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Original Article

Metabolomics Approach towards the Chemical Distribution in the Sponge Penares cf. nux

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Abstract

A metabolomics workflow is used to trace the connection between the chemical allocation and the bud differentiation in the sponge *Penares cf. nux*. The sponge specimens collected from two locations; Koh-Tao, Surat-Thani Province, and Saiburi, Pattani Province, were separated into two parts, the budding capitums and the bases. **The geographic locations had no apparent effects on the sponge’s chemical profiles.** PCA of the CHCl$_3$-extracts, however, discriminated the capitums from the bases, and emphasized the impacts from the trisoxazole macrolides and the sterols. The results indicated that the sponge was able to allocate the secondary metabolites towards the capitums. On the other hand, the dataset of the aq MeOH-extracts, representing the water-soluble primary metabolites, scattered throughout the score plot and suggested that there were no differences between the primary metabolisms in the two parts.

**Keywords:** *Penares nux*; trisoxazole macrolides; kabiramides; metabolomics; chemical allocation
1. Introduction

The sponge *Penares* cf. *nux* de Laubenfels (Figure 1) is among the common sponge species distributing widely in the tropical coral reefs. The sponge has been documented – under the epithet *Penares nux* and other synonyms – to spread throughout the West-Central Pacific, from Palau and Guam to Malaysia and Thailand (Van Soest, Beglinger, & De Voogd, 2010). Chemically, the sponge *P. nux* is one of the major sources of trisoxazole macrolides of the kabiramide family, among which kabiramides C and G (Figure 2) constituted the major macrolide composition (Petchprayoon et al, 2006; Sirirak et al, 2011a; Sirirak, Brecker, & Plubrukarn, 2013). Most of the trisoxazole macrolides showed a wide range of biological activities, from antiproliferative activities against cancer cell lines to antifungal and antiparasitic activities (Matsunaga, 2006). This is due to the mechanisms of actin binding, therefore disrupting the functions of actin, which is the main cytoskeleton of eukaryotic cells (Klechin et al, 2003; Tanaka et al, 2003; Petchprayoon et al, 2005). Whereas the actin-binding mechanism leads to the indiscriminating toxicity, trisoxazole macrolides are on the other hand known to be employed by the sponges and other dietary sequestering invertebrates as a chemical defense against predating fish (Pawlik, Kernan, Molinski, Harper, & Faulkner, 1988; Matsunaga, 2006; Dalisay, Rogers, Edison, & Molinski, 2009).

Besides being the source of the trisoxazole macrolides, the sponge *P. nux* also has an unusual, characteristic morphological feature. Unlike most of other sponge species whose asymmetric and irregular-shaped bodies may bud into morphologically indistinctive formations, the sponge *P. nux* can bud into the tree-shaped branch(es) sprouting randomly from the substratum-attached base (Figure 1b). The tree-shaped buds – called hereinafter the capitums – associate with the specific allocation of the trisoxazole
macrolides. Kabiramides C and G were found to accumulate significantly more in the capitums of the sponge than in the bases (Sirirak et al, 2011b). It was proposed that the specific allocation of the trisoxazole macrolides was a chemical defensive mechanism that the sponge used to protect the protruding and vulnerable parts of its body (Sirirak et al, 2011b; Olatunji, Petchoubon, Thawai, & Plubrukarn, 2018).

Upon realizing the specific distribution of the trisoxazole macrolides in the sponge \textit{P. nux}, new questions have arisen. Apart from the trisoxazole macrolides, are other classes of compounds, whether primary or secondary metabolites, allocated also specifically? And if so, is such an allocation parallel or opposite to that of trisoxazole macrolides? To extend the scope of this investigation to cover both primary and secondary metabolites, here we adopted an NMR-based metabolomics workflow to investigate the chemical profiles of the sponge \textit{P. nux} in a simultaneous and holistic manner, and to examine whether the resulting profiles may have a specific connection to the morphological differentiation of the sponge.

