue was purified by flash column chromatography (hexane/EtOAc 2:1) to afford the product 4-mercaptobenzyl alcohol (**SI-1**) as a white solid (904 mg, 6.45 mmol, 77%).[72] R_f = 0.30 (hexane/EtOAc 2:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.22–7.19 (m, 2H), 7.18–7.15 (m, 2H), 4.57 (s, 2H), 3.38 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 138.6, 130.2, 129.7, 128.0, 65.0. IR (film): 3327 (br, OH), 2561 (SH) cm^{-1}. HRMS (ESI): $C_7H_7OS^-$ $[M-H]^-$, *m/z* = 139.02231, found 139.02223.

4-Mercaptobenzyl alcohol (**SI-1**) (0.500 g, 3.57 mmol) was dissolved in AcOH/EtOH (1:20, 8.7 mL, degassed with N_2 for 5 min) and added dropwise to a solution of 2,2′-dithiobispyridine (1.60 g, 7.25 mmol) in AcOH/EtOH (1:20, 8.7 mL, degassed with N_2 for 5 min) over 10 min under inert atmosphere, and the mixture was stirred for 18 h at r.t. The solvent was then evaporated to dryness at reduced pressure and the residue was purified by a flash column chromatography (hexane/EtOAc, 2:1) resulting in disulfide **3** (612 mg, 2.45 mmol, 69%) as a yellowish oil.[73] **R_f** = 0.26 (hexane/EtOAc 1:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.51–8.47 (m, 1H), 7.80–7.69 (m, 2H), 7.56–7.48 (m, 2H), 7.35–7.28 (m, 2H), 7.21 (m, 1H), 4.66 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 159.3, 147.7, 141.2, 139.3, 134.4, 128.4, 128.0, 121.5, 120.9, 64.6. IR (film): 3360 (br, OH) cm^{-1}. HRMS (ESI): calcd. for $C_{12}H_{12}ONS_2^+$ $[M+H]^+$, *m/z* = 250.03548, found 250.03515.

2-((4-((methoxymethoxy)methyl)phenyl)disulfaneyl)-2-methylpropan-1-amine (***5***): MOM-Cl (114 μL, 1.50 mmol) was added dropwise to a stirred solution of DIPEA (209 μL, 1.20 mmol) and compound **3** (150 mg, 0.602 mmol) in DCM (5 mL) at 0 °C. The color of the reaction mixture turned yellow, it was allowed to reach r.t., and continued to be stirred under inert atmosphere for 14 h. After the completion of the reaction, the mixture was diluted with Et_2O (15 mL), washed with 10% aq. HCl solution (2×10 mL), and brine (2×10 mL). The organic phase was collected, dried over $MgSO_4$, filtered, and the solvent was evaporated at reduced pressure. The product 2-((4-((methoxymethoxy)methyl)phenyl)disulfaneyl) pyridine (**SI-2**) was obtained after column chromatography (hexane/EtOAc 4:1) in 69% yield (121.5 mg, 0.414 mmol) as a colorless oil. R_f = 0.29 (hexane/EtOAc 4:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.52–8.47 (m, 1H), 7.74–7.66 (m, 2H), 7.52 (d, *J* = 8.4 Hz, 2H), 7.30 (d, *J* = 8.0 Hz, 2H), 7.17 (m, 1H), 4.68 (s, 2H), 4.55 (s, 2H), 3.39 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 159.4, 148.4, 138.4, 137.7, 134.9, 128.7, 127.8, 121.2, 120.3, 95.8, 77.4, 77.0, 76.7, 68.5, 55.4. IR (film): 2944, 2884, 1573, 1561, 1491, 1446, 1418, 1377, 1211, 1149, 1102, 1082, 1044, 1015, 986, 958, 919, 843, 806, 760, 718 cm^{-1}. HRMS (ESI): calcd. for $C_{14}H_{16}O_2NS_2^+$ $[M+H]^+$, *m/z* = 294.06170, found 294.06138.

