JOURNAL OF TROPICAL BIOLOGY AND CONSERVATION 9(2) : 200 – 205, 2012

Research Article

Isopropyl(ene)-type cembrene diterpene an important secondary metabolite in soft coral *Sinularia flexibilis* of Tun Sakaran Marine Park, Malaysia

Charles S. Vairappan[1*], Intan Irna Zanil[1] and Takashi Kamada[1] and M. Palaniappan[2]

[1]Institute for Tropical Biology and Conservation,
*[2]Borneo Marine Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia. *email:csv@ums.edu.my*

ABSTRACT. Two cembrane diterpenes; (3*S*,4*S*,11*S*,12*S*,1*E*,7*E*)-3,4:11,12-bisepoxycembra-1,7-diene (**1**) and (1*E*,3*E*,7*E*)-11,12-epoxycembra-1,3,7-triene (**2**) were isolated from a population of *Sinularia flexibilis* collected from Kapikan Reef, Semporna, Sabah. The chemical structures were elucidated based on ^{1}H-NMR and ^{13}C-NMR spectroscopic data. This is the first record of cembranoid diterpenes isolated from the Malaysian soft coral genus *Sinularia*. Thesecembrenes could be used as chemotaxonomical markers and have also exhibited potent anti-bacterial activity against pathogenic *Escherichia coli* and *Staphylococcus aureus*.

Keywords: Soft coral, *Sinularia*, Kapikan Reef, cembranoid diterpenes, chemotaxonomic, antibacterial activity.

INTRODUCTION

The soft coral genus *Sinularia* (Alcyonacea, Alcyoniidae) is known as a prolific producer of secondary metabolites among marine organisms (Yu *et al.*, 2006). A total of 30 of the 96 known species of *Sinularia* have been chemically examined and contain diterpenoids, sesquiterpenoids and steroids as their secondary metabolites (Goud *et al.*, 2002; Jin *et al.*, 2005;Amira *et al.*, 2006;Liang *et al.*, 2010). Due to their soft bodies and sedentary life, soft corals are known to biosynthesize secondary metabolites to chemically protect themselves from predators and colonizers (Li *et al.*, 2006; Bonnard *et al.*, 2010). In the last 30 years, there have been more than 15,000 novel secondary metabolites discovered from these organisms (Li *et al.*, 2006).

Soft corals are known to synthesize and accumulate terpenes, particularly sesquiterpenes and diterpenes of eudesmane and cembranoid types (Matthee *et al.*, 1998). It has been suggested that some of these terpenes are used as chemical defence compounds with ichthyotoxicity activity to avoid predatory fishes (Iwagawa *et al.*, 1999). In light of some recent findings, these compounds have also shown anti-fungal, anti-bacterial and anti-bleaching potentials in nature, and for this reason have been suggested as ecological chemicals of importance for these organisms (Chao *et al.*, 2006; Ishii *et al.*, 2010a). In addition, these compounds are also actively evaluated for biological activities of pharmacological significance (Lin *et al.*, 2009).

To date, biomedical and pharmaceutical studies have shown interesting activities such as in anti-cancer, anti-microbial, anti-fungal, anti-inflammatory, anti-coagulant, anti-platelet and anti-viral (Kamel *et al.*, 2005; Chao *et al.*, 2006; Arepalli *et al.*, 2009; Chen *et al.*, 2010). In addition to their importance as test metabolites for pharmacological assay,

Received 19 July 2012. Revision accepted 14 September 2012

information pertaining to the distribution of secondary metabolites based on their chemical structures could be used to assist the systematics of soft corals. There has been some success in the application of cembrane-type diterpenes in the chemotaxonomy of major soft coral genus such as *Sinularia, Lobophytum* and *Nepthea* (Longeon *et al.*, 2002; Lin *et al.*, 2009; Cheng *et al.*, 2010).

Despite these advances in the chemistry of soft corals, there is shortage of information pertaining to the chemistry of soft corals in Malaysia. The only available to date are on the chemistry of the soft coral genus *Nepthea* collected from the coastal waters of Kota Kinabalu, Sabah. This genus was found to synthesis compounds such as sterols, norsesquiterpenoids and cembranoid diterpenes (Ishii *et al.*, 2009a, b; 2010a, b). Therefore, this paper reports the discovery of cembranoid diterpenes from the population of soft coral genus *Sinularia* at the Kapikan Reef, Semporna, Sabah.

