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Glycerols and fatty acids isolated from *Micractinium* sp. KSF0031

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ABSTRACT

From the collected extract from King Sejong Antarctic Station, strain *Micractinium* sp. KSF0031, led to the isolation of one new monoacyldigalactosyl glycerol (**1**) and seven known compounds (**2–8**). Their chemical structures were established using extensive spectroscopic techniques, including 1D, 2D-NMR, and MS, and compared with the published data. To the best of our knowledge, this is the first report to investigate the secondary metabolites from genus *Micractinium*. The monoacyldigalactosyl glycerol in *Micractinium* could serve as its chemotaxonomic markers.

1. Subject and source

Microalgae are a polyphyletic group of unicellular photosynthetic eukaryotes comprising at least 40,000–70,000 species from various phyla such as Cyanophyta (blue algae), Rhodophyta (red algae), Chlorophyta (green algae), Pyrrophyta, Cryptophyta, Haptophyta, Heterokontophyta, and Streptophyta. *Micractinium* is a genus containing many species of green algae in the globally-distributed family, Chlorellaceae (Tran et al., 2019). The extract-producing actinomycete strain collected from freshwater of King Sejong Antarctic Station (62° 13′ S, 58° 47′ W) in 2006.

This strain was maintained in Bold's basal medium (BBM) consist of 10 mL $NaNO_3$ 2.94 mM, 10 mL $CaCl_2.2H_2O$ 0.17 mM, 10 mL $MgSO_4.7H_2O$ 0.3 mM, 10 mL K_2HPO_4 0.43 mM, 10 mL KH_2PO_4 1.29 mM, 10 mL NaCl 0.43 mM, 1 mL mixture of alkaline EDTA solution 17.10 mM and EDTA (Titriples III) 55.30 mM, 1 mL $FeSO_4.7H_2O$ 0.18 mM, 1 mL H_3BO_3 18.50 mM, 1 mL mixture of $ZnSO_4.7H_2O$ 8.82 mM, $MnCl_2.4H_2O$ 1.44 mM, MoO_3 0.71 mM, $CuSO_4.5H_2O$ 1.57 mM, and $Co(NO_3)_2.6H_2O$ 0.49 mM and 1000 mL of distilled water in a Erlenmeyer flask keeping at pH value of 6.6 and incubated at 2–3 °C under continuous lighting from light-emitting diodes at an intensity of 35 μmol photons $m^{-2}s^{-1}$. The species identification was performed by Professor Sanghee Kim and a voucher specimen, *Micractinum* sp. KSF0031 (KPRI-KSF0031) was deposited at the herbarium belong with the Division of Polar Life Sciences, Korea Polar Research Institute, Korea.

2. Previous work

The previous chemical investigation of the algae revealed the presence of carotenoids, fatty acids, sterols, mycosporine-like amino acids, phycobilins, polyketides, pectins, halogenated compounds, and toxins (Tran et al., 2019). However, investigation into the chemical composition of extracts from the genus *Micractinium* has been limited. Recently, we have collected several samples from the unexplored unique environment in King Sejong Antarctic Station.

3. Present study

3.1. Experimental

Specific optical rotation was measured using a JASCO DIP 1000 digital polarimeter. UV spectrum was recorded using a Thermo spectrometer. IR spectra were recorded using a JASCO FT/IR-4100 spectrometer. The 1D and 2D-NMR spectra were obtained using Varian Unity Inova 400 MHz and a Bruker Ascend™ 500 MHz spectrometer with tetramethylsilane (TMS) as an internal standard and the chemical shifts were recorded in δ values (ppm). Mass spectra were recorded using JEOL JMS-AX 300L and JEOL JMS-700 MODEL spectrometer. Silica gel (Merck, 63–200 mm particle size) and RP-18 (Merck, 75 mm particle size) were used for open column chromatography. TLC was performed using Merck silica gel 60 F_{254} and RP-18 F_{254} plates. Compounds were visualized after spraying with aqueous 10% H_2SO_4 and heating for 3–5 min.

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Table 1
^{1}H-NMR (500 MHz) and^{13}C-NMR (125 MHz) spectroscopic data of **1** in methanol-d_4.

