Science of The Total Environment
March 2022, Volume 812 Pages 152487 (11p.)
https://doi.org/10.1016/j.scitotenv.2021.152487
https://archimer.ifremer.fr/doc/00741/85330/

Towards eco-friendly marine antifouling biocides – Nature inspired tetrasubstituted 2,5-diketopiperazines

Grant Thomas M. [1], Rennison David [1], Cervin Gunnar [2], Pavia Henrik [2], Hellio Claire [3], Foulon Valentin [3], Brimble Margaret A. [1], Cahill Patrick [4], Svenson Johan [4, *]

[1] School of Chemical Sciences, University of Auckland, 23 Symonds Street, Auckland, New Zealand
[2] Department of Marine Sciences, Tjärnö Marine Laboratory, University of Gothenburg, SE-452 96 Strömstad, Sweden
[3] Univ. Brest, Laboratoire des Sciences de l'Environnement MARin (LEMAR), CNRS, IRD, IFREMER, Brest 29285, France
[4] Cawthron Institute, 98 Halifax Street, Nelson, New Zealand

* Corresponding author : Johan Svenson, email address : johan.svenson@cawthron.org.nz

Abstract :

Marine biofouling plagues all maritime industries at vast economic and environmental cost. Previous and most current methods to control biofouling have employed highly persistent toxins and heavy metals, including tin, copper, and zinc. These toxic methods are resulting in unacceptable environmental harm and are coming under immense regulatory pressure. Eco-friendly alternatives are urgently required to effectively mitigate the negative consequence of biofouling without causing collateral harm. Amphiphilic micropeptides have recently been shown to exhibit excellent broad-spectrum antifouling activity, with a non-toxic mode of action and innate biodegradability. The present work focused on incorporating the pharmacophore derived from amphiphilic micropeptides into a 2,5-diketopiperazine (DKP) scaffold. This privileged structure is present in a vast number of natural products, including marine natural product antifoulants, and provides advantages of synthetic accessibility and adaptability. A novel route to symmetrical tetrasubstituted DKPs was developed and a library of amphiphilic 2,5-DKPs were subsequently synthesised. These biodegradable compounds were demonstrated to be potent marine antifoulants displaying broad-spectrum activity in the low micromolar range against a range of common marine fouling organisms. The outcome of planned coating and field trials will dictate the future development of the lead compounds.

Graphical abstract

Highlights

► Antifouling biocides that are effective but environmentally safe are urgently needed. ► Novel synthetic route for mixed functionality 2,5-diketopiperazines was developed. ► Libraries of tetrasubstituted 2,5-diketopiperazines were synthesised and screened. ► Broad-spectrum antifouling was dictated by both hydrophobicity and cationic charge. ► These biodegradable molecules show promise for effective yet safe antifouling.

Keywords : Diketopiperazine, Antifouling, Marine, Non-toxic, Broad-spectrum, Synthesis

Abbreviations. DKP. Diketopiperazine; AMP. Antimicrobial peptide; CSV. Connolly solvent excluded volume; QAS. Quaternary ammonium salt; Gpp. 4-Guanidinophenylalanine; Bip. 4-Biphenylalanine;

1. Introduction

Marine biofouling – the settlement and accumulation of marine organisms on surfaces submerged in the sea – is a ubiquitous process that results in huge economic and

environmental costs.[1,2] Marine biofouling can be split into two major classes: microfouling, which largely consists of bacteria and microalgae; and macrofouling, which comprises larger organisms such as barnacles, seaweeds, and mussels. The hydrodynamic penalties, biodeterioration and biosecurity risks caused by biofouling negatively impact shipping, aquaculture, and energy production and a range of other maritime activities.[3–7] Although microfouling makes an important contribution to the biofouling process, larger macrofouling organisms have a disproportionately impact in most scenarios.[7] For example, light calcareous biofouling decreases the power coefficient of cross-flow turbines by up to 15%, while heavier fouling can render turbines incapable of producing power.[8] Biofouling increases green-house gas emissions of ships by up to 40% due hydrodynamic drag[2,9,10] and is increasingly recognised as a primary means for global spread of invasive marine organisms.[11,12] Current estimates place the combined cost of these diverse impacts of marine biofouling at approximately US$60 billion per annum.[13,14]

A key challenge in addressing the biofouling problem is the vast taxonomic diversity of marine biofouling organisms. Over 4000 fouling species have been identified, covering many different taxonomic classes and thus physiologies.[15] To account for this taxonomic diversity, the default approach to biofouling management has been coatings laced with broad-spectrum biocides.[15] Such coatings can be highly effective, but pose high collateral risk to the environment due to their propensity to bioaccumulate.[4] The best-known example is tributyltin, which is a highly effective antifouling biocide but has been banned due to off-target impacts.[16,17] There is urgent need for replacement technologies that are effective but do not cause collateral harm in the environment.[18] Non-biocidal approaches show promise for some scenarios, and foul-release coatings are commercially available.[19] However, the foul-release mechanism is only applicable for fast moving vessels in near constant use[19,20] and other non-biocidal approaches have failed to translate beyond primary research (e.g., surface

microtopography) or are prohibitively labour intensive (e.g., manual cleaning).[7,21]

Chemical biocides remain as an essential component of a holistic toolbox of antifouling technologies, and the natural chemical antifouling defences of marine and terrestrial organisms provides valuable inspiration for effective yet safe chemistry.[22–24] Antifoulants inspired by marine natural product can exhibit targeted efficacy[25–27], with examples including derivatives of barettin (**1**) from the sponge *Geodia barretti*, phidianidine A and B (**2**) from the ophisthobranch mollusk *Phidiana militaris*, and synoxazolidinone A (**3**) from the ascidian *Synoicum pulmonaria* (Figure 1).[28–3031] Terrestrial natural product leads include chalcone flavonoids,[32] the sesquiterpene dialdehyde polygodial (**4**) (Figure 1),[23,33] bile acid derivatives,[34] and allelopathic batatasin from Arctic crow berries (*Empetrum nigrum*).[35] Structure-activity studies of marine and terrestrial natural products have generated understanding of mode of action and tuned bioactivity and toxicity[23,32,34,35], however, the native compounds are often seen as unrealistic for commercial use due to limited natural supply and/or synthetic inaccessibility.

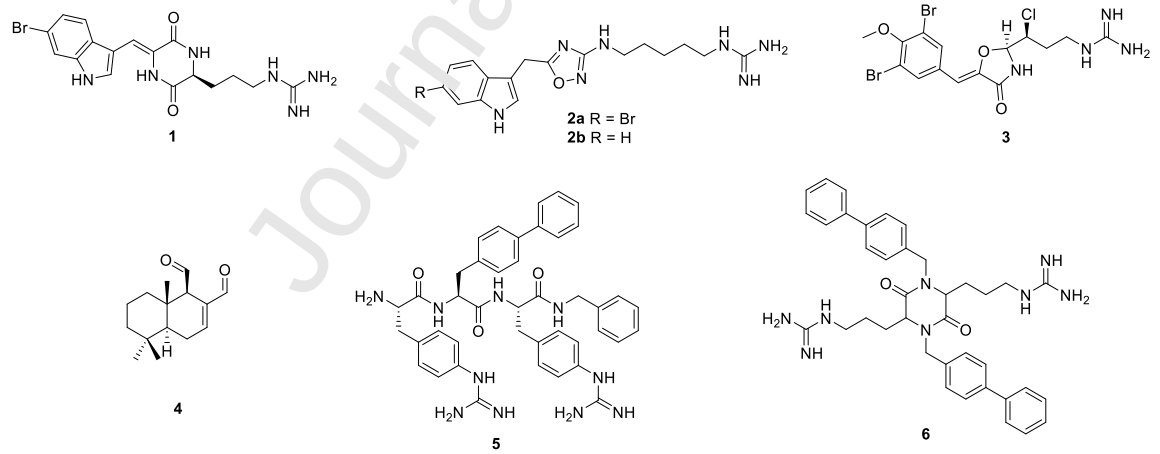

Figure 1. Antifouling marine natural products barettin (**1**), phidianidine A (**2a**) and B (**2b**), synoxazolidinone A (**3**). Terrestrial natural product polygodial (**4**), antifouling tripeptide Gpp-Bip-GppNHBn (**5**) and lead 2,5-diketopiperazine (DKP) (**6**).

One option to overcome the commercial limitations of native natural product antifoulants is

the production of simplified mimics via peptide chemistry. This approach has been employed to generate efficient analogues of both barettin and the synoxazolidinones.[30,36] Peptide synthesis is innately scalable and adaptable and, as peptides are targets for a multitude of natural proteolytic enzymes, the mimics are unlikely to persistent in the environment.[37,38] Peptide chemistry is increasingly employed for drug development,[39] and advances from that field can be used to incorporate functionalities from antifouling natural products to further improve or augment antifouling activity. Synthetic truncation of the lactoferricin peptide[40], and the incorporation of unnatural amino acids led to the generation of highly potent antimicrobial peptides (AMPs) comprising as few as 3 amino acids.[41–44] Trepos *et al.* evaluated 13 AMPs for marine antifouling, and a tripeptide (**5**; Figure 1) was identified that exhibited broad-spectrum activity against a range of marine fouling organisms via a non-toxic mechanism.[45] Subsequently, Labrière *et al.* revealed a series of 2,5-diketopiperazines (DKPs) adopting the design principles of the aforementioned linear tripeptides, and DKP **6** (Figure 1) displayed potent antibacterial activity against both Gram-positive and Gram-negative terrestrial bacteria.[46] DKPs are privileged structures with the ability to bind to a range of receptors and represent a particularly interesting structural platform to develop effective yet safe antifouling agents.[47] DKPs are non-persistent due to their peptidic nature[46,48–50] and the core scaffold is amenable to extensive derivatisation at four positions. The later factor allows for simultaneous tuning of multiple physicochemical parameters inspired by emerging advances from both antifouling natural products and antibacterial micropeptides,[24,46,51]

The current study rationally designs and assesses four distinct libraries of 2,5-DKPs to develop effective yet safe marine antifouling biocides (Figure 2). The DKPs were designed from an established amphiphilic antimicrobial peptide pharmacophore, often referred to as the "2+2 pharmacophore" combined with elements from marine antifouling natural

products.[24,51] Through the synthesis of DKPs **6**-**27** (Figure 2), this study probes the demands of this pharmacophore, examining 1) the hydrophobic bulk (X) requirement (in terms of both lipophilicity and hydrophobic volume), 2) the constitution of the cationic groups (Y), and 3) the role of the spacer units (Z) between the DKP scaffold and the terminal charged groups. The compounds were then evaluated against a comprehensive panel of marine fouling organisms to evaluate their antifouling potential.

