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| **Preprint Title** | Three new *O*-isocrotonyl-3-hydroxybutyric acid congeners produced by a sea anemone-derived marine bacterium of the genus *Vibrio* |
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# Three new *O*-isocrotonyl-3-hydroxybutyric acid congeners produced by a sea anemone-derived marine bacterium of the genus *Vibrio*

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# Abstract

Liquid cultures of *Vibrio* sp. SI9, isolated from outer tissue of sea anemone *Radianthus crispus*, was found to produce three new *O*-isocrotonyl-3-hydroxybutyric acid derivatives, *O*-isocrotonyl-3-hydroxypentanoic acid (**1**), *O*-isocrotonyl-3-hydroxyhexanoic acid (**2**), and *O*-(*Z*)-2-hexenoyl-3-hydroxybutyric acid (**3**), together with known *O*-isocrotonyl-3-hydroxybutyric acid (**4**). The structures of **1-3** were established by spectroscopic analyses of NMR and mass spectrometry coupled with anisotropy-based chiral analysis, revealing the same (*R*)-configurations for all congeners **1-4**. Compounds **1-4** were weakly growth-inhibitory against a marine fish ulcer pathogenic bacterium, *Tenacibaculum maritimum* NBRC16015.



# Keywords

3-hydroxybutyric acid; polyhydroxyalkanoate; sea anemone;*Tenacibaculum maritimum*; *Vibrio*

# Introduction

The genus *Vibrio*¸ within the class *Gammaproteobacteria*, are a group of Gram-negative, halophilic, facultatively anaerobic, rod-shaped bacteria, which are motile with sheathed polar flagella [1]. This group is one of the most widespread bacterial genera of marine origin, cataloging 128 species at the time of writing [2], of which more than 12 are known to cause enteritis, marine food poisoning, bacteremia, septicemia, cellulitis, or other infectious diseases in human and aquatic animals [3,4]. Others can fix nitrogen [5], have phototrophy [6], or produce plant hormone [7], thus showing higher metabolic versatility, which is also represented by 150 and more secondary metabolites discovered from this genus [8].

As part of our continuing study on the secondary metabolites of marine bacteria, *Vibrio* sp. SI9, isolated from the sea anemone *Radianthus crispus*, was found to produce a known ester **4** and its new congeners **1**-**3** (Figure 1). Compound **4** is the shortest among the five oligomers of *O*-isocrotonyl-oligo(3-hydroxybutyrate) previously discovered from *Vibrio* [9]. In this study, we describe isolation, structure elucidation including the absolute configuration, and bioactivity of **1**-**4**.

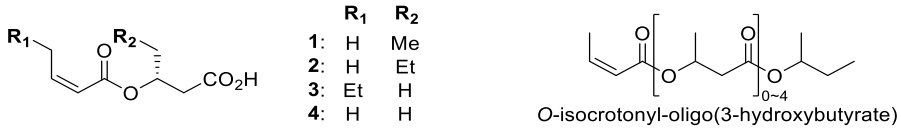


**Figure 1:** Structures of the compounds.

# Results and Discussion

The producing strain was cultured in sea water-based medium and then extracted with *n*-BuOH. The extract was successively fractionated by silica gel chromatography eluted by stepwise gradient of MeOH in $CHCl_3$ and ODS flash chromatography eluted by acidic aqueous MeCN. One of the mid-polar fractions was purified by revered-phase HPLC to give **1** (1.5 mg), **2** (11.2 mg), **3** (4.3 mg), and **4** (135.6 mg) from a 3L culture.

$^1$H NMR spectra of **1**-**4** similarly showed three deshielded resonances (H3, H2´, and H3´) and a pair of mutually coupled double-doublet resonances ($H_2$2), indicating a shared core structure (see Supporting Information and Tables 1 and 2). In fact, $^{13}$C NMR spectra exhibited two carboxy (carboxamide), two olefinic, and one oxygenated carbon resonances in common (see Supporting Information), and analysis of an HSQC spectrum added two methyl groups and one to three aliphatic methylene(s) to this composition. The molecular formula was determined to be $C_9H_{14}O_4$ for **1**, $C_{10}H_{16}O_4$ for **2** and **3**, and $C_8H_{12}O_4$ for **4** by HRESITOFMS measurements, differing by a factor of one to two methylene unit(s) but giving the same three degrees of unsaturation, which are explained by two carboxy groups and one double bond. Thus, **1**-**4** were confirmed to be a series of acyclic compounds with varying length of aliphatic chains.