Position	H δ_H mult., (J in Hz)	C δ_C, type	Position	H δ_H mult., (J in Hz)	C δ_C, type
1	4.12, dd (6.3, 11.5)	66.5, CH_2	1″	4.30, d (7.5)	104.9, CH
	4.18, dd (4.5, 11.5)		2″	3.86, m	72.3, CH
2	4.03, m	69.4, CH	3″	3.54, m	74.3, CH
3	3.67, dd (6.0, 10.0)	71.9, CH_2	4″	3.91, m	71.2, CH
	3.90, m		5″	3.79, m	74.4, CH
1′	4.82, ovl[a]	100.1, CH	6″	3.72, m	62.5, CH_2
2′	3.91, m	72.3, CH	1‴		176.3, C
3′	3.91, m	69.2, CH	2‴	2.37, t (7.5)	34.8, CH_2
4′	3.79, m	70.8, CH	3‴	1.60, m	25.7, CH_2
5′	3.79, m	69.9, CH	$(CH_2)_n$	1.26–1.30, m	23.4, 29.9, 30.0, 30.1, 30.2, 30.3, 30.4, 32.7
6′	3.71, m	67.6, CH_2	14‴	0.87, t (7.0)	14.3, CH_3

Assignments were supported with COSY, HMQC and HMBC NMR spectra.
[a] Overlapped to the other signal.

3.2. Extraction and isolation

M. sp. KSF0031 was refermented on a large scale in the same medium previously used to prepare the fractionation. Freeze dried *M.* sp. KSF0031 (110.0 g) was absolutely extracted three times (3 h × 500 mL) with methanol. After removing the solvent under reduced pressure, the methanol extraction (14.3 g) was suspended in H_2O and then successively partitioned with *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH. The combination of *n*-BuOH and H_2O fractions (5.4 g) was subjected to separation on a silica gel column (60 × 3.5 cm) using a gradient solvent system of CH_2Cl_2–MeOH–H_2O (60:1:0.1 to 0:100:1, v/v). Subsequently, the eluents were separated using a semi-preparative Waters HPLC systems with an isocratic solvent system of 92% MeOH in H_2O + 0.1% trifluoroacetic acid (flow rate 5 mL/min) over 90 min, with UV detection at 220 and 205 nm. This resulted in the isolation of compounds **1** (4.5 mg), **2** (3.8 mg), **3** (5.1 mg), and **4** (6.0 mg). In addition, the combination of CH_2Cl_2 and EtOAc fractions (1.4 g) was subjected to silica gel column chromatography (60 × 2.5 cm) with *n*-hexane-EtOAc (2:1, v/v) to yield 6 sub-fractions (Fr.1 to Fr.6). Fraction 4 (300.0 mg) was further purified using a YMC RP-18 column with a MeOH–H_2O mobile phase (4:1 to 6:1, v/v), and a semi-preparative Waters HPLC system with an isocratic solvent system of 90% MeOH in H_2O + 0.1% trifluoroacetic acid (flow rate 5 mL/min) over 90 min as an eluent, UV detection at 254 and 210 nm to yield **7** (27.0 mg) and **8** (3.5 mg). Furthermore, the *n*-hexane soluble fraction (7.5 g) was eluted chromatographically on a silica gel column (60 × 4.5 cm) using a stepwise gradient of *n*-hexane-EtOAc (40:1 to 0:1, each 500 mL, v/v) to yield eight fractions (Fr.7 to Fr.14), distinguishable by their TLC profiles. Fraction 9 (2.5 g) was subsequently subjected to silica gel column chromatography (60 × 2.5 cm) and eluted with *n*-hexane-EtOAc (30:1, v/v) to yield 4 sub-fractions (Fr. 9.1 to 9.4). Fraction 9.3 (1.5 g) was chromatographed on a silica gel column (60 × 2.5 cm) using a gradient solvent system of *n*-hexane-acetone (15:1 to 0:1, v/v) to yield compounds **5** (5.0 mg) and **6** (7.0 mg).

