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Bioactive Secondary Metabolites from the Borneon Soft Corals of the Genus *Nephthea*

Takahiro Ishii[1], Reiko Ueoka[2], Shigeki Matsunaga[2] and Charles S. Vairappan[1*]

[1]Laboratory of Natural Products Chemistry, Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia.

[2]Laboratory of Aquatic Natural Products Chemistry, Graduate School of Agriculture and Life Sciences, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

* csv@ums.edu.my (*Corresponding author*)

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ABSTRACT As part of our continuous interest in the presence of secondary metabolites in marine organisms of Borneo, we investigated the chemical constituents of the soft corals of the genus *Nephthea*. Four secondary metabolites were isolated from the organic extracts of three Borneon soft corals *Nephthea* species and these compounds were identified as 24-methylenecholesterol (**1**), 5β,8β-epidioxy-11-hydroxy-6-eudesmene (**2**), 5β,8β-epidioxy-11-hydroperoxy-6-eudesmene(**3**)and24-methyl-cholesta-5,24(28)-diene-3β,19-diol-7β-monoacetate (**4**). Their structures were established by NMR spectroscopic data analysis and comparison of the data with those of the literature.

ABSTRAK Sehubungan dengan minat dalam kajian kandungan sebatian sekunder dalam organisma marin dari perairan Borneo, kami telah menyelidik kandungan kimia karang lembut daripada genus *Nepthea*. Sebanyak empat sebatian sekunder telah dipencilkan daripada ekstrak organik tiga spesis *Nepthea*, dan sebatian tersebut telah dikenalpasti sebagai 24-methylenecholesterol (**1**), 5β,8β-epidioxy-11-hydroxy-6-eudesmene (**2**), 5β,8β-epidioxy-11-hydroperoxy-6-eudesmene(**3**)dan24-methyl-cholesta-5,24(28)-diene-3β,19-diol-7β-monoacetate (**4**). Struktur kimia untuk keempat-empat sebatian ini dikenalpasti berdasarkan analisa data spektroskopi NMR dan perbandingan data yang telah dilaporkan dalam literatur.

(**Keywords**: Secondary Metabolites, Antibacterial activities, Cytotoxic activities, *Nephthea* sp., Nephtheidae, Soft coral)

INTRODUCTION

Soft corals (Octocorallia: Alcyonacea) are soft-bodied invertebrates that are widely distributed in the tropical marine ecosystems like coral reefs, mangrove forest and inter-coastal channels. Since they are not protected by a hard exoskeleton, most soft corals defend their space on the reef by exuding mucus with traces of defensive compounds that repel other organisms such as algae, fishes, gastropods and mollusk. Based on the literature available soft corals are known to produce and concentrate a wide diversity of structurally interesting secondary metabolites [1-15]. Therefore, soft corals have the potential to be the source of useful chemicals, such as biomedical drugs and eco-pesticides. In this context,

soft corals of the genus *Nephthea* (Family Nephtheidae) are known as a rich source of terpenoids and steroids with unique chemical structures and interesting biological activities. They are found abundantly in the coastal waters of Sabah, Malaysia.

However, since research in the area of marine natural products chemistry is still in its infancy in Malaysia, there are only a few reports on chemical investigation of Malaysian soft corals [16]. One of our previous chemical investigations on the soft coral species belonging to genus *Nephthea* led to the isolation and identification of a new sterol [16]. Besides sterol, bioactive germacrane-type norsesquiterpene and cembrane diterpene were isolated from two different specimens from Tun Sakaran Marine Park and Layangan Island, Kota Kinabalu, Sabah [17, 18].

In the course of our interest to further discover interesting chemicals from marine soft corals, we investigated three *Nephthea* sp. specimens with similar morphological features from different locations; Dinawan (Kota Kinabalu, Sabah), Lankayan (Sandakan, Sabah) and Sebangkat (Semporna, Sabah). A total of four bioactive compounds were isolated and identified. A sterol, 24-methylenecholesterol (**1**) [14,19-21] was isolated from the Dinawan specimen. The Lankayan specimen contained two sesquiterpenes, 5β,8β-epidioxy-11-hydroxy-6-eudesmene (**2**) and 5β,8β-epidioxy-11-hydroperoxy-6-eudesmene (**3**) [12]. On the other hand, the Sebangkat specimen afforded a 19-oxygenated sterol, 24-methyl-cholesta-5,24(28)-diene-3β,19-diol-7β-monoacetate (**4**) [6,10,22]. In the present paper, we describe the isolation and the antimicrobial and cytotoxic activities of these metabolites.