2. Materials and Methods

Commercially available starting materials were obtained from Sigma-Aldrich, Merck, or AK scientific (USA) and were used as received unless otherwise noted. Reactions performed at low temperature were cooled either with a Julabo FT402 immersion cooler. Reactions were monitored by thin-layer chromatography (TLC) carried out on silica gel plates (Merck) using UV light as a visualising agent and an ethanolic solution of vanillin, potassium permanganate or ninhydrin and heat as developing agents. The purity and retention factor of all target compounds was assigned using achiral reverse-phase HPLC (Dionex P680 system using a Phenomenex Gemini C18-Si column (150 mm × 4.6 mm, 5 µm)) – eluted using an isocratic gradient of 26:74 A:B over 90 min at 1 mL/min; where solvent A was water (+0.1% v/v trifluoroacetic acid) and solvent B was acetonitrile (+0.1% v/v trifluoroacetic acid), or a gradient of 100:0 A:B to 0:100 A:B over 10 min at 1 mL/min; where solvent A was water (+0.1% v/v trifluoroacetic acid) and solvent B was acetonitrile (+0.1% v/v trifluoroacetic acid), unless stated otherwise. Accompanying *in silico* derived log *D* values were generated using ACD labs version 12.0, and Connolly solvent excluded volumes for the hydrophobic (X) groups generated using Chem3D Ultra 19.1. NMR spectra were recorded at room temperature in $CDCl_3$, MeOH-d_4, or DMSO-d_6, solutions, on either Bruker DRX400 spectrometers operating at 400 MHz for ^{1}H nuclei and 100 MHz for ^{13}C nuclei or using a Bruker DRX-500 spectrometer operating at 500 MHz for ^{1}H nuclei and 125 MHz for ^{13}C

nuclei. Chemical shifts were reported in parts per million (ppm) and were measured relative to the solvent in which the sample was analysed ($CDCl_3$: δH 7.26 ppm or δH 0.00 ppm (TMS), δC 77.16 ppm; MeOH-d_4: δH 3.31 ppm, δC 49.00 ppm; DMSO-d_6: δH 2.50 ppm, δC 39.52 ppm). High-resolution mass spectra (HRMS) were obtained using a micrOTOF-Q II mass spectrometer.

2.1 Chemical Synthesis

Noting earlier observations around the generally superior antimicrobial activity exhibited by the arginine-derived AMPs over their corresponding ornithine/lysine counterparts,[44,46] our initial library (Figure 2, library 1) focused on DKPs based on lead compound **6** incorporating three carbon spacer units (Z) terminating with guanidines (Y), while varying the hydrophobic unit (X) (DKPs **6**-**20**).

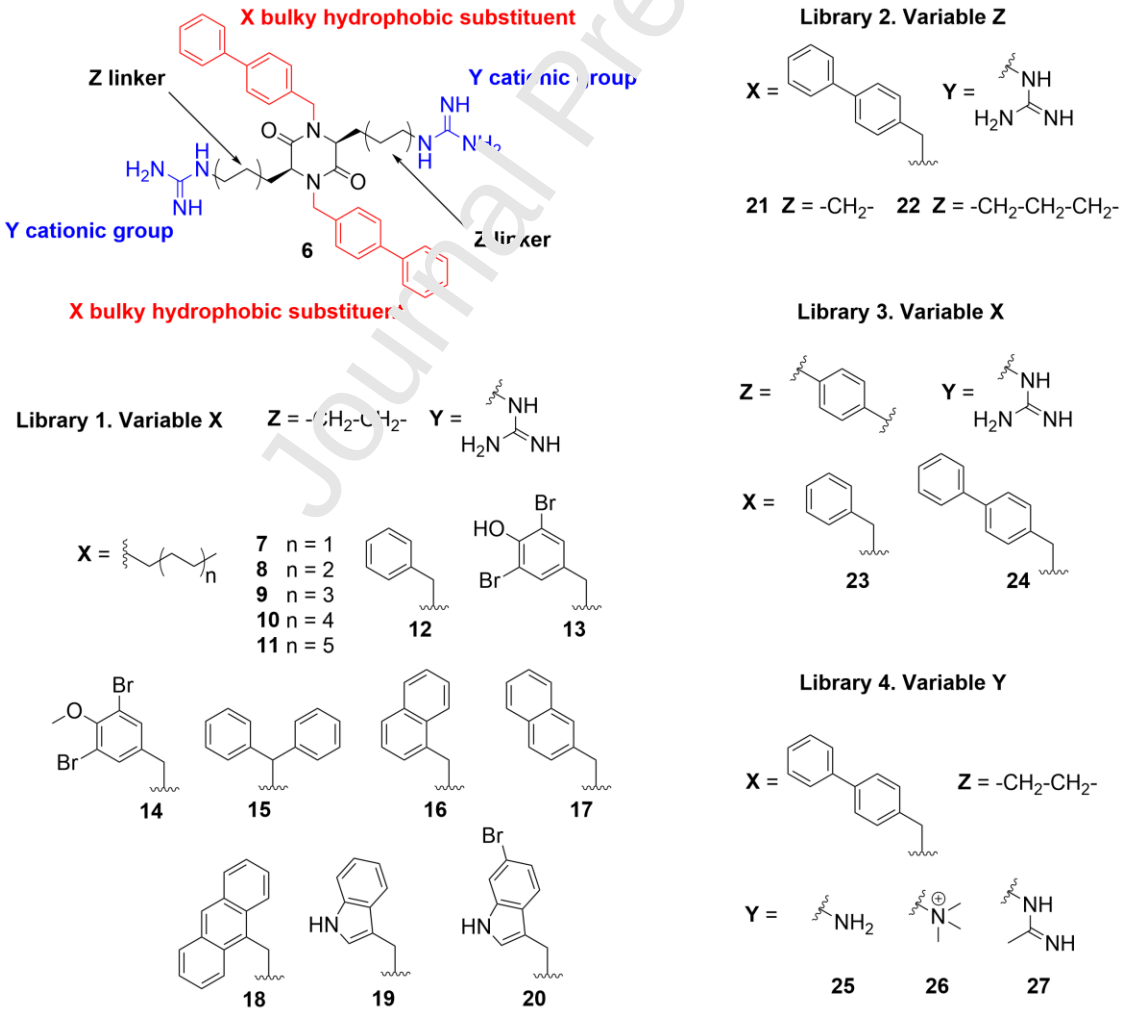

Figure 2. General compound design and selection of variable building blocks used for the generation of the DKPs reported in this study.

Drawing inspiration from the natural product literature[28–30,52], it was suggested that through the exploitation of hydrophobic moieties found in natural antifouling compounds, potent antifoulants exhibiting reduced off-target toxicity could potentially be developed. Other hydrophobic moieties were also included following literature examples of synthetic peptide antimicrobials.[24,53] Subsequent libraries shifted focus to the linker (Z) (libraries 2 and 3) and the cationic unit (Y) (library 4), to probe the charge requirements, through the inclusion of synthetic linkers from previous tripeptide libraries, and cationic units inspired by other natural products, respectively (Figure 3).[30,44] DKPs **6-20** were all synthesised from a common di-Boc arginine protected DKP intermediate (***40***) which itself was synthesised through known chemistry that allowed for the retention of stereochemistry (Figure 3).[54]

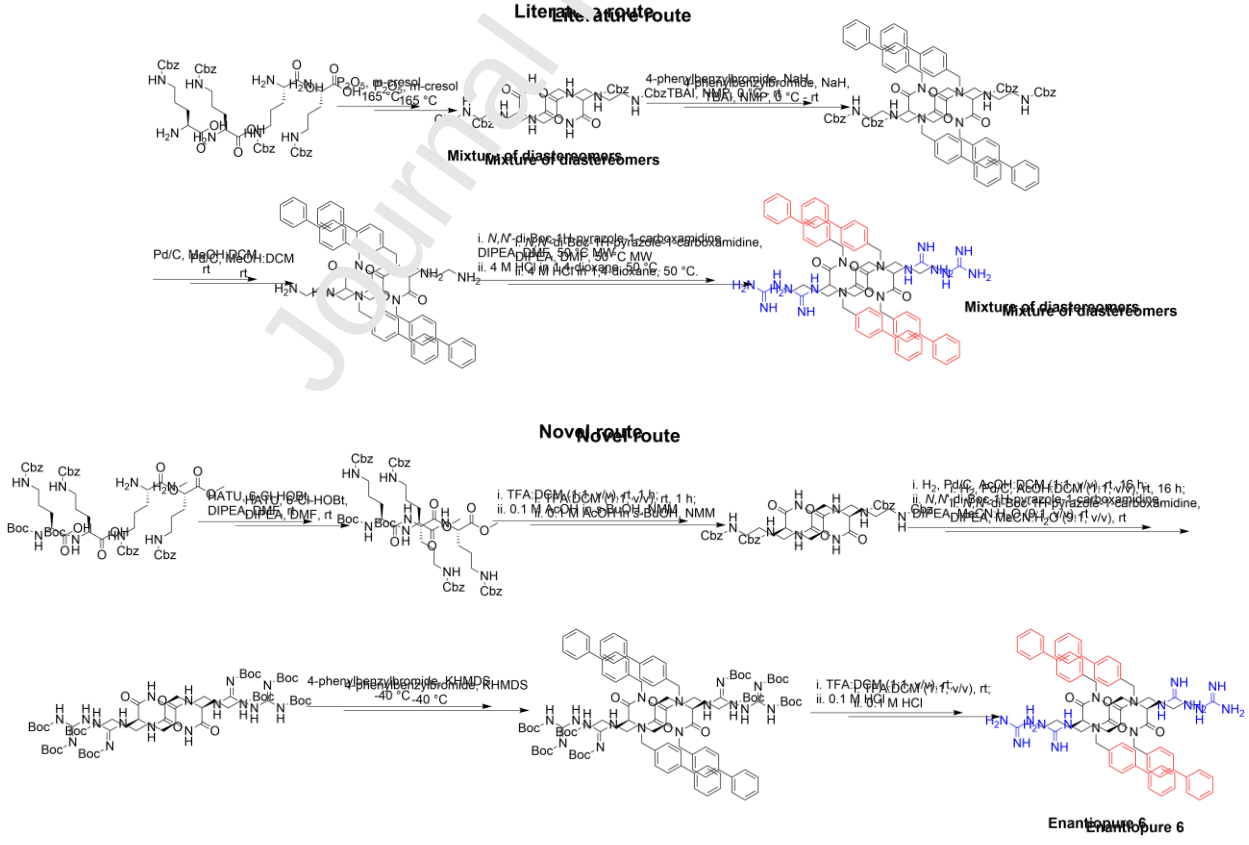

Figure 3. Literature route and novel route to lead DKP **6**. With the inclusion of the dipeptide cyclisation step the

stereochemistry is able to be controlled allowing for the enantiopure generation of DKP **6**.