**Table 1:** NMR data for **1** and **2** in $CDCl_3$

| | **1** | | | | **2** | | | |
|---|---|---|---|---|---|---|---|---|
| No. | $^{13}$C | $^1$H (*J* in Hz), integr. | COSY | HMBC[a] | $^{13}$C | $^1$H (*J* in Hz), integr. | COSY | HMBC[a] |
| 3-hydroxy-4-methylbutyric acid | | | | | 4-ethyl-3-hydroxybutyric acid | | | |
| 1 | 175.0 | | | | 176.2 | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2 | 38.4 | 2.68, dd (7.3, 15.8), 1H | 3 | 1, 3, 4 | 39.0 | 2.68, dd (7.2, 15.9), 1H | 3 | 1, 3, 4 |
| | | 2.61, dd (5.5, 15.9), 1H | 3 | 1, 3, 4 | | 2.60, dd (5.6, 15.8), 1H | 3 | 1, 3, 4 |
| 3 | 70.8 | 5.22, brqui (6.3), 1H | 2, 4 | 1, 2, 4, 5, 1´ | 69.5 | 5.25, m, 1H | 2, 4 | 1, 2, 4, 5, 1´ |
| 4 | 26.9 | 1.71, m, 2H | 3, 5 | 2, 3, 5 | 36.1 | 1.61, m, 2H | 3, 5 | 2, 3, 5, 6 |
| 5 | 9.4 | 0.94, t (7.4), 3H | 4 | 3, 4 | 18.4 | 1.35, m, 2H | 4, 6 | 3, 4, 6 |
| 6 | | | | | 13.8 | 0.91, t (7.4), 3H | 5 | 3, 4, 5 |
| isocrotonic acid | | | | | isocrotonic acid | | | |
| 1´ | 165.9 | | | | 165.8 | | | |
| 2´ | 120.5 | 5.78, dq (11.5, 1.8), 1H | 3´ | 1´, 4´ | 120.5 | 5.74, qd (11.4, 1.8), 1H | 3´ | 1´, 4´ |
| 3´ | 145.6 | 6.34, dq (11.5, 7.3), 1H | 2´, 4´ | 1´, 4´ | 145.5 | 6.30, m, 1H | 2´, 4´ | 1´, 4´ |
| 4´ | 15.4 | 2.13 dd (7.3, 1.8) 3H | 3´ | 1´, 2´, 3´ | 15.4 | 2.10, dd (7.3, 1.6), 3H | 3´ | 1´, 2´, 3´ |

[a]HMBC correlations from proton to indicated carbons.

