Conference Paper

Use of Poly (ϵ -Lysine) Dendrons: A Strategy Targeting Bacterial Quorum Sensing and Biofilm Formation

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Received 6 September 2013; Accepted 10 February 2014; Published 11 March 2014

Academic Editors: J. Gough, R. Sammons, and C. Scotchford

This Conference Paper is based on a presentation given by Rahaf Issa at “UK Society for Biomaterials Annual Conference 2013” held from 24 June 2013 to 25 June 2013 in Birmingham, United Kingdom.

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Pseudomonas aeruginosa is recognised as a major aetiological agent of nosocomial infections, which are associated with multiple-antibiotic resistance. Among many of its important virulence factors is its ability to form biofilms on the surfaces of implantable medical devices and to produce toxic metabolites, pyocyanin, via an intercellular cell density-dependent signalling system of communication. In this study, poly (ϵ -lysine) dendrons composed of increasingly branching generations were synthesised, characterised, and examined for their effects on virulence factor production in *P. aeruginosa*. The results show that these hyperbranched poly (ϵ -lysine) dendrons, in particular the 3rd generation, can increase the efficacy of a conventional antibiotic, ciprofloxacin, and reduce pyocyanin production, with marginal effects on the rate of bacterial replication, suggesting that the observed effects are not due to dendron toxicity. Furthermore, dendron and ciprofloxacin coadministration was identified as the most effective strategy which highlights the potential of peptide-based dendrons as quorum sensing inhibitors.

1. Introduction

The emergence of multiple-antibiotic resistant bacterial strains is one of the greatest contemporary challenges in modern medical science and is increasing at a rate that far exceeds the pace of the development of new drugs [1, 2]. This rise in antimicrobial resistance allows infections to develop into chronic conditions, which are estimated to account for approximately 25,000 excess mortalities in the EU annually and cost the national healthcare providers in excess of £1 billion per annum [2]. Among the most prevalent human pathogens noted for antibiotic resistance is *Pseudomonas aeruginosa*. This Gram-negative, opportunistic bacterium accounts for an estimated 6% of all nosocomial infection reports [1] and is also the primary cause of respiratory deterioration and mortality in patients with cystic fibrosis [3, 4].

The ability of many pathogens to negate the effects of antibiotics is mediated in part by the formation of surface-attached, structured communities of bacterial cells through

a process termed biofilm formation [5]. The development of these microbial communities has been shown to provide an altered microenvironment, whereby an intrinsic physical barrier is formed which therefore protects underlying organisms from external stresses (such as antibiotic penetration) [6]. Given the latter, it is not surprising that approximately 65% of all human bacterial infections involve biofilms [7]. Furthermore, biofilms regularly impede the ability of medical implants to function, which results in device failure [8].

Although the complete mechanism of biofilm formation remains unclear, it is known that this process is under the influence of an intercellular cell density-dependent signalling system of communication, termed quorum sensing (QS) [9]. In addition, downregulation of QS has been shown to attenuate bacterial virulence and pathogenicity, as evidenced by studies with mutants lacking functional QS systems [10–12]. Quorum sensing has therefore emerged as a prime therapeutic target and a promising alternative strategy for controlling *P. aeruginosa* biofilm infections.

To date, research has been focused on designing QS inhibitors capable of modifying the structure of signalling molecules, *N*-acylated L-homoserine lactones (AHL) [13] or acting as antagonists for AHL [14, 15]. At present, only a few have been tested in animal models with the majority of compounds exhibiting stability and toxicity complications for eukaryotic cells [16, 17] and, therefore, are regarded as unsuitable for human use. However, a novel avenue of antimicrobial research involving the use of hyperbranched macromolecules (such as peptide-based dendrons) has been proposed [18–20]. These hyperbranched peptide-based dendrons are emerging as particularly useful molecules for a broad-spectrum of biological applications [21, 22], mainly due to their biocompatibility, ease of manipulation and derivatisation, compact structure, stability, and reproducibility. In addition, the outermost branching generation of each dendron offers a high local concentration of functional groups, which can be exploited for targeting multiple QS systems and therefore infections caused by multiple species. It is indeed for these features that dendrons should be explored for antibacterial applications.