MATERIALS AND METHODS

Sample Collection

Specimens of *Nephthea* sp. were collected from three different locations; (1) sample of *Nephthea* sp. from Dinawan Island, Sabah (5°50'750"N, 115°59'585"E), collected on April 18, 2008, (2) sample of *Nephthea* sp. from Lankayan Island, Sabah (6°29'995"N, 117°55'604"E), collected on May 27, 2008 and (3) sample of *Nephthea* sp. from Sebangkat, Sabah (4°33'152"N, 118°39'418"E), collected on November 26, 2008. The voucher specimens were deposited in BORNEENSIS collection of Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah.

Separation and Isolation of the Dinawan Specimen

The fresh soft coral (270 g wet wt) was extracted with MeOH. The crude extract was evaporated under reduced pressure and the residue was partitioned between EtOAc and H_2O. The EtOAc fraction was further partitioned with hexane and 90% MeOH. The hexane fraction (608 mg) was fractionated by Si gel column chromatography with a step gradient of hexane and EtOAc. The fraction (20.0 mg) eluted with hexane/EtOAc (7:3) was further separated by preparative TLC with $CHCl_3$ to afford compound **1** (10.2 mg).

Separation and Isolation of the Lankayan Specimen

The fresh soft coral (991 g wet wt) was treated as described above. The hexane fraction (3.03 g) was chromatographed on a Si gel column using hexane and EtOAc system of increasing polarity as eluant.

The fraction (70.0 mg) eluted with hexane/EtOAc (7:3) was further subjected to reversed-phase HPLC (Luna 5m C18(2) 100A) with 70% MeCN to give compounds **2** (7.8 mg) and **3** (5.4 mg).

Separation and Isolation of the Sebangkat Specimen

The fresh soft coral (1.20 kg wet wt) was treated as described above. The 90% MeOH fraction (820 mg) was chromatographed on a Si gel column using a gradient solvent system of hexane and EtOAc. The fraction (25.0 mg) eluted with EtOAc was further submitted to repeated preparative TLC with EtOAc and toluene/MeOH (9:1) to yield compound **4** (6.7 mg).

SPECTROSCOPIC PROCEDURES

General Experimental Procedures

Optical rotations were measured on an AUTOPOL IV automatic polarimeter (Rudolph Research Analytical). ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were recorded with a JEOL ECA 600, with TMS as internal standard. HPLC was conducted on a Waters 600 using UV detector and Luna 5m C18(2) 100A (10.0 x 250 mm). Preparative TLC was performed with silica gel plate (Merck, Kieselgel 60 F_{254}). Silica gel (Merck, Kieselgel 60, 70-230 mesh) was used for column chromatography. Analytical TLC was performed on Merck Kieselgel 60 F_{254}. Spots were visualized by UV light or by spraying with a 5% phosphomolybdic acid ethanol solution.

24-Methylenecholesterol (1) – white solid; $[a]^{25}_D$ –41.3 (*c* 0.45, $CHCl_3$); $C_{28}H_{46}O$. ^{1}H-NMR ($CDCl_3$, 600 MHz) d 0.67 (3H, s, H_3-18), 0.94 (3H, d, *J* = 6.8 Hz, H_3-21), 1.00 (3H, s, H_3-19), 1.01 (3H, d, *J* = 2.8 Hz, H_3-27), 1.02 (3H, d, *J* = 2.8 Hz, H_3-26), 3.51 (1H, m, H-3), 4.64 (1H, s, H-28a), 4.70 (1H, s, H-28b), 5.34 (1H, m, H-6); ^{13}C NMR (150 MHz, $CDCl_3$) d 11.9 (C-18), 18.8 (C-21), 19.5 (C-19), 21.2 (C-11), 21.9 (C-26), 22.1 (C-27), 24.4 (C-15), 28.3 (C-16), 31.1 (C-23), 31.7(C-2), 32.0 (C-7), 32.0 (C-8), 33.9 (C-25), 34.8 (C-22), 35.8 (C-20), 36.6 (C-10), 37.3 (C-1), 39.9 (C-12), 42.4 (C-4), 42.4 (C-13), 50.2 (C-9), 56.1 (C-17), 56.8 (C-14), 71.9 (C-3), 106.0 (C-28), 121.8 (C-6), 140.8 (C-5), 157.0 (C-24). Spectroscopy data corresponds with data published by Iguchi *et al.* and Su *et al.* [18,19].

