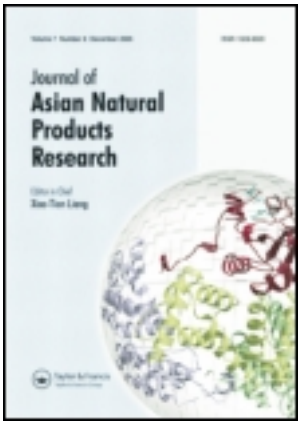

Journal of Asian Natural Products Research

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ganp20

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Available online: 29 Apr 2011

To cite this article: Lin Chen, Xi-Xiang Tang, Min Zheng, Zhi-Wei Yi, Xiang Xiao, Ying-Kun Qiu & Zhen Wu (2011): A novel indole alkaloid from deep-sea sediment metagenomic clone-derived *Escherichia coli* fermentation broth, Journal of Asian Natural Products Research, 13:05, 444-448

To link to this article: http://dx.doi.org/10.1080/10286020.2011.570262

Journal of Asian Natural Products Research
Vol. 13, No. 5, May 2011, 444–448

A novel indole alkaloid from deep-sea sediment metagenomic clone-derived *Escherichia coli* fermentation broth

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(*Received 1 November 2010; final version received 7 March 2011*)

To explore secondary metabolites in deep-sea sediment metagenomic clone-derived *Escherichia coli* fermentation broth, different kinds of chromatography methods were used in the isolation procedures, while the structures of the isolated compounds were assigned based on the MS analysis and their ^{1}H and ^{13}C NMR spectra including 2D NMR techniques such as COSY, HMQC, and HMBC experiments. As a result, a novel compound was isolated and characterized as *N*-{1-[4-(acetylamino)phenyl]-3-hydroxy-1-(1*H*-indol-3-yl)propan-2-yl}-2,2-dichloroacetamide (**1**). In addition, eight known compounds were also obtained. Fatty acid amide hydrolase and monoacylglycerol lipase were used to screen analgesic activity, and the new compound showed analgesic activity to some extent in pharmacological test.

Keywords: deep-sea sediment; metagenomic clone; *N*-{1-[4-(acetylamino)phenyl]-3-hydroxy-1-(1*H*-indol-3-yl)propan-2-yl}-2,2-dichloroacetamide; indole alkaloids

1. Introduction

Deep marine subsurface sediments are one of the most extensive microbial habitats on Earth. Marine sediments cover more than two-thirds of the Earth's surface, and microbial cells and microbial activity appear to be widespread in these sediments [1]. And the deep sea is usually characterized by extremely high salinity, darkness, high pressure, and high/low temperature [2]. Because of the particularity of the marine environment, marine microorganisms have unique metabolic ways, producing many novel chemical structures with great complexity and diversity [3]. This untapped potential has resulted in the recent acceleration in the interest of studying the marine microorganisms. However, the dilemma is that the vast majority of the microorganisms cannot be cultivated presently. Given that the metagenomic approach clones the total microbial genome (the metagenome) in culturable bacteria (such as *Escherichia coli*) to discover novel microbial resources, it represents an efficient method of isolating novel and useful genes [4].

Endocannabinoid system played an important role in pain signal transduction, and different kinds of endocannabinoids had been isolated in brain, including anandamide (AEA), 2-arachidonoylglycerol, and

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ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020.2011.570262
http://www.informaworld.com

2-oleoylglycerol (2-OG), which can be hydrolyzed by degrading enzyme (fatty acid amide hydrolase, FAAH and monoacylglycerol lipase, MGL) [5]. To find new degrading enzyme inhibitor with analgesic activity, we investigated the extract from deep-sea sediment metagenomic clone. As a result, a clone-coded 11F6 showed potent enzyme inhibitory activity. The clone was transformed into *E. coli* and fermented with large scale. Studies on the chemical constituents in 11F6-derived *E. coli* fermentation broth resulted in the isolation of one new compound *N*-{1-[4-(acetylamino) phenyl]-3-hydroxy-1-(1*H*-indol-3-yl) propan-2-yl}-2,2-dichloroacetamide (**1**), together with eight known compounds. This article deals with the isolation, structural elucidation, and enzyme inhibitory activities of the new compound.

