

SUPPLEMENTAL MATERIAL

Allocation of trioxazole macrolides in the sponge *Penares cf. nux* and the impact on epiphytic bacterial cohabitants

Oyenike O. Olatunji^a, Aekaphon Petchoubon^a, Chitti Thawai^{b,c}, and Anuchit Plubrukarn^{a*}

^a*Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand;* ^b*Department of Biology, and* ^c*Actinobacterial Research Unit, Faculty of Science, King Mongkut's Institute of Technology, Ladkrabang, Bangkok 10520, Thailand*

*corresponding author; Anuchit Plubrukarn; email; anuchit.pl@psu.ac.th

Abstract

The correlation between the allocation of trisoxazole macrolides in the capitums, appendages, and bases of the sponge *Penares cf. nux* and the surface-attached bacteria on the corresponding parts was examined. The kabiramide contents were highest in the capitums, followed by the appendages and bases. Conversely, direct counts of cultivable surface-attached bacteria showed that the bacteria aggregate more densely on the surfaces of the bases. This suggested the repelling effects of the kabiramides against the fouling bacteria, particularly on the capitums and appendages. Twenty-two bacterial strains were isolated and identified to 15 species; however, none has shown the potentials as a producer of any secondary metabolites in the sponge *P. nux*.

Contents

Experimental	S-3
1. Specimen collection and preparation	S-3
2. Quantitation of kabiramides B, C, and G	S-3
3. Scanning electron microscopes	S-5
4. Surface-attached bacterial count	S-5
5. Identification of sponge surface-attached bacteria	S-6
6. Statistical analyses	S-6
References	S-7

Tables

Table S-1 Specimen dry weights and extraction yields from each part of the sponge <i>P. nux</i>	S-9
Table S-2 Contents of kabiramides B, C, and G, and plate counts of surface-attached bacteria in each part of the sponge specimens	S-10
Table S-3 Identification to closely related strains of bacterial isolates from the surface of the sponge <i>P. nux</i>	S-11

Figures

Figure S-1. (a-c) Contents of kabiramides B,C, and G (mg/g sponge dry weight) from 11 selected colonies; (b) plate-count of surface-attached bacteria (10^6 CFU/cm ² sponge surface) from corresponding colonies as in (a)	S-14
Figure S-2. Scanning electron micrographs of <i>P. nux</i> 's surfaces of three selected colonies, showing the capitums (a-c) and the bases (d-f) of respective colonies	S-15
Figure S-3. (a-c) Correlations between the plate-counts of surface-attached bacteria and the contents of kabiramides B, C, and G, respectively	S-16

Experimental

1. Specimen collection and preparation

The sponge *Penares* cf. *nux* de Laubenfels were collected on an individual and separated colony basis from Hin-Wong Pinnacle, Surat-Thani Province, Thailand (10° 7.140' N, 99° 50.948' E) in May, 2013. The taxonomic identification was performed by Dr.Sumaitt Putchakarn, Research Institute of Marine Sciences, Burapha University, Chonburi, Thailand. The sponge was identified in the earlier publications (Petchprayoon et al. 2006; Sirirak, Intaraksa, et al. 2011; Sirirak, Kittiwisut, et al. 2011; Sirirak et al. 2013) as *Pachastrissa nux*, but is transferred here as *Penares* cf. *nux* de Laubenfel (Van Soest et al. 2010). A voucher specimen (AP04-002-01) is deposited at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. All the specimens were preserved in an ice chest upon surfacing (0 °C) until reaching the laboratory.

Forty-two sponge colonies on which the capitums or appendages or both are clearly identified were selected for the chemical allocation and plate count experiments. Eleven collected colonies have all the three parts of capitums, appendages, and bases, 16 have only capitums and bases, and 15 have appendages and bases. Once arriving at the laboratory, each part (at a minimum of 2 cm in all dimensions) was removed, tagged, and subjected to the plate count experiment, after which they were all available for the chemical investigation. The specimens were preserved separately at -20°C until the time of extraction.

2. Quantitation of kabiramides B, C, and G

Standard kabramides B, C, and G were prepared in house using the isolation protocol described previously (Sirirak, Kittiwisut, et al. 2011). All the isolated compounds were

identified based on the spectroscopic analyses, including MS, UV, IR, and NMR spectroscopies, and were all compared with the data previously reported (Sirirak, Kittiwisut, et al. 2011; Sirirak et al. 2013). The purity of each compound was referred to the ^1H NMR spectrum, in which the resonances of any impurities were unobservable.

