Bioprospecting of the Balinese marine sponges and nudibranchs

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Abstract. Secondary metabolites play an invaluable role as a starting point in drug discovery process. Among marine organisms, sponges and nudibranch mollusc (Mollusca: Gastropoda: Nudibranchia) have been the prolific sources of bioactive secondary metabolites with diverse chemical structures and promising bioactivities. This report presents the isolation and structure elucidation of bioactive natural products from the Balinese marine sponge Aplysinella strongylata and the nudibranchs Hypselodoris infucata and Glossodoris hikuerensis. The samples were collected from Tulamben beach Bali, an unexplored site but rich in marine biodiversity. The separation and purification of the metabolites were achieved by mean of NP-flash column chromatography and HPLC. Structure elucidations of the isolated compounds were completed by analysis of spectroscopic dataset including 1H and 2D NMR. A series of bromotyrosine-derived alkaloid possessing a spirooxepinisoxazoline moiety were identified from the extract of A. strongylata. One of the metabolites, 19-hydroxypsammaplysin E (1), showed the best antimalarial activity, with an IC50 value of 6.4 μM. The absolute configuration of psammaplysin series compounds has been assigned as (6R, 7R) using experimental and calculated electronic circular dichroism (ECD) data and NMR analysis of MPA esters prepared from the acetamide derivative of psammaplysin A. A furanosesquiterpen, (–)-furodysinin (2), was identified for the first time from the nudibranch H. infucata. The compound inhibited the growth of HeLa cell line at IC50 102.7 μg/mL. Two new scalarane sesterterpenes (3-4) were characterised from an organic extract of a single specimen of the nudibranch G. hikuerensis.

1. Introduction
Marine animals have been known to produce myriad of secondary metabolites (or natural products) with diverse chemical structures and an array biological properties [1,2]. Studies of the chemical properties of these secondary metabolites have led to discovery of lead compounds some of which have entered clinical trials, particularly as anticancer and antimicrobial agents [3]. Currently, the number of bioactive natural products isolated from marine organisms now exceeds 28,000 with hundreds of new compounds being discovered every year [4].

Among marine organisms, sponges and nudibranchs (Mollusca: nudibranchia) have been prolific sources of bioactive secondary metabolites, several of which have inspired the development of new classes of therapeutic agents. Recently, the active compounds of four out of seven new FDA-approved drugs were originally collected from sponges and nudibranchs. For example, the drug marketed as Cytarabine (anticancer), Vidarabine (antiviral), and Eribulin Mesylate (anticancer) were originally isolated from Caribbean sponge Cryptotheca crypta and Halichondria okadai respectively [5].

Here we presented chemical studies of marine natural products from sponges and nudibranchs collected from Bali, an under-exploited marine hot spot but rich in species diversity. Although there
have been several reports on chemical studies of marine sponges and sponge-associated fungi collected from West Bali [6-7], this is an early study carried out of specimens from East Bali including Tulamben. A single specimen of sponge Aplysinella strongylata and two nudibranch extracts including Hypselodoris infucata and Glossodoris hikuerensis were investigated.

The isolation and purification of secondary metabolites from the extracts of these specimens were achieved by means chromatography including flash column chromatography and high performance liquid chromatography (HPLC). Accordingly, the structure elucidations of the isolated metabolites were completed by analysis of 1D and 2D nuclear magnetic resonance (NMR) data. Furthermore, biological activities of the metabolites were tested in vitro against chloroquine-sensitive (3D7) P. falciparum malaria parasites and HeLa cell line.

Herein reported the isolation and structure elucidation of 19-hydroxypsammaplysin E (1) from the extract of Balinese marine sponge Aplysinella strongylata. It showed the best antimalarial activity, with an IC₅₀ value of 6.4 μM. The absolute configuration of psammaplysin series compounds were assigned as (6R, 7R) using experimental and calculated electronic circular dichroism (ECD) data and NMR analysis of MPA esters prepared from the acetamide derivative of psammaplysin A. A furanosesquiterpen, (−)-furodysinin (2), was identified for the first time from the nudibranch H. infucata. Two new scalarane sesterterpenes (3-4) were characterised from an organic extract of a single specimen of the nudibranch G. hikuerensis.