3.3. Structural elucidation

Compound **1** was amorphous white powder with a specific optical rotation $[\alpha]_D^{24}$ +19.9. The UV–visible spectrum absorption maximum was observed at 203.0 nm. The molecular formula of **1** was determined to be $C_{29}H_{54}O_{14}Na$ based on the high resolution fast-atom bombardment mass spectrometry (HR-FABMS) with a molecular ion peak at *m/z* 649.3414 ($[M+Na]^+$) (Calcd for $C_{29}H_{54}O_{14}Na$, 649.3411). The infrared (IR) spectrum displayed absorption bands at 3329 and 1743 cm^{-1}, indicating the presence of OH and ester functionalities, respectively. The ^{1}H-NMR spectrum of **1** indicated the existence of a triplet methyl signal at δ_H 0.87 (3H, t, J = 7.0, H-14‴), a mass of oxymethylene and oxymethine hydrogen signals between δ_H 3.54 and 4.19, and long chain signals at δ_H 1.26–1.60. These spectral features revealed that the glycolipid moiety was connected to a saturated fatty acid unit. In addition, the signal of protons at δ_H 4.82 (1H, overlapped, H-1′)/4.30 (1H, d, J = 7.5, H-1") and chemical shift of carbons at δ_C 100.1/104.9 attributed to the presence of two α- and β-glycosidic linkages, respectively. From carbon-13 nuclear magnetic resonance (^{13}C-NMR) analysis and distortionless enhancement by polarization transfer (DEPT) spectra of **1**, carbon signal of sugars and glycerol moiety were assigned (Table 1). By comparing the NMR data of **1** with those of 1′-*O*-palmitoyl-3′-*O*-[6-*O*-α-D-galactopyranoside-β-D-galactopyranosyl]-*sn*-glycerol isolated from *Ulva pertusa* (Fusetani and Hashimoto, 1975), the structure of **1** was assigned as a monoacyldigalactosyl glycerol, possessing modification of two sugars and myristoyl unit instead of palmitoyl unit in **1**. Acid hydrolysis of **1** by CF_3COOH confirmed the presence of galactose, which was further authenticated by comparison of thin-layer chromatography (TLC) migration with that of the authentic sample. The heteronuclear multiple-bond correlations (HMBC) spectrum, exhibited a significant correlation between the signals at δ_H 4.12/4.18 (H-1) and δ_C 176.3 (C-1‴), indicating acylation of the compound at C-1 (see Fig. 2). The absolute configuration of C-2 was determined by proton chemical shifts with the signal of H-1 (δ_H 4.12 and 4.18) methylene protons and the coupling constant value between H-2 and H-3a as 6.0 Hz, as compared with the published value of H-1 signals (4.12 and 4.16) and H-3a (J = 4.5 Hz) (Kim et al., 2004); apparently, **1** was assigned the 2*S* configuration. To confirm the structure, a fatty acid ester (**1a**, 0.4 mg) and digalactosyl glycerol (**1b**, 0.5 mg) were obtained by the treatment of **1** with NaOMe–MeOH solution by following the reported method. Fatty acid ester (**1a**) was determined by electron impact mass spectrometric (EIMS) analysis at *m/z* 242 $[M]^+$ and found to be methyl myristate. In addition, the characteristics of digalactosyl glycerol (**1b**), $[\alpha]_D^{20}$ +83.3 (c = 0.1, H_2O), resembled with the specific optical rotation and ^{1}H-NMR data of (2*R*)-1-*O*-[α-D-galactopyranosyl-(1"→6′)-*O*-β-D-galactopyranoside] glycerol (Jung et al., 1996). Considering the aforementioned indication, the structure of **1** was identified as (2*S*)-1-*O*-myristoyl-3-*O*-[6-*O*-β-D-galactopyranosyl-α-D-galactopyranoside]-*sn*-glycerol and the compound was named micractinin A.

The chemical structure of the known compounds were identified as lilioside D (**2**) (Miyuki et al., 1984), glycerol (**3**) (Kalal et al., 1988), lilioside E (**4**) (Miyuki et al., 1984), 5*Z*,8*Z*,11*Z*-tetradecatrienoic acid (**5**) (Kock et al., 1998), 4*Z*-heptenoic acid (**6**) (Sato et al., 1981), 5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid (**7**) (Sacchi et al., 1994), and 5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid-2,3-dihydroxypropyl ester (**8**) (Chang et al., 2008) (Fig. 1) based on the comparison of their spectroscopic data with those reported in the literature.

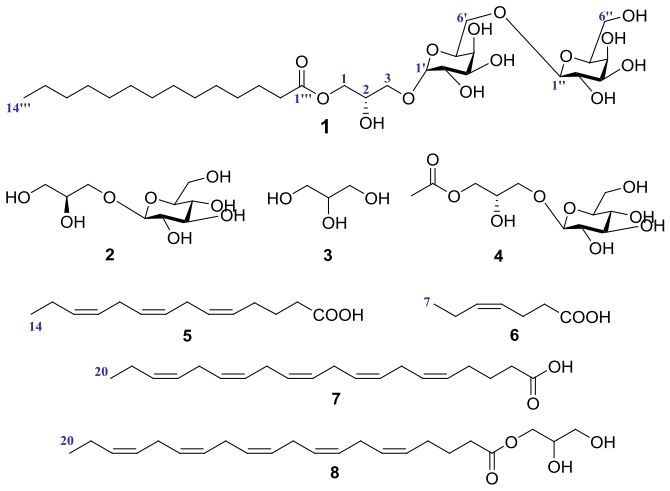

Fig. 1. The structure of compounds (**1**–**8**) isolated from *M.* sp. KSF0031.

