The Journal of Antibiotics (2015) 68, 628–632
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www.nature.com/ja

ORIGINAL ARTICLE

# Trichoketides A and B, two new protein tyrosine phosphatase 1B inhibitors from the marine-derived fungus *Trichoderma* sp.

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**Two new octaketides, trichoketides A (1) and B (2), were isolated from a culture broth of the seawater-derived fungus *Trichoderma* sp. TPU1237 together with two known analogs, trichodermaketones C (3) and D (4), by ODS column chromatography followed by preparative ODS and chiral HPLC. The structures of 1 and 2 were elucidated on the basis of their spectroscopic data, and absolute configurations were assigned by comparing their experimental electronic circular dichroism (ECD) spectra with the calculated ECD spectra. Compounds 1 and 2 were epimers at the C-8 position (α-position of dihydrofuran ring). The $IC_{50}$ values of compounds 1–4 against protein tyrosine phosphatase 1B were 53.1, 65.1, 68.0 and 55.9 μM, respectively.**
*The Journal of Antibiotics* (2015) **68,** 628–632; doi:10.1038/ja.2015.44; published online 22 April 2015

## INTRODUCTION

Protein tyrosine phosphatases (PTPs) are a large family of signaling enzymes that regulate several cell functions. Disordered PTP activity has been implicated in various diseases including diabetes, cancer and dysfunctions in the immune system.[1] Among the members of the PTP family, PTP1B has been shown to play a key role as a negative regulator in the insulin and leptin signaling pathways.[2–4] Accordingly, the development of PTP1B inhibitors is expected to provide new treatments for type 2 diabetes and obesity.[2–4] Although a number of PTP1B inhibitors have been obtained from natural sources and by chemical syntheses, a clinically efficient drug has not yet been developed because the activity and selectivity of these candidates were not satisfactory.[5] Therefore, the search for potent and selective PTP1B inhibitors is important in the field of natural products chemistry.

During the course of our research on new types of PTP1B inhibitors from marine organisms such as ascidians, sponges and microorganisms, we have reported the isolation, structures and inhibitory activities against PTP1B of polybromodiphenyl ethers, dehydroeurypspongin and hyattellactones.[6–9] Further screening bioassays revealed that a culture broth of the marine-derived fungus *Trichoderma* sp. TPU1237 inhibited PTP1B activity. Bioassay-guided separation of the ethyl acetate (EtOAc) extract from the culture broth led to the isolation of two new octaketides, trichoketides A (**1**) and B (**2**), together with two known polyketides,[10] trichodermaketones C (**3**) and D (**4**) (Figure 1). We herein described the isolation, structural elucidation including absolute configurations and biological activities of **1**–**4**.

## RESULTS AND DISCUSSION

### Identification of the producing fungus

Fungal strain TPU1237 was isolated from a seawater sample collected at Aomori, Japan, and its ITS1 rDNA sequence (239 nucleotides) was investigated, revealing 100% similarity to 71 known *Trichoderma* species including *T. koningiopsis*, *T. trixiae*, *T. gamsii*, *T. viridescens*, *T. koningii*, *T. viride* and *T. ovalisporum*.

### Production of compounds 1 and 2

The quantitative production of two new metabolites (**1** and **2**) was monitored by HPLC. Compounds **1** and **2** were detected in the culture broth from 4 to 6 days after the inoculation, and the production of **1** and **2** peaked (6.4 and 6.1 mg $l^{-1}$, respectively) 14 days after of the inoculation.

### Isolation of compounds 1–4

The TPU1237 strain was cultured under static conditions for 14 days, and acetone was added to the culture broth. After filtration, the aqueous acetone solution was concentrated and extracted with EtOAc. The EtOAc extract was subjected to ODS column chromatography followed by preparative HPLC to give a mixture of **1** and **2** (3.2 mg), **3** (1.1 mg) and **4** (1.4 mg). Compounds **1** and **2** were separated from the mixture by HPLC using a chiral column after the examination of various HPLC conditions.