From this intermediate, a series of amide nitrogen derivatised DKPs (compounds **6**-**20**, Figure 2, library 1) was synthesised using standard alkylation chemistry,[55] with subsequent Boc-deprotection of the guanidine groups (using TFA) revealing the target compounds. Having successfully synthesised a series of N_α-derivatised DKPs bearing different hydrophobic substituents, focus shifted onto modifying the spacer unit (Z) between the DKP scaffold and the terminal guanidine group (DKPs **21**-**24**, library 2, Figure 2).[44,56] DKPs **21** (two-methylene spacer) and **22** (four-methylene spacer) were synthesised following procedures analogous to those used in the preparation of DKP **6** (Figure 2). To complement the aliphatic-based spacer series, DKPs **23** and **24** (library 3, Figure 2), prepared as examples incorporating aromatic spacer groups, were synthesised, building on the design principles of lead linear Gpp-Bip-GppNHBn tripeptide **5** (Figure 1).[45] Finally, through DKPs **25** (amine), **26** (quaternary ammonium salt) and **27** (acetamidine), a focused selection of DKPs bearing different cationic groups (Y) were synthesised to probe the requirements of the positively charged moiety (library 4, Figure 2). To the authors knowledge this is the first example of an acetamidine being incorporated into an antimicrobial peptide/peptide mimic, the synthesis of which may aid the development of future libraries.[57]

2.1.1 Synthesis of example DKP 6 cyclo(N_α-Bip-L-Arg-N_α-Bip-L-Arg).2HCl (6)

To a solution of cyclo(Arg(di Boc)-Arg(di Boc)) (0.1 g, 0.14 mmol) in DMF (2 mL) under nitrogen at -40 °C was added dropwise KHMDS (0.70 mL, 0.70 mmol, 1 M in THF), and the mixture stirred for 1 h. A solution of 4-phenylbenzyl bromide (0.17 g, 0.70 mmol) in DMF (1 mL) was then added slowly, and the mixture stirred at -40 °C for a further 16 h. The mixture was then poured into sat. aq. NH_4Cl (15 mL) and extracted with DCM (3 × 5 mL). The combined organic layers were washed with brine (2 × 15 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Purification by flash chromatography (Pet. Ether-EtOAc

4:1) afforded DKP **6a** (0.065 g) as a colourless solid. To a solution of DKP **6a** (0.065 g) in DCM (2.5 mL) was added TFA (2.5 mL), and the mixture stirred at rt for 1 h. The mixture was then concentrated, triturated with Et_2O, and the resulting solid collected *via* vacuum filtration. The solid was then redissolved in aq. HCl (2 mL, 0.1 M) and lyophilized to afford *title compound* **6** (colourless solid, 0.042 g, 41%) as the dihydrochloride salt. $[\alpha]_D^{26.0}$ -104.9 (*c* 0.13, MeOH); **^{1}H NMR** (400 MHz; MeOD): δ 7.61 – 7.30 (m, 18H, H-Ar), 5.17 (d, J = 15.1 Hz, 2H, H_a-1′), 4.32 (d, J = 15.1 Hz, 2H, H_b-1′), 4.04 (ABX, J = 9.4, 4.6 Hz, 2H, H-2), 3.29 – 3.16 (m, 4H, H-5), 2.18 – 2.09 (m, 2H, H_a-3), 1.98 – 1.72 (m, 6H, H_b-3, H-4); **^{13}C NMR** (100 MHz; MeOD): δ 168.1 (2 × C, C-1), 158.7 (2 × C, C-6), 142.3 (2 × C, C-Ar), 141.6 (2 × C, C-Ar), 136.4 (2 × C, C-Ar), 129.9 (4 × CH, C-Ar), 129.5 (4 × CH, C-Ar), 128.6 (2 × CH, C-Ar), 128.5 (4 × CH, C-Ar), 127.9 (4 × CH, C-Ar), 60.8 (2 × CH, C-2), 48.7 (2 × CH_2, C-1′), 41.7 (2 × CH_2, C-5), 31.8 (2 × CH_2, C-3), 26.8 (2 × CH_2, C-4); **HRMS** (ESI +) *m/z*: $[M + H]^+$ calcd for $C_{38}H_{45}N_8O_2$, 645.3660; found, 645.3642; **Retention time** 8.24 min (purity at $\lambda_{254\ nm}$ >99% (AUC)). Full details of synthesis and characterisation of all compounds is provided in supplementary information.

2.2 Physicochemical properties

Retention factor (R_f) was used as a proxy for the hydrophobic contribution of the N_α-substituent using high-throughput HPLC for determining compound lipophilicity.[58] R_f is a ratio between the retention time of the desired product and an unretained compound, eliminating the "dead-volume" in the measurement $R_f = \frac{Retention\ of\ desired\ peak - Retention\ of\ unretained\ peak}{Retention\ of\ unretained\ peak}$. R_f has previously been shown to closely correlate to biological activity for other structurally related AMPs.[59]

2.3 Antifouling evaluation

Compounds were screened for antifouling activity using a comprehensive range of model macrofouling and microfouling species (Supporting information Table 1). For the

macrofoulers, brood stock of the transparent sea squirt *Ciona savignyi*, the blue tubeworm *Spirobranchus cariniferus,* the blue mussel *Mytilus galloprovincialis*, and the Asian kelp *Undaria pinnatifida* were sourced from coastal populations in the Nelson region of New Zealand. Brood stock of these species were held in a recirculating seawater system (18 ± 1 ºC, 33 ± 1 PSU) and fed bulk-cultured *Isochrysis galbana* until ready to spawn, except for *U. pinnatifida* which was spawned immediately after collection.[60] Brood stock of the bay barnacle *Amphibalanus improvisus* were collected from Tjärnö, Sweden and were held in a flow-through seawater system at ambient conditions (20-25 °C, 25 PSU). Larval spawning and rearing procedures followed previously described methods for *C. savignyi*,[61] *S. cariniferus*,[62] *M. galloprovincialis*,[63] *U. pinnatifida*,[64] and *A. improvisus*.[65] In all instances, competent larvae, or gametophytes in the case of *U. pinnatifida*, were diluted in artificial seawater to prescribed concentrations (SI Table 1). Aliquots of these larval suspensions were added to multi-well plates containing serial dilutions of the test compound in small amounts of ethanol or dimethyl sulfoxide (DMSO) to yield desired concentration ranges (SI Table 1). Blank (artificial seawater only) and solvent (artificial seawater plus ethanol or DMSO) controls were also included, and three replicates were performed in all cases (N = 3). All multi-well plates were incubated for 5 days, after which the number of successfully settled and metamorphosed individuals were counted, endpoints shown in Figure 4. Dose-responses were modelled using Weibull or logistic curve fitting (as dictated by model fit) and nominal concentration estimates that reduced settlement and metamorphosis relative to the controls by 50% (IC_{50}) calculated using R statistical software.[66] For the microfoulers, two marine bacteria (*Vibrio harveyi* DSM 19623, *Halomonas aquamarina* DSM 30161) and two microalgae (*Porphyridium purpureum* AC122, *Exanthemachrysis gayraliae* AC15) were used to assess the growth inhibition activity of the DKPs employing methodology described previously.[67] Microorganism suspensions for use in the assays were made up to prescribed densities in

peptone marine broth for bacteria and F/2 medium for microalgae (SI Table 1). Stock solutions (10 µg/mL) of the test compounds were prepared in 20% ethanol and added to wells in 100 µL-aliquots in six replicates wells. Ethanol was evaporated from the wells at 25 °C under vacuum prior to adding aliquots of the respective microorganism suspensions. Bacterial growth was monitored via absorption (620 nm) after incubating for 48 h at 25 °C. The microalgae assays were incubated for 5 days at 20 °C and growth was quantified via chlorophyll-a fluorescence (485 nm excitation, 645 nm emission) released upon addition of 100 µL of methanol to the wells. Data analysis quantified percent inhibition of bacterial or microalgal growth relative to blank controls.

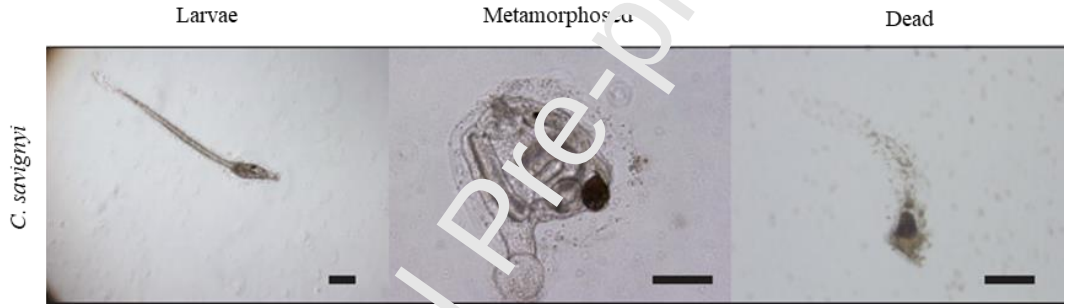

Figure 4. Visual endpoints for the evaluation of the DKPs towards the settlement and metamorphosis of *C. savignyi*.

2.4 Statistical analyses

The macrofouling assays were performed in triplicate and the microfouling were studied in six replicates, and blank and solvent controls were run in all instances. The dose-responses were modelled using Weibull or logistic curve fitting (as dictated by model fit). The nominal concentration estimates that reduced settlement and metamorphosis relative to the controls by 50% were used as IC50-values and were calculated using R statistical software.[66] Seawater, and seawater + DMSO were employed as controls. All synthetic compounds were prepared and tested at >95% purity as verified using NMR, HRMS and HPLC as presented in supplementary information.

