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Article

Ecological Niche-Inspired Genome Mining Leads to the Discovery of Crop-Protecting Nonribosomal Lipopeptides Featuring a Transient Amino Acid Building Block

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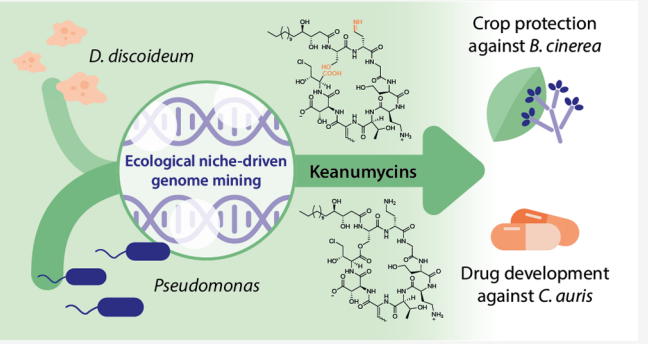
ABSTRACT: Investigating the ecological context of microbial predator–prey interactions enables the identification of microorganisms, which produce multiple secondary metabolites to evade predation or to kill the predator. In addition, genome mining combined with molecular biology methods can be used to identify further biosynthetic gene clusters that yield new antimicrobials to fight the antimicrobial crisis. In contrast, classical screening-based approaches have limitations since they do not aim to unlock the entire biosynthetic potential of a given organism. Here, we describe the genomics-based identification of keanumycins A–C. These nonribosomal peptides enable bacteria of the genus *Pseudomonas* to evade amoebal predation. While being amoebicidal at a nanomolar level, these compounds also exhibit a strong antimycotic activity in particular against the devastating plant pathogen *Botrytis cinerea* and they drastically inhibit the infection of *Hydrangea macrophylla* leaves using only supernatants of *Pseudomonas* cultures. The structures of the keanumycins were fully elucidated through a combination of nuclear magnetic resonance, tandem mass spectrometry, and degradation experiments revealing an unprecedented terminal imine motif in keanumycin C extending the family of nonribosomal amino acids by a highly reactive building block. In addition, chemical synthesis unveiled the absolute configuration of the unusual dihydroxylated fatty acid of keanumycin A, which has not yet been reported for this lipodepsipeptide class. Finally, a detailed genome-wide microarray analysis of *Candida albicans* exposed to keanumycin A shed light on the mode-of-action of this potential natural product lead, which will aid the development of new pharmaceutical and agrochemical antifungals.



INTRODUCTION

Over- and misuse of anti-infectives in health care,[1] livestock,[2] and plant[3,4] agriculture has led to the increase and global distribution of antimicrobial resistance in microorganisms. The worrisome prevalence of multidrug-resistant pathogens is the cause of the antimicrobial resistance crisis, which has severe socioeconomic implications for human society.[5,6] In the United States alone, approximately 3 million individuals are infected with antimicrobial-resistant germs each year, causing 35,000 causalities.[7] In addition, many patients will suffer from severely debilitating and often long-term side effects from the protracted illness and intensive treatment. Combating antimicrobial resistance relies on a combination of strategies ranging from the reduction of anti-infective use in general, as well as creating new antimicrobials. Importantly, treatment options for stockbreeding and plant agriculture that do not rely on antimicrobials used in human medicine are desperately needed. Unfortunately, the development of antimicrobials has been discontinued by a majority of pharmaceutical companies as it was deemed unprofitable. One of the main reasons for this decline can be attributed to the fact that the majority of all marketed antibiotics are natural products or their derivatives. Unfortunately, the costs associated with finding new natural product-based lead structures have increased significantly, due to extraordinary rediscovery rates of already known substances or compound classes.[8,9]

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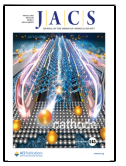

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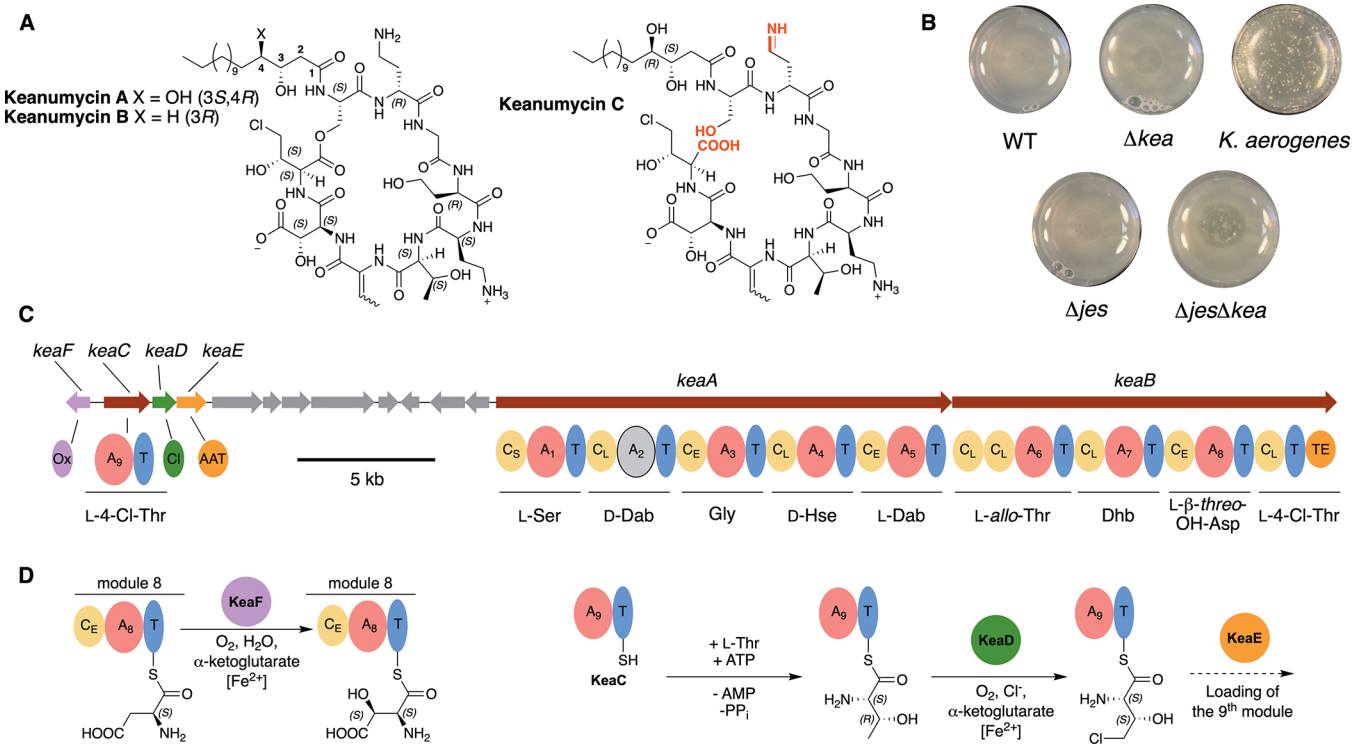