In the present paper, the synthesis, characterisation and *in vitro* antibacterial potential of hyperbranched poly (ϵ -lysine) dendrons of various generation numbers are described. Their ability to interfere with two quorum sensing-controlled phenotypes, biofilm formation on abiotic surfaces, and pyocyanin production is also presented.

2. Materials and Methods

2.1. Bacterial Strains and Growth Media. *Pseudomonas aeruginosa* strain NCTC 10662 and the wild-type strain PAO1 were used throughout this study. All strains were stored in 15% (v/v) glycerol (Fisher Scientific, UK) stocks and stored at -80°C . For each experiment, subcultures were prepared from these stocks, where cells were maintained on nutrient agar (NA) plates and in nutrient broth (NB) (Oxoid, UK) to produce overnight cultures at 37°C with agitation.

2.2. Minimal Inhibitory Concentration. The MIC of ciprofloxacin (Sigma-Aldrich, UK) was determined using the broth macrodilution method. Briefly, overnight cultures of *P. aeruginosa* containing 4.7×10^9 colony forming units per mL (CFU/mL) were added to iso-sensitest broth (Oxoid, UK) supplemented with the serially diluted antibiotic to attain the final concentration and incubated at 37°C for 24 h without agitation. The lowest concentration of antibiotic for which a similar optical density (OD) at 600 nm was observed in the inoculated and the blank negative controls was recorded as the MIC. This concentration was used for all subsequent experiments.

2.3. Dendron Synthesis. Peptide-based dendrons consisting of an arginine (R) residue, onto which were assembled up to three branching generations of poly (ϵ -lysine) (K) G(1, 2 or 3)K, were synthesised using solid-phase peptide synthesis in a fourfold molar excess of Fmoc-protected amino acids [21]. All organic solvents used were HPLC

grade (Fisher scientific, UK). Amino acids were purchased from Novabiochem, UK, with a purity of $>98\%$. Tenta gel S-NH₂ resin beads (0.5 g, 0.26 mmol/g, Iris Biotech GmbH, Germany) were swollen in N,N-dimethylformamide (DMF) in a 10 mL polypropylene syringe for 15 minutes. To allow cleavage of the dendron once the synthesis was complete, an acid labile linker molecule, Fmoc-protected Rink amide linker (Iris Biotech GmbH, Germany), was first attached. A solution containing 3 mL DMF, 0.4 mmol O-benzotriazole-N,N,N',N'-tetramethyluronium-hexafluorophosphate (HBTU, Novabiochem, Germany), 0.8 mmol N,N-diisopropylethylamine (DIPEA, Iris Biochem GmnH, Germany), and 0.4 mmol Fmoc-protected Rink amide linker was prepared, briefly sonicated and then added to the syringe containing the resin and allowed to react for 30 minutes. The solvent was then expelled from the syringe and the contents were washed with 3×6 mL DMF. Fmoc-protecting groups on the Rink amide linker were deprotected by the addition of 20% (v/v) piperidine (Sigma-Aldrich, UK, 99%) in DMF using 3×2 minute washes, before the resin was washed with 5×6 mL of DMF. All Fmoc-protected amino acids (Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Fmoc)-OH) were then sequentially assembled using the aforementioned coupling and deprotection steps to produce dendrons of branching generations RG(1, 2 or 3)K. At the desired generation, the resin was washed with 40 mL dichloromethane followed by washes with 40 mL methanol and finally 40 mL diethyl ether. The dendrons were then cleaved from the resin by incubation for 3 hours in 88% (v/v) trifluoroacetic acid (Fisher Scientific, UK, 99%), 2% (v/v) deionised water, 5% (v/v) triisopropylsilane (Sigma-Aldrich, UK, 99%), and 5% (w/v) phenol (Sigma-Aldrich, UK, 99%). The products were precipitated in ice-cold 15 mL diethyl ether and collected through a series of washing and centrifugation steps at 3500 rpm for 5 minutes. The final obtained products (dissolved in methanol) were analysed by analytical HPLC (Waters/Millipore 717plus Autosampler, Shimadzu SPD 6A UV spectrophotometric detector, PerkinElmer 2000 series lc binary gradient pump) with a Phenomenex Luna C18 (150 mm $\times$ 4.6 mm $\times$ 3 μm) column. Dendrons were also characterised using micro-TOF mass spectrometry (Bruker).