3. Results and Discussion

The synthesis of DKPs **6-20** centred around a common di-Boc protected arginine DKP which was synthesised over four steps from commercially available protected amino acids. This route allowed for the rapid generation of an enantiomerically pure common intermediate in good yield (>70%), from which an extensive array of desired derivatives was accessed. Previous syntheses of DKP libraries have been hampered by epimerisation, thus previous antimicrobial evaluation of related amphiphilic DKPs focused on diastereomeric mixtures.[46,68] The ability of the synthetic approach developed here to retain the stereochemistry throughout the synthesis of the DKPs represents a significant advance that, for example, enables reliable access to enantiomerically pure (*S,S*)-DKPs previously shown to be more active than the corresponding diastereomeric mixture.[46] With the common DKP intermediate in hand, a focused series of amide nitrogen derivatized DKPs (compounds **6-27**, Figure 3) was synthesized using standard alkylation chemistry,[55] with subsequent Boc-deprotection of the guanidine groups (using TFA) revealing the target compounds. This is the first example of the synthesis of a comprehensive series of libraries of symmetric, enantiopure, 2,5-DKPs adhering to the 2 + 2 pharmacophore.

3.1 Macrofouling activity

On account of marine biofouling being an immensely complex process involving many different organisms, assessment using a single model assay is fraught and poses a high risk for incomplete understanding of bioactivity and poor translation of results from lab to field.[69] A broad range of both micro- and macrofouling marine organisms when evaluating novel marine antifoulants is essential, an imperative which is rarely met in this field.[70,71] In this study, bioassays using five problematic macrofouling and four microfouling organisms spanning diverse taxonomy underpinned the comprehensive examination of the potential antifouling properties of the investigated DKPs.[61,72,73] *Ciona savignyi*, a well-studied

ascidian, was selected as the primary test organism for this study on account of both its rapid development and its ability to spawn year-round in temperate climates.[61] *Mytilus galloprovincialis*, a blue mussel, *Spirobranchus cariniferus*, a tube worm, *Undaria pinnatifida*, a macroalgae, and cyprids of the barnacle *Amphibalanus improvisus* were also included as further examples of marine macrofoulers. This panel represents an extensive examination of macrofouling organisms which allowed for a more in-depth analysis of the antifouling potential of the synthesised compounds. All bioassays were performed on competent larvae (gametophytes in the case of *U. pinnatifida*) of the given taxa, enabling direct assessment of bioactivity during settlement and metamorphic processes. Assays for *C. savignyi*, *M. galloprovincialis*, *S. cariniferus*, and *A. improvisus* were performed according to previously published methods,[61,74] with serial dilutions from 0.1 to 20 ug/mL.

The majority of the DKPs prepared (compounds **6**-**27**) demonstrated broad-spectrum activity, in several instances registering IC_{50} values in the low to sub-µg/mL range (Table 1). This activity was encouraging given that this was the first instance in which this class of compound had been evaluated for antifouling potential with the activity appearing to be high compared to other evaluated libraries.[75] DKPs **6**, **9**, **10**, **16**, **18** and **24** all exhibited IC_{50} values <2 µg/mL against *C. savignyi*, *M. galloprovincialis*, *S. cariniferus* and *U. pinnatifida*, representing the most potent, broad-acting compounds within this series. Conversely, DKPs **7**, **12** and **23** were inactive against all species tested (IC_{50} ≥20 µg/mL,). In terms of activity against a particular organism, DKPs **15** (IC_{50} 0.11±0.0 µg/mL), **10** (0.15±0.1 µg/mL), **26** (0.27±0.05 µg/mL) and **16**-**18** (1.0±0.5 µg/mL) recorded the lowest individual IC_{50} values against *C. savignyi*, *M. galloprovincialis*, *S. cariniferus* and *U. pinnatifida*, respectively. Lead DKP **6** exhibited superior activity compared to its linear counterpart, which was largely inactive,[45] and its broad-spectrum activity against both macro- and (to a lesser extent) microfouling organisms was encouraging.[28,30,36,75] These potency values compare favourably

to a range of marine natural product antifoulants, with a meta-analysis of 198 marine natural product antifoulants concluding that 50% lethal (LC_{50}) or effective (EC_{50}) concentration < 15 μg/mL correspond to 'medium to high bioactivity'.[75] Furthermore their inhibitory properties are comparable to that of commercial booster biocide SeaNine.[23]

Table 1. Activity of compounds **6**-**27** against *C. savignyi*, *M. galloprovincialis*, *S. cariniferus*, *U. pinnatifida*, and *A. improvisus*.

		IC_{50} (μg/mL)				
Entry	**R_f**	*C. savignyi*	*M. galloprovincialis*	*S. cariniferus*	*U. pinnatifida*	*A. improvisus*
6	19.4	0.3±0.2	0.8±0.3	0.3±0.1	1.3±0.4	>5.0
7	0.4	>20	>20	>20	>20	>5.0
8	2.4	>20	6.63±0.16	3.95±0.28	>20	>5.0
9	32.4	0.54±0.1	0.8±0.3	1.1±0.5	1.1±0.0	>5.0
10	49.0	1.9±0.4	0.15±0.1	0.38±0.0	2.98±0.18	>5.0
11	>50	2.4±1.4	2.1±0.9	4.7±3.6	>20	5.0
12	0.6	>20	>20	>20	>20	>5.0
13	1.1	>20	2.3±0.95	n.t[a]	4.24±0.35	>5.0
14	9.8	10.2±2.2	2.8±0.23	n.t	2.79±0.44	5.0
15	4.3	0.11±0.0	>20	n.t	2.6±0.7	0.5
16	3.7	0.21±0.02	1.7±0.4	0.49±0.07	1.0±0.2	5.0
17	3.7	0.14±0.01	2.5±1.3	0.34±0.03	1.0±0.4	>5.0
18	23.1	0.2±0.06	1.1±0.1	n.t	1.0±0.5	0.5
19	0.8	>20	7.97±0.41	3.68±0.91	12.91±2.88	5.0
20	4.7	5.0±0.3	1.7±0.1	0.85±0.16	3.08±0.33	2.5
21	11.9	1.8±0.9	5.7±0.7	1.22±0.13	4.98±1.08	>5.0
22	21.5	2.5±0.8	2.4±0.5	2.1±0.6	1.2±0.3	5.0
23	1.2	>20	>20	n.t	>20	2.5
24	45.0	1.54±0.68	0.17±0.06	0.47±0.14	1.99±0.08	0.5
25	8.5	2.31±0.2	0.91±0.34	n.t	1.47±0.05	1.0
26	12.1	7.2±2.3	3.88±0.13	0.27±0.05	1.51±0.21	>5.0
27	15.7	4.3±0.26	1.02±0.07	n.t	1.39±0.07	>5.0

[a] n.t = not tested due to limited access to brood stock.

The design process for the initial library began with the removal of the *para*-phenyl substituent present in DKP **6** (0.3±0.2 μg/mL), to reveal unsubstituted N_α-benzyl DKP **12** (>20 μg/mL), and resulted in a complete loss of activity against *C. savignyi*. By incorporating the hydrophobic moieties present in synoxazolidinone A, it was found that moderate activity could be restored through the introduction of hydrophobic substituents onto the phenyl ring of DKP **12** (e.g. through the preparation of dibromoanisole DKP **14**, 10.2±2.2 μg/mL);

conversely, dibromophenol DKP **13** (>20 μg/mL) was inactive, in line with previous literature on antimicrobial tripeptides due to an insufficient hydrophobic element.[41] The incorporation of a phenyl group at the benzylic position of DKP **12**, as found in DKP **15** (0.11±0.0 μg/mL), similarly restored a level of activity comparable to that of lead DKP **6**. Exchanging the biphenyl moiety of DKP **6** for planar polyaromatic moieties such as naphthyl (DKPs **16**, 0.21±0.02 μg/mL and **17**, 0.14±0.01 μg/mL) and anthranyl (DKP **18**, 0.2±0.06 μg/mL) maintained a high level of potency but did not improve upon the lead compound.[24,43] As examples of planar heterocyclic groups found in natural antifoulants,[28,29] bromoindole DKP **20** (5.0±0.3 μg/mL) was subsequently demonstrated to be moderately active, whereas its unsubstituted counterpart (DKP **19**, >20 μg/mL) was found to be inactive.

Barnacles are a major contributor to fouling pressure and cost maritime industries billions of dollars as a result of increased maintenance demands.[5] When assessed against *A. improvisus* cyprids (Figure 5), several of the DKPs were shown to inhibit cyprid settlement at a compound concentration of 5 μg/mL (Table 1).

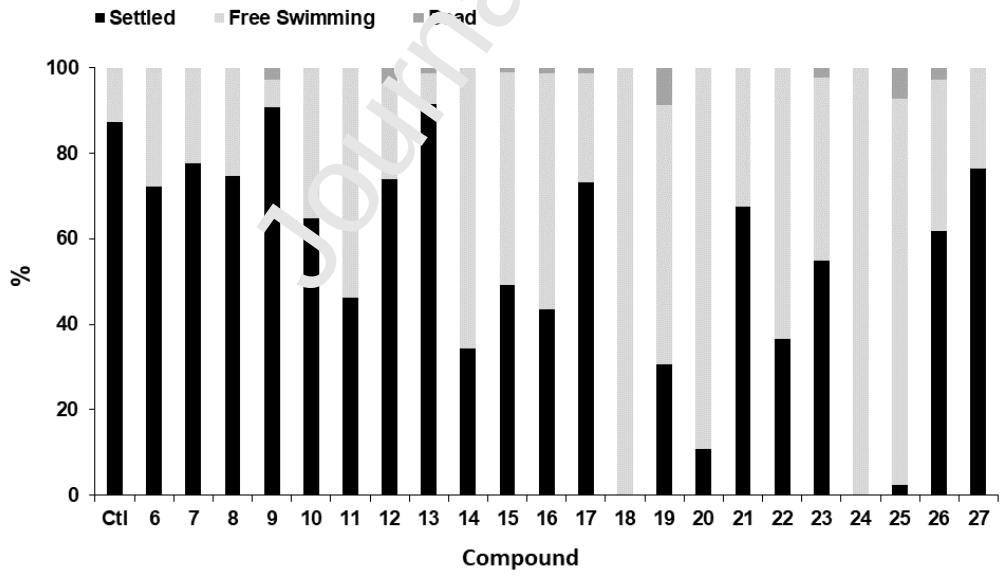

Figure 5. Effects of 2,5-diketopiperazines **6-27** on *A. improvisus* cyprid settlement, shown as percentage of settled, free swimming, and dead cyprids.