5b,8b-Epidioxy-11-hydroxy-6-eudesmene (2) – colorless oil, $[a]^{25}_D$ +3.8 (*c* 0.37, $CHCl_3$); $C_{15}H_{24}O_3$. ^{1}H-NMR ($CDCl_3$, 600 MHz) d 0.89 (3H, s, H_3-14), 1.00 (3H, d, *J* = 6.9 Hz, H_3-15), 1.27 (1H, dd, *J* = 13.4, 2.0 Hz, H_a-9), 1.40 (3H, s, H_3-12), 1.43 (3H, s, H_3-13), 1.47 (2H, m, H_2-3), 1.49 (1H, m, H_a-1), 1.54 (2H, m, H_2-2), 1.94 (1H, dd, *J* = 13.4, 3.4 Hz, H_b-9), 2.00 (1H, m, H_b-1), 2.06 (1H, m, H-4), 4.76 (1H, ddd, *J* = 3.4, 2.0, 1.7 Hz, H-8), 6.20 (1H, d, *J* = 1.7 Hz, H-6); ^{13}C-NMR ($CDCl_3$, 150 MHz) d 16.1 (C-15), 21.0 (C-2), 25.5 (C-14), 28.1 (C-12), 28.2 (C-13), 29.4 (C-3), 32.7 (C-4), 35.1 (C-10), 35.7 (C-1), 41.7 (C-9), 70.7 (C-11), 71.5 (C-8), 81.6 (C-5), 124.2 (C-6), 149.6 (C-7) (Fig 1). Spectroscopy data corresponds with data published by Cheng *et al.* [12].

5b,8b-Epidioxy-11-hydroperoxy-6-eudesmene (3) – colorless oil, $[a]^{25}_D$ +5.0 (*c* 0.32, $CHCl_3$); $C_{15}H_{24}O_4$. ^{1}H-NMR ($CDCl_3$, 600 MHz) d 0.90 (3H, s, H_3-14), 0.99 (3H, d, *J* = 6.9 Hz, H_3-15), 1.35 (1H, dd, *J* = 13.3, 2.1 Hz, H_a-9), 1.36 (3H, s, H_3-12), 1.48 (2H, m, H_2-3), 1.49 (3H, s, H_3-13), 1.50 (1H, m, H_a-1), 1.54 (2H, m, H_2-2), 1.93 (1H, dd, *J* = 13.3, 3.6 Hz, H_b-9), 2.00 (1H, m, H_b-1), 2.08 (1H, m, H-4), 4.78 (1H, ddd,

J = 3.6, 2.1, 1.7 Hz, H-8), 6.30 (1H, d, J = 1.7 Hz, H-6), 7.67 (1H, brs, -OOH); ^{13}C-NMR ($CDCl_3$, 150 MHz) d 16.1 (C-15), 21.0 (C-2), 22.7 (C-12), 25.4 (C-14), 29.4 (C-13), 29.7 (C-3), 32.7 (C-4), 35.0 (C-10), 35.7 (C-1), 41.1 (C-9), 71.0 (C-8), 81.6 (C-5), 81.8 (C-11), 128.9 (C-6), 145.8 (C-7). Spectroscopy data corresponds with data published by Cheng *et al.* [12].