Figure 1. *Pseudomonas* sp. QS1027 produces keanumycins. (A) Structure of keanumycins A−C. Main differences between keanumycin A−B and keanumycin C are highlighted in red. (B) Comparison of edibility using *D. discoideum* as a predator. *Pseudomonas* sp. QS1027 WT and corresponding mutants, which lack the ability to produce different natural products, were tested. *K. aerogenes* served as a positive control. (C) Architecture of the *kea* BGC, and deduced NRPS assembly line. Depicted amino acid selectivity and stereochemistry are derived from experimental data and do not represent the bioinformatics prediction, which is L-Ser-D-Dab-Gly-D-Xxx-L-Dab-L-Thr-D-Thr-L-Asp-L-Thr. The gray A domain (second module) was deleted to generate mutant Δ*kea*. (D) Proposed enzymatic transformations necessary for the modification of the last two C-terminal amino acids of the keanumycins. For a full biosynthetic pathway, please see the Supporting Information (Figure S57).

Instead of brute force screenings of microbial monocultures,[10] tapping into the metabolome associated with mutualistic or antagonistic ecological interactions between prokaryotes and eukaryotes is an effective approach to unlock the antibiotic treasure trove of nature.[11−15] Especially when in combination with genome mining, structure prediction of natural products from genetic information using bioinformatics tools is greatly facilitated.[16] As a consequence, the biosynthetic potential of symbiotic bacteria can be uncovered in a timely fashion.[17] Notably, amoeba−bacteria predator−prey interactions have been fruitful sources of novel natural products. Amoeba are single-celled eukaryotes that prey on smaller organisms and are ubiquitous in soil and freshwater. Therefore, bacteria sharing the same habitat as amoeba are exposed to a high selection pressure that fosters either evolution of defense mechanism against grazing or coevolution to form a symbiotic bond with the predator.[18] Various amoeba species engage in mutual[19] and antagonistic[20−23] relationships with bacteria that are mediated by natural products.

Here, we give a detailed account on how genome mining of a *Pseudomonas* strain associated with the social amoeba *Dictyostelium discoideum* led to the discovery and isolation of potent antimicrobial nonribosomal lipopeptides (NRLPs) belonging to the heterogenous syringomycin group.[24−26] Using a combination of nuclear magnetic resonance (NMR), mass spectrometry (MS), degradation experiments, and organic synthesis, we were not only able to elucidate the structure of two new NRLPs but also identify a transient derivative, which shines light on previously unreported biochemical aspects of nonribosomal peptide synthetases (NRPSs). These natural products are powerful pest control agents protecting plants from the devastating fungal phytopathogen *Botrytis cinerea,* and their antimycotic properties make them good lead structure candidates for the development of antifungal drugs. In fact, the new NRLPs are so efficient at killing different microbial species that we named them keanumycins in tribute to the actor Keanu Reeves, who played many iconic killers in his cinematic career (Figure 1A).

RESULTS

Bioinformatics Analysis of *Pseudomonas* sp. QS1027.

In a previous study, we found that the strain *Pseudomonas* sp. QS1027, which was isolated from fruiting bodies of the social amoeba *D. discoideum*,[27] produces the amoebicidal natural product jessenipeptin.[28] Interestingly, the null mutant Δ*jes*, which is not able to produce jessenipeptin, was still toxic for *D. discoideum* (Figure 1B). In contrast to the food source *Klebsiella aerogenes*, which is used for the propagation of *D. discoideum*, the amoebae were not able to graze on the Δ*jes* strain and to form fruiting bodies. Consequently, we concluded that *Pseudomonas* sp. QS1027 is able to biosynthesize additional amoebicidal natural products. Mining of the corresponding genome (GenBank accession number PHSU00000000) using antiSMASH[29] revealed multiple biosynthetic gene clusters (BGCs). Manual curation (see Supporting Information Figures S1 and S2) uncovered a BGC that was split between two contigs and displayed high similarity with a nunamycin gene cluster, which is required