2.4. Treatments. For all strains the bactericidal effects of ciprofloxacin alone or in combination with the dendrons was determined. Both antibiotic and dendrons were dissolved in the experimental media (nutrient broth) to attain a stock concentration of 15 $\mu\text{g/mL}$ and 6 mM, respectively. Cells of *P. aeruginosa* were exposed to one of four treatments (Table 1), in order to determine the bacterium's capacity to produce quorum-sensing controlled phenotypes in the presence of each.

2.5. Biofilm Formation. Biofilms of *P. aeruginosa* (NCTC 10662) were allowed to develop on the surface of medical grade stainless steel discs (diameter 20 mm) placed in the wells of a polystyrene 6-well microtiter cell culture plate under dynamic conditions (90 rpm) at 37°C . Briefly, overnight cultures containing 6.1×10^8 – 1.7×10^9 CFU/mL

TABLE 1: Treatments used against cultures of *Pseudomonas aeruginosa*.

Treatment	Comments
RGnK*	Equimolar final concentration of NH ₂ outermost groups, where RG1K was applied at 1600 μ M, RG2K at 800 μ M, and RG3K 400 μ M.
Ciprofloxacin	Minimal inhibitory concentration of 0.5 μ g/mL.
Administration of RGnK followed by ciprofloxacin (0.5 μ g/mL)	<i>Dendron</i> : equimolar final concentration of NH ₂ outermost groups, where RG1K was applied at 1600 μ M, RG2K at 800 μ M, and RG3K 400 μ M. <i>Ciprofloxacin</i> : MIC of 0.5 μ g/mL.
Coadministration of RGnK and ciprofloxacin (0.5 μ g/mL)	<i>Dendron</i> : equimolar final concentration of NH ₂ outermost groups, where RG1K was applied at 1600 μ M, RG2K at 800 μ M, and RG3K 400 μ M. <i>Ciprofloxacin</i> : MIC of 0.5 μ g/mL.
Positive growth control	Only cells.
Negative growth control	Only medium (NB).

*RGnK: branching generation number (*n*) of a poly (ϵ -lysine) dendron with an arginine residue.

were used for inoculation where 200 μ L per disc was added to each well containing 100% NB to a final volume of 3 mL. To initiate cellular adhesion, cultures were maintained under static conditions for 1 hour at 37°C. The media were thereafter replaced with 3 mL 10% NB. Discs were then incubated under dynamic conditions at 37°C, 5% CO₂, for different times which were reflective of initial cellular colonisation and a maturing biofilm, 30 minutes and 48 hours, respectively. To remove nonadherent cells, discs were washed with PBS and the media was replaced at 24 h intervals with fresh 10% NB (w/v). The ability of each treatment to disperse preformed biofilms was then assessed. Cultures of *P. aeruginosa* were subsequently exposed to each of the four treatments (Table 1) for 1 h without agitation (in a final volume of 3 mL) at the aforementioned stages of biofilm development. Blank experimental samples (i.e., wells without cells or with untreated cells) were used as controls. After one hour incubation, discs were washed in PBS. Any adherent cells were dislodged from the material by vortex-mixing with 4 mm glass beads (Fisher Scientific, UK), and viable cells were recovered by spread plating which determined the number of CFU/mL.

2.6. Pyocyanin Production. The influence of generation 3 dendrons was assessed against pyocyanin production in planktonic cultures of *P. aeruginosa* (PAO1) [23]. The wild type strain, PAO1, was selected, as it is capable of producing significantly higher concentrations of pyocyanin than the isolate *P. aeruginosa* NCTC 10662 (data not shown). Overnight cultures of *P. aeruginosa* were diluted and then grown to an OD_{600nm} of approximately 0.5 (midlogarithmic phase), before being diluted again to 0.05 in 100 mL NB. Culture aliquots were then grown in the presence of each probe (Table 1) in a final volume of 7 mL. The samples were incubated for 20 h with agitation (120 rpm) at 37°C. To extract the produced pigment, pyocyanin, cell-free 0.2 μ m filtered *P. aeruginosa* supernatants were harvested and exposed to an

equal volume of chloroform (Fisher Scientific, UK). Samples were mixed vigorously and incubated at 37°C for 20 minutes. The organic phase was then collected and pyocyanin was re-extracted by adding 0.2 M HCl (Fisher Scientific, UK). Absorbance of the aqueous layer was then determined using a spectrophotometer (Jenway 6300, UK) at 520 nm; where a sample of sterile NB exposed to chloroform and HCl was used as a blank. The percentage of pyocyanin production relative to untreated control samples was calculated.