2. Materials and Methods

2.1 Sponge specimen collection and authentication

The sponge \textit{Penares} cf. \textit{nux} de Laubenfels was collected from two locations; from Koh-Tao, Surat-Thani Province (10.1040, 99.8460) in June 2011, and from Saiburi coast, Pattani Province (6.7080, 101.6600) in August 2013. Specifically for the sponge specimens to be subjected to the metabolomics studies, only the colonies that had at least one fully developed capitum were selected. The remaining specimens were combined and were subjected to the extraction for the standard kabiramides.
The sponge specimens were collected by SCUBA diving with gloved hands at the depths of 10-15 m. Both in situ and upon surfacing, the sponge has a charcoal black outer color, with a lighter brownish gray and soft inner core. The colony (1-2 cm thick, 5-10 cm long) has an encrusting, irregular-shaped mass. Occasionally and randomly, branching tree-like capitum(s) (2-5 cm tall) are found budding from the substratum-attached base.

All the collected specimens were preserved in an ice chest (0°C) upon surfacing, and at -20°C once arrived at the lab until extraction. The specimens from both locations were identified to be Penares cf. nux de Laubenfels (Van Soest et al, 2010) by Dr. Sumaitt Putchakarn, Institute of Marine Science, Burapha University, Chonburi, Thailand. The voucher specimens from both location are deposited at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.

2.2 Extraction and sample preparations

The standard kabiramide C was prepared in-house from the specimens described above using the modified isolation protocols (Sirirak et al, 2011a). The authentication was performed by means of complete spectroscopic analysis, and was compared to be identical to the spectroscopic data reported previously (Sirirak et al, 2011a).

The extraction and sample preparations for the metabolomics study were performed separately on an individual part, individual colony basis. Each selected sponge colony was separated into two parts, the capitum and base. In a case that the selected colony may develop more than one distinctive piece of capitum, or the base was very large, more than one specimen of either part were collected accordingly. Each specimen was freeze-dried, ground with the aid of liquid N₂, and extracted as a retrieved whole
specimen with a mixture of CHCl$_3$/MeOH/water (4:2:2). The weights of ground specimens subjected to the extraction ranged 150-200 mg. The mixture was vortexed for 10 minutes, and set aside for an additional 30 minutes. Once clearly separated, a 3-mL portion from each CHCl$_3$- and aq MeOH-phase was brought to a complete dryness. The dry extracts were stored at -20°C until the spectroscopic measurement.

2.3 NMR acquisition and data handling

The $^1$H NMR spectrum of the standard kabiramide C was performed on a Varian Unity Inova 500 MHz NMR spectrometer, using a standard spectroscopic acquisition. The spectrum was obtained in CDCl$_3$, referencing the solvent signals at $\delta$ 7.24 (residual CHCl$_3$) for the chemical shift assignment.

For the metabolomics experiments, the NMR measurements were performed on a Bruker Avance 600 FT-NMR spectrometer at 600.13 MHz for $^1$H, using TopSpin 3.1 for Fourier transformation and spectral processing. For each sample, 128 scans were recorded with the following parameters; 124998 datapoints per FID, 90° flip angle (pulse width 11-13 ms per sample), 2.0 s relaxation delay, 7812.5 Hz (13 ppm) spectral width with 5.498 ppm offset (Kim, Choi, & Verpoorte, 2010).

Each extract obtained as described above was re-constituted whole with 0.8 mL of either CDCl$_3$ (freshly filtered through an alumina patch prior to use) or D$_2$O. The weights of CHCl$_3$-extracts to be investigated ranged 0.02-0.04 mg, and those of aq MeOH-extracts ranged 0.10-0.20 mg. The chemical shifts in ppm were referred to that of the solvent signals ($\delta$ 7.24, residual CHCl$_3$, or 4.28, residual HOD). An erratic signal of succinic acid (7.62 mM) was digitally inserted to each spectrum (arbitrarily placed at 10.01 ppm, erratic line width 2 Hz, correction as equivalent to 1 H), and was referred to as an internal standard for the signal integration. Each spectrum was manually phased.
and baseline-corrected, and bucketed with a 0.02 ppm interval. The bucketed integrals, normalized to a per 100 mg dried sponge weight basis, were used as variables for the statistical analysis. The spectral widths to be analyzed were narrowed to 8.5-0.5 ppm for those in CDCl₃, and to 7.5-0.5 ppm for the ones in D₂O. The solvent signals at δ 7.44-7.38, 7.28-7.20, and 7.10-7.04 (CHCl₃ and ¹³CHCl₃ satellites) were dismissed from the spectra obtained from CDCl₃, and those at δ 4.50-4.46 (HOD) were from the ones in D₂O.