24-Methyl-cholesta-5,24(28)-diene-3b,19-diol-7b-monoacetate (4) – colorless oil, $[a]^{25}_{D}$ +19.2 (c 0.50, $CHCl_3$); $C_{30}H_{48}O_4$. ^{1}H-NMR ($CDCl_3$, 600 MHz) d 0.75 (3H, s, H_3-18), 0.95 (3H, d, J = 6.5 Hz, H_3-21), 1.02 (3H, d, J = 6.6 Hz, H_3-26), 1.02 (3H, d, J = 6.6 Hz, H_3-27), 2.03 (3H, s, $-COCH_3$), 3.60 (1H, m, H-3), 3.66 (1H, d, J = 11.6 Hz, H-19a), 3.88 (1H, d, J = 11.6 Hz, H-19b), 4.65 (1H, s, H-28a), 4.71 (1H, s, H-28b), 4.97 (1H, d, J = 8.3 Hz, H-7), 5.58 (1H, brs, H-6); ^{13}C-NMR ($CDCl_3$, 150 MHz) d 12.2 (C-18), 18.8 (C-21), 21.7 (OCOCH3), 21.9 (C-26), 21.9 (C-11), 22.1 (C-27), 25.0 (C-15), 28.4 (C-16), 31.1 (C-23), 31.9 (C-2), 33.3 (C-1), 33.9 (C-25), 34.8 (C-22), 35.7 (C-20), 37.9 (C-8), 39.8 (C-12), 41.5 (C-10), 41.7 (C-4), 43.2 (C-13), 48.7 (C-9), 55.4 (C-17), 56.6 (C-14), 63.0 (C-19), 71.0 (C-3), 75.4 (C-7), 106.1 (C-28), 126.8 (C-6), 140.1 (C-5), 156.8 (C-24), 171.4 ($OCOCH_3$). Spectroscopy data corresponds with data published by Jia *et al.* [20].

Antibacterial Bioassay

The antimicrobial bioassays for the isolated compounds were carried out using 10 strains of human pathogenic bacteria. Details of the test organisms are given in Table 1. Assays were performed as previously described by Vairappan [21].

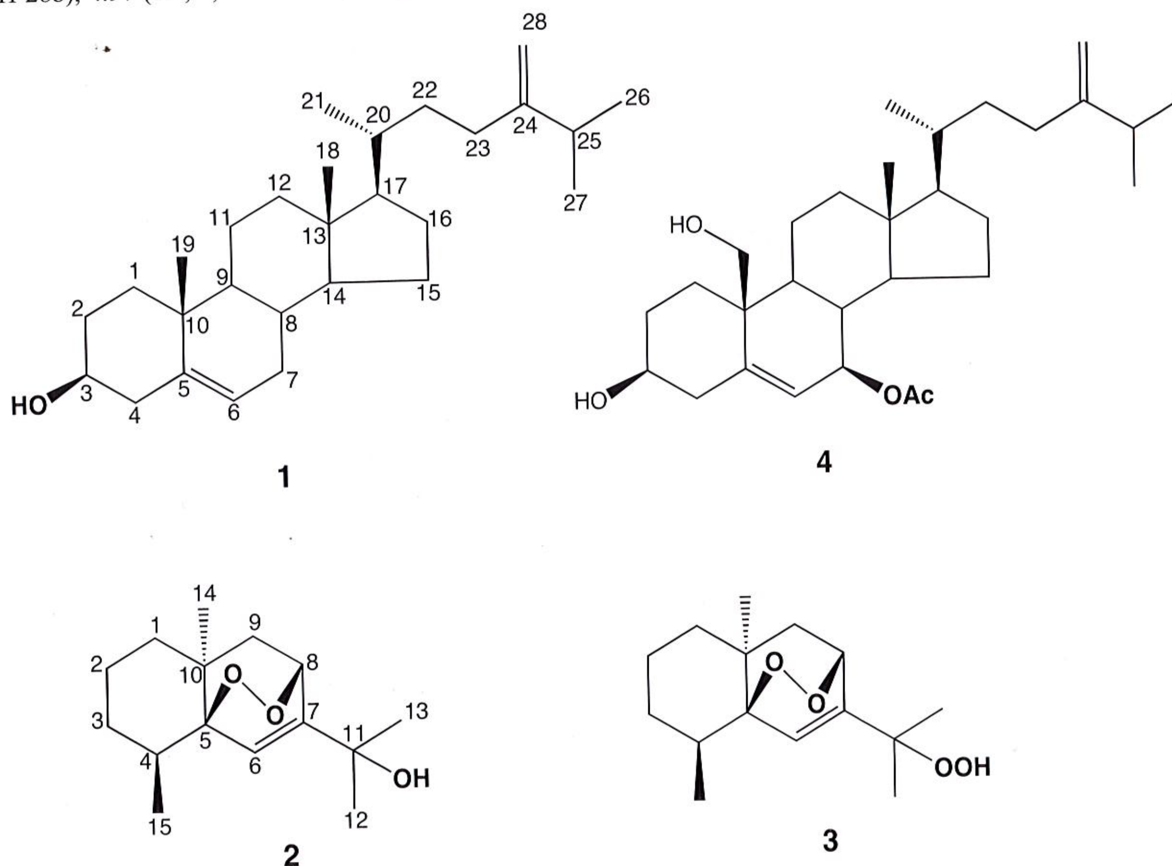

Figure 1. Structures of compounds **1-4**.