**Table 2:** NMR data for **3** and **4** in $CDCl_3$

| | **3** | | | | **4** | | | |
|---|---|---|---|---|---|---|---|---|
| No. | $^{13}C$ | $^{1}H$ (*J* in Hz), integr. | COSY | HMBC[a] | $^{13}C$ | $^{1}H$ (*J* in Hz), integr. | COSY | HMBC[a] |
| 3-hydroxybutyric acid | | | | | 3-hydroxybutyric acid | | | |
| 1 | 175.6 | | | | 175.4 | | | |
| 2 | 40.5 | 2.73, dd (7.2, 15.9), 1H | 3 | 1, 3, 4 | 40.5 | 2.73, dd (7.1, 15.9), 1H | 3 | 1, 3, 4 |
| | | 2.56, dd (5.7, 15.9), 1H | 3 | 1, 3, 4 | | 2.57, dd (5.5, 15.9), 1H | 3 | 1, 3, 4 |
| 3 | 66.5 | 5.30, brsep (6.3), 1H | 2, 4 | 1, 2, 4, 1´ | 66.5 | 5.32, brsep (6.3), 1H | 2, 4 | 1, 2, 4, 1´ |
| 4 | 19.9 | 1.35, d (6.2), 3H | 3 | 2, 3 | 19.9 | 1.35, d (6.3), 3H | 3 | 2, 3 |
| (*Z*)-2-hexenoic acid | | | | | isocrotonic acid | | | |
| 1´ | 165.6 | | | | 165.6 | | | |
| 2´ | 109.6 | 5.73, dt (11.6, 1.6), 1H | 3´ | 1´, 4´ | 120.5 | 5.56, dq (11.5, 1.6), 1H | 3´ | 1´, 4´ |
| 3´ | 150.9 | 6.23, dt (11.5, 7.5), 1H | 2´, 4´ | 1´, 4´, 5´ | 145.5 | 6.33, dq (11.5, 7.3), 1H | 2´, 4´ | 1´, 4´ |
| 4´ | 31.0 | 2.61, qd (7.4, 1.6), 2H | 3´, 5´ | 2´, 3´, 5´, 6´ | 15.4 | 2.13, dd (7.2, 1.9), 3H | 3´ | 2´, 3´ |
| 5´ | 22.3 | 1.46, sex (7.4), 2H | 4´, 6´ | 3´, 4´, 6´ | | | | |
| 6´ | 13.7 | 0.94, t (7.3), 3H | 5´ | 4´, 5´ | | | | |

[a]HMBC correlations from proton to indicated carbons.

Analysis of a COSY spectrum of the smallest congener **4** revealed two $C_3$ fragments $H_2$2-H3(O)-$H_2$4 and H2´=H3´-$H_3$4´. The geometry at C2´ was determined to be *Z* based on a vicinal coupling constant between the olefinic protons H2´ and H3´ (*J*=11.5 Hz; Table 2). Both of the fragments showed HMBC correlations to the same carboxy carbon C1 ($\delta_C$ 165.6), revealing an intervening ester linkage. Finally, HMBC

correlations from methylene proton $H_2$2 and oxymethine proton H3 to the other carboxy carbon C1 ($\delta_C$ 175.3) placed a carboxylic acid functionality on the methylene group, which completed the structure of **4** as *O*-isocrotonyl-3-hydroxybutyric acid (Figure 2).

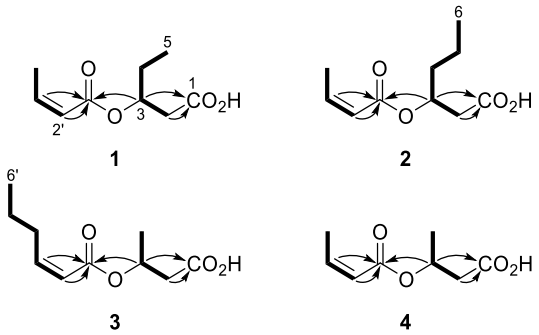


**Figure 2:** COSY (bold lines) and selected HMBC (arrows) correlations for **1**-**4**.

A close similarity of NMR data for **1**-**3** (Tables 1 and 2) allowed the same sequence of structure analysis. Compounds **1** and **2** were found to have extra $C_1$ and $C_2$ extensions on the butyric acid units, while in **3** an extra $C_2$ extension occurred on the isocrotonyl group, as shown by the connectivity established by the analysis of COSY spectra (Figure 2). Thus, **1**-**3** were concluded to be *O*-isocrotonyl-3-hydroxypentanoic acid, *O*-isocrotonyl-3-hydroxyhexanoic acid, and *O*-(*Z*)-2-hexenoyl-3-hydroxybutyric acid, respectively (Figure 1).

A database search identified the planar structure of **4** in a patent that described a series of *O*-isocrotonyl-oligo(3-hydroxybutyrate) (Figure 1) from marine obligate *Vibrio* sp. C-984 [9]. To determine the absolute configuration of C3 in **1**-**4**, an anisotropy-based chiral analysis using chiral derivatization reagents, phenylglycine