Quantitative analysis was carried out using LC-MS on an Agilent 1260 Infinity LC system, equipped with a binary pump (G1312B), an autosampler (G1367E), a column compartment (G1316A), and a degasser (G4225A), and coupled to a mass detector API 3200TM LC/MS/MS (AB Sciex). The chromatographic conditions were as followed; RP-C18 column (VertiSepTM UPS, 5 μm , 150 \times 2.1 mm), 45°C, 1% aq AcOH/CH₃CN (45:55), 0.8 mL/min. MS detection was operated in a positive ESI mode, from which peaks at m/z 929 (kabiramide B, t_R 5.3 min), 943 (kabiramide C, t_R 6.8 min), and 900 (kabiramide G, t_R 8.3 min) were extracted and calculated. (The peak at m/z 900 of kabiramide G was the base peak, and was selected instead of the pseudomolecular peak due to a weak relative intensity of the MH^+). The protocol was validated on the topics of linearity, limits of detection and quantitation, and intra-day and inter-day precision and accuracy, as dictated by ICH. The validations of the three standards are as followed; kabiramide B, linearity range 0.3-10 $\mu\text{g/mL}$ (R^2 0.999), LOD 0.01 $\mu\text{g/mL}$, LOQ 0.03 $\mu\text{g/mL}$, recovery percentages 99.36-100.3% (%*RSD* 0.6-1.0%); kabiramide C, linearity range 0.01-30 $\mu\text{g/mL}$ (R^2 0.9999), LOD 0.004 $\mu\text{g/mL}$, LOQ 0.01 $\mu\text{g/mL}$, recovery percentages 98.70-100.6% (%*RSD* 1.2-2.2%); and kabiramide G, linearity range 0.3-10 $\mu\text{g/mL}$ (R^2 0.999), LOD 0.01 $\mu\text{g/mL}$, LOQ 0.03 $\mu\text{g/mL}$, recovery percentages 98.75-101.9% (%*RSD* 0.8-1.8%).

The determination of the three kabiramides was carried out on a per-part, per-colony basis. Each part from each colony was separately freeze-dried and refluxed in THF (15 mL, 1 h), and the resulting solution was evaporated to dryness (Sirirak,

Intaraksa, et al. 2011). Each extract was diluted quantitatively to 500 µg/mL in CH₃CN, and subjected to the LC protocol as described with no further purification. The content of each kabiramide analogue in each part was calculated and reported in a mg/g sponge dry weight basis.

3. Scanning electron microscope

The capitums and bases (approximately 1.5 cm in all dimensions) were removed from selected colonies of the sponge *P. nux* that had been set aside for scanning electron microscope. Each specimen was rinsed extensively with artificial seawater, and fixed with 2.5% glutaraldehyde in 0.2 N PBS (2h), then rinsed with 0.1 N PBS (×3). The specimens were dehydrated with ethanol series, and the liquid was removed with critical point drier (Polaron CPD7501). Each dried specimen was stubbed and sputter coated with gold mix. The micrographs were viewed in a high vacuum mode on a Quanta400 FEI scanning electron microscope.

4. Surface-attached bacterial count

Determination of colony forming unit of surface-attached bacteria was carried out by directly swabbing the sponge surface (Chelossi et al. 2004). Each specimen was rinsed extensively with sterile artificial seawater to rid most of the dirt and loosely attached fouling organisms. The specimen was swabbed with a sterile cotton swab within an area of 1×1 cm² frame. Specifically for any capitum and appendage specimens of which a single branch is smaller than the required area, several branches from such a specimen were placed tightly to comply with the swabbing frame. The cotton swab was then soaked in sterile artificial seawater (2 mL), and the solution was diluted quantitatively to a serial dilution. A 100-mL of each dilution (×6) was plated onto

marine nutrient agar 2216 (Difco), and the plates were incubated at 25°C for 7 days. CFU was counted and calculated (as CFU/cm²) from the dilution that yielded 30-300 clearly observable colonies.

5 Identification of sponge surface-attached bacteria

The identification of selected bacterial isolates from the plate count experiment described above was performed using 16S rRNA sequencing and phylogenetic analysis. The bacterial isolates subjected to the identification were selected on a basis of distinctive pigmentation and morphology of appearing colonies, yielding 22 bacterial isolates. Each isolate was primarily purified by cross streaking on a marine nutrient agar, and preserved at -80°C in a marine broth supplemented with 40% glycerol.