METHODOLOGY

Collection

The *Sinularia flexibilis* population was collected from a coral reef at 10 m depth in the waters of Kapikan Reef (Semporna, Sabah) (N 04⁰06' 53.35" E 118⁰37' 41.60"). The samples were photographed underwater, collected by SCUBA divers and a representative specimen (SC022011SI) is deposited at the Laboratory of Natural Products Chemistry, Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah. The samples were weighed and cut into pieces. Then the samples were brought back to the laboratory under cool conditions (4⁰C) and processed according to the procedures described by Ishii *et al.* (2009a).

Extraction and isolation

The specimen was soaked in methanol (MeOH) at room temperature for approximately seven days. The resulting MeOH extract was concentrated in *vacuo* and the concentrate was partitioned between ethyl acetate (EtOAc) and H_2O. The EtOAc layer was further concentrated and partitioned between hexane (Hex) and 90% MeOH. This procedure yielded Hex and 90% MeOH extracts. The hexane extract was then fractionated using Silica gel column chromatography with a step gradient of Hex and EtOAc with the ratio 9:1, 8:2, 7:3, 6:4, 1:1 and $CHCl_3$:MeOH:H_2O (65:25:4). The first fraction was then further isolated using Preparative Thin Layer Chromatography (PTLC) using toluene (Tol) and EtoAc with the ratio 98:2 to obtain **1** while fraction 2 was further isolated using Hex and EtoAC with the ratio 9:1 to obtain **2**. Compounds **1** and **2** were subjected to ^{1}H-NMR, ^{13}C-NMR and 2D NMR measurements such as ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY. High-resolution mass spectroscopy data was measured using Shimadzu LC-MS-IT-TOF. Other physical characteristics were obtained as described by Vairappan *et al.* (2001) and Ishii *et al.* (2009b).

Antibacterial assay

Six strains of bacteria, *Clostridium sordelli, Clostridium novyi, Pseudomonas aurelis, Escherichia coli, Staphylococcus aureus* and *Vibrio parahaemolyticus,* were used to test the anti-bacterial activity of compounds **1** and **2**. One loopful of bacteria was inoculated in 10 ml of nutrient broth and incubated for 24 hours. The optical density of the inoculated bacteria was adjusted to 0.5 McFarland (0.5 McFarland = 0.105±0.005A@625 nm). Then, 100 µl of the adjusted bacteria were seeded and spread evenly on pre-prepared nutrient agar plates using a cell spreader. Compounds **1** and **2** were then loaded onto paper discs (Whatman, 6 mm) and the impregnated discs were placed on the seeded agar plates. The diameters of the inhibitory zones were measured after the plates were incubated at 28°C for 24 hours. Minimum inhibitory concentration was determined upon serial dilution of the compound coupled with the observation of the inhibition zone disappearance (Vairappan *et al.*, 2001).