Compounds **3** and **4** were identified by comparing their spectroscopic data with those of the reported values for trichodermaketones C and D, respectively.[10]

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Received 4 February 2015; revised 18 March 2015; accepted 30 March 2015; published online 22 April 2015

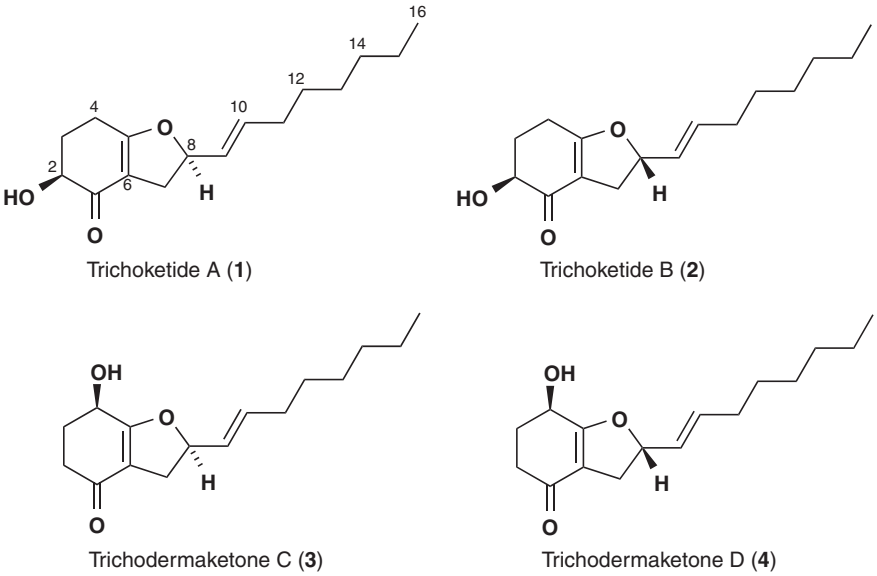


**Figure 1** Structures of trichoketides A (**1**) and B (**2**) and trichodermaketones C (**3**) and D (**4**) produced by the marine-derived fungus *Trichoderma* sp. TPU1237.

**Structure elucidation of compounds 1 and 2**

Trichoketide A (**1**) was obtained as a colorless oil, and its molecular formula was deduced to be $C_{16}H_{24}O_3$ from the HREIMS data. The $^1$H and $^{13}$C NMR spectra (in acetone-$d_6$) showed 23 proton and 16 carbon signals (Table 1) that were classified into one methyl, eight sp$^3$ methylene, two sp$^3$ oxygenated methine, two sp$^2$ methine, two sp$^2$ quaternary and one carbonyl carbons through an analysis of heteronuclear multiple quantum correlation and DEPT spectra. IR absorption at 3421 cm$^{-1}$ revealed the presence of a hydroxyl group. The $^1$H–$^1$H COSY spectrum of **1** revealed connectivities from C-7 to C-16 and C-2 to C-4 (Figure 2). The connection of the above two partial structures and the positions of two sp$^2$ quaternary carbons and a carbonyl group were elucidated from heteronuclear multiple-bond correlation (HMBC) data for **1** (Figure 2). $H_2$-4 (δ 2.50, 2.63) and $H_2$-7 (δ 2.44, 3.00) showed HMBC to C-5 (δ 178.0) and C-6 (δ 110.8). HMBC was detected from $H_2$-3 (δ 1.79, 2.30) to C-1 (δ 195.2) and C-5, and an HMBC was observed from H-2 (δ 4.01) to C-1. The *E*-orientation of the double bond in **1** was assigned from the large coupling constant (15.3 Hz) between H-9 and H-10. Thus, the planar structure of **1** was assigned, as shown in Figure 2.

Trichoketide B (**2**) was isolated as a colorless oil, and the HREIMS data indicated the molecular formula of **2** as $C_{16}H_{24}O_3$, which was the same as that of **1**. $^1$H and $^{13}$C NMR data (in acetone-$d_6$) for **2** were very similar to those for **1** (Table 1). Moreover, the correlation peaks detected in the 2D NMR spectra of **1** and **2** were very similar to each other. The planar structure of **2** (Figure 2) was elucidated to be the same as that of **1** by a similar analysis of spectroscopic data.

A marked difference was observed in the specific rotations of **1** and **2**. Compound **1** showed a negative rotation ($[\alpha]_D$ –79.3, *c* 0.10, $CH_3OH$), whereas **2** had a positive sign ($[\alpha]_D$ = +114.4, *c* 0.10, $CH_3OH$). The optical properties of **1** and **2** indicated that these compounds were diastereomers.