Cytotoxic assay

P388 murine leukemia cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 5 mL of Penicillin/Streptmycin solution (10,000 units of penicillin/mL, 10 mg of streptomycin/mL) at 37°C under an atmosphere of 5% CO_2. To each well of the 96-well microplate containing 100 mL of tumor cell suspension ($1x10^4$ cells/mL) was added 100 mL of test solution dissolved in RPMI-1640 medium then the plate was incubated in a CO_2 incubator at 37°C for 96 h. After addition of 50 mL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) saline solution (1 mg/mL) to each well, the plate was incubated for 3 h under the same condition to stain live cells. After the incubation, the plate was centrifuged and the supernatants were removed and cells were dissolved in 150 mL of DMSO to determine the IC_{50} values.

Table 1. Antimicrobial activities of the isolated compounds against human pathogenic bacteria.

Tested Bacteria	Compounds Tested			
	1	**2**	**3**	**4**
Pseudomonas aurelis	-	-	8	-
Salmonella sp.	-	-	-	-
Proteus mirabilis	7	7	7	-
Escherichia coli	-	-	10	-
Salmonella typii	-	-	-	-
Salmonella enteridis	7	7	8	-
Salmonella thyphymunium	7	7	7	-
Staphylococcus aereus	-	-	8	-
Listeria monocytogenes	-	-	-	-
Vibrio cholerae	-	-	9	-

Inhibition zone diameter: mm, -: no inhibition. Compound concentration: 30 g $disc^{-1}$.

RESULTS AND DISCUSSION

Each specimen was cut into small pieces, subsequently macerated and extracted in MeOH for 7 days. The obtained MeOH extracts were concentrated *in vacuo* and partitioned between EtOAc and H_2O. The EtOAc-soluble fraction was further partitioned between hexane and 90% MeOH. Each lipophilic fraction from the MeOH extracts was purified by silica gel column chromatography, as well as preparative TLC and reversed-phase HPLC, to yield compounds **1-4**. 24-Methylenecholesterol (**1**) was isolated from the Dinawan collection. The Lankayan collection afforded 5β,8β-epidioxy-11-hydroxy-6-eudesmene (**2**) and 5β,8β-epidioxy-11-hydroperoxy-6-eudesmene (**3**). On the other hand, the Sebangkat collection contained a 19-oxygenated sterol, 24-methyl-cholesta-5,24(28)-diene-3β,19-diol-7β-monoacetate (**4**). All known compounds were identified by comparison of their spectral data with those reported [12,18-20].

A well-known sterol, 24-methylenecholesterol (**1**) is widely distributed in marine organisms and was first isolated in 1955 by Idlea and Fagerlund from mollusks, the oyster *Ostrea gigas* and the clam *Saxidomus giganteus* [20]. In addition, Su et al. have described the first isolation of **1** in 1989 among soft corals [21]. Two sesquiterpenoids **2** and **3** were previously only isolated from the Formosan soft coral *Nephthea erecta* [12]. The 19-oxygenated sterols are very rare in nature and have been shown to exhibit siginificant cytotoxity against cancer cells *in vitro* [6,10-13,20]. A 19-hydroxylated steroid (**4**) has been isolated from several soft corals of the genus *Nephthea* and *Sinularia* [6,10,22].

All the isolated metabolites were tested for their antimicrobial and cytotoxic potentials. Table 1 shows the activities of the tested compounds against pathogenic bacteria. Compound **3** showed antimicrobial activities against a wide range of pathogenic microorganisms. The activities of compounds **1** and **2** were lower than that of **3**. In contrast, compound **4** was inactive on inhibition of bacterial growth.

On the other hand, Compounds **2** and **4** exhibited moderate cytotoxity against P-388 murine leukemia cells with IC_{50} values of 2.2 mg/mL, respectively. Compound **3** showed stronger cytotoxity (IC_{50} = 0.36 mg/mL) than that of **2** and **4**, while **1** was not cytotoxic to P-388 cells. As a result, compound **3** had the strongest antimicrobial activity and cytotoxity among these metabolites. This result indicates that the hydroperoxy moiety play an important role in the activity. These findings suggest a possibility that these compounds play a role in the soft coral as defensive substances to evade other organisms.

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