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Polyoxygenated Diterpene Produced by the Indonesian Marine Sponge *Callyspongia* sp. as an Inhibitor of the Human Pancreatic Cancer Cells

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The isolation of bioactive compound from the Indonesian Marine Sponge *Callyspongia* sp. based on bioassay-guided separation with several steps of chromatography has been done. There were 4 major compounds known as isocopalane diterpene derivatives together with a plausible new compound, isocopalanol, a polyoxygenated isocopalane diterpene. Isocopalanol has a molecular weight $[M+H]^+$ 412.576 m/z indicate that the compound has molecular formula $C_{24}H_{44}O_5$ determined by LCMS-ESI. This compound has bioactivity IC_{50} = 0.1 μg mL^{-1} against human pancreatic cancer cell.

Key words: bioactivity, *Callyspongia* sp., cancer, diterpene, isocopalane

Senyawa bioaktif dari bunga karang laut Indonesia *Callyspongia* sp. telah diisolasi secara kromatografi dengan panduan bioasai. Ada empat senyawa utama yang dikenal sebagai turunan diterpen isocopalan bersama dengan senyawa yang kemungkinan baru yaitu, isocopalanol, suatu senyawa diterpen isocopalan polioksigen. Isocopalanol berdasarkan data LCMS-ESI memiliki berat molekul $[M+H]^+$ 412,576 m/z yang menunjukkan senyawa tersebut memiliki rumus molekul $C_{24}H_{44}O_5$. Senyawa ini memiliki bioaktivitas IC_{50} = 0,1 μg mL^{-1} terhadap sel kanker pankreas manusia.

Kata kunci: bioaktivitas, *Callyspongia* sp., diterpen, isocopalan, kanker

Pancreatic cancer is a disease in which there is an increase in the number of abnormal cells in the pancreas. Pancreatic cancer is one cancer that is very malignant, difficult to diagnose and carry (Coombs *et al*. 2014; Sanli *et al*. 2017; Hanada *et al*. 2017). Generally, cancer was found at an advanced stage without obvious symptoms except by medical checkups. On the other hand, scenario in advanced cancer administration requires high-cost treatment such as surgery, chemotherapy, and radiotherapy although there is no guarantee of recovery (Donadelli *et al*. 2007; Zigeuner 2017).

The appropriate treatment options can be chosen by doctors based on the patient's health status and the progress of cancer, one of which is chemotherapy (Morimoto *et al*. 2003; Romuald *et al*. 2010; Kenner *et al*. 2017). To get rid of cancer cells in the body or prevent growth, patients can do chemotherapy with anticancer drugs. Chemotherapy can be done before or after surgery, or even if surgery cannot be performed. Chemotherapy drugs have two forms, namely those consumed directly and those given by infusion (Tsukamoto *et al*. 2000; Shah *et al*. 2007; Zeng *et al*. 2014).

The treatment with chemotherapy makes the patients receive chemicals substances continuously for a long period of time which has side effects such as decrease the body weight (Moutinho-Ribeiro *et al*. 2017). Therefore, alternative methods are needed in the treatment of cancer.

The results of previous studies have reported that the secondary metabolites from the Indonesian marine organisms have the activity against anticancer (Romuald *et al*. 2010; Kurnianda *et al*. 2018). In this study, we tried to find bioactive compounds as pancreatic cancer cell inhibitors from the Indonesian's marine sponge *Callyspongia* sp.

MATERIALS AND METHODS

Extraction and Isolation. Bioactive compound was extracted from *Callyspongia* sp. (600 g wet weight, Fig 1) by acetone and partitioned with ethyl acetate and

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Fig 1 *Callyspongia* sp.

water to obtain 23.86 mg of ethyl acetate portion. Furthermore, it was fractionated by an open column chromatography silica gel using EtOAc gradient produced three major fractions. The results of viability showed that the third fraction showed the strongest activity against PANC-1 [15.62 mg (IC_{50} = 30 µg mL^{-1})]. Then, the third fraction (15.62 mg) was purified using $^{5}C_{18}$-MS II HPLC (EtOAc:DCM) yielding seven fractions. The fifth fraction indicated as potential bioactive compound [7.6 mg (IC_{50} = 5 µg mL^{-1})] was purified using column $^{5}C_{18}$-MS III HPLC using MeOH:H_2O (1:1) produced four fractions, the fraction a (0.8 mg), fraction b (0.5 mg), fraction c (0.3 mg), and fraction d (0.8 mg). The fraction d [0.8 mg (IC_{50} = 0.1 µg mL^{-1})] showed cytotoxic to PANC-1 cells.