A solution of dimethyl cysteamine (44.0 mg, 0.311 mmol) in H_2O (1.5 mL) was added to the solution of compound **SI-2** (70.1 mg, 0.239 mmol) in CH_3CN (1.5 mL) under inert atmosphere and the mixture was stirred for 1.3 h at r.t. The solvent was then evaporated at reduced pressure, and the reaction mixture was diluted with saturated aqueous solution of $NaHCO_3$ and extracted with EtOAc (5×15 mL). The organic layers were combined, dried over anhydrous $MgSO_4$, filtered, and concentrated at reduced pressure. The residue was purified by column chromatography (CH_2Cl_2/MeOH 19:1) resulting in desired product **5** (38.1 mg, 0.133 mmol, 56%) as a yellowish oil. R_f = 0.11 (CH_2Cl_2/MeOH 19:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.49 (s, 3H), 7.54 (m, 2H), 7.32–7.29 (m, 2H), 4.70 (s, 2H), 4.56 (s, 2H), 3.40 (s, 3H), 3.14 (d, *J* = 5.6 Hz, 2H), 1.41 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 137.4, 136.8, 128.7, 128.0, 95.9, 68.7, 55.57, 49.8, 48.0, 25.6, 25.1. IR (film): 2923, 2883, 2264, 1510, 1491, 1459, 1148, 1101, 1045, 1014, 918, 805 cm^{-1}. HRMS (ESI): calcd. for $C_{13}H_{22}O_2NS_2^+$ $[M+H]^+$, *m/z* = 288.10865, found 288.10844.

3-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-N-(2-((4-(hydroxymethyl)phenyl) disulfaneyl)-2-methylpropyl)propenamide (**6**): A solution of compound **5** (34.9 mg, 0.121 mmol) in dry DMF (0.90 mL) was added to the mixture of DIPEA (62.0 μL, 0.366 mmol), azido-PEG_3-acid (48.3 mg, 0.195 mmol) and HATU (92.8 mg, 0.224 mmol) in dry DMF (0.50 mL) under inert atmosphere. The reaction mixture was stirred for 1 h at r.t. Afterwards, the solvent was evaporated at reduced pressure, and the residue was dissolved in CH_2Cl_2, and washed with H_2O (3×20 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated at reduced pressure. The residue was purified by column chromatography (CH_2Cl_2/MeOH 24:1) to afford compound **SI-3** in 80% yield (50.2 mg, 0.097 mmol) as a yellowish oil. **R_f** = 0.23 (CH_2Cl_2/MeOH 24:1). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.57–7.53 (m, 2H), 7.32–7.28 (m, 2H), 6.40 (t, *J* = 6.3 Hz, 1H), 4.69 (s, 2H), 4.56 (s, 2H), 3.72–3.63 (m, 12H), 3.40 (s, 3H), 3.37 (m, 4H), 2.42 (t, *J* = 5.8 Hz, 2H), 1.24 (s, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 171.8, 137.6, 137.1, 128.7, 128.0, 95.9, 70.9, 70.8, 70.7, 70.6, 70.2, 68.7, 67.5, 55.6, 52.8, 50.8, 47.0, 37.1, 25.5. **IR** (film): 3334 (N–H), 2103 (N_3), 1658 (C=O), 1536, 1148, 1103, 1046 cm^{-1}. **HRMS (ESI)**: calcd. for $C_{22}H_{37}O_6N_4S_2^+$ $[M+H]^+$, *m/z* = 517.21490, found 517.24196.

To a cooled (0 °C) solution of compound **SI-3** (50.2 mg, 1.40 mmol) in THF (0.5 mL), a solution of HCl in dioxane (4 M, 0.5 mL) was added dropwise over 5 min and the reaction mixture was stirred for 24 h at r.t. After the completion of the reaction, the mixture was quenched with saturated solution of aq. $NaHCO_3$ (1.3 mL) and extracted with EtOAc (2×3 mL). The combined organic layers were washed with H_2O (2×5 mL), and brine (1×5 mL), dried over $MgSO_4$, filtered, and the solvent was evaporated at reduced pressure. The residue was triturated three times with pentane yielding azide **6** (27.7 mg, 0.059 mmol, 60%) as a colorless oil, which was used for the next step without additional purification. R_f = 0.2 (CH_2Cl_2/MeOH 19:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58–7.54 (d, *J* = 8.3 Hz, 2H), 7.32 (d, *J* = 8.3 Hz, 2H), 6.12 (br, 1H), 4.64 (s, 2H), 3.68–3.63 (m, 12H), 3.40–3.35 (m, 2H), 3.31 (d, *J* = 6.0 Hz, 2H), 2.31 (t, *J* = 5.9 Hz, 2H), 1.24 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 171.6, 140.8, 137.4, 128.7, 127.9, 70.8, 70.7, 70.6, 70.5, 70.2, 67.4, 64.6, 52.6, 50.8, 46.9, 37.0, 25.5. IR (film): 3331 (br, O–H), 2921, 2868, 2101 (N_3), 1654 (C=O), 1544, 1285, 1120 cm^{-1}. HRMS (ESI): calcd. for $C_{20}H_{33}O_5N_4S_2^+$ $[M+H]^+$, *m/z* = 473.18869, found 473.18857.

