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Neomacrophorin I, II, and III, novel drimenyl cyclohexanes with hydroxylated butanoates from *Trichoderma* sp. 1212-03

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ABSTRACT

Neomacrophorins I (**1**), II (**2**), and III (**3**) were isolated from the culture broth of *Trichoderma* sp. 1212-03, which was collected at Shirakami Mountainous area in Japan. Structural analyses disclosed that these resemble known macrophorins but possess *axial*-hydroxy group at C3 as well as different side chains at C7′. These are diastereomeric forms of macrophorins for 5′,6′-epoxide functionality. The NMR analyses suggested their relative configurational relationship between the C1–C15 drimene and C1′–C7′ epoxyquinone moieties. ECD spectral discussions verified them particularly for C5′,C6′-epoxyquinone (**1**), C5′,C6′-epoxysemiquinone (**2** and **3**), and 2″,3″-dihydroxybutanoate moiety in **1** and **2**. The configuration of C3″-stereocenter of **3** was determined by chiral GC–MS after converting into methyl (*S*)-3″-hydroxybutanoate by basic of **3** methanolysis. Biological assays disclosed that **1** induces hyphal branching of *Cochliobolus miyabeanus* as well as cytotoxicity against human colorectal cancer COLO 201.

1. Introduction

Shirakami mountains in Japan is covered by virgin forest with Siebold's beech trees (locally known as Buna), and it has been registered as one of UNESCO world heritage sites since 1993.[1] With the assumption that microorganisms in this area had not been investigated as the genetic resources, we explored biologically active and novel metabolites from ecologically unique fungi in this area, which led us to disclose several novel daucanes.[2] Continuous exploration with *Trichoderma* sp. 1212-03 collected in that area disclosed neomacrophorins I (**1**), II (**2**), and III (**3**). These are structurally related to macrophorins isolated from *Macrophoma*[3,4] and *Eupenicillium*,[5] but these feature 3-α-hydroxy group in the C1–C15 drimene framework as well as 5′,6′-α-epoxide on the C1′–C7′ epoxyquinone moiety, while macrophorins possess the β-epoxide. These involve different side chains at C7′. The biological activities are also discussed.

2. Results and discussion

Trichoderma sp. 1212-03 collected from the soil at Shirakami Natural Science Park of Hirosaki University, Aomori Prefecture Japan was cultured with potato–dextrose medium at 26 °C for 14 days (1.0 L) and its ethyl acetate extracts were fractionated with a series of chromatographic separations to yield neomacrophorins I (**1**, 14.3 mg), II (**2**, 39.5 mg), and III (**3**, 7.2 mg) as well as known trichodermin (20.4 mg).[6] The ^{1}H and ^{13}C NMR data of **1**–**3** are summarized in Table 1.

2.1. Neomacrophorin I (1)

Neomacrophorin I (**1**) provided 26 resonances in the ^{13}C NMR spectrum and the protonated molecular ion peak at *m*/*z* 477.2493 in the ESIMS. The HMQC spectrum classified the carbons into four methyls, seven methylenes, seven methines, and eight quaternary carbons, disclosing 33 hydrogens directly linked with carbons. The ^{13}C NMR indicated three carbonyls (172.55, 192.25, 192.73 ppm), four oxymethines (58.93, 68.69, 74.60, 75.78 ppm), and an oxymethylene (60.47 ppm). These results suggested the molecular formula as $C_{26}H_{36}O_8$ (M+H: calcd 477.2488). These suggested that three hydrogens should be attributed to alcohol functionalities. Two broaden singlets (4.37, 4.81 ppm) correlated with a carbon resonance at 107.83 ppm in HMQC, which revealed an exomethylene in the molecule. The three singlet methyls appeared at characteristically lower frequencies (0.75, 0.84, and 0.96 ppm) in the ^{1}H NMR, thus we assumed that **1** should be a cyclic terpenoid. Further detailed NMR analyses involving COSY, HMQC, and HMBC revealed the planar structure, which is comprised of 3-hydroxy-$\Delta^{8,12}$-drimene (C1–C15), epoxyquinone (C1′–C7′), and 2,3-dihydroxybutanoate (C1″–C4″) units (Fig. 1).