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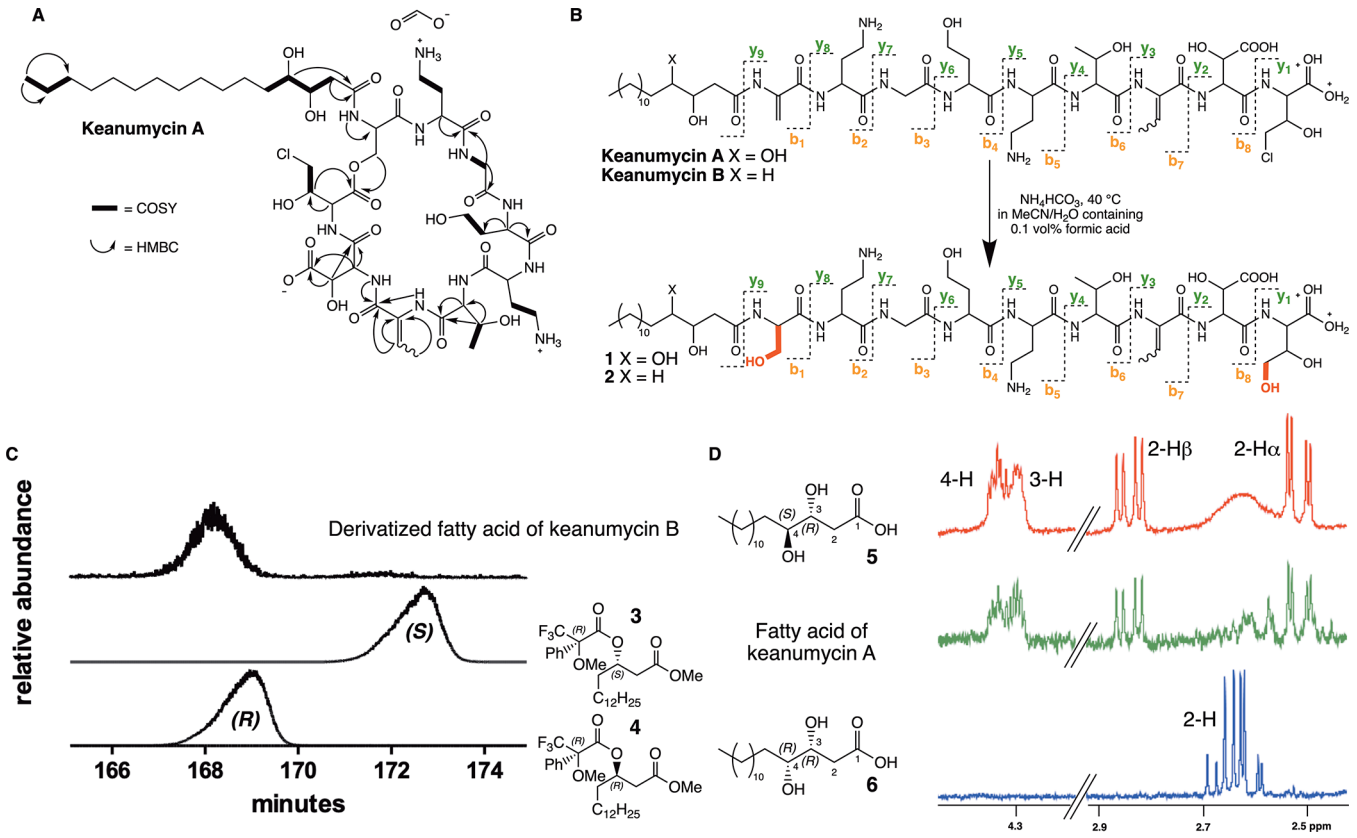

Figure 2. Structure elucidation of keanumycin A and B. (A) Key COSY and HMBC correlations observed during NMR analysis of keanumycin A. (B) Fragmentation pattern of keanumycin A and B as well as of their corresponding degradation products **1** and **2**. (C) GC−MS traces of derivatized fatty acid moiety of keanumycin B and traces of the corresponding synthetic (*S*)- and (*R*)-configured 3-hydroxyhexadecanoic acid derivatives **3** and **4**. Relative abundances of the extracted-ion chromatogram representing a diagnostic fragment (*m*/*z* 237) are shown. (D) Selected sections of the ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of the two carboxylic acid standards **5** and **6** and the isolated fatty acid of keanumycin A including annotation of diagnostic peaks.

for the production of antifungal NRLPs belonging to the syringomycin family.[30] Since fungi and social amoebae share many similarities with respect to cellular structures, and many antifungal agents target *D. discoideum*, we reasoned that this NRPS gene cluster may be responsible for the biosynthesis of another amoebicidal natural product.[31]

To gain insight into the function of this NRPS gene, we first closed the gap between the two contigs by PCR amplification of the intracontig region and subsequent Sanger sequencing of the amplicons. Thus, we obtained the complete NRPS BGC sequence (see the Supporting Information for detailed procedures). The resulting contig (GenBank accession number MW331495) shows 100% similarity with the nunamycin BGC (Figure S4). The keanumycin BGC consists of six genes (*keaA*−*F*), which code for two NRPSs (KeaA and B) and four enzymes (KeaC−F) involved in the modification of the two C-terminal amino acids (Figure 1C). KeaA and B contain five and three full modules, respectively, which consist of a condensation (C), adenylation (A), and thiolation (T) domain and select for individual amino acids. KeaB contains a fourth module composed of only a C and a T domain followed by a thioesterase domain, which may catalyze the macrolactonization of the oligopeptide after assembly.[32] The last incomplete module of KeaB is therefore likely loaded by *trans*-acting enzymes in analogy to the syringomycin biosynthetic machinery (Figure 1D). KeaC would activate L-Thr via its A domain and would covalently attach the amino acid through a thioester to its T domain.[33] The amino acid would then subsequently be chlorinated by the nonheme Fe^{2+} α-ketoglutarate-dependent enzyme KeaD to generate 4-Cl-L-Thr, which would then be transferred by the amino-acyltransferase KeaE to the T domain of the last module of KeaB.[34] KeaF probably oxidizes Asp to L-*β*-*threo*-OH-Asp.[35]

Molecular Biological Evaluation of the *kea* BGC. To test if the keanumycin BGC allows the production of an amoebicidal compound, we generated an in-frame deletion of the gene fragment coding for the A domain of the second module of *keaA* resulting in the mutant Δ*kea* (Figure 1C). The Δ*kea* mutant itself is still toxic; however, *D. discoideum* is able to graze on the double mutant Δ*jes*Δ*kea* (Figure 1B). Interestingly, comparison of the metabolic profiles of an ethyl acetate extract of wild type (WT) and Δ*kea* mutant cultures revealed no major differences and showed no bioactivity against *D. discoideum*. After realizing that the new natural products could be too hydrophilic and are therefore not extractable with our standard method, we screened different absorbent resins (Amberlite XAD4 extracts keanumycin A and B) and solvents (*n*-butanol can extract all keanumycins) to develop a suitable extraction protocol. Using the new method, we were able to see an absence of metabolites in the Δ*kea* mutant compared to the WT strain, which turned out to be the keanumycins (Figure S8). Hence, the combination of genome mining with a functional bioassay was crucial to identify these hydrophilic NRLPs, which,

