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# Identification of a Novel Chlorinated Fatty Acid from the Marine *Rhodopirellula baltica*

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*Rhodopirellula baltica* is an attached-living marine bacterium spending most of their lifetime attached on the surface of other marine organisms or organic aggregates. As a general technique to isolate the new strains of attached-living bacteria is not yet available,[1] genomic and proteomic information of this species which revealed the presence of useful proteins such as enzymes important for the metabolism of sulfated carbohydrates was obtained only recently.[2] Secondary metabolites produced by *R. baltica*, however, have never been investigated probably due to the difficulties in its cultivation.

During the course of our search for biologically active constituents from tropical marine organisms,[3] we have encountered a colonized cluster of *R. baltica* attached to the surface of the brown algae, *Pylaiella littoralis* in the Federated States of Micronesia. Directed by the results of $^1$H NMR analyses, the crude extract was separated by solvent partitioning followed by reversed-phase vacuum flash chromatography and $C_{18}$ HPLC to afford two secondary metabolites. Herein, we describe the isolation and structure elucidation of a new chlorinated fatty acid (**1**) along with a known malyngic acid (**2**) (Figure 1).

Frozen specimens of *R. baltica* attached on the surface of *P. littoralis* were extracted with MeOH and $CH_2Cl_2$. The combined extract was partitioned between *n*-BuOH and $H_2O$. The butanol layer was dissolved in 15% aqueous MeOH and extracted with hexane. Further separations of the methanol layer by ODS columns, followed by reversed-phase HPLC, afforded two fatty acids. Compound **1** was obtained as a colorless oil, of which the negative LRESIMS showed two molecular ion peaks at *m*/*z* 215.19 and *m*/*z* 217.23 suggesting the presence of a chloride in the structure. The molecular formula $C_{11}H_{17}ClO_2$ was established based on a combination of HREIMS and $^{13}$C NMR spectrometry. Several characteristic features of the unsaturated fatty acid appeared in the $^1$H NMR spectral data, which showed the presence of a terminal methyl group (δ 0.92), five methylenes (δ 1.45, 2.18, 2.30, 2.34 and 2.76), a disubstituted double bond (δ 5.43 and 5.53), and an additional olefinic proton (δ 5.89) (Table 1). The corresponding carbon signals were observed in the $^{13}$C NMR spectra, which showed the presence of a methyl group (δ 14.2), five methylenes (δ 21.2, 28.9, 33.0, 34.9 and 38.8), two double bonds (δ 114.1, 130.6, 134.1 and 142.9), and an additional carbonyl (δ 176.9) carbon. The structure of **1** was elucidated further by 2D NMR experiments. In the $^1$H-$^1$H COSY experiment, the methylene proton signal at δ 1.45 showed correlations with the methyl signal at δ 0.92 and the methylene signal at δ 2.18. The olefinic proton signals at δ 5.43 and 5.53 showed correlations with the methylene signals at δ 2.76 and 2.30, respectively. A combination of the HSQC and HMBC data allowed assignments of the characteristic $^{13}$C NMR signals, and the key HMBC correlations from H-11 ($\delta_H$ 5.89) to C-6, C-7, and C-8 and from H-3 ($\delta_H$ 2.30) to C-1 ($\delta_C$ 176.9) confirmed the location of chloroalkene group and carboxylic acid units (Figure 2).

The geometry of the chloroalkene residue was determined by the measurement of vicinal $^{13}$C-$^1$H coupling constants. It was probable that the geometry of the double bond between C-7

**Table 1.** NMR Data (500 MHz, $CD_3OD$) for **1**

| Position | $\delta_C$, mult | $\delta_H$ (*J* in Hz) | HMBC[a] |
|---|---|---|---|
| 1 | 176.9, C | | |
| 2 | 34.9, $CH_2$ | 2.34, t (6.0) | 1, 3, 4 |
| 3 | 28.9, $CH_2$ | 2.30, q (6.0) | 1, 2, 4, 5 |
| 4 | 132.4, CH | 5.53, dt (15.1, 6.0) | |
| 5 | 129.0, CH | 5.43, dt (15.1, 6.8) | |
| 6 | 38.8, $CH_2$ | 2.76, d (6.8) | 4, 5, 7, 8 |
| 7 | 142.9, C | | |
| 8 | 33.0, $CH_2$ | 2.18, t (7.6) | 6, 7, 9, 10 |
| 9 | 21.2, $CH_2$ | 1.45, tq (7.6, 7.3) | 7, 8, 10 |
| 10 | 14.2, $CH_3$ | 0.92, t (7.3) | 8, 9 |
| 11 | 114.1, CH | 5.89, s | 6, 7, 8 |

[a]HMBC correlations are from proton(s) stated to the indicated carbon.