Materials. The framework of skeleton from bioactive compound analyzed by NMR JEOL ECA-500 MHz. The isolation sample based on bioassay-guided separation using the ESI-TOF-MS brand Q-Tof Ultima (Waters Co., MA, A.S.A). The IR spectrum was analyzed using the JASCO FT/IR-5300 tool. The UV spectrum was analyzed using a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan). The bioactive compounds purified using HPLC Cosmosil $^{5}C_{18}$-MS-II (ID 10 mm × 250 mm, Nacalai Tesque) and Cosmosil ODS ($^{75}C_{18}$- OPN, Nacalai Tesque, Kyoto, Japan). The bioassay test was conducted using bioradic spectroscopy, bioradic plates, and 96-well plastic plates. For the toxicity test a CO_2 incubator was used with the following reagents, Kanamycin solution 50 µg mL^{-1}, trypsin Mc-Coys medium, trypan blue, human cell PANC-1, phosphate buffer buffer (PBS), Fetal Bovine Serum (FBS), and Modified Dulbecco Eagle medium (DMEM).

Cell Culture and Toxicity Test. Cell culture and toxicity test were operated with reference to the procedure developed by Arai et al. (2016). Cultivation of PANC-1 (human pancreatic carcinoma) cells was preserved in DMEM (Dulbecco's Modified Eagle Medium) on heat-deactivated 10% Fetal Bovine Serum (FBS) and kanamycin (50 µg mL^{-1}) in a moistened ambiance of 5% CO_2 at 37°C. The growth of PANC-1 cells was carried out in two different media, namely a glucose-deficient medium and a glucose medium. In means of nutrient hunger, cultivation PANC-1 cells could be carried out in the glucose deficient medium containing of Basal Medium buffer (pH 7.4), *i.e.* (25 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES), enhanced 6.4 g/L NaCl, 700 mg L^{-1} $NaHCO_3$, 400 mg L^{-1} KCl, 265 mg L^{-1} $CaCl_2.2H_2O$, 200 mg L^{-1} $MgSO_4.7H_2O$, 125 mg L^{-1} NaH_2PO_4, 0.1 mg L^{-1} $Fe(NO_3).9H_2O$, 15 mg L^{-1} Phenol red, 10 mL vitamin solution (X100) (GIBCO, Carlsbad, CA, USA), 200 mmol L^{-1} L-glutamine (GIBCO), and 50 mg L^{-1} kanamycin holding 10% dialyzed FBS. The glucose medium was prepared by mixing basal medium enriched with 10% FBS and 2.0 g L^{-1} glucose (final concentration of 25 mM). The bioassay was carried out by comparing PANC-1 cells growth in the glucose-deficient medium with the glucose medium (Kurnianda *et al*. 2018; Romuald *et al*. 2010).

The cells were pre-incubated with the DMEM containing as much as 10% FBS for 24 hours. To regulate nutritional hunger, the glucose deficient medium or the glucose medium was replaced. The medium was incubated for 12 hours, then the diluted fraction d were added, after that the incubation was continued for another 12 hours under 5% CO_2 at 37°C. Detection of cell proliferation was exploited the WTS-8 colorimetric reagent. In determining IC_{50} values can be done using the growth inhibition curve. The difference of IC_{50} values between the glucose deficient medium and the glucose medium was determined selectivity in the anti-proliferation (S.I) activity (Kurnianda *et al*. 2017; Coombs *et al*. 2014).

Table 1. ^{1}H (500 MHz) dan ^{13}C (125 MHz) data NMR ($CDCl_3$) of compound **1**

No	^{13}C	^{1}H (mult. *J* in Hz)
1	36.7 (t)	1.56 (m, 1H)
		1.3 (m, 1H)
2	19.6 (t)	1.46 (m, 1H)
		1.53 (m, 1H)
3	41.2 (t)	1.06 (m, 1H)
		1.29 (m, 1H)
4	34.1 (s)	
5	55.9 (d)	0.69 (m, 1H)
6	15.08 (t)	1.62 (m, 1H)
		1.64 (m, 1H)
7	40.1 (t)	1.33 (m, 1H)
		1.56 (m, 1H)
8	35.2 (s)	
9	45.5 (d)	1.36 (m, 1H)
10	37.7 (s)	
11	25.2 (t)	1.67 (m, 1H)
		1.82 (m, 1H)
12	78.8 (d)	3.05 (br t, 1H, 6.8; 2.0)
13	75.7 (s)	
14	57.4 (d)	1.46 (m, 1H)
15	57.7 (t)	2.71 (br d, 1H, 12.0)
16	27.8 (q)	1.12 (s, 3H)
17	25.2 (q)	1.49 (s, 3H)
18	21.9 (q)	0.82 (s, 3H)
19	29.3 (q)	0.85 (s, 3H)
20	12.6 (q)	0.84 (s, 3H)
1'	45.4 (d)	4.74 (br t, 1H, 7.0; 2.7)
2'	15.4 (q)	1.17 (d, 3H, 7.0)
1"	43.9 (d)	4.81 (br t, 1H, 7.0; 2.5)
2"	15.4 (q)	1.15 (s, 3H, 7.0)