2. Results and discussion

The molecular formula of $C_{21}H_{21}Cl_2N_3O_3$ of compound **1** was deduced from the pseudo-molecular ion peak at *m/z* 456.0859 $[M + Na]^+$ in HR-EI-MS and NMR spectral data, whose peak intensities between the molecular ion and the isotopic ion were M: M + 2: M + 4 = 9: 6: 1 due to the presence of two Cl atoms. The IR absorption bands at 3323 and 1683 cm^{-1} showed the existence of amide group, which were affirmed by ^1H NMR spectral data at δ 8.44 (1H, br. d, $J = 8.3$ Hz) and 9.85 (1H, br. s) and ^{13}C NMR spectral data at δ 163.1 and 168.1. The ^1H NMR spectrum showed an olefinic proton at δ 7.20 (1H, d, $J = 2.4$ Hz), a set of four-spin proton system signals at δ 7.26 (1 H, br. d, $J = 8.1$ Hz), 7.32 (1H, br. d, $J = 8.1$ Hz), 6.85 (1H, td, $J = 8.1$, 1.0 Hz), and 6.98 (1H, td, $J = 8.1$, 1.0 Hz), and a downfield labile proton singlet at δ 10.86 (1H, br. d, $J = 2.0$ Hz), and these data suggested that **1** might have a *mono*-substituted indole moiety. The ^1H NMR spectrum also showed the presence of a 1,4-disubstituted benzene ring system at δ 7.23 (2H, d, $J = 8.3$ Hz) and 7.42 (2H, d, $J = 8.3$ Hz). Other proton signals belong to the oxymethylene protons at δ 3.17 (1H, m) and 3.37 (1H, m), two methine protons at δ 4.42 (1H, overlapped) and 4.42 (1H, overlapped), one methylene proton at δ 6.32 (1H, s), and one methyl proton at δ 1.97 (3H, s). The ^{13}C NMR signal at δ 66.9 located in lower field might link to an electron-drawing group. And it was due to a dichloromethylene fragment, based on the chemical shift of the proton attaching on it was δ 6.32 (1H, s). $^1H-^1H$ COSY correlations between H-7 and H-8, H-8 and H-9, 8-NH and H-8, and 9-OH and H-9 indicated a fragment of 2,3,3-trisubstituted propanol. Correlations from H-2′ to C-3′, C-3a′, and C-7a′ in the HMBC spectrum confirmed the presence of a 3-indolyl unit. Correlations from H-11 and H-8 to C-10 indicated 2,2-dichloroacetamide was attached to C-8. Correlations from H-13 to C-12 confirmed the presence of an acetyl moiety. Finally, key HMBC correlations from H-7 to C-2, C-3′, and C-1 and from H-2 to C-7 suggested that the substituted benzene was attached to C-3′ of the indole moiety via a C—C linkage. Up till now, the structure was elucidated as *N*-{1-[4-(acetylamino)-phenyl]-3-hydroxy-1-(1*H*-indol-3-yl)propan-2-yl}-2,2-dichloroacetamide, whose key HMBC and $^1H-^1H$ COSY correlations are shown in Figure 1, and its ^1H and ^{13}C NMR spectral data (DMSO-d_6) are summarized in Table 1.

Based on the 1D and 2D NMR spectroscopic analysis (including COSY, HSQC, and HMBC) and comparison of their spectroscopic data with those reported in literature, other compounds were identified as 3,3-di-1*H*-indol-3-yl-1,2-propandiol (**2**) [6], 1*H*-indole-3-carbaldehyde (**3**) [7], cyclo (Pro-Tyr) (**4**) [8], cyclo (Trp-Tyr) (**5**) [9], cyclo (*L*-Val-*D*-Pro) (**6**) and cyclo (*L*-Val-*L*-Pro) (**7**) [10–12], and cyclo (*L*-Ala-*D*-Pro) (**8**) and cyclo (*L*-Ala-*L*-Pro) (**9**) [10–12].