methyl ester (PGME) was conducted [10]. Compounds **1**-**4** were derivatized with (*S*)- or (*R*)-PGME by the action of *N*,*N*´-diisopropylcarbodiimide (DIC) and *N*,*N*-dimethylaminopyridine (DMAP) in $CH_2Cl_2$, followed by reversed phase HPLC to give respective (*S*)- or (*R*)-PGME amides **1a**, **1b**, **2a**, **2b**, **3a**, **3b**, **4a**, and **4b**. Calculation of $^1$H NMR chemical shift differences $\Delta\delta_{(S-R)}$ beyond C3 by subtracting the chemical shift of each proton in the (*R*)-isomer (**1b**-**4b**) from those in the (*S*)-isomer (**1a**-**4a**) arranged positive values at C4, C5, C6 and negative values for C2´ and C3´ for all four compounds (Figure 3). Note that the sign distribution of $\Delta\delta_{(S-R)}$ values in β,β-substituted carboxylic acids is inverted from that observed in α,α-substituted counterparts, because the PGME anisotropy group is flipped upside down due to the insertion of an extra methylene group between the chiral center and carboxylic acid functionality [11]. Thus, *R*-configurations were concluded for all four compounds **1**-**4**.

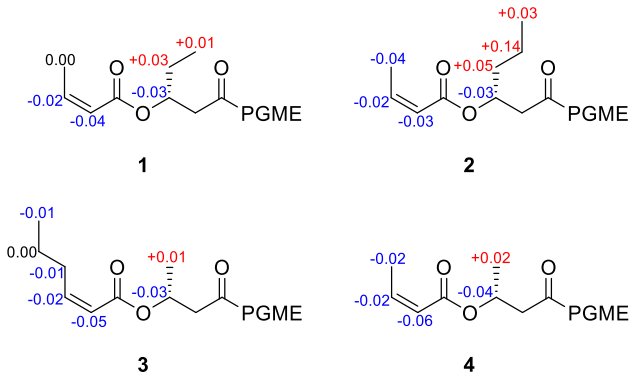


**Figure 3:** Distribution of positive (red) and negative (blue) $\Delta\delta_{(S-R)}$ values (in ppm) calculated from $^1$H NMR chemical shifts of (*S*)- and (*R*)-PGME amides of **1**-**4**.

Compounds **1**-**4** are closely related to polyhydroxyalkanoates (PHAs), the energy reserve substances for eubacteria and some species of archea [12]. Both groups are composed of (*R*)-configured 3-hydroxy fatty acids [13], and 3-hydroxybutyric acid and 3-hydroxyhexanoic acid are two most common building blocks for PHAs [14]. However, the degrees of polymerization as low as **1**-**4** and dehydrative modification have not be precedented besides *O*-isocrotonyl-oligo(3-hydroxybutyrate) [9].

Because PHAs are by nature biodegradable, can be produced from renewable bioresources, and have material properties close to the conventional petroleum-derived plastics, commercial production and market development are actively pursued by several companies amid the growing plastic waste crisis [14,15]. *Vibrio* are perhaps the first to be known as producers of PHAs among marine microbes [16], and still be isolated predominantly in the screening of PHA productivity [17]. Intriguingly, aquatic farmed animals feeded with poly(3-hydroxybutyrate) showed reduced mortality than those not feeded upon challenge by pathogenic *Vibrio* species, suggesting the application of PHAs as a biocontrol agent [18]. Although the toxicity of **1**-**4** toward the producing strain was not tested, they weakly inhibited the growth of *Tenacibaculum maritimum*, a causative agent of skin ulcer in marine fish at MIC values of 25, 50, 50, 25 μg/mL, respectively. None of the compounds showed cytotoxicity against 3Y1 rat embryonic fibroblastic cells below 50 μg/mL.

# Experimental

## General experimental procedures

Optical rotations were recorded on a JASCO P-1030 polarimeter. UV and IR absorption spectra were recorded on Shimadzu UV-1800 and Perkin Elmer Spectrum 100 spectrophotometers, respectively. NMR spectra were obtained on a Bruker AVANCE 500 spectrometer referencing to residual solvent peaks at $\delta_H$ 7.26 and $\delta_C$ 77.0 for $CDCl_3$. HR-ESI-TOF-MS spectra were measured using a Bruker micrOTOF focus mass spectrometer. Absorbance of formazan solution at 540 nm was measured on a ThermoFisher Scientific Multiskan Sky microplate reader.