RESULTS

Structure Identification. Substances obtained from the fractions a, b, and c have been identified as isocopalane diterpene derivative compounds. The compound derived from the fraction d (compound **1**) is thought to be a new compound of a polyoxygenated isocopalane diterpene. The compound **1** (colorless amorphous) has the molecular formula $C_{24}H_{44}O_5$ determined by LCMS-ESI with molecular weight $[M+H]^+$ 412.576 m/z. Based on the FTIR spectrum shows that the active metabolite has functional groups at 3419 cm^{-1} as O-H streaching, 2995 cm^{-1} and 2853 cm^{-1} as alkane skeleton of hydrocarbon, and the fingerprint region of ether functional group at 1261 cm^{-1} as C-O (Fig 2). The results show that the active compound is an polyoxygenated hydrocarbon.

DISCUSSION

The ^{1}H and ^{13}C NMR of compound **1** (Table 1) showed the characteristics signal of methyl group observed at 0.82 (s, 3H), 0.84 (s, 3H), 0.85 (s, 3H), 1.12 (s, 3H) 1.15 (d, 3H), 1.17 (d, 3H), 1.49 (s, 3H) as the previous reported (Wyk *et al*. 2007). The presences of the oxymethylene appears at 2.71 (br d, 1H, C-15). The signal from oxymethane appears at 3.05 (br t, 1H, C-12), 4.74 (br t, 1H, C-1'), 4.81 (br t, 1H, C-1"). Furthermore, COSY and HMBC correlation indicate that the active compound contain polyoxygenated moiety with diterpene skeleton (Takekawa *et al*. 2006; Kubota *et al*. 2007; Kurnianda *et al*. 2017).

The analysis of COSY spectrum from compound active revealed the proton-proton sequences of carbon 1-2–3, 5-6-7, 9-11-12, 1'-2' and 1"-2". The correlation

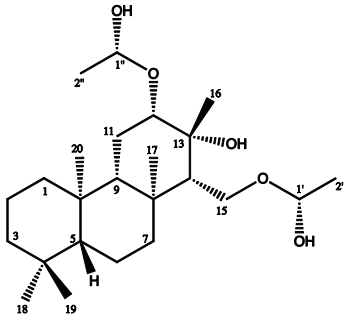

Fig 2 Structure of bioactive compound **1**.

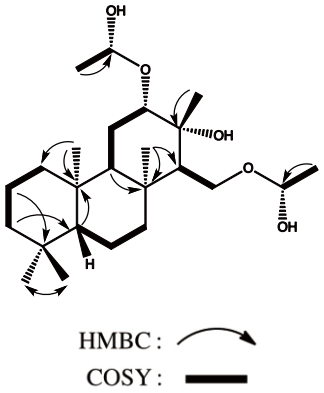

Fig 3 COSY and HMBC correlation of bioactive compound **1**.

of proton-carbon from HMBC spectrum showed that the characteristics correlation at C-20 to C-1, C-17 to C8 indicated methyl group attach to bridge from 2 six-membered rings moiety. Furthermore, correlation between C-18 and C-19 as methyl singlet attached on quaternary sp^3 carbon at C-4. The presence of 2 ether moieties confirmed by HMBC and COSY correlation at C-12 to 1" and C-15 to 1' as no cross link between them (Fig 3). We assumed this position contain ether functional group moiety confirmed by IR data and proton chemical shift between C-12 to C-1" and C-15 to C-1' (Shah *et al*. 2007; Zeng *et al*. 2014). Furthermore, the absolute stereochemistry of secondary alcohol moiety determined by Mosher's method. The relative configuration indicate as (5*R**, 8*S**, 9*S**, 10*R**, 12*S**, 13*R**, 14*S**,1'*R**, 1"*R**)-isocopalane diol.

The viability against human pancreatic cancer cell in glucose deficiency medium from Marine sponge *Callyspongia* sp. showed a potential inhibitor of cell proliferation analyzed by WST-8 colorimetric reagents. The linear interpolation of the viability curve was used for determine of the IC_{50} value in glucose deficiency medium. The bioactive compound indicated as anti-proliferative activity in glucose deficiency medium (Romuald *et al*. 2010).

The bioactive compound from the Indonesian Marine sponge *Callyspongia* sp. has potential anti-proliferative against PANC-1 cell with $IC_{50} = 0.1\ \mu g\ mL^{-1}$. The bioactive compound indicated as secondary metabolite which has activity for alternative drug models against commercial drug antimycin = $0.1\ \mu g\ mL^{-1}$.

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