2.7. Statistical Analysis. Data obtained from each experiment were analysed by ANOVA and Student's *t*-test (where appropriate) to reveal statistical differences between treatments. *P* values of <0.05 were considered statistically significant, using Excel 2010 (USA) statistical software package.

3. Results

3.1. Mass Spectrometry of Dendrons. The synthesis of all generations of poly (ϵ -lysine) dendrons was confirmed by mass spectrometry. Dendrons became increasingly branched in line with the generation number, exposing a large number of functional amine groups on the outermost branching generation (Figures 1(a)–1(c)). Each completed reaction produced four batches yielding up to 70 mg individually. These products were obtained regardless of the synthesised generation number. Despite this difference in yield, a purity of at least 85% was confirmed for all products using HPLC (data not shown).

3.2. Biofilm Development. To show that these molecules do not interfere with primary metabolic functions such as bacterial replication, the growth response of *P. aeruginosa* biofilms was monitored. Although the presence and concentration of RGnK appeared to initially cause a change in the growth rate, it is important to note that the endpoint cell density was not affected (Figure 2). In addition, the number of

FIGURE 1: Chemical structures of hyperbranched poly (ε-lysine) dendrons consisting of an arginine (R) root and various generation numbers of lysine (GnK). (a) Generation 1 poly (ε-lysine) dendrons (RG1K), $C_{24}H_{50}N_{10}O_5$, calculated $m/z = 558.40$, found 558.43 [RG1K + 1H]¹⁺; (b) generation 2 poly (ε-lysine) dendrons (RG2K), $C_{48}H_{98}N_{18}O_9$, calculated $m/z = 1070.78$, found 1070.80 [RG2K + 1H]¹⁺, 535.91 [RG2K + 2H]²⁺; and (c) generation 3 poly (ε-lysine) dendrons (RG3K), $C_{96}H_{194}N_{34}O_{17}$, calculated $m/z = 2095.54$, Found 357.61 [RG2K + 3H]³⁺; 699.53 [RG3K + 3H]³⁺; and 524.90 [RG3K + 4H]⁴⁺. Chemical structures were generated using ChemBioDraw Ultra 12.0.

viable cells recovered from *P. aeruginosa* biofilms following treatment with increasingly branching generations of poly (ε-lysine) dendrons was not statistically different for biofilms supplemented with RGnK than for untreated biofilms over a test period of 48 h ($P \geq 0.9$, ANOVA). This suggests that the observed effects are not due to toxicity of the poly (ε-lysine) dendrons.

3.3. Biofilm Dispersion. The activity of all three generations of poly (ε-lysine) dendrons was investigated against 30 minutes and 48 h-old biofilms of *P. aeruginosa*, using the stainless steel disc assay. Then, sensitivity to ciprofloxacin, a second-generation fluoroquinolone antibiotic, was evaluated.

Biofilm disruption assays showed that exposure to RGnK renders biofilm bacteria more susceptible to this conventional antibiotic (Figure 3). The coadministration of RGnK and ciprofloxacin appeared to be more effective at disrupting preformed biofilms than the pretreatment strategy. This was indicated by a greater reduction in the percentage of viable cells recovered.

Pretreatment of immature biofilms (30-minutes old) with either RG1K or RG2K and subsequent exposure to ciprofloxacin partially enhanced susceptibility to the antibiotic by 1.4 and 2%, respectively (Figures 3(a)–3(b)). Whilst, in mature pre-RG1K or RG2K-treated biofilms, this enhancement rose to 10.8 and 4.9%, respectively. However, compared to ciprofloxacin alone, treatment of 48 h-old biofilms with

FIGURE 2: Influence of hyperbranched poly (ϵ -lysine) dendrons on growth of *Pseudomonas aeruginosa* NCTC 10662 (semilogarithmic graph). Dendrons of increasing lysine generation numbers were added (at an equimolar concentration of NH_2 groups, Table 1) during initial stages of cellular attachment (at approximately 1 h after setup) to stainless steel discs, as indicated by the arrow. The data represent mean values of experiments performed in duplicates. Error bars are means $\pm$ SD. $P \geq 0.9$, ANOVA.

a combination of either RG1K or RG2K and ciprofloxacin exhibited a significantly greater reduction in the percentage of viable cells recovered (17 and 18.1%, resp., $P \leq 0.02$, t -test).