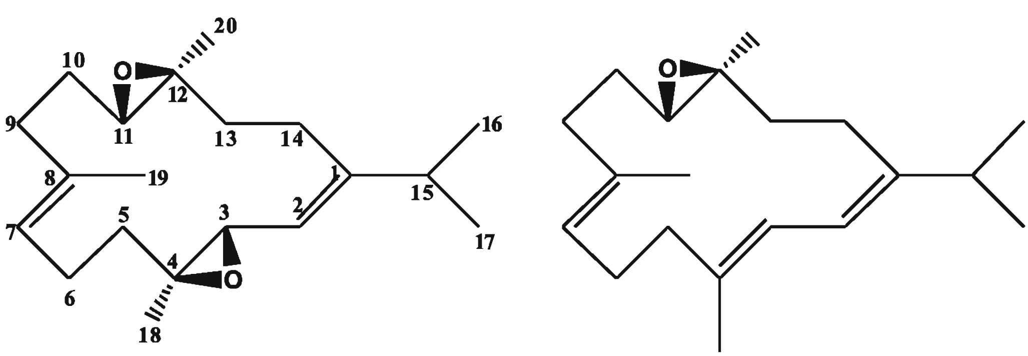
RESULTS AND DISCUSSION

A total of 500 g fresh *Sinularia flexibilis* was homogenized in 2 L of methanol and left to soak for seven days. Upon an extensive extraction and partition process as described above, 1.8 g of Hex extract and 4.6 g of 90 % MeOH extract were obtained. Repetitive preparative thin layer chromatography of fraction 1 gave compound **1** in the amount of 3.7 %, while fraction 2 gave compound **2** as 3.1 % of the fresh soft coral biomass. The structure of **1** and **2** were elucidated independently and the resulting planar structure was compared to the data available in Marin Lit (version 2012) and were found to be (3*S*,4*S*,11*S*,12*S*,1*E*,7*E*)-3,4:11,12-bisepoxycembra-1,7-diene (bisepoxy) (**1**) and (1*E*,3*E*,7*E*)-11,12-epoxycembra-1,3,7-triene (**2**) as reported by Bowden *et al.* (1983 &1978), respectively.

Both compounds revealed a similar 14-member ring with terminal isopropyl functionality as shown in Figure 1. Compound **1** has four olifinic carbons (120.3, 127.1, 135.3 and 152.0 ppm) resulting in two pairs of double bond. ^{13}C-DEPT experiments revealed the presence of five methyl carbons (δ 1.64 (*s*), 1.27 (*s*), 1.23 (*s*), 1.03 (*d*) and 1.03 (*d*) ppm), two epoxides (62.8 (*d*), 62.2 (*s*), 61.9 (*s*) and 60.2 (*d*)) and six methylene carbons. Presence of an allylic epoxide proton (3.33 (*d*)) and a nonallylic epoxide proton (δ 2.66 (*dd*)) further confirms the presence of two epoxide functionality.

On the other hand, detailed spectroscopic data analysis of compound **2** revealed the presence of six olifinic carbons (δ 147.8, 136.6, 134.1, 127.8, 121.6, 119.1) resulting in three pairs of double bond. ^{13}C-DEPT experiments revealed the presence of five methyl carbons (δ 1.72 (*s*), 1.61 (*s*), 1.26 (*s*), 1.03 (*d*) and 1.03 (*d*)), and six methylene carbons. Presence of secondary methyl indicates the presence of isopropyl functionality and this was confirmed by HMBC correlations. An epoxy methane proton was observed at δ 2.70. In the earlier publication of these compounds, complete ^{1}H and ^{13}C assignments were lacking and these are given in this report. The detailed spectroscopy data are given below:

Compound 1, colourless oil, $C_{20}H_{32}O_2$: $[\alpha]_D$ +48.0°, [^{13}C NMR ($CDCl_3$, 150 MHz), 152.0 (C-1), 135.3 (C-8), 127.1 (C-7), 120.3 (C-2), 62.8 (C-11), 62.2 (C-4), 61.9 (C-12), 60.2 (C-3), 39.7 (C-13), 38.1 (C-5), 37.5 (C-9), 34.8 (C-15), 28.3 (C-14), 25.3 (C-10), 23.1 (C-16), 23.0 (C-6), 22.6 (C-17), 18.9 (C-18), 17.0 (C-20), 15.6 (C-19); ^{1}H-NMR ($CDCl_3$, 600 MHz), 5.29 (1H, *t*, H-7), 4.97 (1H, *d*, H-2), 3.33 (1H, *d*, H-3) 2.66 (1H, *dd*, H-11), 2.28 (1H, *m*, H-9), 2.23 (1H, *m*, H-15), 2.21 (1H, *m*, H-14), 2.17 (1H, *m*, H-6), 2.13 (1H, *m*, H-13), 2.11 (1H, *m*, H-10), 2.08 (1H, *m*, H-14), 2.06, (1H, *m*, H-9),



1 2

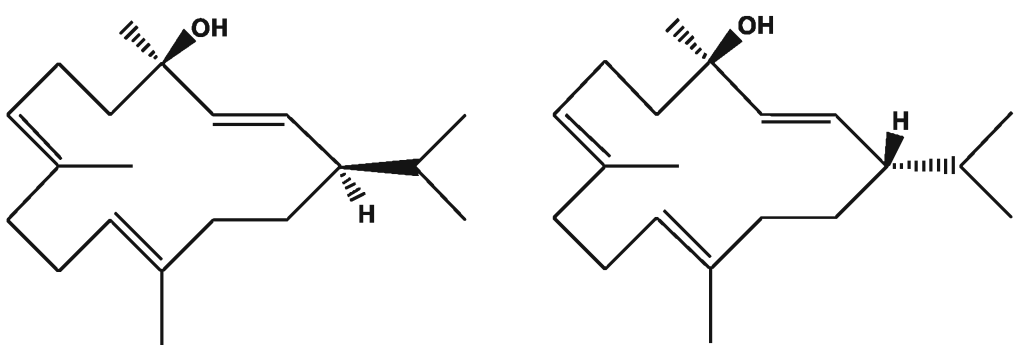
Figure 1. Isopropyl cembrene diterpenes (**1, 2**) from Borneon *Sinularia* sp.