## Biological material

Sea anemone *Radianthus crispus* was purchased from an aquarium vendor in Nagasaki, Japan. Strain SI9 was isolated from its outer tissue specimen according to the method described previously [19], and identified as a member of genus *Vibrio* on the basis of 98.6% similarity in the 16S rRNA gene sequence (1458 nucleotides; DDBJ accession number LC498627) to *Vibrio nereis* DSM 19584$^T$ (accession number LHPJ01000025).

## Fermentation and isolation of **1**-**4**

Colonies of strain SI9, recovered on a Marine Agar plate, were transferred into a 500 mL K-1 flask containing 100 mL 1/3 strength of simplified Marine Broth, which was prepared from 0.5% peptone, 0.1% yeast extract, and 3 L seawater with pH adjusted to 7.6. After fermented at 30 ℃ at 200 rpm for 2 days, 3 mL aliquots of seed culture thus prepared were dispensed into 500 mL K-1 flasks each containing 100 mL A16

production medium consisted of 2% glucose, 1% Pharmamedia, 0.5% $CaCO_3$ and 1% Diaion HP-20 in natural seawater. After shake-cultured at 30 ℃ at 200 rpm for 5 days, each production culture received 100 mL *n*-butanol, and the flasks were shaken for additional 1 h for extraction. The emulsified broth was centrifuged at 6000 rpm for 10 min, and the resulting butanol layers were collected and concentrated *in vacuo* to give 5.8 g of an extract from 3 L culture. This was first fractionated on a silica gel column with a stepwise elution by $CHCl_3$-MeOH mixtures (1:0, 20:1, 10:1, 4:1, 2:1, 1:1, and 0:1 v/v). The third fraction (1.35 g) was further fractionated by ODS column chromatography eluting with MeCN-0.1% HCOOH (2:8, 3:7, 4:6, 5:5, 6:4, 7:3, and 8:2 v/v). The fourth fraction (0.28 g) was subjected to reversed-phase HPLC on a Cosmosil AR-II column (10 × 250 mm) eluted with 32% MeCN in 0.1% $HCO_2H$ at 4 mL/min to give **1** (1.5 mg; $t_R$ 16.1 min), **2** (11.2 mg; $t_R$ 17.6 min) and **3** (4.3 mg; $t_R$ 18.3 min), and **4** (135.6 mg; $t_R$ 9.8 min).

(*R*)-*O*-Isocrotonyl-3-hydroxypentanoic acid (**1**): colorless amorphous solid; $[\alpha]^{24}_D$ -53 (*c* 0.10, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 208 (3.67) nm; IR (ATR) $\nu_{max}$: 2973, 2938, 2641, 1716, 1646, 1438, 1415, 1368, 1179, 1135, 1057, 1033, 997, 927, 815 $cm^{-1}$; $^1H$ and $^{13}C$ NMR data see Table 1; HRESITOFMS *m/z* 209.0784 $[M+Na]^+$ (calcd for $C_9H_{14}O_4Na$, 209.0784).

(*R*)-*O*-Isocrotonyl-3-hydroxyhexanoic acid (**2**): colorless amorphous solid; $[\alpha]^{24}_D$ -67 (*c* 0.10, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 207 (4.15) nm; IR (ATR) $\nu_{max}$: 2962, 2937,

1717, 1646, 1438, 1415, 1178, 1009, 970, 815 $cm^{-1}$; $^{1}H$ and $^{13}C$ NMR data see Table 1; HRESITOFMS *m/z* 223.0941 $[M+Na]^+$ (calcd for $C_{10}H_{16}O_4Na$, 223.0941).

(*R*)-*O*-(*Z*)-2-Hexenoyl -3-hydroxybutyric acid (**3**): colorless amorphous solid; $[\alpha]^{24}_D$ -38 (*c* 0.10, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 209 (4.17) nm; IR (ATR) $\nu_{max}$: 2962, 2934, 2874, 1717, 1640, 1456, 1414, 1178, 1059, 816 $cm^{-1}$; $^{1}H$ and $^{13}C$ NMR data see Table 2; HRESITOFMS *m/z* 223.0941 $[M+Na]^+$ (calcd for $C_{10}H_{16}O_4Na$, 223.0941).