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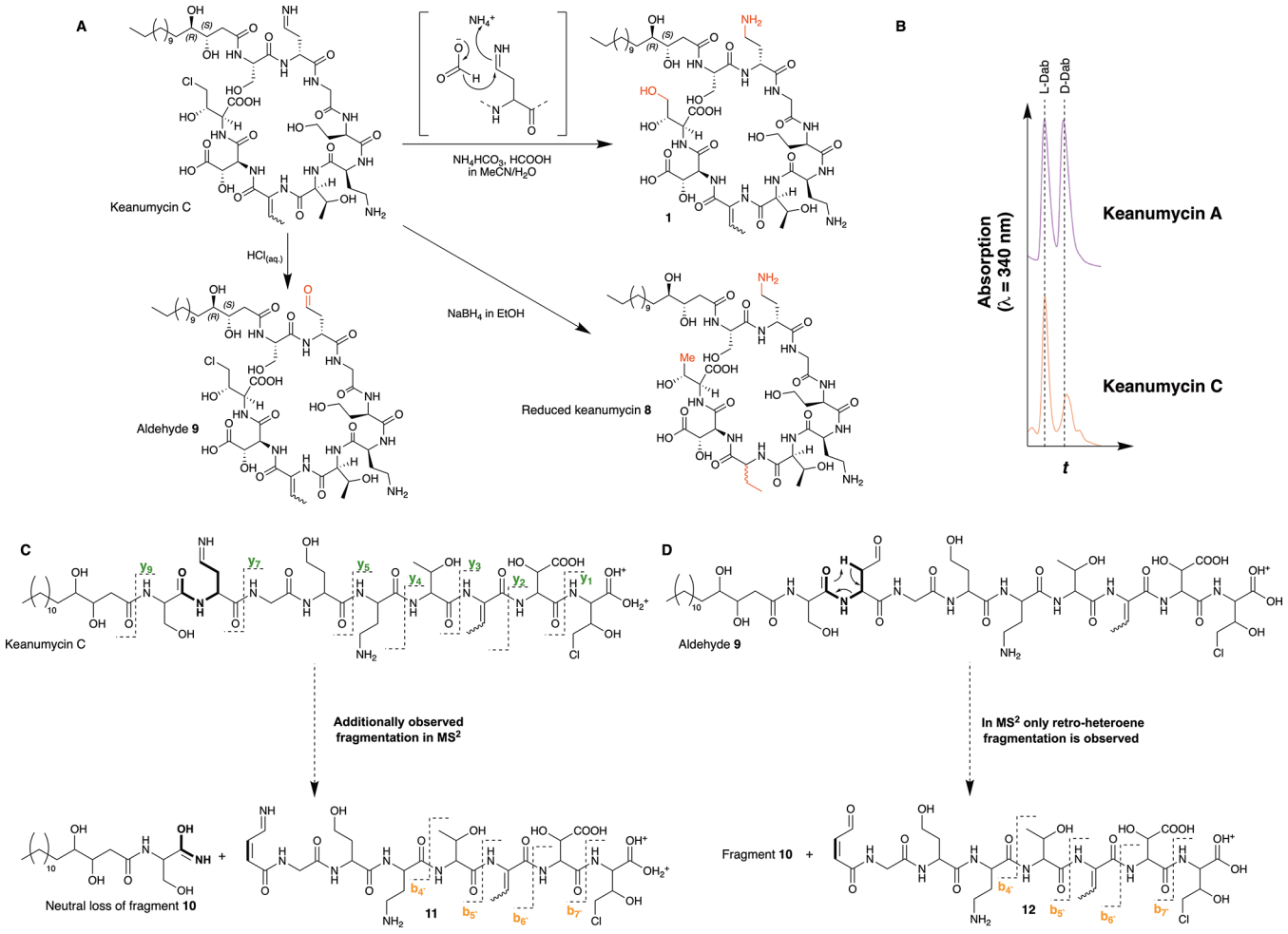

Figure 3. Structure elucidation of keanumycin C. (A) Degradation experiments of keanumycin C. Functional groups are highlighted in red to clarify chemical transformations after degradation experiments (see Figure S7 for a full account). (B) Comparison of a section of liquid chromatography-MS traces of Marfey's reagent derivatized amino acids derived from keanumycin A and C. (C) Fragmentation pattern of keanumycin C. (D) Fragmentation pattern of aldehyde **9**.

otherwise, would have been overlooked based on bioassay-guided fractionation.

Since the keanumycin BGC is adjacent to the jessenipeptin BGC, we hypothesized that both BGCs are regulated by the same LuxI/LuxR-type quorum sensing (QS) system previously described for the biosynthesis of jessenipeptin.[28] Indeed, using a set of regulatory gene knockout mutants and the signaling molecule *N*-hexanoyl-homoserine lactone, we were able to show that the production of jessenipeptin and the keanumycins is regulated by the same QS system (Figure S9). Furthermore, the production of the virginiafactins, which are NRLPs also produced by this strain, is not under the control of this particular QS system and is not impaired by the in-frame deletion of gene fragments that were used to generate the Δ*kea* and Δ*jes*Δ*kea* mutants (Figures S8 and S9).[36]

Structure Elucidation of the Keanumycins A and B. We isolated keanumycin A (*m/z* 583.7892 $[M + 2H]^{2+}$ consistent with the molecular formula $C_{49}H_{84}ClN_{11}O_{19}$) and impure keanumycin B (*m/z* 575.7900 $[M + 2H]^{2+}$; $C_{49}H_{84}ClN_{11}O_{18}$), which could not be separated from an unknown metabolite (Figures 1A and S10). We were also able to isolate keanumycin C (*m/z* 591.7866 $[M + 2H]^{2+}$; $C_{49}H_{84}ClN_{11}O_{20}$). Unfortunately, keanumycin C slowly degrades in solution or when concentrated to dryness, yielding a poorly soluble solid, which prevented us from recording NMR spectra. Therefore, a detailed NMR analysis was only performed on keanumycin A. Important correlations in the $^{1}H,^{1}H$-correlation spectroscopy (COSY) experiment disclosed the presence of a 3,4-dihydroxylated linear fatty acid, which is a rare motif in NRLPs and only described for two other members of the syringomycin family (Figures 2A and S31).[24,37] The $^{1}H,^{13}C$-heteronuclear multiple bond correlation (HMBC) experiment allowed us to establish a majority of the linear amino acid sequence, the attachment point of the fatty acid and provided evidence for the position of the lactone ring between the carbonyl moiety of the 4-Cl-L-Thr and the alcohol side chain of the Ser (for a full assignment of all ^{1}H and ^{13}C signals please see Table S20). These data were confirmed by high-resolution tandem mass spectrometry ($HRMS^2$) experiments, which showed a fragmentation pattern in accordance with the NMR data and the bioinformatics prediction (Figures 2B and S15). Saponification of keanumycin A under mild conditions led to the expected dechlorinated and linear lipopeptide **1**, whose planar structure was confirmed by further $HRMS^2$ experiments.