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Table 1
NMR spectral data of neomacrophorins I (**1**), II (**2**), and III (**3**) in $CDCl_3$

	Neomacrophorin I (**1**)		Neomacrophorin II (**2**)		Neomacrophorin III (**3**)	
Position	δ ^{13}C	δ ^{1}H [intensity, multiplicity *J*(Hz)]	δ ^{13}C	δ ^{1}H [intensity, multiplicity *J*(Hz)]	δ ^{13}C	δ ^{1}H [intensity, multiplicity *J*(Hz)]
1	31.47	α: 1.50 (1H, m) β: 1.45 (1H, m)	31.50	1.46 (1H, m)	31.54	1.50 (2H, m)
2α	25.77	1.67 (1H, m)	25.72	α: 1.62 (1H, m)	25.85	α: 1.64 (1H, m)
2β		1.93 (1H, ddt, 2.2, 4.2, 14.4)		β: 1.91 (1H, m)		β: 1.94 (1H, ddt, 2.3, 5.5, 12.8)
3	75.78	3.44 (1H, dd, 2.2, 2.6)	76.12	3.43 (1H, t, 2.3)	75.96	3.44 (1H, t, 2.3)
4	37.79	—	37.52	—	37.81	—
5	48.26	1.58 (1H, 2.2, 13.0)	48.34	α: 1.59 (1H, m)	48.42	1.60 (1H, m)
6	23.58	α: 1.67 (1H, m) β: 1.38 (1H, dq, 4.2, 13.0)	23.54	β: 1.64 (1H, m) 1.38 (1H, dq. 3.3, 12.4)	23.66	α: 1.66 (1H, m) β: 1.38 (1H, dq, 4.2, 12.7)
7α	37.42	α: 2.02 (1H, ddd, 5.0, 13.0, 13.0)	37.77	α: 2.02 (1H, dt, 5.2, 11.9)	37.59	α: 2.03 (1H, m)
7β		β: 2.42 (1H, ddd, 2.0, 3.7, 13.0)		β: 2.40 (1H, dd, 2.2, 11.9)		α: 2.42 (1H, ddd, 2.4, 4.1, 13.9)
8	148.46	—	148.71	—	149.24	—
9	48.79	1.54 (1H, dd, 3.6, 9.8)	49.08	1.60 (m)	49.4	1.62 (1H, m)
10	39.11	—	39.03	—	39.13	—
11	19.17	2.29 (2H, m)	19.91	2.17 (2H. m)	19.71	2.16 (1H, dd, 1.5, 16.0) 2.27 (1H, dd, 11.3, 16.0)
12	107.83	4.37 (1H, br s) 4.81 (1H, br s)	107.92	4.48 (1H, br s) 4.81 (1H, br s)	107.7	4.43 (1H, br s) 4.83 (1H, br s)
13	28.50	0.96 (3H, s)	28.54	0.95 (3H, s)	28.52	0.97 (3H, s)
14	22.17	0.84 (3H, s)	22.19	0.83 (3H, s)	22.2	0.84 (3H, s)
15	14.63	0.75 (3H, s)	14.64	0.73 (3H, s)	14.64	0.75 (3H, s)
1′	192.73	—	194.91	—	194.28	—
2′	132.75	6.64 (1H, t, 1.6)	123.44	5.99 (1H, s)	123.76	6.00 (t, 1.3)
3′	142.08	—	150.84	—	150.6	—
4′	192.25	—	63.66	4.50 (1H, br s)	64.06	4.51 (1H, br s)
5′	58.93	3.52 (1H, s)	61.89	3.54 (1H, br s)	61.72	3.54 (1H, d, 1.5)
6′	62.26	—	59.61	—	59.42	—
7′	60.47	4.89 (1H, dd, 1.6, 16.4) 5.11 (1H, dd, 1.6, 16.4)	64.72	4.76 (1H, d, 15.3) 5.07 (1H, d, 15.3)	63.82	4.80 (1H, dd, 1.5, 15.0) 4.91 (1H, dd, 1.5, 15.0)
1″	172.55	—	172.90	—	171.99	—
2″	74.60	4.11 (d, 2.3 Hz)	74.69	4.11 (1H, d, 1.5)	42.99	2.50 (1H, dd, 9.0, 16.0) 2.57 (1H, dd, 3.4, 16.0)
3″	68.69	4.16 (1H, dq, 2.3, 6.4)	68.76	4.19 (1H, dq, 1.5, 6.5)	64.54	4.25 (1H, m)
4″	19.51	1.33 (3H, d, 6.4)	19.34	1.30 (3H, d, 6.5)	22.85	1.26 (3H, d, 6.3)

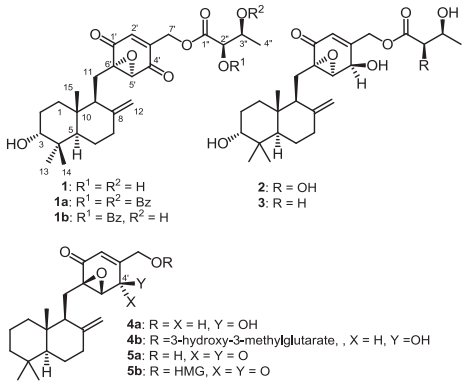

Fig. 1. Structures of neomacrophorins I (**1**), II (**2**), III (**3**), macrophorins (**4a**–**5b**).