2.03 (1H, *m*, H-5), 1.98 (1H, *m*, H-6), 1.64 (3H, *s*, 8-CH_3), 1.51 (1H, *m*, H-5), 1.32 (1H, *m*, H-10), 1.27 (3H, *s*, 12-CH_3), 1.23 (3H, *s*, 4-CH_3), 1.11 (1H, *m*, H-13), 1.03 (6H, *d*, $CH(CH_3)_2$].

Compound 2, colourless oil, $C_{20}H_{32}O$: $[\alpha]_D$ +110°, [^{13}C NMR ($CDCl_3$, 150 MHz), 147.8 (C-1), 136.6 (C-4), 134.1 (C-8), 127.8 (C-7), 121.6 (C-3), 119.1 (C-2), 61.7 (C-12), 61.1 (C-11), 39.1 (C-5), 37.7 (C-13), 37.5 (C-9), 34.8 (C-15), 26.0 (C-6), 25.2 (C-10), 25.1 (C-14), 23.0 (C-16), 23.0 (C-17), 18.7 (C-20), 18.1 (C-18), 15.7 (C-19); ^{1}H-NMR ($CDCl_3$, 600 MHz), 5.93 (1H, *d*, H-2), 5.85 (1H, *d*, H-3), 5.23 (1H, *t*, H-7), 2.88 (1H, *dd*, H-11), 2.32 (1H, *m*, H-15), 2.27 (1H, *m*, H-9), 2.23 (2H, *m*, H-6), 2.16 (2H, *m*, H-5), 2.15 (1H, *m*, H-14), 2.14 (1H, *m*, H-9), 2.03 (1H, *m*, H-13), 2.00 (1H, *m*, H-14), 1.78 (1H, *m*, H-10), 1.72 (3H, *s*, 4-CH_3), 1.61 (3H, *s*, 8-CH_3), 1.58 (1H, *m*, H-10), 1.43 (1H, *m*, H-13), 1.26 (3H, *s*, 12-CH_3), 1.03 (6H, *d*, $CH(CH_3)_2$].

Bowden and coworkers (1978) published the first report on the chemistry of compound **2** from *Sinularia grayi*. Five years later they discovered both compounds **1** and **2** in a population of *Lobophytum* sp. Present discovery of both these compounds as major metabolites in *Sinularia flexibilis* confirms their existence and importance in these compounds in this genus. Although cembranoid diterpenes are known secondary metabolites in soft corals, the presence of the 14-membered ring with an isopropyl terminal functionality is unique to soft corals genus *Sinularia* (Li *et al.*, 2006; Lin *et al.*, 2009). Similar enantiomers of isopropyl cembrene with alcohol functionality were isolated from another population of *Sinularia facile* by Bowden *et al.* (1981). These compounds were identified as (1*R*,4*R*,2*E*,7*E*,11*E*)-cembra-2,7,11-trien-4-ol (**3**) and (1*E*,4*R*,2*E*,7*E*,11*E*)-cembra-2,7,11-trien-4-ol or thunbergol (**4**) (Figure 2).

The soft coral genus *Sinularia* is known to be a prolific producer of diterpene secondary metabolites. Cyclization of a geranylgeraniol-derived precursor between one and 14 generates a 14 membered diterpenoid called cembrene. Members of this genus are known to mainly biosynthesize isopropyl(ene) type cembranoid diterpenes as reported in this paper. Therefore, the basic structure is characterized by terminal isopropyl functionality and three methyl-substituted 14-membered ring. Further structural changes in the position of double bonds, epoxidation, allylic and isopropyl oxidation, and carbon cyclization leads to another four rearranged cembrenes such as -lactone-type cembrene, -lactone-type cembrene, -lactone-type and casbane-type.