Acid-mediated degradation of keanumycin A followed by derivatization of the resulting amino acids with Marfey's reagent and comparison with commercial or synthetic

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standards[38−40] established the presence of the following amino acids: 1× Gly, 1× L-Ser, 1× L-Dab, 1× D-Dab, 1× D-Hse, 1× L-*allo*-Thr, 1× 4-Cl-L-Thr, 1× L-*β*-*threo*-OH-Asp (Figure S32). With these data in hand, the configurations of all amino acids, with the exception of the L- and D-Dab moieties, are unambiguously determined. Bioinformatics analysis of the keanumycin BGC indicated that the Dab at position two of the peptide chain should be assigned the D-configuration because the third module of *keaA* is predicted to contain a condensation/epimerization (C_E) domain, which not only condenses a Dab with a Gly but also epimerizes the preceding L- to a D-amino acid.[41] In contrast, the Dab at position five should be L-configured, since the first module of KeaB is predicted to contain a tandem C_L domain, which lacks epimerase activity and should not introduce a D-amino acid into the nascent peptide chain. The presence of the tandem C_L domain in the NRPS assembly line has been reported for other members of the syringomycin class[42] of cyclic lipopeptides, such as thanamycin[43] or nunamycin.[44] To our knowledge, the biosynthetic details of the condensation domain duplication have not yet been investigated, and it remains elusive, if one or both domains are crucial for the condensation reaction.

Before focusing on the stereochemistry of the fatty acid of keanumycin A, we first investigated the structure of keanumycin B. The molecular mass difference of 16 Da between keanumycin A and B indicates that both natural products differ by a single oxygen atom. Hence, we concluded that both natural products presumably share the same amino acid sequence but vary in the oxidation state of their fatty acid comparable to pseudomycin A and B.[37] Hydrolysis of keanumycin B followed by derivatization with Marfey's reagent indeed yielded the same amino acid composition as found for keanumycin A (Figure S34). $HRMS^2$ analysis of intact and saponified keanumycin B **2** confirmed the amino acid sequence and hinted toward a monohydroxylated fatty acid connected to the C-terminal Ser, which was further validated by a COSY experiment of the impure keanumycin B (Figures 2B and S31).

To determine the stereochemistry at the *β*-position of the fatty acid of keanumycin B, we hydrolyzed the NRLP, isolated the lipid, converted the acid into a methyl ester using trimethylsilyldiazomethane, and esterified the alcohol function using Mosher's acid chloride (see Figures S43 and S44). The derivatized fatty acid was subjected to gas chromatography (GC)-MS and compared to synthetic standards **3** and **4** (see Figure 2C and Scheme S1). Thus, we established the nature of the fatty acid of keanumycin B to be (*R*)-3-hydroxyhexadecanoic acid.

With one stereogenic center of the lipid moiety elucidated, we went on to deduce the absolute configuration of the 3,4-dihydroxy moiety of keanumycin A, whose stereochemistry has never been assigned in related compounds (e.g., pseudomycin A and syringostatin B). To this end, we modified a previously published method for the synthesis of the Japanese orange fly lactone.[45] Starting from the chiral pool material D-gluconolactone, we synthesized (3*R*,4*S*)- and (3*R*,4*R*)-3,4-dihydroxyhexadecanoic acid, **5** and **6**, respectively (see Scheme S2).[46] Comparison of the 1D ^{1}H-NMR spectra of **5** and **6** with a sample derived from basic hydrolysis (acidic conditions led to decomposition) of keanumycin A clearly showed that the vicinal system is *trans* configured. Thus, we concluded that keanumycin A contains a (3*S*,4*R*)-3,4-dihydroxyhexadecanoic acid moiety.

Structure Elucidation of Keanumycin C. Since keanumycin A and C have a mass difference of 16 Da, we first thought that keanumycin C could bear a trihydroxylated lipid chain. However, after subjecting keanumycin C to various degradation and MS experiments, it became evident that this NRPS product does not contain a macrolactone moiety. Instead, keanumycin C is the first described linear NRLP with a terminal imine moiety. This adds 2-amino-4-iminobutanoic acid to the amino acid building block repertoire, found in NRPs (in this particular case at position 2). Hydrolysis of keanumycin C under mild basic conditions in the presence of formate leads to the linear and dechlorinated degradation product **1**, which is the same degradation product obtained after saponification of keanumycin A (Figure 3A). This observation can only be explained by an Eschweiler−Clarke type reaction using formate as the reducing agent and keanumycin C being a linear lipopeptide.[47] Reduction of keanumycin C with $NaBH_4$ generated the expected reduced derivative **8**. Treatment with diluted HCl led to the formation of the corresponding aldehyde **9**. Acidic hydrolysis of keanumycin C followed by derivatization with Marfey's reagent yielded the following amino acid composition:1× Gly, 1× L-Ser, 1× L-Dab, 1× D-Hse, 1× L-*allo*-Thr, 1× 4-Cl-L-Thr, 1× L-*β*-*threo*-OH-Asp. Compared to keanumycin A, keanumycin C lacks 1× D-Dab moiety but generated a derivatized aspartate-4-semialdehyde (Figures 3B, S41 and S42). In contrast, when the reduced compound **8** was subjected to the same derivatization procedure 1× L-Dab and 1× D-Dab could be detected (Figure S40).

$HRMS^2$ experiments of keanumycin C further validated the presence of an imine moiety as the fragmentation pattern distinctly differs from keanumycin A and B. Two y fragments (y_8 and y_6) and all b fragments were absent in the spectrum of keanumycin C when using the previously applied conditions (Figure 3C). Furthermore, a retro-heteroene reaction leading to the neutral loss of fragment **10** was observed.[48] The remaining ion **11** fragmented further into prominent b' fragments (Figure S18). Aldehyde **9** yielded an even more unique fragmentation pattern, as no conventional b or y fragments were detected (Figure 3D). The retro-heteroene reaction was dominant and exclusively yielded the main fragment **12**, which further fragmented in analogy to ion **11** (Figure S29).