DKPs **18** and **24** were the most active, completely inhibiting cyprid settlement (where the cyprid population remained in the swimming phase), while DKPs **20** and **25** inhibited settlement at levels >90%. Moreover, DKPs **6**-**27** were also shown to display no significant toxicity (<5% death at 5 μg/mL) against the cyprids, similar to other natural products and their derivatives.[29,31] This non-toxic mechanism provides a promising platform for the future development of new antifoulants, as any new product must be shown to be environmentally benign. This lack of toxicity is also in line with previous literature on related micropeptides, which were shown to simply "deactivate" the cyprids (which could later then be revived through incubation in fresh seawater).[45] IC_{50} values were subsequently determined for those DKPs which inhibited cyprid settlement by at least 50% at 5 μg/mL (Table 1), with DKPs **15**, **18** and **24** revealed to be the most active with IC_{50} values of 0.5 μg/mL. This activity is highly potent when compared to related marine natural product antifoulants such as synoxazolidinone A (7.44 μg/mL)[30] or manzamine (3.0 μg/mL),[76] and is comparable to the natural product barettin (0.9 μg/mL)[26] and the commercially available antifoulant SeaNine™ (0.25 μg/mL).[77] This non-toxic activity implies that the DKPs examined may operate via multiple mechanisms of action with barnacles potentially being impacted by mechanisms other than anticipated membrane disruption. This likelihood is consistent with other literature that has revealed multiple intracellular mechanisms of action of DKPs alongside generic membrane disruption, as well as the increased resilience of the cyprids to the DKPs as evidenced by the increase in IC_{50}.[48] Coupled with the efficient and cost effective synthesis of the DKPs, these results provide a promising lead for a commercially viable alternative.

In an effort to rationalize the aforementioned trends in structure-activity, attempts were next made to tie observed biological activity to the hydrophobic contribution of the N_{α}-substituent of the DKP. An HPLC method was subsequently developed which revealed lead DKP **6** to have a R_f of 19.4. Of the compounds which similarly exhibited sub-μg/mL activity against *C.*

savignyi, DKPs **9** (32.4), **15** (4.3), **16** (3.7), **17** (3.7), and **18** (23.1) revealed a broad range of R_f values. Conversely, inactive (>20 μg/mL) DKPs **7** (0.4), **8** (2.4), **12** (0.6), **13** (1.1), and **19** (0.8) were all demonstrated to be significantly less hydrophobic. A strong correlation between the $\log_{10}(R_f)$ and the *in silico* generated $clogD_{8.1}$ was also observed, with an R^2 value = 0.73, indicating a strong relationship and predictability through *in silico* methods (Supplementary information). Previous studies have also shown a strong correlation between the computationally generated Connolly solvent excluded volume (CSV) parameter and activity,[24,46] thus as a second estimate of hydrophobicity, the CSV parameter was adopted (SI Table 2); where the biphenyl group present in lead DKP **6** was subsequently calculated to have a CSV of 145.6 $Å^3$. Again, of the compounds that were most potent against *C. savignyi*, namely DKPs **9** (143.8 $Å^3$), **15** (151.0 $Å^3$), **16** (125.9 $Å^3$), **17** (126.3 $Å^3$), and **18** (162.7 $Å^3$), compared to those which were revealed to be completely inactive (>20 μg/mL), namely DKPs **7** (75.3 $Å^3$), **8** (109.5 $Å^3$) **12** (87.9 $Å^3$), **13** (133.4 $Å^3$) and **19** (112.8 $Å^3$), only DKPs **13** and **14** were revealed as outliers (■=DKPs **13** and **14** (144.8 $Å^3$)); although the boundaries were again observed to be tight (Figure 6).

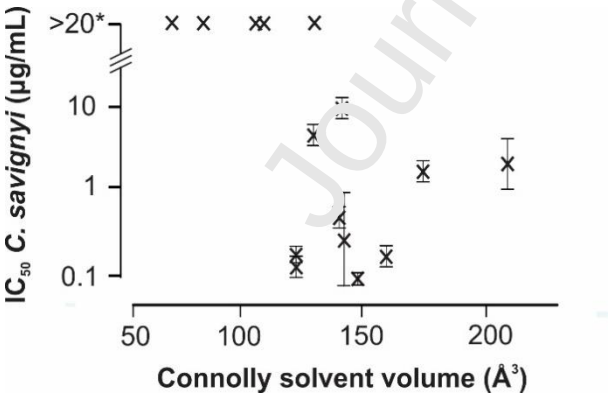

Figure 6. Correlation between the IC_{50} of DKPs **6**-**20** towards *C. savignyi* and the calculated Connolly solvent excluded volume (CSV).

Based on these findings it could be tentatively argued that a minimum hydrophobic requirement was in operation within the 2+2 pharmacophore, with a delicately poised

hydrophobic cut-off lying somewhere between an R_f of 2.4 and 3.7 or a CSV of 112 and 120. In reality though it would appear that, within this particular series of DKPs, these high-throughput methods cannot reliably be utilized as an accurate means of determining compound activity, as has been done previously for pathogenic bacteria.[59]

Similar trends in activity were observed across the other macrofouling organisms (*M. galloprovincialis, S. cariniferus* and *U. pinnatifida*) studied (Supplementary information). Some deviation from the initial SAR was noted for DKPs **13** and **19**, which were previously observed to be inactive against *C. savignyi* yet exhibited moderate activity against some of the other macrofouling organisms. Conversely, DKP **15**, which was demonstrated to be active against *C. savignyi*, exhibited no activity against *M. galloprovincialis*. The trend against *A. improvisus* cyprids was less well defined, however, a common feature across the series was the non-toxic nature of the DKPs *per se*, with only DKP **19** displaying toxicity (>5% death at 5 μg/mL). Overall DKP **18** exhibited the broadest spectrum of activity against the macrofouling organisms screened, also inhibiting cyprid settlement by up to 50% at 0.5 μg/mL; an inhibition value lower than that of many reported antifouling natural products, and at levels comparable to the market antifoulant SeaNine™.[45,76,78]

Trends within the linear alkyl substituted DKP series (**7**-**11**) were found to be better defined than the poly-aromatic substituted DKPs (Table 1). DKPs **7** (N_α-butyl) and **8** (N_α-hexyl) were demonstrated to be inactive (>20 μg/mL), whereas DKPs **9** (N_α-octyl, 0.54±0.1 μg/mL) and **10** (N_α-decyl, 1.90±0.4 μg/mL) showed good activity against *C. savignyi*. Activity was again compared to both the R_f and CSV parameters, where inactive DKPs **7** (R_f = 0.4; CSV = 75.3) and **8** (R_f = 2.4; CSV = 109.5) revealed significantly distinct values compared to their more active counterparts, DKPs **9** (R_f = 32.4; CSV = 143.8) and **10** (R_f = 49.0; CSV = 178.0); supporting the idea of a minimum hydrophobic requirement within the pharmacophore. Similar trends were observed against *M. galloprovincialis, S. cariniferus* and *U. pinnatifida*.

Interestingly, a fall in activity was observed for DKP **11** (N_α-dodecyl) (R_f > 50.0; CSV = 212.3) across all tested organisms, particularly against *U. pinnatifida*, where it was rendered inactive; which could equally imply an upper limit to the hydrophobic requirement, which could also influence compound solubility.

The spacer group between the terminal cationic moiety and the DKP scaffold was also found to be a contributing factor towards activity (Table 1). DKPs **21** (*C. savignyi*, 1.8±0.9 μg/mL) and **22** (*C. savignyi*, 2.5±0.8 μg/mL), incorporating two and four-methylene unit spacers respectively, revealed a notable drop in activity across most macrofouling species, when compared to lead DKP **6** (0.3±0.2 μg/mL); such a decrease in activity has similarly been reported against terrestrial bacteria.[46] Conversely, DKP **24**, incorporating a benzyl-spacer group, in combination with Bip as the hydrophobic unit demonstrated excellent activity across all tested species, including cyprid settlement (IC_{50} 0.5 μg/mL), which is in line with the linear lead tripeptide **5**.[45]

With regards to the contribution of the cationic group, free amine **25** (*C. savignyi*, 2.31±0.22 μg/mL), quaternary ammonium salt **26** (*C. savignyi*, 7.2±2.0 μg/mL) and acetamidine **27** (*C. savignyi*, 4.3±0.26 μg/mL) containing DKPs all displayed a reduction in activity against *C. savignyi*, with similar or reduced activity against the other screened organisms (when compared directly to DKP **6**). Such findings were largely in line with previous literature observations due to the decreased capacity for hydrogen bonding between the active compound and the target phospholipid membrane.[24] However, an unexpected result was noted in the cyprid settlement assay, with DKP **25** exhibiting an IC_{50} of 1 μg/mL, a significant improvement on lead DKP **6**. Figure 7 highlights the dose response of arginine-derived DKP **6** compared to its less active quaternary ammonium salt (QAS) counterpart, DKP **26**.

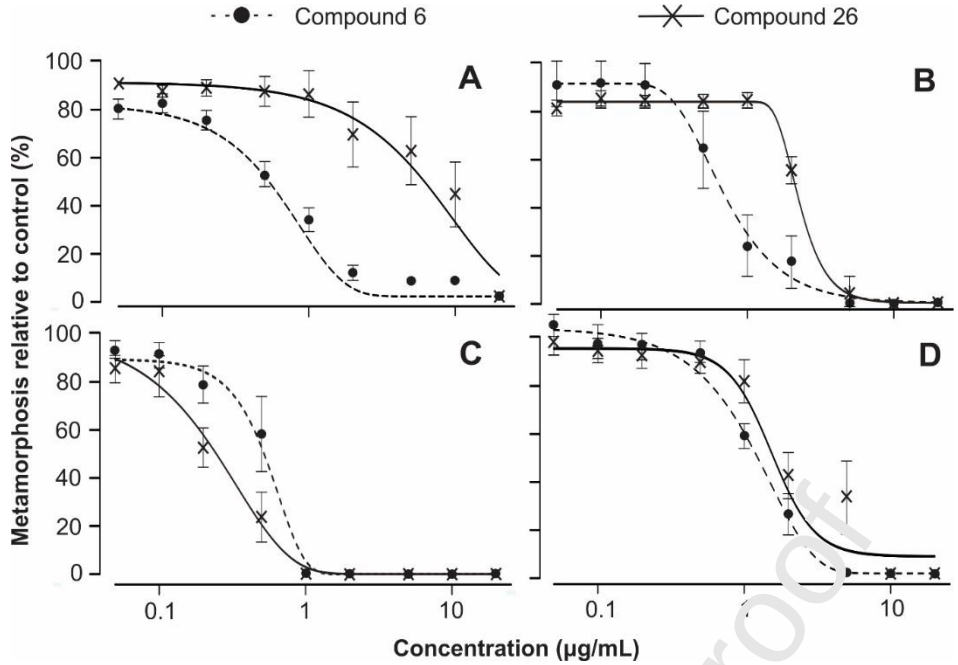

Figure 7. Dose-response curves for compounds **6** and **26** against A) *C. savignyi*, B) *M. galloprovincialis*, C) *S. cariniferus*, D) *U. pinnatifida*.