This molecule has unique *axial*-hydroxy group at C3 position based on its ^{1}H signal profile (3.44 ppm, dd, *J*=2.2, 2.6 Hz). *trans*-Decalin system was established by observing an NOE at 14-H_3 (0.84 ppm), as shown in Fig. 2, when 15-H_3 (0.75 ppm) was irradiated.[7] Both 14-H_3 and 15-H_3 gave NOEs also with 2-Hβ (1.93 ppm) and 6-Hβ (1.38 ppm) to confirm the above discussions. Methylene substituent at C11 took β-orientation because of an NOE between 15-H_3 and one of the 11-H_2 (2.29 ppm). Other NOEs, the other 11-H (2.29 ppm)↔one of 12-H_2 (4.37 ppm) and 9-H (1.54 ppm)↔7-Hα (2.02 ppm), supported above configurational assignment.

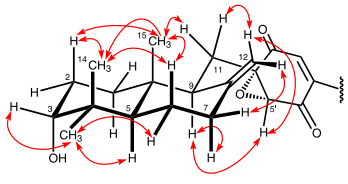

Fig. 2. Characteristic NOE correlations of neomacrophorin I (**1**).

The suggested C1–C15 drimene+C1′–C7′ epoxyquinone substructure closely resembles that of 4′-oxomarcophorins A (**5a**) and D (**5b**) except that **1** has hydroxy group at C3.[5] However, the chemical shifts for 5′-H (3.52 ppm) of **1** appeared at considerably lower frequency than the corresponding protons of **5a**,**b** (3.72 and 3.77 ppm, respectively) in $CDCl_3$. The C3-hydroxy group is geometrically far from 5′-H that it hardly affects the 5′-H chemical shift. We assumed that **1** is a diastereomeric form of **5a**,**b** at the 5′,6′-epoxy functionality. The 5′-H was found to provide NOEs with 9-H and to one of the H_2-12 (4.37 ppm). The NOE between H-9 and H-5′ was very intense. When a virtual α-C5′,C6′-epoxide **model X** was analyzed with EDF2/6-31G*,[8] all stable conformers satisfy these NOEs (Fig. 3). These conformers are estimated to occupy more than 95% abundance at room temperature based on their steric energies. The distances 9-H↔5′-H and 12-H↔5′-H were estimated to be around 2.4 and 3.1 Å, respectively, in the most stable conformer. The C1″–C4″ side chain was omitted in the **model X** to reduce the number of conformers. In contrast, the C5′C6′-diastereomeric isomer, the model corresponding to **5a**,**b**, does not take the similar conformations. These conformations cause steric hindrance between the C12 exomethylene and the C5′,C6′-epoxide oxygen (see Supplementary data). Theoretical chemical shift calculations[9] suggested that H-5′ signal in **model X** appears 0.4 ppm

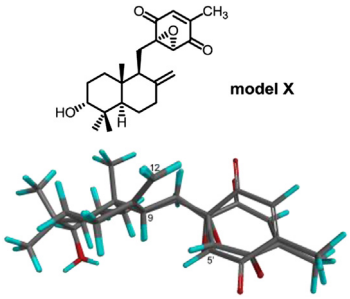

Fig. 3. Structure of **model X** and its stable conformers obtained by EDF2/6-31G*.

lower frequency (3.14 ppm) than that of the β-epoxide (3.46 ppm), which is the similar tendency to the experimental data. These findings revealed the relative configurational relationship for the C1–C15 drimene framework and C1′–C7′ epoxyquinone moieties as (3*R**,5*R**,9*S**10*R**,5′*R**,6′*S**)-form.

Since both chiral forms have also been reported for the drimane framework,[10] the absolute configuration of **1** could not be concluded simply with its structural similarity to macrophorins. Since **1** showed the optical rotation [−53 (*c* 0.58, MeOH)] with the opposite sign to those of 4′-oxomarcophorins **5a**,**b**[5] [+35.5 (*c* 0.30, MeOH), +10.2 (*c* 0.30, MeOH), respectively], comparison of the specific rotation values were not conclusive. We then studied that with the electronic circular dichroism (ECD). Neomacrophorin I (**1**) gave ECD Cotton Effects at 386 nm (Δε −0.64), 261 nm (Δε +2.89), 238 nm (Δε −2.16), 228 nm (Δε −0.16), and 214 nm (Δε −5.04), which is approximately the inversed profile of **5a**,**b**. Since the Cotton effect at 261 nm corresponds to the λ_{max} (260 nm, sh, ε 2800) of this molecule and it was assigned due to π→π* transition (K-band) of the C1′–C7′ epoxyquinone moiety, the Cotton effect at this wavelength should attribute to its chirality. These suggested that the C1′–C7′ epoxyquinone moiety in **1** is the mirror image of **5a**,**b**. In fact, the ECD profile of **1** resembles that of phyllostine, which is the same chirality for the epoxyquinone part.[11] These established the absolute configurations as (3*R*,5*R*,9*S*,10*R*,5′*R*,6′*S*)-form.[12]