(*R*)-*O*-Isocrotonyl-3-hydroxybutyric acid (**4**): colorless oil; $[\alpha]^{23}_D$ -43 (*c* 0.10, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 208 (4.07) 274 (2.47) nm; IR (ATR) $\nu_{max}$: 2985, 2937, 2643, 1710, 1644, 1435, 1414, 1291, 1175, 1057, 970, 815 $cm^{-1}$; $^{1}H$ and $^{13}C$ NMR data see Table 2; HRESITOFMS *m/z* 195.0627 $[M+Na]^+$ (calcd for $C_8H_{12}O_4Na$, 195.0628).

## Preparation of (*S*)- and (*R*)-Phenylglycine Methyl Ester Amides

To a solution of **4** (1.7 mg, 10 μmol) in anhydrous $CH_2Cl_2$ (0.5 mL) in a dried vial were added (*S*)-phenylglycine methyl ester (PGME, 3.3 mg, 16.4 μmol), DMAP (1.2 mg, 10 μM), and DIC (3.0 μL, 19.5 μmol), and the mixture was stirred for 30 min at room temperature. Production of the amide was monitored by thin layer chromatography (TLC) developed by ethyl acetate-*n*-hexane (1:1) followed by heating with phosphomolybdic acid. After removing the solvent, the residue was subjected to HPLC on a Cosmosil π-NAP column (10 x 250 mm) eluted by a gradient

method (MeCN-0.1% HCOOH) at 4 mL/min with monitoring at 254 nm to give (*S*)-PGME amide **4a** (2.9 mg, 93%) as colorless oil.

(*S*)-PGME amide of **4** (**4a**): $^1$H NMR (500 MHz, $CDCl_3$) δ 7.34 (4H, m), 6.75 (1H, d), 6.31 (1H, m), 5.70 (1H, qd), 5.57 (1H, d), 5.25 (1H, m), 3.72 (3H, s), 2.54 (2H, m), 2.09 (3H, dd), 1.34 (3H, d); HRESITOFMS *m/z* 342.1312 (calcd for $C_{17}H_{21}NO_5Na$ 342.1312).

Other PGME amides **1a**, **1b**, **2a**, **2b**, **3a**, **3b**, and **4b** were prepared by the same procedure with replacing the starting material and chiral reagent.

(*S*)-PGME amide of **1** (**1a**): $^1$H NMR (500 MHz, $CDCl_3$) δ 7.34 (4H, m), 6.75 (1H, d), 6.33 (1H, t), 5.57 (1H, d), 5.56 (1H, qd), 5.25 (1H, m), 5.14 (1H, t), 3.72 (3H, s), 2.11 (3H, d), 1.70 (2H, m), 0.92 (3H, t), ; HRESITOFMS *m/z* 356.1468 $[M+Na]^+$ (calcd for $C_{18}H_{23}NO_5Na$, 356.1468).

(*R*)-PGME amide of **1** (**1b**): $^1$H NMR (500 MHz, $CDCl_3$) δ 7.34 (4H, m), 6.75 (1H, d), 6.35 (1H, t), 5.57 (1H, d), 5.56 (1H, qd), 5.25 (1H, m), 5.17 (1H, t), 3.72 (3H, s), 2.11 (3H, d), 1.67 (2H, m), 0.91 (3H, t); HRESITOFMS *m/z* 356.1495.

(*S*)-PGME amide of **2** (**2a**): $^1$H NMR (500 MHz, $CDCl_3$) δ 7.34 (4H, m), 6.75 (1H, d), 6.34 (1H, m), 5.75 (1H, m), 5.57 (1H, d), 5.25 (1H, m), 5.23 (1H, m), 3.72 (3H, s),

2.13 (3H, m), 1.51 (2H, m), 1.35 (2H, overlap), 0.93 (3H, t); HRESITOFMS *m/z* 370.1625 $[M+Na]^+$ (calcd for $C_{19}H_{25}NO_5Na$, 370.1625).