The absolute configurations at the C-2 position of **1** and **2** were elucidated by an analysis of $^1$H coupling constants, NOESY data and electronic circular dichroism (ECD) spectra. The H-2 signal (δ 4.01) in the $^1$H NMR spectrum of **1** showed a large coupling constant (12.0 Hz) with an axial proton at C-3 (H-3a, δ 1.79) (Table 1), and an NOE correlation was detected between H-2 and the axial proton at C-4 (H-4a, δ 2.50) in the NOESY spectrum of **1**. A similar coupling constant (12.0 Hz) between H-2 (δ 4.03) and H-3a (δ 1.78) and an NOE correlation between H-2 and H-4a (δ 2.50) were observed in the $^1$H NMR and NOESY spectra of **2**. Therefore, the hydroxyl group at the C-2 position in **1** and **2** was assigned as equatorial. The solid lines in Figure 3 show the experimental ECD spectra of **1** (a) and **2** (b) that were related to the conformation of the cyclohexene ring.[10,11] Positive cotton effects, correlating to the $n \rightarrow \pi^*$ transition, were observed at 297 nm (Δε +3.45) and 267 nm (Δε +11.5) in the ECD spectra of **1** and **2**, respectively, suggesting that the absolute configurations at the C-2 positions of **1** and **2** were *S*. Consequently, compounds **1** and **2** were epimers at the C-8 position.

**Absolute configurations of compounds 1–4**

As no NOE correlation was detected between six- and five-membered rings in the NOESY spectra of **1** and **2**, the absolute configurations at the C-8 positions in **1** and **2** were elucidated by comparing the experimental ECD spectra of **1** and **2** with the ECD spectra calculated for (2*S*, 8*R*)- and (2*S*, 8*S*)-isomers (Figure 3).[8,9,12] The experimental ECD spectra of **1** and **2** matched the calculated spectra of the (2 *S*, 8 *R*)-isomer (Figure 3a, dashed line) and (2*S*, 8*S*)-isomer (Figure 3b, dashed line), respectively. Thus, the absolute configurations of compounds **1** and **2** were assigned as shown in Figure 1.

The absolute configurations of trichodermaketones C (**3**) and D (**4**) were also elucidated by a comparison of their specific rotations with those of **1** and **2**, as the configurations at the C-8 positions of **3** and **4** had not yet been determined.[10] The specific rotations of **3** ($[\alpha]_D$ –152.2, *c* 0.10, $CH_3OH$) and **1** ($[\alpha]_D$ = − 79.3, *c* 0.10, $CH_3OH$) had negative signs, whereas **4** ($[\alpha]_D$ +196.6, *c* 0.10, $CH_3OH$) showed the same positive sign as **2** ($[\alpha]_D$ = +114.4.0, *c* 0.10, $CH_3OH$). Therefore, the absolute configurations of **3** and **4** were assigned as shown in Figure 1.

**Biological activities**

Recent studies indicated that PTP1B will be an effective and attractive target for the discovery of drugs to treat type 2 diabetes and obesity because it is the main negative regulator of insulin and leptin signaling cascades.[2–4] The isolated compounds **1–4** were tested for their effects

**Table 1** $^{13}C$ (100 MHz) and $^{1}H$ (400 MHz) NMR data for 1 and 2 (acetone-$d_6$)

| | 1 | | 2 | |
|---|---|---|---|---|
| *Position* | $\delta_C$ | $\delta_H$(*J in Hz*) | $\delta_C$ | $\delta_H$(*J in Hz*) |
| 1 | 195.2 | | 195.3 | |
| 2 | 72.1 | 4.01 d (12.0, 5.0) | 72.1 | 4.03 d (12.0, 5.0) |
| 3a | 30.9 | 1.79 dddd (12.4, 12.0, 12.0, 6.0) | 31.0 | 1.78 dddd (12.2, 12.0, 12.0, 5.0) |
| 3b | | 2.30 m | | 2.31 dddd (12.2, 5.0, 5.0, 2.5) |
| 4a | 23.2 | 2.50 m | 23.2 | 2.50 m |
| 4b | | 2.63 m | | 2.63 m |
| 5 | 178.0 | | 178.0 | |
| 6 | 110.8 | | 111.0 | |
| 7a | 32.7 | 2.44 m | 32.8 | 2.55 m |
| 7b | | 3.00 dd (14.2, 10.0) | | 2.89 ddd (13.9, 10.0, 2.0) |
| 8 | 87.7 | 5.32 dt (10.0, 7.6) | 88.1 | 5.29 dt (10.0, 8.0) |
| 9 | 129.5 | 5.61 ddt (15.3, 7.6, 1.5) | 129.5 | 5.65 ddt (15.6, 8.0, 2.0) |
| 10 | 135.5 | 5.83 dt (15.3, 6.8) | 135.9 | 5.85 dt (15.6, 7.0) |
| 11 | 32.7 | 2.08 m | 32.7 | 2.09 m |
| 12 | 29.6 | 1.39 m | 29.6 | 1.40 m |
| 13 | 32.4 | 1.29 m | 32.4 | 1.31 m |
| 14 | 29.5 | 1.29 m | 29.5 | 1.31 m |
| 15 | 23.2 | 1.29 m | 23.2 | 1.31 m |
| 16 | 14.3 | 0.88 t (7.0) | 14.3 | 0.88 t (7.0) |