Next, we studied the configuration of the 2,3-dihydroxy butanoate (C1″–C4″) moiety. The coupling constant $^3J_{HC2''HC3''}$ was 2.3 Hz, indicating *gauche*-conformational relationship between H-2″ and H-3″. The two hydroxy groups should also take *gauche*-conformation due to hydrogen bonding between them. A combined analysis with HMBC and ^{1}H non-decoupled ^{13}C NMR spectra revealed the $^3J_{CH}$ values for 1″-C/3″-H and 2″-H/4″-C to be ca. 1.0 and 2.0 Hz, respectively. These small $^3J_{CH}$ values suggested *gauche*-relationships between 1″-C and 3″-H as well as 2″-H and 4″-C as shown in Fig. 4.[13] Thus, we successfully assigned *threo*-2,3-dihydroxybutanoate unit in **1**. A strong NOESY correlation between 2″-H and 4″-H_3 may support the above discussions. This assignment was verified with the δ value at the 4″–C (19.51 ppm). Methyl carbons in *threo*-2,3-dihydroxybutanoic acid and its esters have been observed at 18–20 ppm,[14–16] while these in the *erythro*-isomers appear at 16–18 ppm.[17–19] Theoretical chemical shifts[9] showed the similar tendency, when *threo*- and *erythro*-methyl 2,3-dihydroxybutanoates were calculated with EDF2/6-31G*

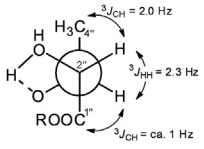

Fig. 4. Conformations of *threo*-2″,3″-dihydroxybutanoate moiety of **1**.

(*threo*: 19.4 ppm, *erythro*: 17.3 ppm).[20] These chemical shift differences can be explained by steric compression[21] in the *erythro*-isomer, because the methyl group should take *gauche*-conformation with bulky C1-carboxy group.

Absolute configuration of the C1″–C4″ side chain was successfully established by ECD exciton chirality method.[22,23] Benzoylation of **1** under conventional conditions gave 2″,3″-*O*-dibenzoate **1a** along with 2″-*O*-monobenzoate **1b**. The 3-OH group was retained under the conditions because of steric hindrance by the C4 *gem*-dimethyl group. Dibenzoate **1a** resulted in a pair of exciton-split Cotton effects with a negative Cotton effect at 237 nm (Δε −18.7) and a positive one at 220 nm (Δε +16.3) as shown in Fig. 5. Since 2″-*O*-monobenzoate **1b** showed much smaller Cotton effects than **1a**, ECD by chromophores other than these two benzoyl groups can be neglected. Thus, the negatively split ECD spectrum of **1a** suggested a negative chiral relationship between these two benzoyl groups in **1a**. There are three possible staggered conformations of **1a**, namely conformations A, B, and C. Conformations A and C may give opposite ECD. The coupling constant between 2″-H and 3″-H (3.4 Hz) suggest a *gauche*-relationship for these protons, which eliminates the contribution of conformation C. Conformation B should be less effective to the ECD spectrum because of *anti*-periplanar orientation. Thus, only conformation A should be considered for the ECD discussions at present. The (2″*R*,3″*S*)-isomer satisfies the negative ECD couplet. These analyses allowed us to conclude the total structure of **1** as depicted in Fig. 1.

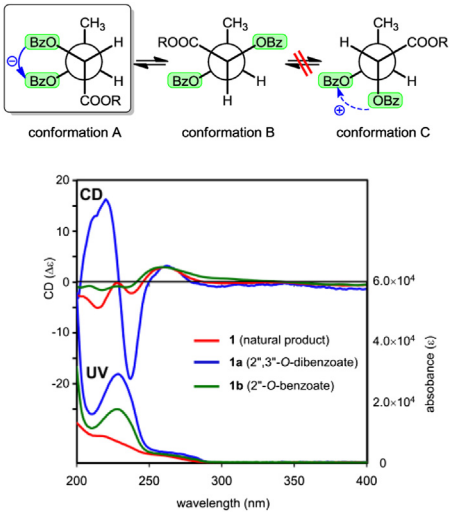

Fig. 5. Staggered conformations of C1″–C4″ side chain moiety of **1b** (upper) and ECD spectra of **1**, **1a**, and **1b**.