2. Methods

**General Experimental Procedures.** All NMR spectra were referenced to solvent signals as follows: δ 7.26 and 77.16 ppm for CDCl₃; δ 2.05 and 29.84 for acetone-d₆; and δ 3.31 and 49.00 ppm for methanol-d₄. 1D and 2D NMR spectra were acquired using a Bruker Avance 400 or a Bruker Avance 500 spectrometer at 298 K. Optical rotations were obtained using a Jasco P2000 polarimeter. Positive ion electrospray mass spectra (LRESIMS) were determined using a Bruker Esquire HCT or (HRESIMS) using a MicroTof Q instruments each with a standard ESI source. Reversed-phase HPLC was carried out on an Agilent 1100 Series instrument fitted with a variable-wavelength UV and refractive index detector, an Agilent D1311A quaternary pump, and a semipreparative Phenomenex C18 Gemini 5 μm 110 Å column (10 mm x 250 mm). Gas chromatography was carried out on a Shimadzu GCMS-QP2010 Plus. Initial temperature was 100 °C, isothermal for 3 min, then ramped 16 °C/min for 10 min. The final temperature was 270 °C, injection temperature 250 °C, and flow rate 1.5 mL/min.
**Biological Material.** The sponge *Aplysinella strongylata* was harvested in Tulamben Bay, Bali, at a depth of approximately 20 m by hand using scuba in November 2010. The sponge was spherical in shape, fleshy to the touch, and compressible. The color in life was milky-colored inside and gray outside, compressible with mucus secretion, turning deep brown in 70% EtOH preservative. The sponge identification was undertaken at the Research Center of Oceanography, Indonesian Institute of Science, where a voucher specimen (TL-20) is deposited. One specimen of *Glossodoris hikuerensis* (crawling length 2 cm) was collected during fieldwork at Tulamben Bay, Bali, in July 2012. Two specimens of *Hypselodoris infucata* (crawling length 2 cm) were collected during fieldwork at Tulamben Bay, Bali in November 2014. The specimens were identified by comparing the surface pictures with that in the encyclopaedia of nudibranch as well as with the online database such as nudipixel (www.nudipixel.net) and WoRM (www.marine-species.org).

**Extraction and Purification.** The freshly collected sponge (147 g wet wt) was frozen before being extracted with 1:1 DCM-MeOH (3×200 mL) at room temperature. The combined extracts were dried under vacuum to produce 64.5 g (wet wt) crude extract. The crude extract was sequentially partitioned between hexanes (3×200 mL) and H2O, followed by EtOAc and H2O (3×200 mL), and finally BuOH and H2O (3×200 mL). This gave hexanes (1.2 g), EtOAc (3.1 g), and BuOH (0.84 g) fractions, respectively. A portion of the EtOAc fraction (3.1 g) was resolved by normal-phase VLC (3×10 cm in diameter) with step gradient elution from 100% hexanes to DCM, EtOAc, and 100% MeOH and gave 11 fractions. Subsequently, each of the fractions was purified by HPLC to give the known and new metabolites.

A single specimen of *Hypselodoris infucata* was diced, extracted in acetone (3×10 mL), and sonicated for 2 min. The combined extracts were partitioned between water and dichloromethane (3×5 mL) and subsequently the organic layer was dried with Na2SO4. Dried organic layer was evaporated to dryness to give a crude extract (5 mg). The extract was passed through a normal-phase Sep-Pak cartridge eluting with 100% hexane (10 mL) to provide compound 2 (1.3 mg).

A single specimen of *Glossodoris hikuerensis* was diced and extracted in acetone (3×10 mL). The combined extracts were dried under reduced pressure to give a crude extract (50 mg). The extracts were passed through a NP flash column eluted with hexanes (100%), hexanes→chloroform (3:1 to 1:3), chloroform→EtOAc (3:1 to 1:3), and EtOAc (100%) to give two terpene-rich fractions coded as F3-4 (17.6 mg) and F5-6 (14.4 mg). The less polar fraction (F3-4) was subjected to NPHPLC with hexanes: EtOAc (80:20) to provide the two new sesterterpenes 3 (3.8 mg) and 4 (~0.1 mg) together with the known compounds.

### 3. Result and Discussion

Twenty-one new psammaplysin derivatives exhibiting a variety of side chains, as well as six previously known psammaplysins including psammaplysin A, B, D, and E and ceratinamides A and B were identified from the extract of the Balinese sponge *Aplysinella strongylata* (order Verongida, family *Aplysinellidae*). Detail spectroscopic data analysis of each of the new metabolites was discussed in the previous publication [8]. Here we only presented an example of structure elucidation of a new compound that possesses the highest bioactivity named as 19-hydroxypsammaplysin D (1).