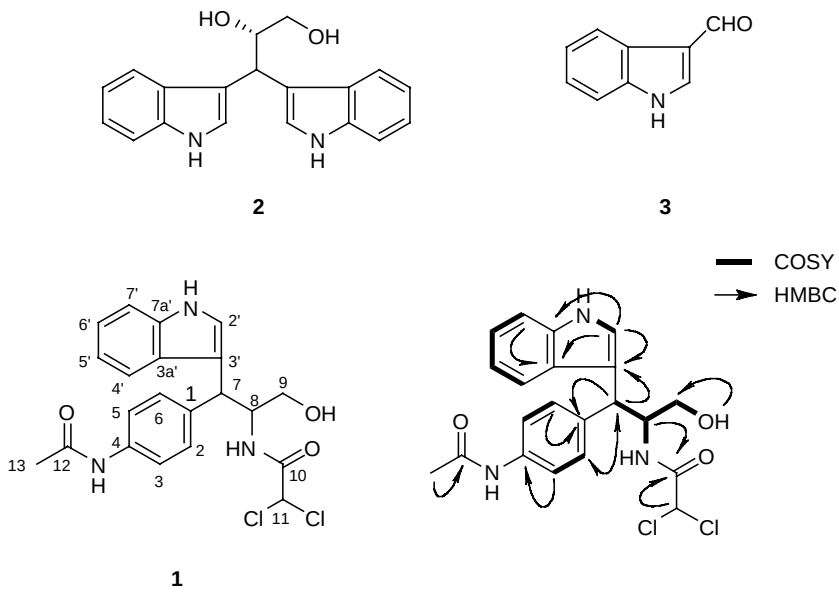

Figure 1. Structures of compounds **1**–**3**, and key $^{1}H-^{1}H$ COSY and HMBC correlations of compound **1**.

3. Experimental

3.1 General experimental procedures

UV spectra were recorded on a Shimadzu UV-260 spectrometer. IR spectra were determined on a Perkin-Elmer 683 infrared spectrometer in KBr pellets. NMR spectra were taken with TMS as internal standard on a Bruker Avance 400 FT-NMR spectrometer. ESI-MS were measured on an AB MD-SCIEX Advantage spectrometer and HR-ESI-MS on a Bruker FT-MS Apex III spectrometer. Column chromatography was performed on silica gel (Yantai Chemical Industry Research Institute, Yantai, China) and Cosmosil 75 C_{18}-OPN (75 μm, Nacalai Tesque Co. Ltd., Kyoto Japan). TLC was conducted on Silica GF254 (Yantai Chemical Industry Research Institute) and RP-18 F254 (Merck, Scandicci, Germany) plates.

Table 1. ^{13}C NMR (100 MHz, DMSO-d_6) and ^{1}H NMR (400 MHz, DMSO-d_6) spectral data of compound **1**.

No.	δ_C	δ_H	No.	δ_C	δ_H
1	137.3		7	42.4	4.42 (1H, overlapped)
4	146.0		8	54.6	4.42 (1H, overlapped)
2, 6	128.4	7.23 (2 H, d, $J = 8.6$ Hz)	9	60.9	3.37 (1H, m) and 3.17 (1H, m)
3, 5	118.9	7.42 (2 H, d, $J = 8.6$ Hz)	10	163.1	
2′	121.1	7.20 (1 H, d, $J = 2.4$ Hz)	11	66.9	6.32 (1 H, s)
3′	115.2		12	168.1	
3a′	126.9		13	23.8	1.97 (3 H, s)
4′	118.4	7.32 (1 H, br. d, $J = 8.1$ Hz)	1′		10.86 (1 H, br. d, $J = 2.0$ Hz)
5′	118.1	6.85 (1 H, td, $J = 8.1$, 1.0 Hz)	4-NH		9.85 (1 H, br. s)
6′	120.8	6.98 (1 H, td, $J = 8.1$, 1.0 Hz)	8-NH		8.44 (1 H, br. d, $J = 8.3$ Hz)
7′	111.1	7.26 (1 H, br. d, $J = 8.1$ Hz)	9-OH		4.80 (1 H, br. t, $J = 4.9$ Hz)
7a′	136.0				

Detection was done by spraying 10% H_2SO_4/ethanol, followed by heating. HPLC was performed with a Varian preparative apparatus using an ODS column (Shimadzu C_{18}, 20 × 250 mm, Kyoto, Japan).

3.2 Fermentation

Sediment samples for DNA extraction were collected from the subsurface sediments at water depths of 3006 m (102.612575°E, 2.022449°N) in southwestern Indian Ocean by the Third Institute of Oceanography. The samples were frozen at 4°C before being processed. They were cultured in artificial seawater medium by the enrichment methods. Then, the DNAs of the enrichment product were extracted and purified, and the size-separated DNAs of 30–40 kb from different locations were pooled equally, and then, the genomic DNA was end-repaired to blunt end and cloned into fosmid vector. The ligation mixture was packed, and the packaged DNA was transformed into *E. coli*, generating a library of 3500 clones, followed by analgesic activity screening. The clone 11F6 producing light-green pigment was selected and characterized. Finally, it was vaccinated to 100 L fermentation tank, and fermentation broth was handled by continuous flow centrifuge after 36 h at the condition of 37°C, 200 rpm, pH 7.0, 60 L/min throughput, and the supernatant fluid was collected. A voucher specimen (11F6) has been deposited at the Third Institute of Oceanography State Oceanic Administration.