For each isolates, the 16S rRNA gene was amplified by PCR using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), and the PCR products were sequenced (First Base, Seri Kembangan, Malaysia) using universal primers (Lane 1991). The 16S rRNA gene sequence as aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases using the CLUSTAL W 1.81 (Thompson et al. 1994). The alignment was manually verified and adjusted prior to the neighbour-joining with genetic distances computed using Kimura's 2-parameter model (Kimura 1980) in the MEGA 5 software (Tamura et al. 2011). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses based on 100 re-samplings. The values for sequence similarity to all bacterial strains were first determined using the ExTaxon-e database (Kim et al. 2012). The 16s rRNA gene sequence similarities among the bacterial strains and the closely related species were calculated manually

after pairwise alignments obtained using the CLUSTAL_X program (Thompson et al. 1997).

6. Statistical analyses

All the statistical analyses were carried out using SOFAstats (1.4.3). Due to the non-parametric nature of the datasets, ranges and medians were used to present the weights of specimens and extracts, kabiramide contents, and CFUs from plate count experiment. Kruskal-Wallis test was used to determine the statistic differences in each comparison. Spearman correlation was used to determine the correlation between kabiramide contents and CFU counts.

References

- Chelossi E, Milanese M, Milano A, Pronzato R, Riccardi G. 2004. Characterisation and antimicrobial activity of epiphytic bacteria from *Petrosia ficiformis* (Porifera, Demospongiae). J Exp Mar Biol Ecol. 309:21-33.
- Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chon J. 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol. 62:716-721.
- Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequence. J Mol Evol. 16:111-120.
- Lane DJ. 1991. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematics. Chichester: John Wiley & Sons; p. 115-148.
- Petchprayoon C, Asato Y, Higa T, Garcia-Fernandez LF, Pedpradub S, Marriott G, Suwanborirux K, Tanaka J. 2006. Four new kabiramides from the Thai sponge, *Pachastrissa nux*. Heterocycles. 69: 447-456.

- Sirirak T, Brecker L, Plubrukarn A. 2013. Kabiramide L, a new antiplasmodial trisoxazole macrolide from the sponge *Pachastrissa nux*. *Nat Prod Res.* 27:1213-1219.
- Sirirak T, Intaraksa N, Kaewsuwan S, Yuenyongsawad S, Suwanborirux K, Plubrukarn A. 2011. Intracolony allocation of trisoxazole macrolides in the sponge *Pachastrissa nux*. *Chem Biodivers.* 8:2238-2246.
- Sirirak T, Kittiwisut S, Janma C, Yuenyongsawad S, Suwanborirux K, Plubrukarn A. 2011. Kabiramides J and K, trisoxazole macrolides from the sponge *Pachastrissa nux*. *J Nat Prod.* 74:1288-1292.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol.* 28:2731-2739.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl Acids Res.* 25:4876-4882.
- Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence through sequence weighing, position-specific gap penalties and weight matrix choice. *Nucl Acids Res.* 22: 4673-4680.
- Van Soest RWM, Beglinger EJ, de Voogd NJ. 2010. Skeletons in confusion: a review of astrophorid sponges with (dicho-)calthrops as structural megastructures (Porifera, Demospongiae, Astrophorida). *Zookeys.* 68:1-88.

Table S-1. Specimen dry weights and extraction yields from each part of the sponge *P.*

nux

Samples	N	Ranges	Medians
Capitum specimens (mg)	27	22.3-365.7	100.8
Extraction yields from capitums (mg/g sponge dry weight)	27	36.6-197.3	71.0
Appendage specimens (mg)	26	274.6-645.3	379.3
Extraction yields from appendages (mg/g sponge dry weight)	26	16.2-67.7	32.7
Base specimens (mg)	42	453.5-2011.3	986.2
Extraction yields from bases (mg/g sponge dry weight)	42	5.4-66.4	11.9

Table S-2. Contents of kabiramides B, C, and G, and plate counts of surface-attached bacteria in each part of the sponge specimens

	N	Ranges	Median s
Kabiramide B (mg/g sponge dry weight)			
Capitum	27	0.057-1.927	0.484
Appendage	26	0.036-1.238	0.164
Base	42	0.001-0.180	0.023
Kruskal-Wallis H 62.645, $P < 0.001$			
Spearman correlation with bacterial population R -0.388, $P < 0.001$			
Kabiramide C (mg/g sponge dry weight))			
Capitum	27	0.043-4.254	0.974
Appendage	26	0.075-1.870	0.292
Base	42	0.001-0.280	0.035
Kruskal-Wallis H 65.298, $P < 0.001$			
Spearman correlation with bacterial population R -0.423, $P < 0.001$			
Kabiramide G (mg/g sponge dry weight))			
Capitum	27	0.068-2.501	0.230
Appendage	26	0.015-0.487	0.080
Base	42	0.001-0.119	0.010
Kruskal-Wallis H 64.765, $P < 0.001$			
Spearman correlation with bacterial population R -0.320,			