***Vancomycin derivative* 7**: *N,N′*-disuccinimidyl carbonate (20.5 mg, 0.08 mmol) and pyridine (12.9 μL, 0.16 mmol) were added to the solution of derivative **6** (25.2 mg, 0.0533 mmol) in anhydrous CH_3CN (1 mL). The reaction mixture was stirred under inert atmosphere for 5 h at r.t., and then the solvent was removed at reduced pressure. The residue was dissolved in EtOAc (2 mL) and washed with a saturated solution of aq. $NaHCO_3$ (3×2 mL), and brine (3×2 mL). The collected organic phase was dried over $MgSO_4$, filtered, and the solvent was evaporated at reduced pressure to afford N-hydroxysuccinimide-activated linker **SI-4** (30.7 mg, 0.050 mmol) as a colorless oil, which was used further without purification. $NaHCO_3$ (3.66 mg, 0.0436 mmol) was added to the solution of vancomycin hydrochloride (58.7 mg, 0.0395 mmol) in H_2O (1.5 mL) at r.t. After stirring for 5 min, this solution was added to the solution of compound **SI-4** (24.2 mg, 0.0394 mmol) in dioxane (1.5 mL) and the reaction mixture was stirred for 3 h 10 min. Afterwards, two more portions of $NaHCO_3$ (3.70 mg, 0.044 mmol) were added to the reaction mixture within 1 h. Dioxane was evaporated at reduced pressure and the residual mixture was lyophilized. The product **7** was afforded in 14% over two steps (19.3 mg, 8 μmol) as a fluffy white powder after purification by preparative RP-HPLC (Gemini-NX): Gradient 10% of B for 18 min; 10%–45% of B for 73 min; 45%-100% of B for 5 min, R_t = 46.5 min. ^{1}H NMR (500 MHz, DMSO): δ 9.29 (br, 1H), 8.87 (br, 1H), 8.57 (br, 1H), 8.42 (br, 1H), 8.23 (br, 1H) 7.91–7.89 (m, 2H), 7.56 (d, *J* = 7.9 Hz, 2H), 7.50–7.49 (m, 1H), 7.46–7.44 (m, 1H), 7.38–7.34 (m, 4H), 7.30–7.27 (m, 1H), 7.26–7.21 (m, 1H), 7.18–7.14 (m, 1H), 7.04 (d, *J* = 8.2 Hz, 1H), 6.82–6.78 (m, 1H), 6.73 (d, *J* = 8.4 Hz, 1H), 6.68 (d, *J* = 8.4 Hz, 1H), 6.58–6.51 (m, 1H), 6.38 (s, 1H), 6.32 (s, 1H), 5.93 (br, 1H), 5.78 (s, 1H), 5.72 (d, *J* = 8.0 Hz, 1H), 5.53 (s, 1H), 5.37 (s, 1H), 5.29–5.26 (m, 1H), 5.25–5.16 (m, 6H), 5.15–5.11 (m, 1H), 4.83 (d, *J* = 9.3 Hz, 1H), 4.69 (d, *J* = 6.6 Hz, 1H), 4.44 (s, 1H), 4.35 (d, *J* = 5.7 Hz, 1H), 4.16 (s, 1H), 3.97 (s, 1H), 3.67 (d, *J* = 10.7 Hz, 1H), 3.58 (t, *J* = 5.2 Hz, 6H), 3.56–3.44 (m, 16H), 3.21 (d, *J* = 6.2 Hz, 4H), 3.16 (br, 1H), 2.82 (s, 3H), 2.54 (s, 1H), 2.34 (t, *J* = 6.4 Hz, 3H), 2.15 (d, *J* = 13.6 Hz, 1H), 1.85 (s, 1H), 1.62 (d, *J* = 13.4 Hz, 2H), 1.57–1.51 (m, 1H), 1.48–1.38 (m, 2H), 1.20 (s, 3H), 1.17–1.13 (m, 7H), 1.08 (d, *J* = 5.9 Hz, 3H), 0.89 (d, *J* = 6.4 Hz, 3H), 0.83 (d, *J* = 6.4 Hz, 3H). HRMS (ESI): calcd. for $C_{87}H_{105}O_{30}N_{13}Cl_2NaS_2^+$ $[M+Na]^+$, *m/z* = 1968.58090, found 1968.58010; calcd. for $C_{87}H_{107}O_{30}N_{13}Cl_2S_2^+$ $[M+2H]^{2+}$, *m/z* = 973.80281, found 973.80271; calcd. for $C_{80}H_{93}O_{28}N_{12}Cl_2S_2^+$ $[M–C_7H_{14}NO_2+H]^+$, *m/z* = 1803.50352, found 1803.50394; calcd. for $C_{74}H_{83}O_{23}N_{12}Cl_2S_2^+$ $[M–C_{13}H_{24}NO_7+H]^+$, *m/z* = 1641.45070, found 1641.45027.