(*R*)-PGME amide of **2** (**2b**): $^1H$ NMR (500 MHz, $CDCl_3$) δ 7.34 (4H, m), 6.75 (1H, d), 6.36 (1H, m), 5.79 (1H, m), 5.26 (1H, m), 5.25 (1H, m), 5.57 (1H, d), 3.72 (3H, s), 2.13 (3H, m), 1.49 (2H, m), 1.35 (2H, overlap), 0.91 (3H, t); HRESITOFMS *m/z* 370.1625 $[M+Na]^+$.

(*S*)-PGME amide of **3** (**3a**): $^1H$ NMR (500 MHz, $CDCl_3$) δ 7.34 (4H, m), 6.75 (1H, d), 6.24 (1H, m), 5.72 (1H, d), 5.57 (1H, d), 5.27 (1H, m), 5.25 (1H, m), 3.72 (3H, s), 2.62 (2H, m), 1.73 (3H, m), 1.49 (2H, m), 0.95 (3H, m); HRESITOFMS *m/z* 370.1625 $[M+Na]^+$.

(*R*)-PGME amide of **3** (**3b**): $^1H$ NMR (500 MHz, $CDCl_3$) δ 7.34 (4H, m), 6.75 (1H, d), 6.26 (1H, m), 5.77 (1H, d), 5.57 (1H, d), 5.30 (1H, m), 5.25 (1H, m), 3.72 (3H, s), 2.63 (2H, m), 1.68 (3H, m), 1.49 (2H, m), 0.96 (3H, m); HRESITOFMS *m/z* 370.1625 $[M+Na]^+$.

(*S*)-PGME amide of **4** (**4a**): $^1H$ NMR (500 MHz, $CDCl_3$) δ 7.34 (4H, m), 6.75 (1H, d), 6.31 (1H, m), 5.70 (1H, qd), 5.57 (1H, d), 5.25 (1H, m), 3.72 (3H, s), 2.54 (2H, m), 2.09 (3H, dd), 1.34 (3H, d); HRESITOFMS *m/z* 342.1312 (calcd for $C_{17}H_{21}NO_5Na$ 342.1312).

## Antibacterial Assay

The antibacterial activity was evaluated by a microculture technique described previously [20] except for 1/100 reduction of the seeding density of *T. maritimum* NBRC16015.

## Cytotoxicity Assay

3Y1 rat embryonic fibroblastic cells were maintained in low-glucose DMEM medium containing L-glutamine and phenol red (Fujifilm Wako Pure Chemical, 041-29775) supplemented with 10% fatal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B (Fujifilm Wako Pure Chemical, 161-23181), and 100 μg/mL gentamicin sulfate (Fujifilm Wako Pure Chemical, 078-06061). Cells were seeded in each well of a 96-well culture plate at a density of 2500 cells/well. Meanwhile, Compounds **1**-**4** and doxorubicin hydrochloride as a positive control were serially diluted 1:3.16 (half-log dilution) by the same medium in a different microtiter plate. After incubating the cell culture for 12 h at 37℃ in an atmosphere of 95% air and 5% $CO_2$ saturated with $H_2O$, the drug solutions were transferred into each cell culture to make 200 μL culture. The highest concentration of vehicle solvents (MeOH or DMSO) was set at 0.5 v/v %, where the growth of cells was not affected. After incubating the test plate for 84 h, 100 μL of medium containing MTT 1 mg/mL was added to each well, and the plate was further incubated for 4 h. The medium was carefully removed by aspiration, and formazan-dye formed

at the bottom of the wells was solubilized by addition of 150 μL DMSO. Respiration of live cells was quantified by measurement of absorption at 540 nm by a microplate reader. The experiment was run in triplicate and rates of cell growth inhibition at each concentration were plotted on a single logarithmic chart to deduce $GI_{50}$ values of the test compounds.

## Supporting Information

Spectroscopic data for **1**-**4** and their PGME amide pairs **1a**/**1b**-**4a**/**4b**

File Name: Li et al. fatty acids SI.pdf

File Format: PDF

Title: Supporting Information for: Three new *O*-isocrotonyl-3-hydroxybutyric acid congeners produced by a sea anemone-derived marine bacterium of the genus *Vibrio*

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