$P < 0.001$

Surface-attached bacteria ($\times 10^6$

CFU/cm²)

Capitum	27	6.667- 115.333	9.133
Appendage	26	6.600-23.833	9.033
Base	42	10.167- 161.667	24.850

Kruskal-Wallis H 52.603, $P < 0.001$

Table S-3. Identification to closely related strains of bacterial isolates from the surface of the sponge *P. nux*

Identification of bacterial isolates (strain[s] and similarity)	Similarity to closely related strain
Class Gammaproteobacteria	
Order Oceanospirillales	
Family Halomonadaceae	
<i>Salinicola</i> sp. PR01	99.5-100%
(= PR14 = PR15 = PR21 = PR22 99.8-100%)	<i>S. salarii</i> (Kim <i>et al.</i> 2007) de la Haba <i>et al.</i> M27
<i>Halomonas</i> sp. PR07	100%
	<i>H. meridiana</i> James <i>et al.</i> DSM 5425
<i>Halomonas</i> sp. ^a PR17	98.6%
	<i>H. qiaohouensis</i> Wang <i>et al.</i> YIM QH88
Family Alcanivoracaceae	
<i>Alcanivorax</i> sp. PR11	100%
	<i>A. xenomutans</i> Rahul <i>et al.</i> JC109
Order Alteromonadales	
Family Idiomarinaceae	
<i>Idiomarina</i> sp. PR05	99.7%
(= PR16 100%)	<i>I. donghaiensis</i> (Wu <i>et al.</i>) Taborda <i>et al.</i> 908033
Family Pseudoalteromonadaceae	
<i>Pseudoalteromonas</i> sp. PR08	100%
	<i>P. issachenkonii</i> Ivanova <i>et al.</i> KMM

(= PR20 100%)	3549
Order Pseudomonadales	
Family Moraxellaceae	
<i>Psychrobacter</i> sp. ^a PR06	98.7%
	<i>P. pacificensis</i> Murayama <i>et al.</i>
	NIBH P2K6
<i>Psychrobacter</i> sp. PR18	99.4%
	<i>P. pacificensis</i> Murayama <i>et al.</i>
	NIBH P2K6

Table S-3 (cont).

Identification of bacterial isolates (strain[s] and similarity)	Similarity to closely related strain
Class Alphaproteobacteria	
Order Sphingomonadales	
Family Erythrobacteraceae	
<i>Erythrobacter</i> sp. PR02	99.6%
(= PR19 100%)	<i>E. pelagi</i> Wu <i>et al.</i> UST081027-248
<i>Erythrobacter</i> sp. PR03	100%
	<i>E. citreus</i> Denner <i>et al.</i> RE35F/1
Order Rhodobacterales	
Family Rhodobacteraceae	
<i>Paracoccus</i> sp. ^a PR04	98.2%
	<i>P. zeaxanthinifaciens</i> Berry <i>et al.</i> ATCC 21588
Order Rhizobiales	
Family Aurantimonadaceae	
<i>Aurantimonas</i> sp. PR13	100%
	<i>A. coralicida</i> Denner <i>et al.</i> DSM 14790
Class Flavobacteriia	
Order Flavobacteriales	
Family Flavobacteriaceae	
<i>Vitellibacter</i> sp. PR10	100%
	<i>V. vladivostokensis</i> Nedashkovskaya

<i>et al.</i> KMM 3516	
Class Actinobacteria	
Order Actinomycetales	
Family Micrococcaceae	
<i>Kocuria</i> sp. PR09	100%
	<i>K. palustris</i> Kovács <i>et al.</i> DSM
	11925

Table S-3 (cont).