3 **4**

Figure 2. Two additional cembranes from *Sinularia facile*.

Both these cembrenes were tested for their anti-bacterial activity against six species of bacteria: *C. sordelli*, *C. novyi*, *P. aurelis*, *E. coli*, *S. aureus* and *V. parahaemolyticus*. Potent anti-bacterial activity was observed against *E. coli* and *S.aureus* with a minimum inhibitory concentration (MIC) value of 20 and 30 mg/disc, respectively. The activity observed with the inhibition against bacteria is not surprising as compounds of this skeleton are known to exhibit biological activities such as anti-fouling and an inhibition of pro-inflammatory proteins in RAW264.7 macrophage cells. They are also known to exhibit very low cytotoxic activities (Ahmed *et al.*, 2008; Lai *et al.*, 2011).

In conclusion, discovery of these two isopropyl cembrenes are an important milestone in the chemical investigation of the Borneon soft corals. These compounds could be regarded as the precursors for the biosynthesis of other cembrene diterpenes in this genus. The anti-bacterial activities exhibited by these compounds are indicators of the potency of these secondary metabolites in soft corals.

ACKNOWLEDGEMENT

The authors would like to acknowledge Sabah Parks for granting a permit for the collection of samples from Kapikan Reef, Tun Sakaran Marine Park, Semporna, Sabah. CSV would like to acknowledge MOSTI for research grant, MOSTI-NOD E-Science-02-01-10-SF0131.

REFERENCES

Ahmed, A.F., S.H. Tai, Z.H. Wen, J.H. Su, Y.C. Wu, W.P. Hu & J.H. Sheu. 2008. A C3 methylated isocembranoid and 10-oxocembranoids from a Formosan soft coral, *Sinularia grandilobata*. *Journal of Natural Products* 71: 946-951.

Amira, R., G. Shmul, Y. Benayahu & Y. Kashman. 2006. Sinularectin, a new diterpenoid from the soft corals *Sinularia erecta*. *Tetrahedron Letters* 47: 2937-2939.

Arepalli, S.K., V. Sridhar, J.V. Rao, P.K. Kennady & Y. Venkateswarlu. 2009. Furano-sesquiterpene from soft coral, *Sinularia kavarattiensis*: induces apoptosis via the mitochondrial-mediated caspase-dependent pathway in THP-1, leukemia cell line. *Apoptosis* 14: 729-740.

Bonnard, I., S.B. Jhaumeer-Laulloo, N. Bontemps, B. Banaigs & M. Aknin. 2010. New lobane and cembrane diterpenes from two Comorian soft corals. *Marine Drugs* 8: 359-372.

Bowden, B.F., J.C. Coll, W. Hicks, R. Kazlaukas & S.J. Mitchell. 1978. Studies of Australian soft corals. X* the isolation of epoxyisoneocembrene-A from *Sinularia grayi* and isonocembrene-A from *Sarcophyton ehrenbegi*. *Australian Journal of Chemistry* 31: 2707-2712.

Bowden, B.F., J.C. Coll, S.J. Mitchell & R. Kazlauskas. 1981. Studies of Australian soft corals. XXIV* two cembranoid diterpenes from the soft coral *Sinularia facile*. *Australian Journal of Chemistry* 34: 1551-1556.

Bowden, B.F., J.C. Coll & D.M. Tapiolas. 1983. Studies of the Australian soft corals. XXXIII* new cembranoid diterpenes from a *Lobophytum* species. *Australian Journal of Chemistry* 36: 2289-2295.

Chao, C.H., C.H. Hsieh, S.P. Chen, C.K. Lu, C.F. Dai & J.H. Sheu. 2006. Sinularianins A and B, novel sesquiterpenoids from the Formosan soft coral *Sinularia* sp. *Tetrahedron Letters* 47: 5889-5891.

Chen, B.W., C.H. Chao, J.H. Su, C.Y. Huang, C.F. Dai, Z.H. Wen & J.H. Sheu. 2010. A novel symmetric sulfur-containing biscembranoid from the Formosan soft coral *Sinularia flexibilis*. *Tetrahedron Letters* 51: 5764-5766.