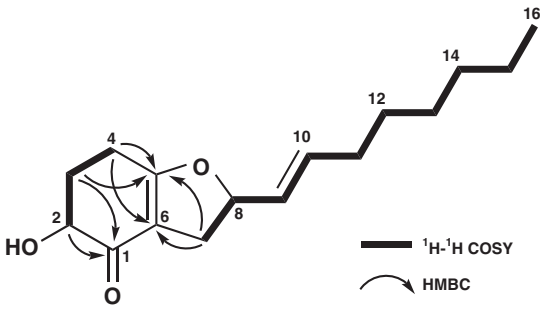


**Figure 2** $^{1}H$-$^{1}H$ COSY and key heteronuclear multiple-bond correlation (HMBC) for trichoketides A (**1**) and B (**2**).

against PTP1B activity using a previously described bioassay method.[6–9] Compounds **1–4** showed moderate inhibitory activities with $IC_{50}$ values of 53.1, 65.1, 68.0 and 55.9 μM, respectively. Oleanolic acid,[13] a positive control, inhibited PTP1B activity with an $IC_{50}$ value of 1.0 μM in the same bioassay. This is the first study to show that trichodermaketones C and D have inhibitory activities against PTP1B. Although the activities of **1–4** were not significant, these compounds may represent new leads for drug candidates as the structures of **1–4** were novel among the PTP1B inhibitors.

## METHODS

### General experimental procedure

EIMS was performed using a JMS-MS 700 mass spectrometer (JEOL, Tokyo, Japan). $^{1}H$ and $^{13}C$ NMR spectra were recorded on a JNM-AL-400 NMR spectrometer (JEOL) at 400 MHz for $^{1}H$ and 100 MHz for $^{13}C$ in acetone-$d_6$ ($\delta_H$ 2.05, $\delta_C$ 29.8 and 206.1). Optical rotations were measured with a JASCO P-2300 digital polarimeter (JASCO, Tokyo, Japan). UV spectra were obtained on a Hitachi U-3310 UV-Visible spectrophotometer (Hitachi, Tokyo, Japan) and IR spectra on a PerkinElmer Spectrum One Fourier transform infrared spectrometer (Waltham, MA, USA). CD spectra were measured with a spectrometer (J-720; JASCO). Preparative HPLC was carried out using the L-6200 system (Hitachi).

### Materials

PTP1B was purchased from Enzo Life Sciences (Farmingdale, NY, USA). *p*-Nitrophenyl phosphate was purchased from Sigma-Aldrich (St Louis, MO, USA). Oleanolic acid was purchased from Tokyo Chemical Industry (Tokyo, Japan). Potato dextrose agar was purchased from BD (Franklin Lakes, NJ, USA). Plastic plates (96-well) were purchased from Corning (Corning, NY, USA). All other chemicals including organic solvents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

### Isolation and identification of the strain

The fungal strain TPU1237 was isolated from a seawater sample collected at Ashizaki-Bay in Mutsu city, Aomori Prefecture, Japan, in November 2012. Approximately 1 ml of sea water was spread on a Potato dextrose agar plate containing 0.005% rose bengal and 0.01% kanamycin, and incubated at 25 °C for 7 days. The strain TPU1237, which grew on the plate, was inoculated and maintained in a LCA slant (0.1% glucose, 0.08% $KH_2PO_4$, 0.02% $K_2HPO_4$, 0.02%, $MgSO_4{\cdot}7H_2O$, 0.02% KCl, 0.2% $NaNO_3$, 0.02% yeast extract, 1.5% agar; adjusted to pH 6.0 before sterilization). The 239 bp ITS1 rDNA sequence of strain TPU1237 was identical to those of 71 known species of the genus *Trichoderma*, and, therefore, the strain was identified as *Trichoderma* sp. TPU1237.