Identification of bacterial isolates (strain[s] and similarity)	Similarity to closely related strain
Family Dermacoccaceae	
<i>Kytococcus</i> sp. PR12	99.6%
	<i>K. sedentarius</i> (ZoBell and Upham)
	Stackebrandt <i>et al.</i> DSM 20547

Note; ^a Potentially novel species

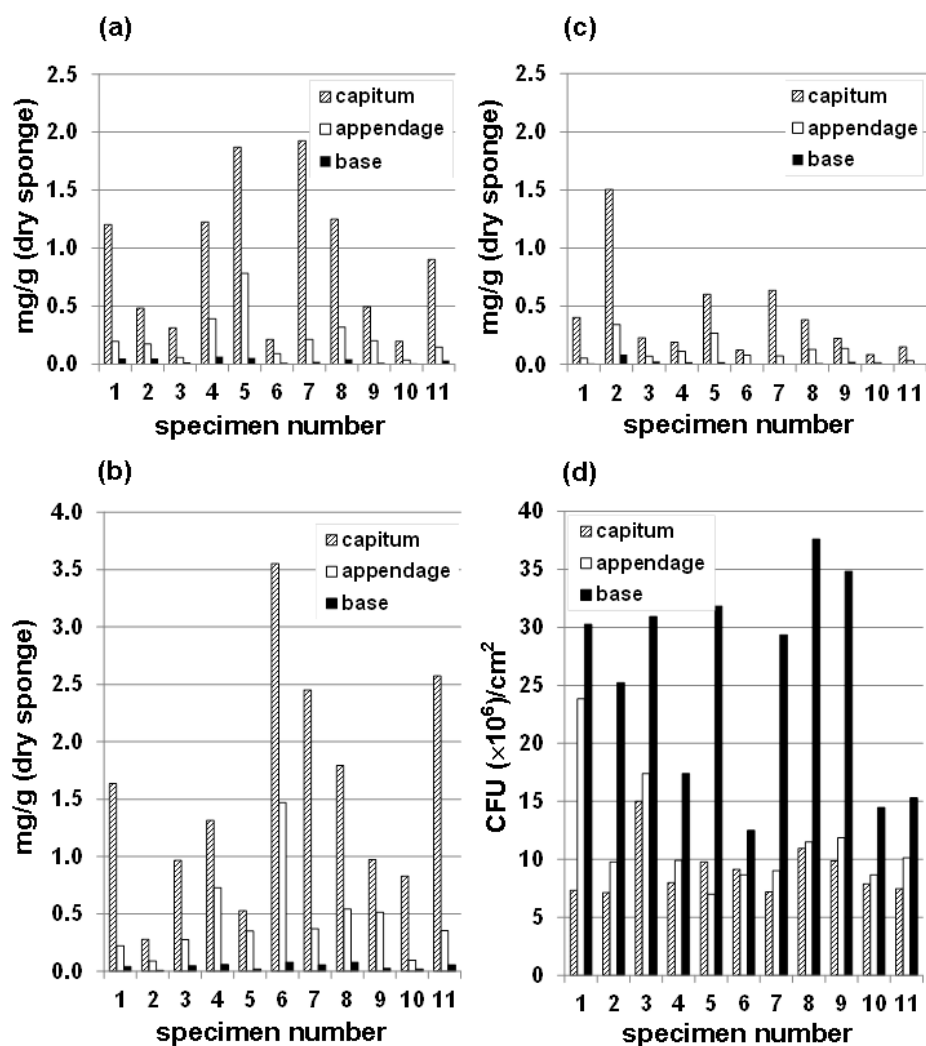


Figure S-1. (a-c) Contents (mg/g sponge dry weight) of kabiramides B,C, and G, respectively, from 11 selected colonies that have all three parts present; (d) plate-count of surface-attached bacteria (10^6 CFU/cm² sponge surface) from corresponding colonies as in (a)