Cheng, S.Y., C.T. Chuang, Z.H. Wen, S.K. Wang, S.F. Chiou, C.H. Hsu, C.F. Dai & C.Y. Duh. 2010. Bioactive norditerpenoids from the soft coral *Sinularia gyrosa*. *Bioorganic & Medicinal Chemistry* 18: 3379-3386.

Goud, T.V., N.S. Reddy, P. Krishnaiah & Y. Venkateswarlu. 2002. Spathulenol: a rare sesquiterpene from soft coral *Sinularia kavarattiensis*. *Biochemical Systematics and Ecology* 30: 493-495.

Ishii, T., H. Matsuura, Z. Zhaoqi & C.S. Vairappan. 2009a. A new 4 -methylated sterol from a *Nephthea* sp. (Nephtheidae) Bornean soft coral. *Molecules* 14: 3360-3366.

Ishii, T., H. Matsuura, Z. Zhaoqi & C.S. Vairappan. 2009b. A new norsesquiterpenoid from the Borneon soft coral, *Nephthea* sp. *Molecules* 14: 4591-4596.

Ishii T., K.L. Tan, R. Ueoka, S. Matsunaga & C.S. Vairappan. 2010a. Bioactive secondary metabolites from the Borneon soft corals of the genus *Nephthea. Malaysian Journal of Science* 29(3): 262-268.

Ishii, T., Z. Zhaoqi & C.S. Vairappan. 2010b. A new cembrane diterpene from the Bornean soft coral *Nephthea* sp. *Molecules* 15: 3857-3862.

Iwagawa, T., R. Nakashima, K. Takayama, H. Okamura, M. Nakatani, M. Doe & K. Shibata. 1999. New cembranes from the soft coral *Sarcophyton* species. *Journal of Natural Products* 62(7): 1046-1049.

Jin, P., Z. Deng, Y. Pei, H. Fu, J. Li, L.v. Ofwegen, P. Proksch & W. Lin. 2005. Polyhydroxylated steroids from the soft coral *Sinularia dissecta*. *Steroids* 70: 487-493.

Kamel, H.N. & M. Slattery. 2005. Terpenoids of *Sinularia*: chemistry and biomedical applications. *Pharmaceutical Biology* 43(3): 253-269.

Lai, D., Y. Li, M. Xu, Z. Deng, L.v. Ofwegen, P. Qian, P. Proksch & W. Lin. 2011. Sinulariols A-S, 19-oxygenated cembranoids from the Chinese soft coral *Sinularia rigida*. *Tetrahedron* 67: 6018-6029.

Liang, C.H., G.H. Wang, J.H. Wei, R.J. Lin, D.L. Cheng & T.H. Chou. 2010. Apoptosis effect of *Sinularia leptoclados, S. depressan* and *S. inflate* extracts in human oral squamous cell carcinomas. *Journal of the Taiwan Institute of Chemical Engineers* 41: 86-91.

Li. K.W.H., S. Jhaumeer-Laulloo, K.Y.R. Choong, I. Bonnard, B. Banaigs & D. Marie. 2006. Biological and chemical study of some soft corals and sponges collected in Mauritian waters. *Western Indian Ocean Journal of Marine Science* 5(2): 115-121.

Lin, Y.S., C.H. Chen, C.C. Liaw, Y.C. Chen, Y.H. Kuo&Y.C. Shen. 2009. Cembrane diterpenoids from the Taiwanese soft coral *Sinularia flexibilis*. *Tetrahedron* 65: 9157-9164.

Longeon, A.,M.L. Bourguet-Kondracki & M. Guyot. 2002. Two new cembrane diterpenes from a Madagascan soft coral of the genus *Sarcophyton*. *Tetrahedron Letters* 43: 5937-5939.

Matthee, G.F., G.M. Konig & A.D. Wright. 1998. Three new diterpenes from the marine soft coral *Lobophytum crassum*. *Journal of Natural Products 61*: 237–240.

Vairappan, C.S., M. Suzuki, T. Abe & M. Masuda. 2001. Halogenated metabolites with antibacterial activity from the Okinawan *Laurencia* species. *Phytochemistry* 58: 517-523.

Yu, S., Z. Deng, L. vOfwegen, P. Proksch & W. Lin. 2006. 5,8-epidioxysterols and related derivatives from a Chinese soft coral *Sinularia flexibilis*. *Steroids* 71: 955-959.