2.4 Principal component analysis

PCA was performed on a SIMCA 13.0.3.0 software (Umetric AB). The bucketed spectral data, once normalized and processed as described above, were imported to SIMCA for the multivariate analysis using a PCA-X model with the pareto scaling approach.

3. Results and Discussion

3.1 Sponge specimen collection and extraction

The specimens of the sponge *P. nux* used in this investigation were collected from two locations; from Koh-Tao, Surat-Thani Province, and from Saiburi coast, Pattani Province. Ten sponge colonies were collected from Surat-Thani, yielding 10 specimens of capitums and 19 of bases, and 20 colonies were from Pattani, yielding 34 capitums and 38 bases. Each specimen was separately extracted as described to yield one CHCl₃- and one aq MeOH-extracts. Totally, 101 extracts from each solvents were obtained. The average weights of the sponge specimens and of the extracts from each solvent are summarized in Table 1.

The extraction protocol with a mixture of inseparable solvents as described is widely used in the metabolomics workflow (Choi et al. 2004; Cho, Kim, & Choi, 2007;
Choi, Yoon, Kim, & Kwon, 2007; Kim, Choi, & Verpoorte, 2010). In addition to an attempt to cover as wide range of chemical compositions as possible, the protocol is based on an assumption that the secondary metabolites, most of which hydrophobic, shall be extracted with the less polar solvent, i.e. CHCl$_3$, whereas the primary metabolites, e.g. sugars and amino acids, shall with the more polar one, i.e. aq MeOH. The mixed solvents therefore allow us to probe intentionally and simultaneously into the differences between the primary and secondary metabolomics profiles (Choi et al. 2004; Cho, Kim, & Choi, 2007; Choi, Yoon, Kim, & Kwon, 2007).

### 3.2 $^1$H NMR spectra of standard kabiramide C and sponge extracts

Kabiramide C, which is the major trisoxazole macrolide present in the sponge $P$. nux, was selected to represent the sponge’s macrolide pool. The compound has the functionalities commonly shared among the kabiramide analogs, and its resonances are clearly visible in the $^1$H NMR spectra of the sponge CHCl$_3$-extracts.

The indicative resonances of the trisoxazole macrolides to be focused here belong to the major cisoid conformer of kabiramide C (Sirirak et al, 2011a). Particularly of interest are the signals in the deshielded region ($\delta$ 8.5-6.0), which separate widely and are well resolved from others signals (Figure 3). These include the resonances at $\delta$ 8.25 ($H$-CONC-35), 8.06 ($H$-14), 8.01 ($H$-17), 7.54 ($H$-11), 6.42 ($H$-35), and 6.26 ($H$-19), all of which are distinctively observable and not overlapped with the signals of other compounds that may be present in the extracts. The resonances of the corresponding protons in other kabiramide analogs shift only slightly from those described above. Also, the amount of other minor kabiramides are generally miniscule and most are overshadowed by that of the cisoid kabiramide C; hence, all are negligible.
Depicted in Figure 4 are the $^1$H NMR spectra – with an expansion in a range of $\delta$ 8.5-6.0 – of the CHCl$_3$- and aq MeOH-extracts from either part of a selected representative colony of the sponge *P. nux*. The intensity in each spectrum illustrated here is not in the same scale, but is arbitrarily resized so that they can be compared pictorially. The indicative signals of kabiramide C described above, albeit minute, are observable unambiguously in the spectrum of the CHCl$_3$-extract from the capitum (Figures 4a and 4e), and to the lesser extent, in the CHCl$_3$-extract from the base (Figures 4b and 4f). The remaining major resonances in the CHCl$_3$-extracts from both parts belong to a mixture of various sterols (not specifically identified). These sterol resonances include those of olefinic protons ($\delta$ 5.50-5.20; either H-4 or H-6, and H-17), the carbonol protons ($\delta$ 3.75-3.25, H-3), and all the alicyclic and aliphatic protons ($\delta$ 2.35-0.75) of the steroid skeletons (Choi et al. 2004; Cho, Kim, & Choi, 2007; Choi, Yoon, Kim, & Kwon, 2007).