3.2 Microfouling activity

In parallel, DKPs **6-27** were evaluated against four different microfouling organisms, including two examples of marine bacteria, *Vibrio harveyi,* and *Halomonas aquamarina*, and two microalgae, *Porphyridium purpureum* and *Exanthemachrysis gayraliae*. The growth inhibitory effect of the compounds on the microfoulers was investigated, and the data from the active DKPs are summarised in Table 2.

Table 2. Activity of 2,5-DKPs **6-27** against microfoulers *H. aquamarine*, and *E. gayraliae*.

Entry	% Inhibition at 10 µg/mL	
	H. aquamarina	*E. gayraliae*
6	33.7±31.8	30.3±8.5
7	0	0
8	23.4±1.0	22.2±6.2
9	97.9±4.0	53.2±2.2
10	88.2±36.0	51.1±4.1
11	82.4±51.2	28.0±3.1
12	0	0
13	0	0
14	43.9±19.0	25.0±7.3
15	35.3±7.5	17.6±1.7
16	61.9±39.6	25.5±6.6

17	100±2.3	37.7±17.3
18	57.1±31.1	0
19	41.1±5.4	27.6±13.2
20	74.7±50.3	40.4±6.4
21	100±11.4	30.2±3.4
22	100±2.2	34.8±6.2
23	12.3±4.8	27.7±5.7
24	8.2±6.0	31.8±5.3
25	0	0
26	19.1±9.5	36.9±9.7
27	0	19.4±6.0

In contrast to the macrofouling activity, the compounds displayed no inhibitory activity against *Vibrio harveyi* at 10 μg/mL (data not shown). *Vibrio* species are particularly resistant marine bacteria and previous studies on linear tripeptides revealed a strong resistance towards this type of compound for all evaluated *Vibrio* strains.[30,45] These results are thus in accordance with the literature, and the cyclic nature of the compounds does not appear to alter their activity towards *Vibrio*. Similarly, low activity was seen against the microalgae *Porphyridium purpureum* (data not included). The activity of the compounds against the bacteria *H. aquamarina* was more pronounced, and several compounds produced near or total growth inhibition at 10 μg/mL. The compounds were only tested at a single concentration, and previous studies report submicromolar IC_{50}-values for Gpp-Bip-GppNHBn (**1**, Figure 1) against a range of marine bacteria, which compares well with the near complete inhibition observed for the most active DKPs in the current library.[45] The effect seen against the microalgae *E. gayraliae* is somewhat less pronounced, and this too is in good correlation with previous studies, suggesting a higher activity towards marine bacteria than against microalgae for this type of small amphiphilic compound.[30,45] The inhibition generated by the most potent compounds range between 20-50% illustrating the reduced ability of these compounds to target algal cells. The microfouling data implies that the cyclic DKPs prepared as part of this current study display similar activity against marine bacteria and microalgae to that observed for related linear structural analogues.[24,45] While there is a notable decrease in activity when

compared to the macrofouling organisms, it can be argued that the economic and environmental impacts of the macrofouling organisms are far greater than that of the microorganisms, thus are a more important target.

Collectively these reported investigations illustrate how naturally inspired 2,5-DKPs can be combined with key functional elements from innate defensive peptides to generate highly active antifouling hybrids in high yields to target an unusually wide array of macrofoulers. The peptidic nature of the compounds opens up for a scalable antifouling approach with low environmental impact. The current study is limited so a specific substitution pattern on the DKP ring and the stereochemistry is also locked. It is expected that refining the substitution pattern and the stereochemistry will lead to optimised analogues with improved activity and selectivity. Further development of these compounds toward commercial reality are underway, including further optimisation of bioactivity, quantification and optimisation of environmental half-life, and tuning compatibility with coating formulations. As with any biocide, subsequent product registration will require in depth ecotoxicological evaluation.

4. Conclusions

The current paper probes the established 2 + 2 antimicrobial pharmacophore for short linear amphiphilic peptides in a cyclic 2,5-DKP format against a series of marine biofouling organisms. With the employed synthetic methodology, control over stereochemistry and substitution pattern can be achieved and the initial studies on enantiopure symmetrically substituted DKPs presented here provide structural insights into their bioactivity. 22 rationally designed 2,5-DKPs were prepared and screened for antifouling potency against a comprehensive selection of model macrofouling and microfouling species. Our results indicate that highly potent cyclic analogues of antifouling linear tripeptides can be prepared with several compounds displaying low micromolar activities against the included macrofouling species. A close relationship between a minimum required hydrophobicity

balanced by two strong cationic charges and a high bioactivity towards the macrofoulers was observed. The effect against microfouling organisms was less pronounced but apparent. Our study is the first describing the antifouling potential of this class of versatile compounds and highlights how the 2 + 2 pharmacophore extends beyond bacteria and other microorganisms and also notes the non-toxic mechanism of action against barnacle cyprids. The optimisation of the naturally antifouling DKP scaffold using insight from the innate defence system has led to a new class of peptidic compounds that can be prepared on scale to offer an antifouling strategy with low environmental impact.

Acknowledgements

This study was funded by the New Zealand Ministry for Business Innovation and Employment (CAWX1805). H.P. and G.C. were supported by the Centre for Marine Chemical Ecology (http://www.cemace.science.gu.se) at the University of Gothenburg. C.H and V. F. were supported by Biogenouest at the University of Brest.

Author contributions

Thomas M. Grant: Methodology, Investigation, Writing-original draft; **David Rennison:** Conceptualisation, Methodology, Supervision, Writing-review and editing; **Gunnar Cervin:** Investigation; **Henrik Pavia.** Investigation; **Claire Hellio:** Investigation, Writing-review and editing; **Valentin Fouchen:** Investigation; **Margaret Brimble:** Conceptualisation, Writing-review and editing, Funding acquisition; **Patrick Cahill:** Conceptualisation, Investigation, Writing-review and editing, Funding acquisition; **Johan Svenson:** Conceptualisation, Writing-review and editing, Project administration.

References

(1) Abbott, A.; Abel, P. D.; Arnold, D. W.; Milne, A. Cost–Benefit Analysis of the Use of TBT: The Case for a Treatment Approach. *Sci. Total Environ.* **2000**, *258* (1–2), 5–19. https://doi.org/10.1016/S0048-9697(00)00505-2.

(2) Champ, M. A. A Review of Organotin Regulatory Strategies, Pending Actions, Related Costs and Benefits. *Sci. Total Environ.* **2000**, *258* (1–2), 21–71. https://doi.org/10.1016/S0048-9697(00)00506-4.

(3) Desher, A. A. Biofouling Impacts on the Environment and Ship Energy Efficiency. Master of Science, World Maritime University, Malmo, Sweden, 2018.

(4) Dafforn, K. A.; Lewis, J. A.; Johnston, E. L. Antifouling Strategies: History and Regulation, Ecological Impacts and Mitigation. *Mar. Pollut. Bull.* **2011**, *62* (3), 453–465. https://doi.org/10.1016/j.marpolbul.2011.01.012.

(5) Schultz, M. P.; Bendick, J. A.; Holm, E. R.; Hertel, W. M. Economic Impact of Biofouling on a Naval Surface Ship. *Biofouling* **2011**, *27* (1), 87–98. https://doi.org/10.1080/08927014.2010.542809.

(6) Want, A.; Crawford, R.; Kakkonen, J.; Kaddue, G.; Miller, S.; Harris, R. E.; Porter, J. S. Biodiversity Characterisation and Hydrodynamic Consequences of Marine Fouling Communities on Marine Renewable Energy Infrastructure in the Orkney Islands Archipelago, Scotland, UK. *Biofouling* **2017**, *33* (7), 567–579. https://doi.org/10.1080/08927014.2017.1336229.

(7) Vinagre, P. A.; Simas, T.; Cruz, E.; Pinori, E.; Svenson, J. Marine Biofouling: A European Database for the Marine Renewable Energy Sector. *J. Mar. Sci. Eng.* **2020**, *8* (7), 495. https://doi.org/10.3390/jmse8070495.

(8) Stringer, C. Implications of Biofouling on Cross-Flow Turbine Performance. Master of Science, University of Washington, 2019.

(9) Walker, J. M.; Flack, K. A.; Lust, E. E.; Schultz, M. P.; Luznik, L. Experimental and Numerical Studies of Blade Roughness and Fouling on Marine Current Turbine Performance. *Renew. Energy* **2014**, *66*, 257–267. https://doi.org/10.1016/j.renene.2013.12.012.

(10) Lewin, R. Microbial Adhesion Is a Sticky Problem. *Science* **1984**, *224* (4647), 375–

377.

(11) Davidson, I. C.; Brown, C. W.; Sytsma, M. D.; Ruiz, G. M. The Role of Containerships as Transfer Mechanisms of Marine Biofouling Species. *Biofouling* **2009**, *25* (7), 645–655. https://doi.org/10.1080/08927010903046268.

(12) Sylvester, F.; Kalaci, O.; Leung, B.; Lacoursière-Roussel, A.; Murray, C. C.; Choi, F. M.; Bravo, M. A.; Therriault, T. W.; MacIsaac, H. J. Hull Fouling as an Invasion Vector: Can Simple Models Explain a Complex Problem?: Hull Fouling Invasion Vector. *J. Appl. Ecol.* **2011**, *48* (2), 415–423. https://doi.org/10.1111/j.1365-2664.2011.01957.x.

(13) Basu, S.; Hanh, B. M.; Isaiah Chua, J. Q.; Daniel, D.; Ismail, M. H.; Marchioro, M.; Amini, S.; Rice, S. A.; Miserez, A. Green Biolubricant Infused Slippery Surfaces to Combat Marine Biofouling. *J. Colloid Interface Sci.* **2020**, *568*, 185–197. https://doi.org/10.1016/j.jcis.2020.02.049.

(14) Bannister, J.; Sievers, M.; Bush, F.; Bloecher, N. Biofouling in Marine Aquaculture: A Review of Recent Research and Developments. *Biofouling* **2019**, *35* (6), 631–648. https://doi.org/10.1080/08927014.2019.1640214.

(15) Yebra, D. M.; Kiil, S.; Dam-Johansen, K. Antifouling Technology—Past, Present and Future Steps towards Efficient and Environmentally Friendly Antifouling Coatings. *Prog. Org. Coat.* **2004**, *50* (2), 75–104. https://doi.org/10.1016/j.porgcoat.2003.06.001.