Finally, these findings also confirm the predicted absolute configuration of the two Dabs, because the imine at position two results in the absence of the D-Dab during amino acid composition analysis of keanumycin C using Marfey's method.

Bioactivity of Keanumycin A. The antimicrobial activity of keanumycin A was first tested against *D. discoideum* (IC_{50} = 4.4 nM) and two human pathogenic acanthamoeba (*A. castellanii* IC_{50} = 2.0 *μ*M and *A. comandoni* IC_{50} = 3.1 *μ*M). Keanumycin A proved to be strongly amoebicidal, especially against the amoeba from which the producer strain was isolated.

To further explore the antimicrobial potential of the keanumycins, a qualitative screen of different microorganisms was performed using a disk diffusion assay (Table S24). Keanumycin A is weakly active against Gram-positive bacteria (*Bacillus subtilis*, *Enterococcus faecalis*, and *Mycobacterium vaccae*) and shows no activity against the Gram-negative bacterium *Pseudomonas aeruginosa* but strongly inhibits the growth of multiple fungi (*Sporobolomyces salmonicolor*, *Candida albicans*, *and Penicillium notatum*), which is a property most

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members of the syringomycin family share.[49,50] Encouraged by these results, we determined minimum inhibitory concentrations (MICs) for keanumycin A against different human pathogenic fungi and fungal phytopathogens (Tables 1 and S25). Keanumycin A is active at low concentrations (0.9 μM or 1 mg L^{-1}) against all *Candida* spp. tested (Table 1). The natural product was even able to inhibit the proliferation of a clinical isolate of *Candida auris*, which is resistant against the antimycotic drug fluconazole (Table S25). In combination with the comparatively low antiproliferative activity (HUVEC: GI_{50} > 43 μM and K-562, GI_{50} = 23 μM) as well as cytotoxicity (HeLa, CC_{50} = 30 μM) and keanumycin A being able to protect human epithelial cells in an in vitro infection model[51] from *C. albicans* induced damage (Figures S51 and S52), this natural product represents a promising lead for the development of new drugs against a broad range of *Candida* spp. infections.[52−58] In contrast, keanumycin A is less active against other opportunistic pathogenic fungi such as *Aspergillus fumigatus* and had no effect on the growth of *Rhizopus arrhizus* at the concentrations tested.[59]

Table 1. MIC Values of Keanumycin A against Different Human Fungal Pathogens and Fungal Phytopathogens[a]

fungal species strain	MIC [μM]
Candida auris NRZ-2021-353	0.86 (1 mg L^{-1})
Candida glabrata NRZ-2021-359	0.86 (1 mg L^{-1})
Candida parapsilosis ATCC 22019	0.86 (1 mg L^{-1})
Candida tropicalis NRZ-2021-360	0.86 (1 mg L^{-1})
Aspergillus fumigatus ATCC 204305	3.42 (4 mg L^{-1})
Rhizopus arrhizus NRZ-2021-366	>6.85 (>8 mg L^{-1})
Scedosporium apiospermum NRZ-2016-079	6.85 (8 mg L^{-1})
Fusarium annulatum NRZ-2021-361	>6.85 (>8 mg L^{-1})
Botrytis cinerea SF011406	0.07 (80 μg L^{-1})
Alternaria solani SF003858	1.07 (1.25 mg L^{-1})

[a]All strains, except for the *B. cinerea* and *A. solani* strain, were incubated according to the procedure of The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 35 °C). The *B. cinerea* and *A. solani* strains were incubated according to the procedure of the National Committee for Clinical Laboratory Standards (NCCLS, 22 °C).

Interestingly, keanumycin A is extremely effective in inhibiting *Botrytis cinerea* (MIC = 69 nM / 80 μg L^{-1}), which is a devastating phytopathogen that causes Botrytis blight, also known as gray mold, in over 1000 plant species[60] and was ranked as the second most important plant-pathogenic fungus.[61] To a lesser extent, it also inhibited the growth of *Alternaria solani*, the causative agent of early blight in tomatoes and potatoes,[62] but not of *Fusarium annulatum*, which can infest different fruits[63,64] and is additionally an opportunistic human pathogen.

Inspired by the inhibition properties of keanumycin A against *B. cinerea,* we wanted to test if the keanumycins can be used as pest control agents and protect plants from this phytopathogen.

Since *B. cinerea* is encountered worldwide, Botrytis blight is one of the most common diseases of greenhouse crops and can cause serious economic damage.[65] As a model organism, we chose *Hydrangea macrophylla*, which is a flowering plant native to Asia that is used in gardening as a very popular ornamental plant and is routinely grown in greenhouses.[66] Unfortunately, isolation of keanumycin A involves multiple purification steps and only yields low amounts of pure natural product (0.1−0.2 mg L^{-1}). Therefore, we reasoned that a sterile filtrate of the fermented supernatant of a *Pseudomonas* sp. QS1027 mutant, which is able to biosynthesize the keanumycins but not capable of producing jessenipeptin and virginifactins, could contain sufficient amounts of highly antifungal NRLPs to inhibit the growth of *B. cinerea*. This assumption was supported by results of coculturing experiments on solid media (Figure S53). In these experiments, the wild type strain was able to inhibit growth of *B. cinerea,* whereas the Δ*jes*Δ*kea* mutant had no effect on growth of the phytopathogen. Interestingly, the Δ*kea* mutant, which still produces jessenipeptin, repressed the growth of the fungus albeit only slightly. In order to validate this result, we determined the MIC of jessenipeptin against *B. cinerea* (10 mg L^{-1}/5.2 μM), which is two orders of magnitude higher than that of keanumycin A.

Indeed, when we applied two different supernatants with varying concentrations of keanumycin A to mycelium plugs of *B. cinerea*, which were used to infect detached leaves of *H. macrophylla*, we could see a drastic inhibition of disease-induced lesions in a concentration-dependent fashion (Figure 4). This experiment shows that the fermentation broth of *Pseudomonas* sp. QS1027 can be used to fight Botrytis blight, which represents a cost-efficient, sustainable, and environmentally friendly alternative to antifungal agrochemicals.