The purity of the compound was analyzed by analytical RP-HPLC (Synergy Hydro): Gradient 10% B for 5 min, 10–100% B for 15 min. The product **7** eluted at R_t = 14.1 min and was detected at 280 nm (see Figure S32).

General Protocol for MIC Value Determination: The minimum inhibitory concentration (MIC) assays were carried out as stated by European Committee of Antimicrobial Susceptibility Testing (EUCAST) utilizing the broth microdilution method for all tested compounds.[75,76]

The stock solutions were prepared by dissolving the tested compounds in Mili-Q water (64 μg/mL). In a 96-well round-bottomed plate, two-fold serial dilutions of the compounds (ranging from 64 μg/mL to 0.125 μg/mL) were prepared in Mueller-Hinton broth 2 (MHB) in a 50 μL end-volume with the addition of 0.002% of Tween 20. The optical density of overnight bacterial culture measured at 600 nm (OD_{600}) was set to a McFarland Standard 0.5 (0.08–0.13) resulting in approximately 1×10^8 CFU/mL. Next, the bacterial cells were diluted with MHB by a factor 1:200 and 1:100 for *B. subtilis* ATCC 6633 and *S. aureus* ATCC 33591, respectively. Afterwards, 50 μL of bacterial suspension was added to the wells containing tested compound solutions resulting in the final inoculum of 5×10^5 CFU/mL. As a positive control, bacteria were also grown without the addition of compounds. The well plates were incubated at 37 °C for 18–20 h in a rotary shaker (200 RPM). The MIC values (determined by visual inspection) were defined as the lowest concentrations of compounds needed to prevent bacterial growth. All experiments were performed in triplicates.

General Protocol for the Microalgae Growth: *Chlamydomonas reinhardtii* (*C. reinhardtii*, 11–32b) were purchased from EPSAG (Experimentelle Phykologie und Sammlung von Algenkulturen der Universität Göttingen) and grown in TAP medium (50 mL) at 25 °C for 4–5 days at 120 rpm shaking on a rotary shaker under constant illumination of 8 μmol/m^{2}s. TAP medium was prepared following the published recipe: 50 mL/L Beijerinck's Solution (8 g/L NH_4Cl, 1 g/L $CaCl_2$*$2H_2O$, 2 g/L $MgSO_4$*$7H_2O$), 8.5 mL/L Phosphate Buffer Solution (11.62 g/L Na_2HPO_4, 7.26 g/L KH_2PO_4), 1 mL/L Hunter's Trace Stock Solution (50 g/L Na_2EDTA*$2H_2O$, 22 g/L $ZnSO_4$*$7H_2O$, 11.4 g/L H_3BO_3, 5.1 g/L $MnCl_2$*$4H_2O$, 5.0 g/L $FeSO_4$*$7H_2O$, 1.6 g/L $CoCl_2$*$6H_2O$, 1.16 g/L $CuSO_4$*$5H_2O$, 1.1 g/L $(NH_4)_6Mo_7O_{24}$*4 H_2O), 10 mL/L Tris Acetate Stock Solution (242 g/L Trisma base, 100 mL/L Glacial AcOH).

***C. reinhardtii* Surface Modification Protocol (CRSMP)**: The microalgae surface functionalization was performed in two steps. The general protocol is described below.