The antimicrobial efficacy of ciprofloxacin was significantly increased in 30 minutes and 48 h-old biofilms exposed to RG3K and subsequently treated with ciprofloxacin by 5 and 25.1%, respectively (Figure 3(c)). However, the most potent effect was observed with a coadministration of RG3K and ciprofloxacin, which revealed the ability of the dendron and antibiotic to synergistically disrupt preformed biofilms. In combination, these treatments significantly increased the inhibitory effects of ciprofloxacin and therefore biofilm dispersion in 30 minutes and 48 h-old biofilms by 17 and 46%, respectively ($P < 0.001$, t -test). This is particularly significant as by day 2, mature biofilms were highly recalcitrant to ciprofloxacin exposure in the absence of RG3K with approximately 96% of cells resilient to treatment.

In all cases (with the exception of RG2K-tested against immature biofilms) the dendrons significantly enhanced antibiotic efficacy ($P \leq 0.04$, t -test). Exposure to RG3K showed consistent as well as maximum dispersion of established *P. aeruginosa* biofilms. However, the effects of RG1K and RG2K on biofilm dispersion (in the presence of ciprofloxacin) were very transient over the 48 h test period and these treatments exhibited minimal population reductions with similarities to ciprofloxacin-treated biofilms. Hence, only RG3K was considered for subsequent experiments.

3.4. Pyocyanin Production. The presence of RG3K within the growth medium was capable of inhibiting the production of pyocyanin by a substantial 86% compared to that of the untreated growth control (Figures 4(a)-4(b)). Importantly,

this dendron had no significant effects on cell growth which removes the possibility of selection pressures leading to the development of resistance ($P > 0.05$, data not shown). RG3K was also capable of reducing the production of this virulence factor by a further 8% in cultures grown in the presence of both dendron and ciprofloxacin, which highlights the quorum sensing disruption synergistic effects of these dendrons.

4. Discussion

The increased knowledge of QS systems and mechanism modulation in *P. aeruginosa* has presented an alternative and more subtle target for the control of infectious and antibiotic-resistant diseases [10, 12]. Interrupting these signalling pathways attenuates the expression of virulence factors that contribute to pathogenicity, without interfering with essential cell processes which, therefore, reduces the selection pressures that arise from the use of conventional antibiotics [11, 13, 15–17].

In the present study, three peptide-based poly (ϵ -lysine) dendrons were screened for antipathogenic activity by determining their effects on biofilm formation and then pyocyanin production. All three molecules showed positive activity against both QS-controlled phenotypes; however, the potency was found to increase in line with the generation number. This observation may be caused by the size of the molecules where dendrons of a higher generation number may be capable of interacting with a larger biofilm surface area (Figures 1(a)–1(c)). In addition, the molecular and chemical properties of all three molecules are similar, with the exception of molecular mass, which further evidences the aforementioned interaction. It is therefore envisaged that the increase in potency (in line with the generation number) is not caused by an increase in the number of amine outer groups as all three dendrons were applied at an equimolar concentration of primary amine groups, but rather by the size of the dendron itself.

Dendrons were shown to enhance the efficacy of ciprofloxacin against biofilms at various stages of development without affecting growth of *P. aeruginosa* (Figures 2 and 3). In agreement with previous studies, cells in the biofilm mode of growth conferred an increased antibiotic resistance, which became more apparent in line with biofilm maturation [6]. However, the simultaneous administration of RG3K and ciprofloxacin was shown to disperse approximately 50% of immature (30-minute-old) and mature (48 h-old) biofilms, where perhaps the maximal activity of RG3K was exhibited (Figure 3(c)).