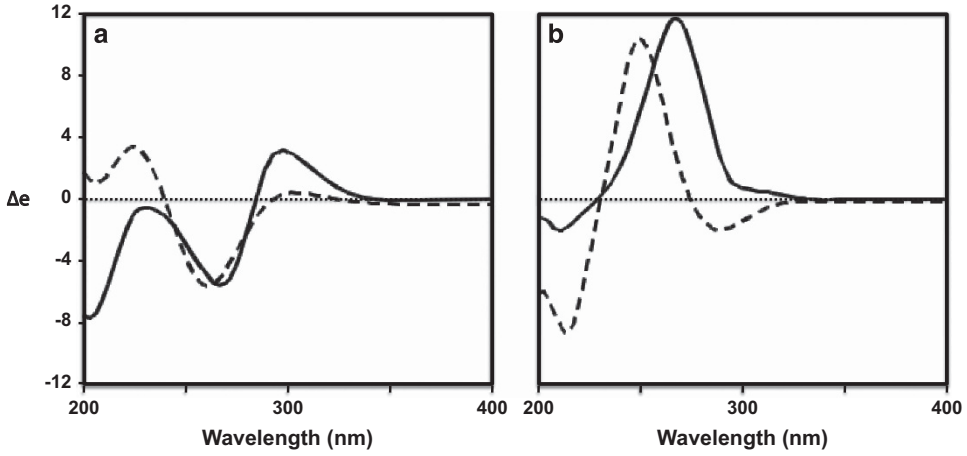


**Figure 3** Experimental (solid lines) and calculated (dashed lines) electronic circular dichroism (ECD) spectra of (**a**) trichoketide A (**1**)/(2*S*, 8*R*)-isomer and (**b**) trichoketide B (**2**)/(2*S*, 8*S*)-isomer.

### Fermentation

A slant culture of strain TPU1237 grown on LCA was inoculated into a 100-ml Erlenmeyer flask containing 50 ml of the seed medium (2.0% glucose, 0.50% polypeptone, 0.050% $MgSO_4 \cdot 7H_2O$, 0.20% yeast extract, 0.10% $KH_2PO_4$ and 0.10% agar; adjusted to pH 6.0 before sterilization). The flask was shaken reciprocally for 3 days at 25 °C to obtain the seed culture that was then transferred to the production medium (3.0% sucrose, 3.0% soluble starch, 1.0% malt extract, 0.30% Ebios (Asahi Food & Healthcare, Tokyo, Japan), 0.50% $KH_2PO_4$ and 0.050% $MgSO_4 \cdot 7H_2O$; adjusted to pH 6.0 before sterilization). The production culture was carried out at 25 °C for 14 days under the static condition.

### Isolation of metabolites

The same volume of acetone was added to the culture broth (2.4 l) after 14 days and filtered. The filtrate was concentrated to remove acetone and extracted with EtOAc. The EtOAc extract was dried over $Na_2SO_4$ and concentrated *in vacuo* to dryness, and the red brown residue (2.6 g) was then suspended in 30% $CH_3OH$ and adsorbed on an ODS column (100 g). The ODS column was eluted stepwise with 200 ml each of 30, 50, 70 and 100% $CH_3OH$ in $H_2O$ to separate into eight fractions (Fr. 1–Fr. 8). Fr. 5 (70% $CH_3OH$ eluate) showed inhibitory activity against PTP1B and was concentrated *in vacuo* to dryness to give a brown oil (160.9 mg). A portion (20 mg) of Fr. 5 was purified by preparative HPLC (column; PEGASIL ODS (Senshu Scientific, Tokyo, Japan), 10 × 250 mm; mobile phase, 60% $CH_3CN$; detection, UV at 254 nm; flow rate, 2.0 ml $min^{-1}$) to give a mixture of **1** and **2** (3.6 mg), **3** (1.1 mg) and **4** (1.4 mg).