Microalgae surface functionalization with dibenzocyclooctyne NHS activated anchor: The microalgae solution (0.5–1 mL, cell density = 1.5×10^6–2.5×10^6 cells/mL, calculated by a counting chamber) was centrifuged (5 min, 4000 RPM), the supernatant was removed, and the cells were washed with PBS (1 mL). After the next centrifugation (5 min, 4000 RPM), the supernatant was removed, and the washing step was repeated one more time. The microalgae were then incubated with NHS-PEG_4-dibenzocyclooctyne (DBCO, BroadPharm) solution (0.250 mL, in PBS) at concentrations 10 times higher than compound **7** for 1 h (r.t., 700 RPM). Next, the cells were centrifuged (3 min, 4000 RPM) and the supernatant was removed. The pellets were washed with PBS (1 mL), centrifuged, and the supernatant was removed. The washing steps were repeated two more times.

Attachment of the Vancomycin Derivative ***7***: The microalgae modified with DBCO were resuspended in the solution of vancomycin derivative **7** (PBS, 250 μL, with 0.002% Tween 20) at the desired concentrations and the mixture was incubated for 1 h (r.t., 700 RPM). Afterwards, the samples were centrifuged (3 min, 4000 RPM), and the supernatant was removed. The algae were washed with PBS (1 mL), centrifuged (3 min, 4000 RPM), and the supernatant was removed. The washing steps were repeated five more times.

Reduction-mediated Release of Vancomycin from Compound 7: PBS (pH = 7.4) and the stock solutions of compound **7** were filtered before use. The solution of a reducing agent (DTT or GSH, 2 mM final concentration, in PBS) was added to the solution of compound **7** (10 μM final concentration, in PBS) at r.t or 37 °C. Aliquots (0.5 mL) of the reaction mixture were taken at different time points and mixed with iodoacetamide solution in PBS (4 mM and 2 mM final concentration for DTT and GSH, respectively) in HPLC vials. The samples were analyzed by UHPLC-MS in SIM mode. The areas under the mass picks were integrated and transformed to the concentrations through the calibration curves method (Figure S1 in Supporting Information). The experiments were performed in triplicate.

Reduction-mediated Release of Vancomycin from *C. reinhardtii* Surface: The microalgae cells were modified according to the protocol described above in CRSMP at the conditions given in the Table 1. After the 2nd modification step, the microalgae were washed with PBS three times in total. The cells were resuspended

Table 1. Conditions used for *C. reinhardtii* surface functionalization with DBCO and vancomycin derivative **7**.

Experiment number	Functionalization step	Concentration [mM]		Solv.	Incubation time [h]
		DBCO	Compound 7		
1	1st	0.1		PBS	1
	2nd		0.01	PBS	1
2	1st	0.1		TAP	1
	2nd		0.01	PBS	1.5

in PBS (205 μL), with the following addition of the solution of Tween 20 in PBS (10 μL, 0.002% final concentration). The microalgae were then incubated with DTT solution in PBS (2 mM or 5 mM final concentration for the experiments 1 and 2, respectively) at 37 °C for different time intervals (20, 40, 60, 120, 180 min) and centrifuged (1 min, 4000 RPM). The collected supernatant was filtered and analyzed by UHPLC-MS in SIM mode. All experiments were performed in triplicates.

Testing of Antibacterial Activity of the System

Bacterial Strains Growth Conditions: Gram-positive *Bacillus subtilis* (*B. subtilis*, ATCC 6633), *Staphylococcus aureus* (*S. aureus*, MRSA strain ATCC 33591) were purchased from American Type Culture Collection (ATCC) and stored at −80 °C. The new bacterial culture was prepared by streaking on Mueller Hinton Agar 2 plate and incubated at 37 °C for 18–20 h. The plates were stored in a fridge at 4 °C. The overnight liquid bacterial culture was prepared by adding a single colony into a sterile Falcon tube (15 mL) which already contained 5 mL of Mueller-Hinton Broth 2 (MHB). The culture was then shaken at 37 °C overnight at 200 RPM.

General Protocol for the Bacterial Experiments (GPBE): An overnight bacterial culture was centrifuged (25 °C, 4000 RPM, 5 min) and resuspended in double concentrated MHB (dcMHB) to an optical density of 0.2 at 600 nm (OD_{600}). PBS aliquots (250 μL) containing Tween 20 (0.004%) were mixed with bacterial suspensions (250 μL) in a 24-well plate and gently mixed by a micropipette (final OD_{600} = 0.1). The bacteria growth was monitored by a plate reader over 14 h at 37 °C (unless stated otherwise) with shaking between the measurements. All experiments were performed in triplicates.