The presence of the psammaplysin carbon framework in 1 could be deduced from 1H and 13C NMR chemical shifts (MeOH-δi) compared to those of psammaplysin E. A signal at δH 7.15 (1H, s) assigned to H-1 had HMBC correlations to signals at δC 103.5 (C-2), 148.9 (C-3), and 122.1 (C-6). Two distinctive geminal protons at δH 3.06 and 3.38 could be attributed to H-5a and H-5b, respectively. This AB system correlated to signals for C-4 (δC 105.7) and C-6 as well as to C-7 at δC 79.3, diagnostic for a hydroxymethylene carbon. The H-7 signal appeared at δH 4.99 as an isolated singlet and correlated to an amide carbon atom at δC 159.1 (C-9). These assignments secured the presence of the spirooxepinoxazoline system in 1 [9]. Furthermore, three mutually coupled methylenes at δH 3.62, 2.13, and 4.07 suggested the presence of a -CH2CH2CH2O- moiety attached to the amide bond of the
spiro ring system. A singlet at δH 7.60 was designated to the symmetric aromatic ring protons H-15 and H-17. HMBC correlation between H-12 and C-13 (δc 153.2) secured the connection between the propyloxy side chain and the substituted aromatic ring.

The 1H NMR spectra of 1 was consistent except for the signals corresponding to H-19 and H-20. In psammaplysin E, H-19 appeared at δH 2.83 (2H) and H-20 at δH 3.55 (2H); in contrast for 4, H-19 was shifted to δH 4.77 (1H, dd, J = 3.7, 7.2 Hz) while H-20 appeared as an AB system [δH 3.42 (1H, dd, J = 7.2, 13.4 Hz) and δH 3.59 (1H, dd, J = 3.7, 13.4 Hz)]; since the H-20 signals were partially obscured by those of H-5b and H-10, they were further resolved by a 1D TOCSY experiment involving irradiation of H-19. The chemical shift of C-19 was also shifted downfield from δc 36.0 in psammaplysin E to δc 71.8 in 1, consistent with a C-19 hydroxy substituent as found previously in both psammaplysins B and D [9-10].

The absolute configuration of psammaplysin A has been assigned as (6R,7R) using experimental and calculated electronic circular dichroism (ECD) data and NMR analysis of MPA esters prepared from the acetamide derivative of psammaplysin A. Detailed conformational analyses of a truncated model compound of psammaplysin A with an in vacuo method and with the PCM solvent model for MeOH have identified the major conformers and factors governing the ECD spectrum of psammaplysin A. The correlation of the ECD data with the stereochemistry of psammaplysin A allows configurational assignment of related psammaplysin analogues on the basis of their ECD spectra (Figure 1)[11].

![Figure 1: Experimental ECD spectrum of psammaplysin A recorded in MeOH (black curve) and PBE0/TZVP PCM/MeOH ECD spectra of (6S,7S)-truncated model for the two lowest-energy conformers obtained by B97D/TZVP (PCM/MeOH) reoptimization (blue and red curves). Bars represent rotational strength values of conformers A and B.](image)

Compound 2 was isolated as a colorless oil ([α]D –51) and showed a single GCMS peak at m/z 216 (M+) with a strong diagnostic base peak at m/z 122 (C₈H₈O) arising from a retro Diels-Alder reaction of the carbocyclic ring adjacent to the furan ring [12]. The 1H NMR (CDCl₃, 500 MHz) spectrum of 2 revealed the presence of diagnostic furan protons at δH 7.21 (1H, d, J = 1.7 Hz, H-1) and δH 6.23 (1H, d, J = 1.7 Hz, H-2) together with an olefinic proton at δH 5.61 (1H, brs, H-10). There were also two geminal methyl groups resonated at δH 1.19 (3H, s, H-15) and δH 1.18 (3H, s, H-14) along with a methyl attached to an on olefinic carbon at δH 1.66 (3H, s, H-13). These 1H NMR data of 2 were in close agreement with those of the synthetic sample of furodysinin reported by Vaillancourt et al. in 1991 [13].
The tricyclic furanosesquiterpene (+)-furodysinin, ([α]D +64) was first reported by Wells et al. in 1978 from an Australian Dysidea herbacea[14] without relative stereochemical assignment detail. Guella et al. subsequently characterised its enantiomer (−)-furodysinin ([α]D −47) [15] from a specimen of D. tupha collected from the Mediterranean. The absolute configuration of (+)-furodysinin has been established as as 6R,11R when optically pure (−)-furodysinin ([α]D −54) was synthesised by Vaillancourt et al. from (+)-9-bromocamphor in four steps [13]. Recently, (−)-(6R,11R)-furodysinin (2) has been isolated from H. herbacea (Fiji) [16], from H. bayeri collected from Cuba [17], and from H. jacksoni from South-East Queensland, Australia. (−)-Furodysinin isolated from H. infucata in the current study has consistently been associated with a 6R,11R configuration, whereas the positive counterpart shows a 6S,11S configuration [18].