3.3 Extraction and isolation

AB-8 macroporous adsorption resin (5 L) was used to handle the fermentation broth (80 L). After eluted with 15 L water, the 95% ethanol eluents (15 L) were collected, followed by evaporating the solvent under reduced pressure to give the total extract (112.5 g). The extract was chromatographed over silica gel (500 g) column, eluting with a gradient system (chloroform → chloroform: methanol, 5: 1), and separated into 19 fractions based on TLC analysis. Fractions 7 → 19 were subjected to further separation using ODS column, eluting with a gradient system (MeOH–H_2O, 10–100%). Each fraction was subjected to preparative HPLC (Shimadzu, C_{18} 20 × 250 mm, 8 ml/min), using MeOH–H_2O as fluent phase for further separation. As a result, fraction 9 (0.9 g) yielded compounds **6** (2 mg) and **7** (4 mg); fraction 10 (2.3 g) produced compounds **2** (5 mg), **8** (7 mg), and **9** (15 mg); fraction 13 (0.8 g) gave compounds **1** (3 mg), **3** (4 mg), and **4** (5 mg); and fraction 17 (0.6 g) yielded compound **5** (12 mg).

3.3.1 N-*{1-[4-(acetylamino)phenyl]-3-hydroxy-1-(1*H*-indol-3-yl)propan-2-yl}-2,2-dichloroacetamide (**1**)*

Colorless oil; $[\alpha]_D^{20}$ +13.5 ($c = 0.0013$, MeOH); UV (MeOH) λ_{max} ($\log \varepsilon$): 219(4.35), 290 (3.39) nm; IR (KBr) ν_{max} (cm^{-1}): 3323, 2921, 1683, 1653, 1635, 1558, and 1540; ^{13}C NMR (100 MHz, DMSO-d_6) and ^{1}H NMR (400 MHz, DMSO-d_6) spectral data are listed in Table 1; ESI-MS: *m/z* 434 $[M + H]^+$; HR-ESI-MS: *m/z* 434.1041 $[M + H]^+$ (calcd for $C_{21}H_{22}Cl_2N_3O_3$, 434.1033); *m/z* 456.0859 $[M + Na]^+$ (calcd for $C_{21}H_{21}Cl_2N_3O_3Na$, 456.0847); *m/z* 472.0585 $[M + K]^+$ (calcd for $C_{21}H_{21}Cl_2N_3O_3K$, 472.0586).

3.4 Enzyme assay

3.4.1 FAAH assay

Tubes containing 50-μg protein derived from FAAH-overexpressing HEK2939 (Human Embryonic Kidney 293) cells with 25 mM AEA as substrate in Tris buffer (50 mM, pH 8.0) containing fatty acid-free bovine serum albumin (0.05%), test compounds in DMSO or DMSO alone for controls (10 μl). After incubation at

37°C for 30 min, reactions were stopped by addition of a mixture of chloroform/-methanol (1:1, vol/vol) followed by vigorous mixing. The hydrolysate was detected using LC–MS [XDB Eclipse C_{18} column (50 × 4.6 mm i.d., 1.8 μm; Zorbax, Agilent Technologies), 95% methanol] in the negative-ion mode using 17:0 fatty acid as internal standard, and the inhibition ratio was calculated by comparing the interior label.

Operational approach of MGL assay was the same as FAAH assay except that the substrate was 2-OG. In all experiments, tubes containing buffer only were used as control for chemical hydrolysis (blank), and this value was systematically subtracted. Using these conditions, URB-597 (IC_{50} = 8 nM) and were positive controls. As a result, 33% FAAH and MGL were inhibited by 50 μM compound **1**.

Acknowledgements

The authors would like to acknowledge the financial support from National High Technology Research and Development Plan (863) of China (2007AA091904) and China Ocean Mineral Resources R&D Association Fund (DYXM-115-02-2-9). Tian-hua Zhong of the Third Institute of Oceanography was appreciated for his kind help in NMR experiments.

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