(16) Champ, M. A. Economic and Environmental Impacts on Ports and Harbors from the Convention to Ban Harmful Marine Anti-Fouling Systems. *Mar. Pollut. Bull.* **2003**, *46* (8), 935–940. https://doi.org/10.1016/S0025-326X(03)00106-1.

(17) Gipperth, L. The Legal Design of the International and European Union Ban on Tributyltin Antifouling Paint: Direct and Indirect Effects. *J. Environ. Manag.* **2009**, *90*, 86–95. https://doi.org/10.1016/j.jenvman.2008.08.013.

(18) Price, A. R. G.; Readman, J. W. Booster Biocide Antifoulants: Is History Repeating

Itself. In *Late lessons from early warning signs: science, precaution, innovation*; European Environment Agency, 2013; pp 265–278.

(19) Lejars, M.; Margaillan, A.; Bressy, C. Fouling Release Coatings: A Nontoxic Alternative to Biocidal Antifouling Coatings. *Chem. Rev.* **2012**, *112* (8), 4347–4390. https://doi.org/10.1021/cr200350v.

(20) Candries, M.; Anderson, C. D.; Atlar, M. Foul Release Systems and Drag: Observations on How the Coatings Work. *JPCL* **2001**, *18* (4), 38–43.

(21) Hopkins, G.; Davidson, I.; Georgiades, E.; Floerl, O.; Morrisey, D.; Cahill, P. Managing Biofouling on Submerged Static Artificial Structures in the Marine Environment – Assessment of Current and Emerging Approaches. *Front. Mar. Sci.* **2021**, *8*, 759194. https://doi.org/10.3389/fmars.2021.759194.

(22) Qian, P.-Y.; Li, Z.; Xu, Y.; Li, Y. Fusetani, N. Mini-Review: Marine Natural Products and Their Synthetic Analogs as Antifouling Compounds: 2009–2014. *Biofouling* **2015**, *31* (1), 101–122. https://doi.org/10.1080/08927014.2014.997226.

(23) Moodie, L. W. K.; Trepos, R.; Cervin, G.; Larsen, L.; Larsen, D. S.; Pavia, H.; Hellio, C.; Cahill, P.; Svenson, J. Probing the Structure–Activity Relationship of the Natural Antifouling Agent Polygodial against Both Micro- and Macrofoulers by Semisynthetic Modification. *J. Nat. Prod.* **2017**, *80* (2), 515–525. https://doi.org/10.1021/acs.jnatprod.6b01056.

(24) Svendsen, J. S. M.; Grant, T. M.; Rennison, D.; Brimble, M. A.; Svenson, J. Very Short and Stable Lactoferricin-Derived Antimicrobial Peptides: Design Principles and Potential Uses. *Acc. Chem. Res.* **2019**, *52* (3), 749–759. https://doi.org/10.1021/acs.accounts.8b00624.

(25) Almeida, J. R.; Vasconcelos, V. Natural Antifouling Compounds: Effectiveness in Preventing Invertebrate Settlement and Adhesion. *Biotechnol. Adv.* **2015**, *33* (3–4), 343–357.

https://doi.org/10.1016/j.biotechadv.2015.01.013.

(26) Liu, L.-L.; Wu, C.-H.; Qian, P.-Y. Marine Natural Products as Antifouling Molecules – a Mini-Review (2014–2020). *Biofouling* **2021**, 1–17. https://doi.org/10.1080/08927014.2020.1864343.

(27) Pinteus, S.; Lemos, M. F. L.; Alves, C.; Silva, J.; Pedrosa, R. The Marine Invasive Seaweeds *Asparagopsis Armata* and *Sargassum Muticum* as Targets for Greener Antifouling Solutions. *Sci. Total Environ.* **2021**, *750*, 141372. https://doi.org/10.1016/j.scitotenv.2020.141372.

(28) Sjögren, M.; Johnson, A.-L.; Hedner, E.; Dahlström, M.; Göransson, U.; Shirani, H.; Bergman, J.; Jonsson, P. R.; Bohlin, L. Antifouling Activity of Synthesized Peptide Analogs of the Sponge Metabolite Barettin. *Peptides* **2006**, *27* (9), 2058–2064. https://doi.org/10.1016/j.peptides.2006.03.027.

(29) Labriere, C.; Elumalai, V.; Staffansson, J.; Cervin, G.; Le Norcy, T.; Denardou, H.; Réhel, K.; Moodie, L. W. K.; Hellio, C.; Pavia, H.; Hansen, J. H.; Svenson, J. Phidianidine A and Synthetic Analogues as Naturally Inspired Marine Antifoulants. *J. Nat. Prod.* **2020**, *83* (11), 3413–3423. https://doi.org/10.1021/acs.jnatprod.0c00881.

(30) Trepos, R.; Cervin, G.; Hellio, C.; Pavia, H.; Stensen, W.; Stensvåg, K.; Svendsen, J.-S.; Haug, T.; Svenson, J. Antifouling Compounds from the Sub-Arctic Ascidian *Synoicum Pulmonaria* : Synoxazolidinones A and C, Pulmonarins A and B, and Synthetic Analogues. *J. Nat. Prod.* **2014**, *77* (9), 2105–2113. https://doi.org/10.1021/np5005032.

(31) Sjögren, M.; Jonsson, P. R.; Dahlström, M.; Lundälv, T.; Burman, R.; Göransson, U.; Bohlin, L. Two Brominated Cyclic Dipeptides Released by the Coldwater Marine Sponge *Geodia Barretti* Act in Synergy As Chemical Defense. *J. Nat. Prod.* **2011**, *74* (3), 449–454. https://doi.org/10.1021/np1008812.

(32) Almeida, J. R.; Moreira, J.; Pereira, D.; Pereira, S.; Antunes, J.; Palmeira, A.;

Vasconcelos, V.; Pinto, M.; Correia-da-Silva, M.; Cidade, H. Potential of Synthetic Chalcone Derivatives to Prevent Marine Biofouling. *Sci. Total Environ.* **2018**, *643*, 98–106. https://doi.org/10.1016/j.scitotenv.2018.06.169.

(33) Cahill, P. L.; Kuhajek, J. M. Polygodial: A Contact Active Antifouling Biocide. *Biofouling* **2014**, *30* (9), 1035–1043. https://doi.org/10.1080/08927014.2014.966305.

(34) Neves, A. R.; Almeida, J. R.; Carvalhal, F.; Câmara, A.; Pereira, S.; Antunes, J.; Vasconcelos, V.; Pinto, M.; Silva, E. R.; Sousa, E.; Correia-da-Silva, M. Overcoming Environmental Problems of Biocides: Synthetic Bile Acid Derivatives as a Sustainable Alternative. *Ecotoxicol. Environ. Saf.* **2020**, *187*, 109812. https://doi.org/10.1016/j.ecoenv.2019.109812.

(35) Moodie, L. W. K.; Trepos, R.; Cervin, G.; Bråthen, K. A.; Lindgård, B.; Reiersen, R.; Cahill, P.; Pavia, H.; Hellio, C.; Svenson, J. Prevention of Marine Biofouling Using the Natural Allelopathic Compound Batatasin-III and Synthetic Analogues. *J. Nat. Prod.* **2017**, *80* (7), 2001–2011. https://doi.org/10.1021/acs.jnatprod.7b00129.

(36) Labriere, C.; Cervin, G.; Pavia, H.; Hansen, J. H.; Svenson, J. Structure–Activity Relationship Probing of the Natural Marine Antifoulant Barettin. *Mar. Biotechnol.* **2021**. https://doi.org/10.1007/s10126-021-10074-z.

(37) Davis, C. A.; Janssen, E. M.-L. Environmental Fate Processes of Antimicrobial Peptides Daptomycin, Bacitracins, and Polymyxins. *Environ. Int.* **2020**, *134*, 105271. https://doi.org/10.1016/j.envint.2019.105271.

(38) Wang, J.; Dou, X.; Song, J.; Lyu, Y.; Zhu, X.; Xu, L.; Li, W.; Shan, A. Antimicrobial Peptides: Promising Alternatives in the Post Feeding Antibiotic Era. *Med. Res. Rev.* **2019**, *39* (3), 831–859. https://doi.org/10.1002/med.21542.

(39) Mcphee, J. B.; Hancock, R. E. W. Function and Therapeutic Potential of Host Defence Peptides. *J. Pept. Sci.* **2005**, *11* (11), 677–687. https://doi.org/10.1002/psc.704.

(40) Hwang, P. M.; Zhou, N.; Shan, X.; Arrowsmith, C. H.; Vogel, H. J. Three-Dimensional Solution Structure of Lactoferricin B, an Antimicrobial Peptide Derived from Bovine Lactoferrin. *Biochemistry* **1998**, *37* (12), 4288–4298. https://doi.org/10.1021/bi972323m.

(41) Strøm, M. B.; Rekdal, Ø.; Svendsen, J. S. Antimicrobial Activity of Short Arginine- and Tryptophan-Rich Peptides. *J. Pept. Sci.* **2002**, *8* (8), 431–437. https://doi.org/10.1002/psc.398.

(42) Haug, B. E.; Stensen, W.; Stiberg, T.; Svendsen, J. S. Bulky Nonproteinogenic Amino Acids Permit the Design of Very Small and Effective Cationic Antibacterial Peptides. *J. Med. Chem.* **2004**, *47* (17), 4159–4162. https://doi.org/10.1021/jm049582b.

(43) Svenson, J.; Stensen, W.; Brandsdal, B.-O.; Haug, B. E.; Monrad, J.; Svendsen, J. S. Antimicrobial Peptides with Stability toward Tryptic Degradation. *Biochemistry* **2008**, *47* (12), 3777–3788. https://doi.org/10.1021/bi7019904.

(44) Karstad, R.; Isaksen, G.; Wyrzendaele, E.; Guttormsen, Y.; De Spiegeleer, B.; Brandsdal, B.-O.; Svendsen, J. S.; Svenson, J. Targeting the S1 and S3 Subsite of Trypsin with Unnatural Cationic Amino Acids Generates Antimicrobial Peptides with Potential for Oral Administration. *J. Med. Chem.* **2012**, *55* (14), 6294–6305. https://doi.org/10.1021/jm3002058.

(45) Trepos, R.; Cervin, G.; Pile, C.; Pavia, H.; Hellio, C.; Svenson, J. Evaluation of Cationic Micropeptides Derived from the Innate Immune System as Inhibitors of Marine Biofouling. *Biofouling* **2015**, *31* (4), 393–403. https://doi.org/10.1080/08927014.2015.1048238.