Compound 3 was isolated as a colorless oil, the molecular formula of which was established to be C27H42O4 from the sodiated (+)-HRESIMS ion at m/z 453.2965. The presence of a 12-hydroxy scalarane framework in 3 was inferred from proton NMR signals (Table 1) for an acetal (δH 6.49, d, J = 2.7 Hz, H-19), the olefinic signal (δH 5.60, brs, H-16), and a carbinolic proton (δH 3.44, dt, J = 4.6, 11.3 Hz, H-12) as well as an AB system [δH 4.38, d, J = 11 Hz (H-20a); 4.24, d, J = 11 Hz (H-20b)]. There were also five methyl singlets at δH 0.89, 0.85, 0.84, 0.80, and 0.76, each linked to their respective carbons at δC 16.3, 16.5, 33.4, 21.4, and 9.1 by HSQC data. The 1H and 13C NMR data of 3 were identical to those of 12-deacetyl-12-epi-deoxoscalaratin isolated in the current study as well as to those reported by Fontana et al., with the exception of the addition of an acetate methyl signal at δH 2.09. The acetate moiety was placed at C-19 based on a key HMBC correlation observed from the signal for H-19 (δH 6.49, d, J = 2.7 Hz) to the carbonyl at δC 171.3. The proton chemical shifts along with the coupling constants of the signals for H-18 (δH 2.43, s) and H-19 (δH 6.49, d, J = 2.7 Hz) in 3 were consistent with those for scalarane isolated in the current study. Further COSY and HMBC correlations secured the gross structure of 3 as 12-deacetyl-12-epi-deoxoscalaratin-19-acetate [21].

Compound 4 was isolated in trace amount and gave a molecular formula of C27H40O4 by HRESIMS. The 1H NMR spectrum displayed an aldehyde signal (δH 10.02, d, J = 2.5 Hz), an olefinic proton (δH 5.89, br s, H-16) as well as a distinctive AB system [δH 5.27, t, J = 14.2 Hz (H-11a); 2.39, dd, J = 13.9, 2.1 Hz (H-11b)]. There were five methyl singlets at δH 0.85 (CH3-21), 0.82 (CH3-22), 1.11 (CH3-23), 0.87 (CH3-24), and 1.16 (CH3-25) that were correlated with their respective carbons at δC 33.4 (C-21), 21.5 (C-22), 16.4 (C-23), 15.8 (C-24), and 15.1 (C-25) in the HSQC spectrum. These NMR data were consistent with the presence of a tetracyclic 12-keto scalarane skeleton, such as in 12-deacetoxy-12-oxo-scalaradial from Glossodoris rufomarginata. The 12-keto moiety in 4 was secured from HMBC correlations from H2-11 and from H3-25 to the carbon at δC 215.5 (C-12). However, the signals for the aldehyde proton at C-17 of 12-deacetoxy-12-oxo-scalaradial were absent in 4, instead replaced by oxymethylene signals [δH 4.48 (s); δC 66.6 (C-20)] [19].
4. Conclusions
The extract of Balinese sponge *Aplysinella strongylata* contained twenty-one new psammaplysin derivatives as well as six previously known psammaplysins. The new metabolite 19-hydroxypsammaplysin D (1) found to be the best antimalarial activity, with an IC$_{50}$ value of 6.4 μM. The absolute configuration of psammaplysin A has been assigned as (6R,7R) using experimental and calculated electronic circular dichroism (ECD) data. Chemical investigation of the extract of Balinese nudibranch *Hypselodoris infucata* yielded the known (−)-furodysinin (2) that exhibited *in vitro* cytotoxic activity of against HeLa cell line with an IC$_{50}$ of 102.7 μg/mL. Two new scalarane sesterterpenes (3, 4) were characterized from an organic extract of a single specimen of the nudibranch *Glossodoris hikuerensis*, and no bioactivity was currently obtained.

ACKNOWLEDGEMENT
We thank to the International Postgraduate Research Scholarship (to I.W.M.). We also appreciated for the financial support provided by Indonesian Directorate General of Higher Education (DIKTI) under Hibah Bersaing research grant scheme 2015-2016 (contract number: 51/UN48.15/LT/2016).

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