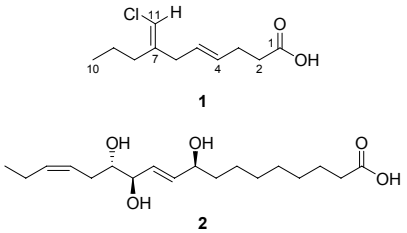


**Figure 1.** Chemical structures of **1** and **2**.

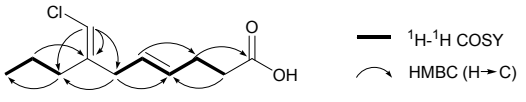


**Figure 2.** Key $^1$H-$^1$H COSY and HMBC interactions of **1**.

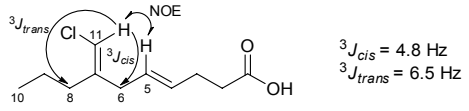


**Figure 3.** Comparison of $^1$H-$^{13}$C couplings in **1**.

and C-11 might be *trans* as a weak NOE interaction between the olefinic proton (H-5) and the proton germinal to the chloride (H-11) was observed. To figure out the geometry clearly, three bond $^{13}$C-$^1$H coupling constants ($^3J_{CH}$) from a $^1$H-coupled $^{13}$C NMR experiment were examined. The J-HMBC spectrum of **1** showed two couplings, i.e., $^3J_{CH}$ = 6.5 Hz from C-8 to H-11 and $^3J_{CH}$ = 4.8 Hz from C-6 (Figure 3). This result unambiguously demonstrated a *trans* relationship between C-8 and H-11, as it is known that the numerical values of the *trans* coupling constants $^3J_{CH}$ are higher than those of the *cis* coupling constants.[4,5,6] The disubstituted alkene between C-4 and C-5 had an *E* configuration, as confirmed by the $^1$H -$^1$H vicinal coupling constant for the olefinic protons ($J$ = 15.1 Hz). On the basis of these results described above, **1** was determined to be (4*E*,7*E*)-7-(chloromethylene)dec-4-enoic acid, which is reported for the first time.

Compound **2**, a more polar constituent, was obtained as a pale-yellow gum. On the basis of the results of combined spectroscopic analyses, it was confirmed that **2** has a structure identical to that of malyngic acid, which was previously reported as a bioactive secondary metabolite of the marine blue-green alga *Lyngbya majuscula*.[7] Spectral data of **2** were in good agreement with those reported previously. The inhibitory activity of **1** against mushroom tyrosinase was examined to result in a 43% reduction in enzyme activity at a concentration of 200 μg/mL.

## Experimental Section

**General procedures.** The $^1$H NMR spectra were recorded on a Variant Unity 500 spectrometer at 500 MHz and $^{13}$C NMR spectra were also recorded on the same instrument at 125 MHz. Chemical shifts were reported on a δ (ppm) scale with the solvent resonance resulting from incomplete deuteration of $CD_3OD$ ($^1$H, 3.30 ppm; $^{13}$C, 49.0 ppm) as the internal reference. The NOE and J-HMBC experiments were performed with Bruker Avance 800 spectrometer. Mass spectra were taken with a Micromass Auto Spec spectrometer. HPLC was performed with an YMC Pack Pro $C_{18}$ column (250 × 10 mm, 5 μm, and 80 Å) using a Shodex RI-101 detector.

**Collection and taxonomic identification.** The bacterial cluster attached on the brown algae was collected by Dr. D.-H. Kang at a depth of 3 - 5 m from Chuuk Atoll, Federated States of Micronesia, in July 2009, and was identified by Dr. C. Oh on the basis of genetic analysis, including the comparison of 16S rRNA gene. A voucher specimen (Registry No. 09CH-101) has been deposited at the Marine Bio-Research Center, Korea Ocean Research & Development Institute, Korea.

**Extraction and isolation.** A colonized cluster of *R. baltica* was immediately frozen and maintained at −25 °C until investigated chemically. The specimens were lyophilized (dry wt 70.3 g) and repeatedly extracted with MeOH (300 mL × 2) and $CH_2Cl_2$ (300 mL). The extract was filtered and concentrated under reduced pressure to afford 14.1 g of the crude extract. The residue was partitioned between $H_2O$ and *n*-BuOH to yield 3.5 g of an organic-soluble material. The *n*-BuOH layer was repartitioned between 15% aqueous MeOH (2.3 g) and *n*-hexane (1.0 g). The residue of the aqueous MeOH layer was subjected to $C_{18}$ reversed-phase flash chromatography using a gradient mixture of MeOH and $H_2O$.

The fraction eluted with 20% aqueous MeOH was dried (70 mg) and separated by reversed-phase HPLC (YMC Pack Pro $C_{18}$ column, 30% aqueous MeOH) to afford 9.6 mg of pure **1** as a major product. The fraction eluted with 30% aqueous MeOH (33 mg) was separated by reversed-phase HPLC (YMC Pack Pro $C_{18}$ column, 55% aqueous MeOH) to yield 1.9 mg of **2**.

**(4*E*,7*E*)-7-(Chloromethylene)dec-4-enoic acid (1):** Colorless oil; IR (neat) $\nu_{max}$ 3436, 2960, 1737, 1629, 1438, 1199 cm$^{-1}$;$^1$H and $^{13}$C NMR data, see Table 1; LRESIMS *m*/*z* 215.19, 217.23 $[M-H]^-$; HREIMS *m*/*z* 216.0919 $[M]^+$ (calcd for $C_{11}H_{17}ClO_2$, 216.0917).

**(9*S*,12*R*,13*S*)-Trihydroxyoctadeca-(10*E*,15*Z*)-dienoic acid (2):** Pale-yellow gum; $[\alpha]_D^{25}$ +10.2° (*c* 0.04, MeOH), [Lit. +7.5°] [7]; LRESIMS *m*/*z* 329.51 $[M+H]^+$.

**Inhibitory effect of mushroom tyrosinase.** The inhibitory activity of tyrosinase was performed according to the method of Vanni *et al*.,[8] with minor modifications. The reaction mixture contained 140 μL of 0.1 M phosphate buffer (pH 6.5), 40 μL of 1.5 mM L-tyrosine, and 10 μL of compound. Then, 10 μL of mushroom tyrosinase (2,100 units/mL) solution was added, and the reaction was incubated at 37 °C for 12 min. After incubation, the amount of dopachrome produced in the reaction mixture was determined by carrying outoptical density at 490 nm in a microplate reader.

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