As expected, the kabiramides were not partitioned into the aqueous methanol; hence the resonances belonging to the kabiramides are absent from the $^1$H NMR spectra of the aq MeOH-extracts from both parts of the sponge (Figures 4c-d, and 4g-h). The major components present in the aq MeOH-extracts are a mixture of amino acids, water-soluble peptides, and amino acid-based nutrients (e.g. choline and betaine). These are indicated by the resonances of the $\alpha$-protons of the amino acids ($\delta$ 4.0-3.5), and the amino acid side chains ($\delta$ 3.50-0.75) (not specifically identified; Lusso et al. 2017; Fotiou et al, 2018; Zhou, Li, Sun, Guan, & Wei, 2019). Noticeably, although certain resonances are visible in the deshielded range of both spectra (Figures 4g and 4h), these belong to the aromatic acids, but not the kabiramides (Fotiou et al, 2018; Zhou, Li, Sun, Guan, & Wei, 2019).
3.3 Metabolomics allocation in the sponge *P. nux*

The multivariate analyses of the metabolomics profiles in the CHCl$_3$- and aq MeOH-extracts from the sponge *P. nux* were performed separately. The $^1$H NMR spectroscopic data of the extracts from each solvent, after normalized to a per-sponge dry weight basis and integrated to 0.02 ppm per bucket, were subjected to the principal component analysis (PCA). The data from the CHCl$_3$-extracts fit well with the PCA model and yielded a good cumulative $R^2$ of 0.84 and $Q^2$ of 0.81 after the first two principal components (PCs). The data from the aq MeOH-extracts also fit and yielded only two significant PCs with cumulative $R^2$ of 0.77 and $Q^2$ of 0.51.

Initially, the resulting score plots from each solvents were screened for the clustering patterns on the basis of geographical locations. This is not to pinpoint the locations(s) that may best facilitate the production of the trisoxazole macrolides. Rather, the main objectives of investigating the sponge specimens from various locations were to observe whether there were any geographical and/or environmental impacts on the macrolide productions and to lay out the metabolomics background. The score plots of the extracts from either solvent, regardless of PC combinations, showed no distinct clusters according to the geographical differences (for examples, PC1-PC2 score plots, Figures 5a and 5b), even when the PCA trials focusing on the trisoxazole macrolide signals (δ 8.5-6.0) were attempted (data not shown).

Focusing on the CHCl$_3$-extracts, the score plots from PCA show that the dataset cluster primarily according to the parts of the sponge, i.e. capitums vs bases. This is true in all possible combinations of the available PCs. For example, depicted in Figure 5c is the score plot between PCs 1 and 2. The data of the capitum extracts aggregate mainly in the (+PC1,+PC2) quadrant, whereas those from the bases do in the (-PC1,-PC2) one.
In the PC1 loading plot, (76.7% contribution; Figure 6a), the indicative signals of the sterols and trisoxazole macrolides score on the positive plane, indicating the positive impact on the cluster of the capitums. In other words, capitum specimens, cluster of which leans towards the +PC1 quadrant, are likely to have more accumulation of either trisoxazole macrolides or sterols, or both.

At first glance, the high scores for the resonances of 5.5-5.2 ppm in the loading plot (Figure 6a) may lead to a conclusion that the sponge sterols strongly dictate the data aggregation. With the dataset from the capitums clustering mainly in the positive PCs quadrants, the capitums suggestively has higher contents of the sterols than do the bases. However, knowing that the trisoxazole macrolides are allocated specifically towards the capitums, our attention was paid on the trisoxazole macrolide signals resonating in the deshielded region (δ 8.5-6.0). Whereas some indicative signals described earlier might be dimmed due to the intrinsic heights and signal broadening, and also to the small proportion of the macrolides in the extracts, a few signals of trisoxazole macrolides can yet be clearly depicted.

Under a close observation, PCA unambiguously picks up and emphasizes the importance of the trisoxazole macrolides. For instance, in the PC1 loading plot (Figure 6a), two indicative signals of the trisoxazole macrolides (δ 8.25, H-CONC-35; and 8.06, H-14) heighten and become easily detectable. Using the bucketed variables at δ 8.25 and 5.33 to represent the trisoxazole macrolides and sterols, respectively, the ratio between the average PC1 loading score of the two selected variables increases approximately 3.3 fold from the ratio between the average bucketed 1H NMR integrals of those two resonances. Similarly, the ratios between the PC1 loading scores of the variables at δ 8.06 and 5.33 increases 3.5 fold from the ratios in the corresponding NMR data. Whereas
this comparison does not intend to dismiss the influences of the sterols, particularly on
the PCA scoring and clustering, it signifies the relevance between the sponge’s bud
differentiation and the accumulation of the trisoxazole macrolides, which are the main
biologically active components in the sponge *P. nux*.