Calculations of the colony forming units (CFU) were performed for bacterial culture (*B. subtills* and *S. aureus*) by its 10-fold dilution prepared in Mueller-Hinton Broth 2 (MHB). The diluted bacterial suspension (100 μL) was drop-plated on the MHB agar plates. The plates with *B. subtilis* were incubated for 7 h at 37 °C followed by additional 13 h of incubation at 26 °C, and then CFU were counted. CFU for *S. aureus* were enumerated after 20 h of incubation at 37 °C. The experiments were performed in triplicates.

Time-resolved Bacterial Growth Experiment in the Presence of Vancomycin Derivative 7: For the stock solution, compound **7** was dissolved in Mili-Q water (128 μg/mL). Two-fold serial dilutions (ranging from 64 μg/mL to 0.125 μg/mL) with MHB were prepared in a 96-well round-bottomed plate with an end-volume of 50 μL. Tween 20 (10 μL, in MHB) was added to each well in 0.002% final concentration. The overnight bacterial suspension (*B. subtillis* ATCC 6633 or *S. aureus* ATCC 33591) was inoculated into each well containing compound **7** resulting in final OD_{600} = 0.1 with approximately 1×10^8 CFU/mL. DTT solution (10 μL, dissolved in H_2O, 2 mM final concentration) was added to the first four horizontal lines of the well plate containing compound **7**. All wells in the plate were filled with MHB to the end volume of 100 μL. A negative control experiment was prepared similarly to the procedure described above, but without the addition of compound **7** or without bacteria. The plate was incubated at 37 °C for 18 h and OD_{600} of the cells was measured every 20 min by a plate reader with shaking between the measurement points (700 RPM). The experiment with *B. subtillis* ATCC 6633 (Figure S2 in Supporting Information) was performed in triplicates and with *S. aureus* ATCC 33591 (Figure S3 in Supporting Information) in quadruplicates.

Release of the Vancomycin from the Biohybrids in the Presence of Bacteria

Vancomycin release in the presence of B. subtilis ATCC 6633: The microalgae were exposed to the same incubation and washing steps as described above in the CRSMP. DBCO (0.2 mM) was added during the 1st modification step and compound 7 (0.02 mM) was added during the 2nd modification step (incubation was prolongated to 1.5 h). The antibacterial activity was recorded by replacing the PBS (0.250 mL) in the GPBE protocol above with the functionalized microalgae resuspended in PBS (0.240 mL) followed by the addition of DTT (10 μL, 2 mM final concentration). For the negative control, functionalized microalgae were dissolved in PBS (0.250 mL) without the addition of DTT (Figure S4 in Supporting Information).

Vancomycin release in the presence of S. aureus ATCC 33591: Two portions of the microalgae were exposed to the modification procedure described in the CRSMP. DBCO (0.3 mM) was added during the 1st modification step and a solution of compound **7** (0.03 mM) was added during the 2nd modification step (incubation was prolongated to 1.5 h). PBS (0.500 mL) was utilized for the functionalization. Two samples of modified microalgae were combined for the experiment. The antibacterial activity was recorded by replacing the PBS (0.250 mL) from the GPBE protocol above with the functionalized microalgae resuspended in PBS (0.240 mL) followed by the addition of DTT (10 μL, 2 mM final concentration). For the negative control, functionalized microalgae were dissolved in PBS (0.250 mL) without the addition of DTT (Figure S5 in Supporting Information).

Motility Experiment: The microalgae cells were modified according to the CRSMP above in PBS or TAP using following conditions: 0.1 mM DBCO, 250 μL, 1 h, then 0.01 mM compound **7**, 250 μL, 1.5 h. Then the microalgae were washed three times with PBS or TAP respectively and placed in water (1 mL) for 1 h. An aliquot of microalgae suspension (10 μL) was added onto a microscopic counting chamber with 0.33 mm square grid. The locomotion abilities of modified with the linker **7** microalgae were analyzed under a microscope, and the observations were recorded on camera (Movies S1 and S2, Supporting Information). The trajectories of algae cells were analyzed using the software Fiji with the TrackMate plugin.[74]

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: antibiotics · chemical biology · drug delivery · microbots · vancomycin

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