Studies on the Mechanism of Action of Keanumycin A. Mode-of-action studies of keanumycin A were facilitated by the observation that it inhibits multiple clinically relevant *Candida* species. Amongst those was *C. albicans*[67] strain SC5314 (MIC = 1.72 μM), which constitutes an important and well-studied model organism in fungal pathogenesis, allowing us to studying the mode of action of keanumycin A.[68,69] We initially compared the transcription rate of *C. albicans* in the presence and absence of a sublethal keanumycin A concentration using a genome-wide microarray (Figure 5).[70] General metabolic pathways were heavily impacted upon exposure to keanumycin A. Ribosome biogenesis was down-regulated, whereas protein as well as fatty acid degradation, endocytosis, and autophagy were increased, indicating a severe general stress response that showed similarities to an unfolded protein response (UPR). The UPR in *C. albicans* can be triggered by the amphiphilic antifungal natural product tunicamycin, which induces endoplasmic reticulum stress by inhibiting *N*-glycosylation causing accumulation of misfolded proteins. The protein kinase Ire1 senses those proteins, activating the UPR via the transcription factor Hac1.[71] To see if keanumycin A triggers the same canonical Ire1-Hac1 UPR pathway, we looked at the splicing of *HAC1* mRNA, which is regulated by Ire1 (Figure S55).[72] Compared to the positive tunicamycin control, we could not observe splicing in *HAC1* mRNA, which makes it unlikely that keanumycin A triggers this pathway.

Since no other specific signaling pathway stood out, we screened homozygous knockout mutants for significant growth differences in the absence and presence of a sublethal concentration of the NRLP. The most prominent growth differences were observed for Δ*mkc1*, Δ*plb5*, and Δ*pmt6*. *MKC1* codes for a crucial protein in the cell wall integrity pathway, which is a central signaling pathway for adapting to multiple cell wall stressors, indicating that keanumycin A induces cell wall stress and is able to trigger this signal transduction cascade (Figure S56).[73] *PLB5*[74] is an important gene for phospholipid homeostasis and *PMT6*[75] codes for a

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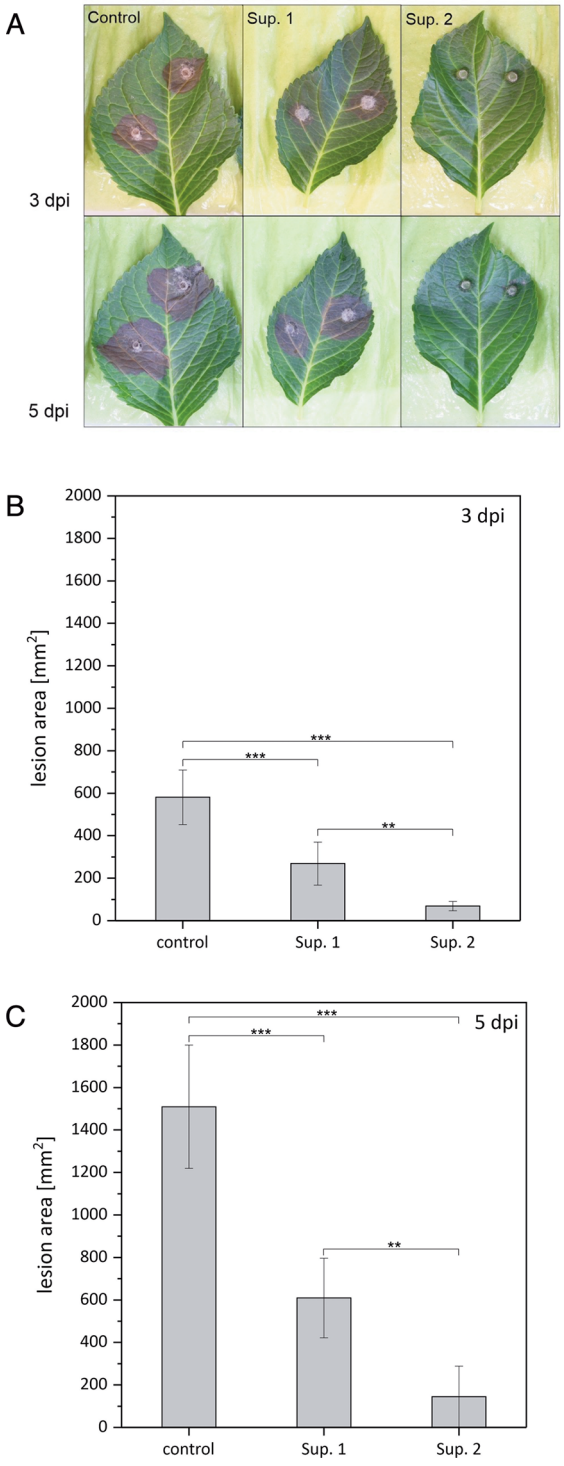

Figure 4. Ability of *Pseudomonas* sp. QS1027 fermentation broth to inhibit Botrytis blight. (A) Representative images of detached *H. macrophylla* leaves infected with mycelium plugs of *B. cinerea* 3- and 5-days post infection (dpi). Control medium contains no keanumycin A, whereas supernatant (Sup.) 1 and 2 contain 0.8 and 2.8 mg L^{-1} keanumycin A, respectively. (B and C) Graphs representing the lesion area induced by *B. cinerea* after 3 and 5 dpi. The mean with standard deviation is presented. Single-factor analysis of variance was performed (Tukey-HSD-test and Bonferroni correction) to evaluate statistical significances between different groups (control, Sup. 1, Sup. 2) at a ***$p < 0.001$ and **$p < 0.01$ ($0.001 \leq p < 0.01$).