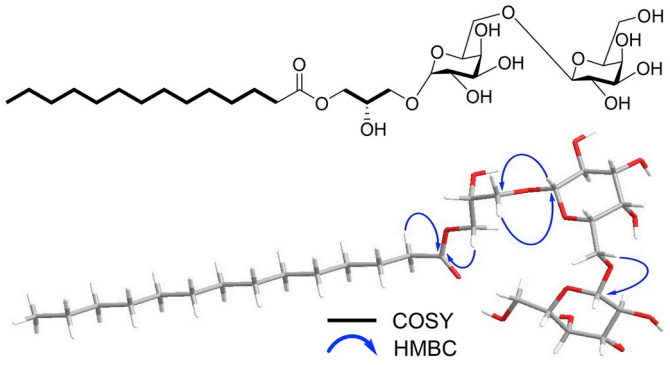

Fig. 2. Key COSY and HMBC correlations for new compound (**1**).

3.4. Micractinin A (1)

Amorphous white powder; $[\alpha]_D^{24}$ +19.9 (c = 0.1, MeOH); UV (MeOH) λ_{max} (logε) nm: 203.0 (0.59); IR ν_{max} cm^{-1}: 3329, 1743, 1448, and 1024; ^1H and ^{13}C-NMR (500 and 125 MHz, methanol-d_4) data, see Table 1; HR-FABMS m/z: 649.3414 $[M+Na]^+$ (Calcd for $C_{29}H_{54}O_{14}Na$: 649.3411).

3.5. Digalatosyl glycerol (1b)

Amorphous white powder; $[\alpha]_D^{20}$ +83.3 (c = 0.1, H_2O); ^1H-NMR (400 MHz, methanol-d_4) δ_H (ppm): 5.04 (d, J = 3.6, H-1′), 4.18 (d, J = 7.2, H-1"), and 3.29–4.00 (m, H-1-3, 2′-6′, and 2"-6").

3.6. Acid hydrolysis

Compound **1** (1.0 mg) was hydrolyzed with 5 mL of 2 N aq. CF_3COOH for 3 h at 95 °C. After extraction was fractionated with CH_2Cl_2 (3 × 5 mL), the water layer was repeatedly evaporated in vacuo to dryness with MeOH, until neutral, and then analyzed by TLC on silica gel (CH_2Cl_2–MeOH–H_2O, 6:2.5:0.5, v/v), and compared with authentic sample (Ngo et al., 2017).

3.7. Methanolysis of 1

A solution of **1** (1.5 mg) and MeOH (1 mL) were treated with 2 mL of 3% NaOMe–MeOH and the mixture was stirred at 40 °C for 2 h. The reaction mixture was neutralized with 2N HCl–MeOH solution and then partitioned with *n*-hexane. The *n*-hexane soluble was evaporated at reduced pressure to give methyl myristate (**1a**). The MeOH layer was evaporated under reduced pressure followed by a RP_{18} column chromatography with MeOH as an eluent to yield digalatosyl glycerol (**1b**) (Kim et al., 2004).

4. Chemotaxonomic significance

Micractinium is a genus consist of many species of green algae in the globally-distributed family Chlorellaceae and strain *M.* sp. KSF0031 was investigated as new species. Herein, 8 compounds (**1** − **8**) including glycerol and its derivatives (**1**–**4** and **8**) and three fatty acid compounds (**5**–**7**) are reported for the first time from *M.* sp. KSF0031. Strain *M.* sp. KSF0031 contains glycerols, which are the chemical marker of the family Chlorellaceae. A new monoacyldigalactosyl glycerol (**1**) identified in this study could be the marker of *M.* sp. KSF0031. The other glycerols (**2**–**4** and **8**) have been isolated from other strains of *Micractinium* (Germond et al., 2013; Li et al., 2012). In addition, fatty acids (**5**–**7**) are commonly found in a variety of microalgae and main constituents of the classes Cyanophyceae, Prymnesiophyceae, Bacillariophyceae, Rhodophyceae, Cryptophyceae, Chlorophyceae, Xantophyceae, and Eustigmatophyceae (Tran et al., 2019). In this study, fatty acids were described as major compounds in the strain *M.* sp. KSF0031. In conclusion, this report increases our knowledge about the secondary metabolites of a species of *Micractinium*. It builds of the fact that the glycerols **2**–**4** and **8** could be markers for the genus, whereas the fatty acids **5**–**7** are common to a range of different microalgae and cannot be used as markers for the genus. However, phytochemistry investigation of the other strains and species of *Micractinium* are required.

CRediT authorship contribution statement

Huynh Nguyen Khanh Tran: Writing - original draft. **Ui Joung Youn:** Writing - original draft. **Jeong Ah Kim:** Writing - original draft. **Hyunsik Chae:** Writing - original draft, Writing - review & editing. **Sanghee Kim:** Writing - original draft, Writing - review & editing. **Byung Sun Min:** Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors have declared that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bse.2019.104000.

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