(46) Labrière, C.; Kondori, N.; Caous, J. S.; Boomgaren, M.; Sandholm, K.; Ekdahl, K. N.; Hansen, J. H.; Svenson, J. Development and Evaluation of Cationic Amphiphilic Antimicrobial 2,5-Diketopiperazines. *J. Pept. Sci.* **2018**, *24* (7), e3090.

https://doi.org/10.1002/psc.3090.

(47) Giessen, T. W.; Marahiel, M. A. Rational and Combinatorial Tailoring of Bioactive Cyclic Dipeptides. *Front. Microbiol.* **2015**, *6*. https://doi.org/10.3389/fmicb.2015.00785.

(48) Borthwick, A. D. 2,5-Diketopiperazines: Synthesis, Reactions, Medicinal Chemistry, and Bioactive Natural Products. *Chem. Rev.* **2012**, *112* (7), 3641–3716. https://doi.org/10.1021/cr200398y.

(49) Tullberg, M.; Grøtli, M.; Luthman, K. Synthesis of Functionalized, Unsymmetrical 1,3,4,6-Tetrasubstituted 2,5-Diketopiperazines. *J. Org. Chem.* **2007**, *72* (1), 195–199. https://doi.org/10.1021/jo0619635.

(50) Labrière, C.; Andersen, J. H.; Albrigtsen, M.; Hansen, J. H.; Svenson, J. Heterocyclic Cellular Lipid Peroxidation Inhibitors Inspired by the Marine Antioxidant Barettin. *Bioorganic Chem.* **2019**, *84*, 106–114. https://doi.org/10.1016/j.bioorg.2018.11.024.

(51) Strøm, M. B.; Haug, B. E.; Skar, M. L.; Stensen, W.; Stiberg, T.; Svendsen, J. S. The Pharmacophore of Short Cationic Antibacterial Peptides. *J. Med. Chem.* **2003**, *46* (9), 1567–1570. https://doi.org/10.1021/jm0240039.

(52) Tian, Y.; Li, Y.-L.; Zhao, F.-C. Secondary Metabolites from Polar Organisms. *Mar. Drugs* **2017**, *15* (3), 28. https://doi.org/10.3390/md15030028.

(53) Haug, B.; Strøm, M.; M. Svendsen, J. The Medicinal Chemistry of Short Lactoferricin-Based Antibacterial Peptides. *Curr. Med. Chem.* **2007**, *14* (1), 1–18. https://doi.org/10.2174/092986707779313435.

(54) Furukawa, T.; Akutagawa, T.; Funatani, H.; Uchida, T.; Hotta, Y.; Niwa, M.; Takaya, Y. Cyclic Dipeptides Exhibit Potency for Scavenging Radicals. *Bioorg. Med. Chem.* **2012**, *20* (6), 2002–2009. https://doi.org/10.1016/j.bmc.2012.01.050.

(55) Marchini, M.; Mingozzi, M.; Colombo, R.; Guzzetti, I.; Belvisi, L.; Vasile, F.; Potenza, D.; Piarulli, U.; Arosio, D.; Gennari, C. Cyclic RGD Peptidomimetics Containing

Bifunctional Diketopiperazine Scaffolds as New Potent Integrin Ligands. *Chem. - Eur. J.* **2012**, *18* (20), 6195–6207. https://doi.org/10.1002/chem.201200457.

(56) Karstad, R.; Isaksen, G.; Brandsdal, B.-O.; Svendsen, J. S.; Svenson, J. Unnatural Amino Acid Side Chains as S1, S1′, and S2′ Probes Yield Cationic Antimicrobial Peptides with Stability toward Chymotryptic Degradation. *J. Med. Chem.* **2010**, *53* (15), 5558–5566. https://doi.org/10.1021/jm1006337.

(57) Shearer, B. G.; Oplinger, J. A.; Lee, S. S-2-Naphthylmethyl Thioacetimidate Hydrobromide: A New Odorless Reagent for the Mild Synthesis of Substituted Acetamidines. *Tetrahedron Lett.* **1997**, *38* (2), 179–182. https://doi.org/10.1016/S0040-4039(96)02268-X.

(58) Dorsey, J. G.; Khaledi, M. G. Hydrophobicity Estimations by Reversed-Phase Liquid Chromatography. *J. Chromatogr. A* **1993**, *656* (1–2), 485–499. https://doi.org/10.1016/0021-9673(93)80815-P.

(59) Flaten, G. E.; Kottra, G.; Stenson, W.; Isaksen, G.; Karstad, R.; Svendsen, J. S.; Daniel, H.; Svenson, J. In Vitro Characterization of Human Peptide Transporter HPEPT1 Interactions and Passive Permeation Studies of Short Cationic Antimicrobial Peptides. *J. Med. Chem.* **2011**, *54* (7), 2422–2432. https://doi.org/10.1021/jm1015704.

(60) Mandrekar, P. Culturing *Undaria Pinnatifida* Gametophytes, Auckland University of Technology, New Zealand, 2018.

(61) Cahill, P. L.; Atalah, J.; Selwood, A. I.; Kuhajek, J. M. Metamorphosis of the Invasive Ascidian *Ciona Savignyi* : Environmental Variables and Chemical Exposure. *PeerJ* **2016**, *4*, e1739. https://doi.org/10.7717/peerj.1739.

(62) Cahill, P. L.; Heasman, K.; Jeffs, A.; Kuhajek, J. Laboratory Assessment of the Antifouling Potential of a Soluble-Matrix Paint Laced with the Natural Compound Polygodial. *Biofouling* **2013**, *29* (8), 967–975. https://doi.org/10.1080/08927014.2013.822488.

(63) Standard Guide for Conducting Static Short-Term Chronic Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs. ASTM International January 15, 2021.

(64) Choi, H. G.; Kim, Y. S.; Lee, S. J.; Park, E. J.; Nam, K. W. Effects of Daylength, Irradiance and Settlement Density on the Growth and Reproduction of *Undaria Pinnatifida* Gametophytes. *J. Appl. Phycol.* **2005**, *17* (5), 423–430. https://doi.org/10.1007/s10811-005-0432-2.

(65) Berntsson, K. M.; Jonsson, P. R.; Lejhall, M.; Gatenholm, P. Analysis of Behavioural Rejection of Micro-Textured Surfaces and Implications for Recruitment by the Barnacle Balanus Improvisus. *J. Exp. Mar. Biol. Ecol.* **2000**, *251* (1), 59–83. https://doi.org/10.1016/S0022-0981(00)00210-0.

(66) Ritz, C.; Baty, F.; Streibig, J. C.; Gerhard, D. Dose-Response Analysis Using R. *PLOS ONE* **2015**, *10* (12), e0146021. https://doi.org/10.1371/journal.pone.0146021.

(67) Hellio, C.; Trepos, R.; Aguila-Ramírez, R. N.; Hernández-Guerrero, C. J. Protocol for Assessing Antifouling Activities of Macroalgal Extracts. In *Natural Products From Marine Algae*; Stengel, D. B., Connan, S., Eds.; Springer New York: New York, NY, 2015; Vol. 1308, pp 421–435. https://doi.org/10.1007/978-1-4939-2684-8_27.

(68) Saavedra, C. J.; Cuevas, F.; Romero- Estudillo, I.; Boto, A. Synthesis of Diketopiperazine Scaffolds with Tailored *N* - and A- Chains by Selective Modification of Customizable Units. *Adv. Synth. Catal.* **2020**, *362* (15), 3158–3169. https://doi.org/10.1002/adsc.202000470.

(69) Bernbom, N.; Ng, Y. Y.; Olsen, S. M.; Gram, L. Pseudoalteromonas Spp. Serve as Initial Bacterial Attractants in Mesocosms of Coastal Waters but Have Subsequent Antifouling Capacity in Mesocosms and When Embedded in Paint. *Appl. Environ. Microbiol.* **2013**, *79* (22), 6885–6893. https://doi.org/10.1128/AEM.01987-13.

(70) John A. Lewis. Marine Biofouling and Its Prevention on Underwater Surfaces. *Mater. Forum* **1998**, *22*, 41–61.

(71) Bressy, C.; Hellio, C.; Maréchal, J. P.; Tanguy, B.; Margaillan, A. Bioassays and Field Immersion Tests: A Comparison of the Antifouling Activity of Copper-Free Poly(Methacrylic)-Based Coatings Containing Tertiary Amines and Ammonium Salt Groups. *Biofouling* **2010**, *26* (7), 769–777. https://doi.org/10.1080/08927014.2010.516392.

(72) Stenton-Dozey, J. M. E.; Heath, P.; Ren, J. S.; Zamora, L. N. New Zealand Aquaculture Industry: Research, Opportunities and Constraints for Integrative Multitrophic Farming. *N. Z. J. Mar. Freshw. Res.* **2020**, 1–21. https://doi.org/10.1080/00288330.2020.1752266.

(73) Jackson, L. *Marine Biofouling: An Assessment of Risks and Management Initiatives.*; Global Invasive Species Program and UNEP, 2008; p 68.

(74) Brooke, D. G.; Cervin, G.; Champeau, O.; Harwood, D. T.; Pavia, H.; Selwood, A. I.; Svenson, J.; Tremblay, L. A.; Cahill, P. L. Antifouling Activity of Portimine, Select Semisynthetic Analogues, and Other Microalga-Derived Spirocyclic Imines. *Biofouling* **2018**, *34* (8), 950–961. https://doi.org/10.1080/08927014.2018.1514461.

(75) Qi, S.-H.; Ma, X. Antifouling Compounds from Marine Invertebrates. *Mar. Drugs* **2017**, *15* (9), 263. https://doi.org/10.3390/md15090263.

(76) Hanssen, K. Ø.; Cervin, G.; Trepos, R.; Petitbois, J.; Haug, T.; Hansen, E.; Andersen, J. H.; Pavia, H.; Hellio, C.; Svenson, J. The Bromotyrosine Derivative Ianthelline Isolated from the Arctic Marine Sponge *Stryphnus Fortis* Inhibits Marine Micro- and Macrobiofouling. *Mar. Biotechnol.* **2014**, *16* (6), 684–694. https://doi.org/10.1007/s10126-014-9583-y.

(77) Moodie, L. W. K.; Cervin, G.; Trepos, R.; Labriere, C.; Hellio, C.; Pavia, H.; Svenson, J. Design and Biological Evaluation of Antifouling Dihydrostilbene Oxime

Hybrids. *Mar. Biotechnol.* **2018**, *20* (2), 257–267. https://doi.org/10.1007/s10126-018-9802-z.

(78) Fusetani, N. Antifouling Marine Natural Products. *Nat. Prod. Rep.* **2011**, *28* (2), 400–410. https://doi.org/10.1039/C0NP00034E.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

.