RG3K effectively reduced the production of the QS-dependent pyocyanin (Figures 4(a)-4(b)), a toxic metabolite of *P. aeruginosa* that accounts for an increase in apoptosis of lung epithelial cells [24]. Perhaps there are two likely mechanisms by which this dendron accomplishes the reduction in pyocyanin production. RG3K might be able to interact with one or two QS pathways, *rhl* and/or *PQS*, by interfering with enzymatic components of the phenazine biosynthesis pathway [9, 10]. Another possibility is that RG3K

FIGURE 3: Effects of hyperbranched poly (ϵ -lysine) dendrons of various lysine generation numbers, in the presence or absence of ciprofloxacin, on biofilm formation of *Pseudomonas aeruginosa* (NCTC 10662). Biofilms of PA10662 were cultured on stainless steel discs for 30 minutes or 48 h. Discs were then treated for 1 h with poly (ϵ -lysine) dendrons (RGnK) at an equimolar concentration of amine groups; (a) RG1K, 1600 μ M; (b), RG2K, 800 μ M; (c), RG3K, 400 μ M; CIP, Ciprofloxacin (0.5 μ g/mL); RGnK >> CIP, administration of RGnK followed by ciprofloxacin (0.5 μ g/mL); RGnK + CIP, coadministration of RGnK and ciprofloxacin (0.5 μ g/mL). Percentage of viable cells recovered from each disc was then determined to assess the ability of each treatment to disperse established biofilms. The data represent mean values of experiments performed in duplicates. Error bars are means $\pm$ SD. * $P \leq 0.02$; ** $P = 0.04$; *** $P < 0.001$ (versus ciprofloxacin), Student's t -test.

might have direct attenuative effects on the expression of *phzM* or *phzS*, genes responsible for pyocyanin synthesis. In addition, there were no significant changes in bacterial replication corresponding to pyocyanin production, which further suggests a quorum sensing inhibition effect as the most plausible scenario.

Given that RG3K exhibited a significant effect on both QS-factors, biofilm formation and pyocyanin production, it suggests that neither a physical inhibition nor repression of components external to QS is probable. In addition, reducing the production of these virulence factors in a wild-type strain is considered a stringent evaluation of inhibitor efficacy. Elucidating the mechanism of action of these dendrons will therefore provide an insight into whether these molecules are global QS regulators or have distinct targets in the QS system.

5. Conclusions

In summary, these findings have highlighted the potential of hyperbranched poly (ϵ -lysine) dendrons, in particular RG3K, as disrupters of two QS-mediated phenotypes (biofilm formation and pyocyanin production) in *P. aeruginosa*. In the presence of a conventional antibiotic, dendrons displayed synergistic effects and increased the efficacy of ciprofloxacin by almost 50% in antibiotic resistant 48 h-old-biofilms of *P. aeruginosa*. In addition, the production of a potent virulence factor (pyocyanin) was reduced by approximately 86% when cells were grown in the presence of the 3rd generation dendron. These therefore support the concept that targeting QS offers a promising alternative strategy in reducing bacterial infections. Such molecules hold promise and with further

FIGURE 4: Quorum sensing inhibitory activity of poly (ϵ -lysine) dendrons against pyocyanin production. Cultures of *Pseudomonas aeruginosa* (PAO1) were grown in the presence or absence of either generation 3 poly (ϵ -lysine) dendrons (RG3K, 400 μ M) and/or ciprofloxacin (CIP, 0.5 μ g/mL). The percentage production of pyocyanin was determined 20 h after treatment. (a) Effect of poly (ϵ -lysine) dendrons alone and in combination with ciprofloxacin on pyocyanin production in PAO1. Cultures were exposed to (i) Nontreated control, (ii) RG3K (400 μ M), (iii) Ciprofloxacin (0.5 μ g/mL), and a combination of RG3K (400 μ M) and ciprofloxacin (0.5 μ g/mL). (b) Quantitative analysis of pyocyanin production in PAO1. The data represent mean values of experiments performed in triplicates. Error bars are means $\pm$ SD. * $P = 0.006$, Student's t -test.

development may provide novel antivirulence agents capable of addressing a major therapeutic target. On-going studies are currently focused on increasing the potency of RG3K.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was funded as part of a University of Brighton, School of Pharmacy and Biomolecular Sciences, funded PhD studentship. The authors are grateful to Howard Dodd for assisting with the mass spectrometry analysis and Cinzia Dedi, Heather Catty, Rowena Berterelli, and Joseph Hawthorne for their technical assistance in microbiology.

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