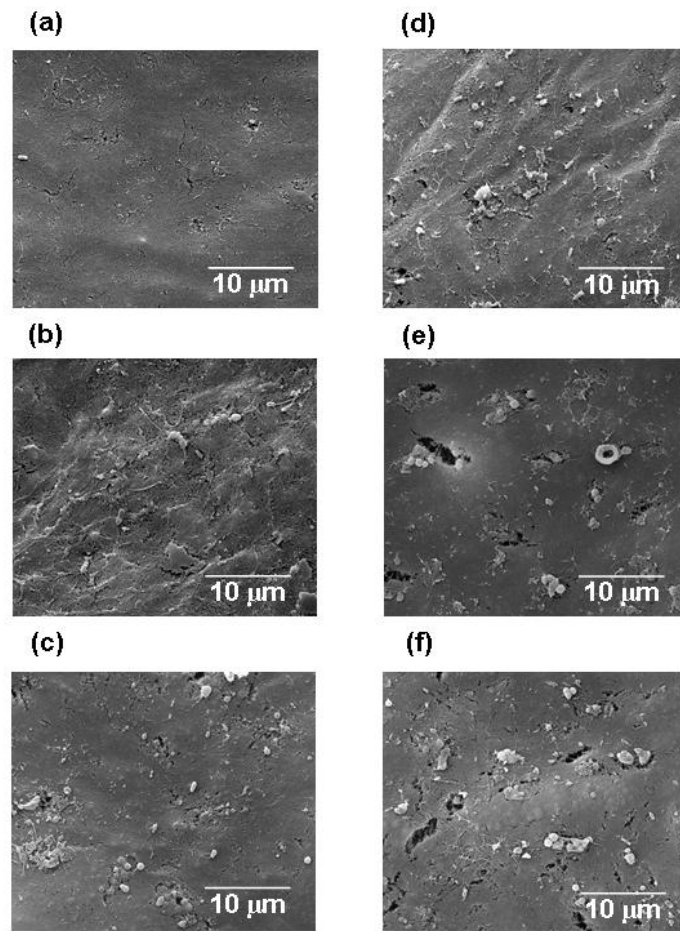


Figure S-2. Scanning electron micrographs of *P. nux*'s surfaces of three selected colonies, showing the capitums (a-c) and the bases (d-f) of respective colonies

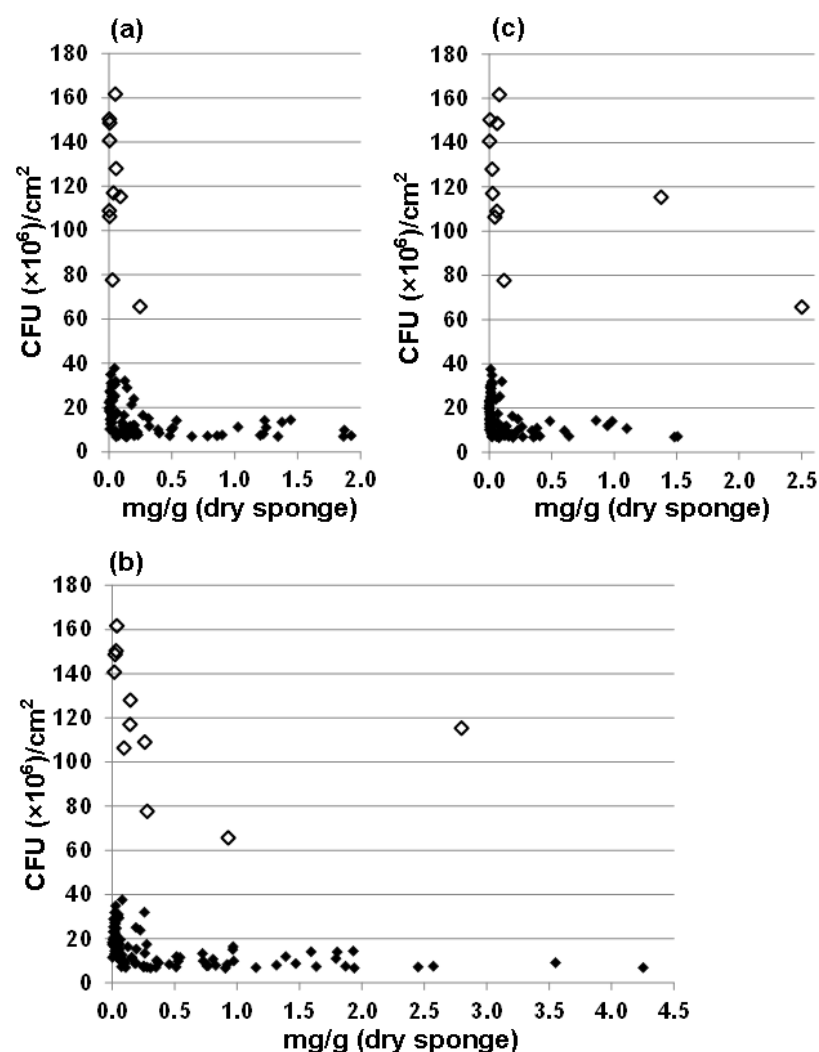


Figure S-3. (a-c) Correlations between the plate-counts of surface-attached bacteria and the contents of kabiramides B, C, and G, respectively; empty diamonds representing 11 outlying samples excluded from Spearman correlation (CFUs exceeding $24.41 - 5.98 \times 10^6 \text{ CFU}/\text{cm}^2$, exclusion criterion based on triple interquartile-range)