The mixture of **1** and **2** (3.6 mg) was further purified by HPLC using the following conditions (column, CHIRAL-PAK AS-RH (DAISEL, Tokyo, Japan), 4.6 × 150 mm; mobile phase, 25% $CH_3CN$; detection, UV 254 nm; flow rate, 0.8 ml $min^{-1}$) to give **1** (0.7 mg) and **2** (0.8 mg).

*Trichoketide A (**1**).* Colorless oil; $[\alpha]^{20}_D$ –79.3 (*c* 0.10, $CH_3OH$); IR (KBr) $\nu_{max}$ 3421, 1728, 1627, 1262 $cm^{-1}$; UV ($CH_3OH$) $\lambda_{max}$ nm (log ε) 274 (4.18); CD ($CH_3CN$) $\lambda_{extermum}$ nm (Δε) 297 (+3.45), 267 (−5.50); EIMS *m/z* 264 $[M]^+$; HREIMS *m/z* 264.1727 ($[M]^+$; calcd for $C_{16}H_{24}O_3$, 264.1725); $^1H$ and $^{13}C$ NMR (acetone-$d_6$), see Table 1.

*Trichoketide B (**2**).* Colorless oil; $[\alpha]^{20}_D$ +114.4 (*c* 0.10, $CH_3OH$); IR (KBr) $\nu_{max}$ 3412, 1725, 1633, 1261 $cm^{-1}$; UV ($CH_3OH$) $\lambda_{max}$ nm (log ε) 271 (4.01); CD ($CH_3CN$) $\lambda_{extermum}$ nm (Δε) 267 (+11.5), 211 (−2.03); EIMS *m/z* 278 $[M]^+$; HREIMS *m/z* 264.1720 ($[M]^+$; calcd for $C_{16}H_{24}O_3$, 264.1725); $^1H$ and $^{13}C$ NMR (acetone-$d_6$), see Table 1.

*Trichodermaketone C (**3**).* Colorless oil; $[\alpha]^{21}_D$ –152.2 (*c* 0.10, $CH_3OH$); UV ($CH_3OH$) $\lambda_{max}$ nm (log ε) 272 (4.04); CD ($CH_3CN$) $\lambda_{extermum}$ nm (Δε) 328 (+0.69), 267 (−5.54); EIMS *m/z* 264 $[M]^+$; $^1H$ NMR (400 MHz, acetone-$d_6$) δ 5.83 (dt, 1H, *J* = 15.2, 6.4 Hz), 5.61 (ddd, 1H, *J* = 15.2, 7.8, 1.8 Hz), 5.23 (dt, 1H, *J* = 10.4, 7.8 Hz), 4.50 (m, 1H), 2.89 (ddd, 1H, *J* = 14.8, 10.4, 1.8 Hz), 2.45 (dd, 1H, *J* = 14.8, 7.8 Hz), 2.40 (m, 1H), 2.24 (m, 1H), 2.20 (m, 1H), 2.07 (m, 2H), 1.94 (m, 1H), 1.39 (m, 2H), 1.29 (m, 6H), 0.87 (t, 3H, *J* = 7.2 Hz); $^{13}C$ NMR (100 MHz, acetone-$d_6$) δ 194.0, 176.5, 135.1, 129.7, 113.0, 86.6, 63.5, 35.0, 33.2, 32.7, 32.4, 32.3, 29.6, 29.5, 23.2, 14.3.

*Trichodermaketone D (**4**).* Colorless oil; $[\alpha]^{21}_D$ +196.6 (*c* 0.10, $CH_3OH$); UV ($CH_3OH$) $\lambda_{max}$ (log ε) 272 (4.24); CD ($CH_3CN$) $\lambda_{extermum}$ (Δε) 298 (−1.81), 265 (+9.37); EIMS *m/z* 264 $[M]^+$; $^1H$ NMR (400 MHz, acetone-$d_6$) δ 5.83 (dt, 1H, *J* = 15.6, 6.8 Hz), 5.61 (ddd, 1H, *J* = 15.6, 8.0, 1.6 Hz), 5.22 (dt, 1H, *J* = 10.0, 8.0 Hz), 4.52 (m, 1H), 2.91 (dd, 1H, *J* = 15.2, 10.0 Hz), 2.45 (ddd, 1H, *J* = 15.2, 8.0, 2.0 Hz), 2.41 (m, 1H), 2.24 (m, 1H), 2.21 (m, 1H), 2.08 (m, 2H), 1.94 (m, 1H), 1.41 (m, 2H), 1.29 (m, 6H), 0.88 (t, 3H, *J* = 7.0 Hz); $^{13}C$ NMR (100 MHz, acetone-$d_6$) δ 194.0, 176.5, 135.5, 129.7, 113.0, 86.8, 63.6, 35.1, 33.2, 32.7, 32.4, 32.3, 29.6, 29.5, 23.2, 14.3.