Interestingly, although the dataset from the aq MeOH-extracts may allow the PCA
model to fit with two significant PCs, the dataset scatter throughout the score plot (Figures
5b and 5d). No patterned clusters were recognized either on the location basis as
described above, or on the basis of parts of the sponge. Except for the variables related
to the amino acids described previously, the loading plot from the aq MeOH-extracts
(Figure 6b) is practically flattened. The results imply that the chemical arrays in the aq
MeOH-extracts of the sponge are either individually unique or indistinguishable from one
another. As described earlier, our assumption for the aq MeOH-extracts was that the
extracts shall represent the composition of the water-soluble primary metabolites such as
sugars and amino acids (Choi et al. 2004; Cho, Kim, & Choi, 2007; Choi, Yoon, Kim, &
Kwon, 2007). The lack in recognizable clustering patterns based on the sponge parts in
the aq MeOH-extracts suggests that the primary metabolisms in the background of the
capitums and bases of the sponge are expressed independently on an individual colony
basis and not parallel to that of the secondary metabolisms. The budding differentiation
into a capitum in the sponge *P. nux* casts the significant impacts only on the production
and/or allocation of the secondary metabolites.

4. Conclusions

In this investigation, we adopted an NMR-based metabolomics workflow to
examine the chemical allocation between two parts, the capitums and bases, of the sponge
Penares cf. nux. The results agreed well with the previous reports (Sirirak et al., 2011b; Olatunji, Petchoubon, Thawai, & Plubrukarn, 2018). Using the multivariate analysis, PCA emphasized the specific allocation of the trisoxazole macrolides towards the capitums of the sponge. Although their specific roles in the buds are unknown to us, other lipophilic components, namely the sterols, expressed high loading scores and suggestively casted their impacts on the clustering patterns in the PCA score plots. The budding differentiation had no observable connection with the water-soluble primary metabolites, as no specific clusters were recognized in the score plots from the dataset of the aq MeOH-extracts. The lack in the distinct clusters from the aq MeOH-extracts suggests that whereas the sponge may allocate the toxic trisoxazole macrolides and other secondary metabolites specifically for the chemical protection of the protruding capitums, its primary metabolisms in the background are indistinguishable throughout the sponge colony.

Acknowledgments

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Figure caption list

Figure 1 The sponge *Penares* cf. *nux* (a) in situ; (b) upon surfacing

Figure 2 Chemical structures of kabiramides C and G

Figure 3 $^1$H NMR spectrum of kabiramide C (500 MHz, CDCl$_3$)

Figure 4 $^1$H NMR spectra of extracts from a selected colony of *P. nux*, (a) CHCl$_3$-extracts from the capitum and (b) from the base (500 MHz, CDCl$_3$); and (c) aq MeOH-extracts from the capitum and (d) from the base (500 MHz, D$_2$O); and the expanded spectra (8.5-6.0 ppm) of (e) CHCl$_3$-extracts from the capitum and (f) from the base, and (g) aq MeOH-extracts from the capitum and (h) from the base

Figure 5 Score plots from PCA on the extracts of the sponge *P. nux*, (a) PC1,PC2 plot from the CHCl$_3$-extracts, and (b) PC1,PC2 plot from the aq MeOH-extracts, dataset colored according to the collecting locations; and (c) PC1,PC2 plot from the CHCl$_3$-extracts, and (b) PC1,PC2 plot from the aq MeOH-extracts, dataset colored according to the parts of the sponge (dataset from the CHCl$_3$-extracts $R^2$(1) 0.767, $R^2$(2) 0.0686; dataset from the aq MeOH-extracts $R^2$(1) 0.639, $R^2$(2) 0.128)

Figure 6 PC1 Loading plots from PCA on the extracts of the sponge *P. nux*, (a) from the CHCl$_3$-extracts, and (b) from the aq MeOH-extracts
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### Table 1: Sponge dry weights and extracts weights (per sponge dry weight)

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<td>174.3 (±10.3)</td>
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