Figure 5. Pathways enriched in *C. albicans* upon treatment with keanumycin A. Global short-term transcriptional response of *C. albicans* to a 1-h sublethal dose exposure of keanumycin A. The graph depicts KEGG pathways (*y*-axis), that manually curated maps of networks of cellular processes, that were significantly enriched and over-represented in a gene set enrichment analysis of genes 2-fold up or down regulated (vs untreated). Abbreviation: KEGG, Kyoto Encyclopedia of Genes and Genomes.

transmembrane protein mannosyltransferase, which is involved in the construction of the outmost mannan cell wall layer in *C. albicans*.[76] This demonstrated that the phospholipid composition of the fungal plasma membrane and the structural integrity of the fungal cell wall are important factors that protect fungi from the toxicity of keanumycin A.

To test our assumption, we focused on the altered expression of individual genes in the microarray data that influence the membrane fluidity via regulation of the biosynthesis of sphingolipids and ergosterol (Table 2). In

Table 2. Transcription Rate Change (log2-Fold Change) of Individual Genes of *C. albicans* Treated with Keanumycin A Compared to Nontreated

transcription rate change	gene	function
0.66	*SYR2/SUR2*[80]	sphingolipid biosynthesis
−1.10	*SCS7/FAH1*[81]	sphingolipid biosynthesis
−0.44	*IPT1*[82]	sphingolipid biosynthesis
0.55	*ORM1*[83]	sphingolipid biosynthesis
−1.83	*ERG3*[84]	ergosterol biosynthesis

particular, the upregulation of *ORM1*, which is a global repressor of the sphingolipid biosynthesis in yeasts, indicates a remodeling of membrane composition upon exposure to keanumycin A.[77] Interestingly, the selected genes also convey resistance to syringomycin E, which belongs to the same natural product class as keanumycin A, in *Saccharomyces cerevisiae*. Consequently, the mechanism of action of keanumycin A is likely comparable to syringomycin E, which is an antifungal that forms ion channels in the plasma membrane leading to the collapse of the membrane potential,[78] as it triggers a similar regulation of *S. cerevisiae* resistance gene homologs in *C. albicans*.[79]

To finally examine if keanumycin A is a membrane-active compound, we performed a membrane permeabilization assay using SYTOX Green (Figure 6). As a cell-impermeant dye, SYTOX Green enters the cell upon loss of membrane integrity

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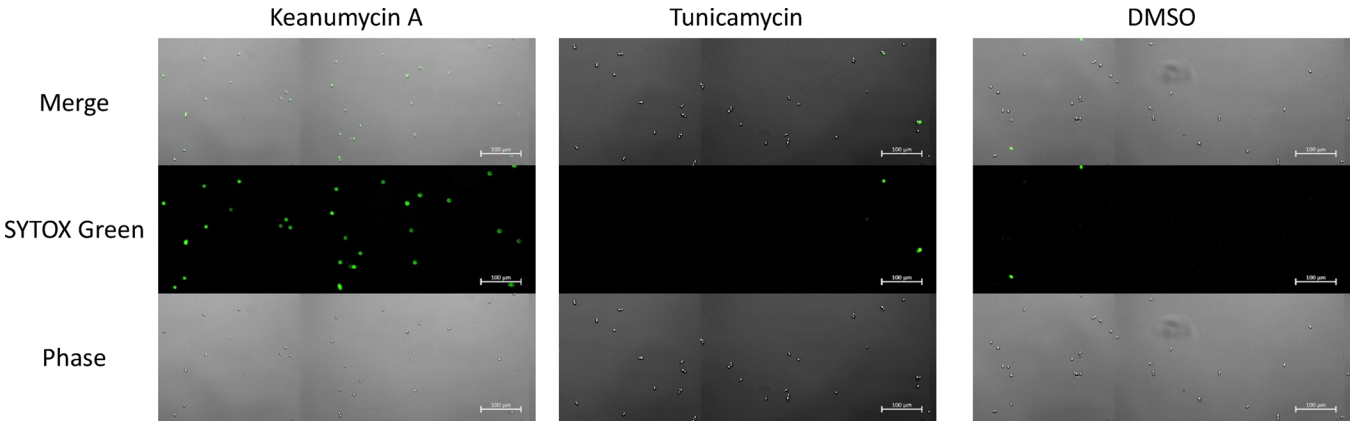

Figure 6. Microscopy images of *C. albicans* incubated with 1% DMSO containing either 4 μg mL^{-1} keanumycin A or tunicamycin. Keanumycin A permeabilizes the cell wall of the fungi as can be seen by the green fluorescent stain of SYTOX Green. In contrast, also tunicamycin was used at the MIC,[88] the membrane integrity of *C. albicans* is not disturbed by this natural product. Only isolated cells are stained, which can be explained by the fact that the dye can also be used to distinguish dead from live cells. Scale bar = 100 μm.

and binds to DNA, which results in fluorescently stained cells. Incubation with keanumycin A led to a staining of all cells showing that this natural product increases membrane permeability. Tunicamycin on the other hand did not cause a loss of membrane integrity as the percentage of stained cells was comparable to the negative DMSO control. Thus, the keanumycins are membrane-active compounds that disturb the structure of the fungal cell wall.

CONCLUSIONS

In summary, we discovered new members of the syringomycin NRLP family using a microbial predator–prey interaction-driven genome mining approach. We elucidated the structure of keanumycins A–C by using a combination of analytical as well as synthetic methods and discovered a novel amino acid building block, namely, 2-amino-4-iminobutanoic acid, which has not been described in an NRP. Keanumycin A is a strong amoebicide and antifungal that shows little cytotoxicity making it a good lead structure for the development of new antimycotics. Furthermore, the fermentation broth of the producing organism containing keanumycins can be directly applied to plants to stop the development of Botrytis blight and has potential for the development of an ecofriendly alternative to antifungal agrochemicals.[85−87] Studies of the mechanism of action revealed that keanumycin A is a membrane-active compound that disrupts the fungal cell wall integrity leading to cell death.

Taken together, our findings show the potential of discovering new natural products in microbial ecological niches that harbor unexplored biochemistry and unprecedented bioactivity, which will help to combat the anti-infective crisis not only from the pharmacological but also from the agricultural side.[42]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c11107.

Supplementary figures and tables, synthetic and molecular biology methods, physical copies of chromatograms, NMR, and mass spectrometry spectra (PDF)

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Notes

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Microarray data are available at ArrayExpress (https://www.ebi.ac.uk/arrayexpress/). The accession number is E-MTAB-12311.

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