### Quantitative analysis of compounds 1 and 2 by HPLC

The amounts of **1** and **2** in the culture broths were measured by analytical HPLC (L-6200 system). A fixed volume of each culture broth was extracted with EtOAc at 2, 4, 6, 8, 10, 12 and 14 days after inoculation. The EtOAc extract was dissolved in $CH_3OH$ and analyzed under the following conditions: column, CHIRAL-PAK AS-RH, 4.6 × 150 mm; mobile phase, 35% $CH_3CN$; detection, UV at 254 nm; flow rate, 0.8 ml $min^{-1}$. Compounds **1** and **2** were eluted at 15.1 and 12.3 min, respectively, and their amounts were calculated by comparing the peak areas with those of authentic **1** and **2**.

### Conformational analysis and calculation of ECD spectra

The most stable conformer of each compound was predicted using Spartan'14 (Wavefunction, Irvine, CA, USA) by a preliminary conformational analysis with the MMFF94 force field, followed by geometry optimization using the density functional theorywith the B3LYP functional and the 6-31G(d,p) basis set. The ECD spectra in acetonitrile were calculated for the predicted most stable conformers using Gaussian 03[14] by the time-dependent density functional theory with the B3LYP functional and cc-pVTZ basis set. The solvent effect was introduced by the polarizable continuum model. Twenty low-lying excited states were calculated, corresponding to the wavelength region down to 170 nm. The calculated spectra were displayed using GaussView 5.0.9,[15] with the peak half-width at half height being 0.333 and 0.25 eV for trichoketides A and B, respectively. The calculated spectrum for trichoketide A was red-shifted by 25 nm to match the experimental spectrum. It should be noted that for both compounds, the calculated low-energy conformers differ each other only in the orientation and conformation of the hexyl group. These differences are not expected to significantly affect the calculated ECD spectra, as indicated by preliminary calculations.

### PTP1B inhibitory assay

PTP1B inhibitory activity was determined by measuring the rate of hydrolysis of a substrate, *p*-Nitrophenyl phosphate, according to the published method with a slight modification.[5–8,16] Briefly, PTP1B (100 μl of 0.5 μg $ml^{-1}$ stock solution) in 50 mM citrate buffer (pH 6.0) containing 0.1 M NaCl, 1 mM dithiothreitol and 1 mM EDTA was added to each well of a 96-well plastic plate. A sample (2.0 μl in $CH_3OH$) was added to each well to make the final concentration from 0 to 189 μM and incubated for 10 min at 37 °C. The reaction was initiated by the addition of *p*-Nitrophenyl phosphate in the citrate buffer (100 μl of 4.0 mM stock solution), incubated at 37 °C for 30 min and the reaction was terminated using 10 μl of a stop solution (10 M NaOH). The optical density of each well was measured at 405 nm using an MTP-500 microplate reader (Corona Electric, Ibaraki, Japan). PTP1B inhibitory activity (%) was defined as $[1-(ABS_{sample}-ABS_{blank})/(ABS_{control}-ABS_{blank})] \times 100$. $ABS_{blank}$ is the absorbance of wells containing only the buffer and *p*-Nitrophenyl phosphate. $ABS_{control}$ is the absorbance of *p*-nitrophenol liberated by the enzyme in the assay system without a test sample, whereas $ABS_{sample}$ is that with a test sample. The assays were performed in two duplicate experiments for all test samples. Oleanolic acid, a known phosphatase inhibitor,[13] was used as a positive control.

## ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research (25870660) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan to HY and by the Foundation for Japanese Chemical Research to HY. We express our thanks to T Matsuki and S Sato of Tohoku Pharmaceutical University for measuring mass and NMR spectra, and K Endo, T Ogasawara, Y Watanabe and M Takahashi of Tohoku Pharmaceutical University for their technical assistance.

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