2.2. Neomacrophorin II (2)

Neomacrophorin II (**2**, $[\alpha]_D^{25}$ −33 (*c* 0.24, MeOH)) was slightly more polar substance than **1** based on its chromatographic behavior. This molecule provided the protonated molecular ion at *m*/*z* 479.2645 (suggesting $C_{26}H_{39}O_8$: 479.2645) in the ESIMS, which was two units larger than that of **1**. The ^{1}H and ^{13}C NMR spectra of **2** resembled those of **1** except for some signals. The 4′-C resonated at 63.66 ppm while the corresponding signal appeared at 192.25 ppm in **1**. This carbon gave HMQC correlation with a proton at 4.50 ppm, which was not found in **1**. Further spectroscopic analyses deduced that **2** is an alcohol form of **1** at C4′. A strong NOE between H-9 (1.60 ppm) and H-5′ (3.54 ppm) was observed also in **2**, which indicated the same α-orientation for

C5′C6′ epoxide as that of **1**. The H-5′ signal appeared as a broaden singlet (half-value width: 3.4 Hz), and this signal became sharper (half-value width: 2.5 Hz) when 4′-H was decoupled. These experiments indicated $^{3}J_{H4'5'H}$ to be ca. 0.9 Hz, suggesting nearly perpendicular dihedral angle for ∠H–C4′–C5′–H. Molecular modeling suggested that β-alcohol (*trans*-epoxyalcohol) would be more plausible based on the expected dihedral angles ∠H–C4′–C5′–H [α-alcohol (*cis*-epoxyalcohol): ca. 60°, β-alcohol (*trans*-epoxyalcohol): ca. 70°]. However, macrophorins A (**4a**) and D (**4b**) also gave small spin couplings between the 4′-H and 5′-H (2.4 and 1.5 Hz, respectively), though these are *cis*-epoxyalcohol. Irradiation at H-5′ gave a potent NOE at H-4′, but both isomers may provide the NOE. Thus, these stereochemical assignments remained ambiguous.

We further investigated it with ECD analysis. Neomacrophorin II (**2**) gave negative Cotton effects at 338 nm ($\Delta\varepsilon$ −3.2) and 243 nm ($\Delta\varepsilon$ −5.4). These can be assigned as R-band ($n\rightarrow\pi^*$ transition) and K-band ($\pi\rightarrow\pi^*$ transition), respectively, of the α,β-unsaturated ketone.[24] Sekiguchi[25] and Sassa[4] suggested that absolute configurations of 5,6-epoxide and 4-hydroxy groups correlate to the Cotton effect at R- and K-bands, respectively in 5,6-epoxy-4-hydroxy-2-cyclohexenones, namely (5*R*,6*R*)-epoxide gives a positive Cotton effect at the R-band (around 340 nm), while (4*R*)-alcohol provides a negative Cotton effect at the K-band (around 245 nm). Configuration of the 5,6-epoxide functionality can also be explained by Snatzke's inverse-octant rule.[26,27] Application of these empirical rules indicated (4′*R*,5′*S*,6′*S*)-configuration for **2**; i.e., 5′,6′-α-epoxy-4-β-alcohol. The configuration thus obtained is consistent with the above discussions.[13]

2.3. Neomacrophorin III (3)

Neomacrophorin III (**3**, $[\alpha]_D^{24}$ −58 (*c* 0.62, $CHCl_3$)) showed similar chromatographic property with **1**. The ESIMS gave the protonated molecular ion *m*/*z* 467.2723 (suggesting $C_{26}H_{39}O_7$: 463.2696), indicating that **3** contains one less oxygen than **2**. NMR spectral comparison revealed that **3** is a 2″-deoxy derivative of **2**. The C2″-methylene protons in **3** were found at 2.50 and 2.57 ppm (each dd, *J*=9.0, 16.0 Hz and *J*=3.4, 16.0 Hz, respectively) in the ^{1}H NMR spectrum, while it was oxymethine (4.11 ppm, d, *J*=1.5 Hz) in **2**. Irradiation of 5′-H (3.54 ppm) in **3** also gave an intense NOE at 9-H (1.62 ppm). This molecule showed the nearly identical ECD spectrum to that of **2** (see Supplementary data). This indicated that **2** and **3** have the same configuration for the C1–C15 drimene+C1′–C7′ epoxysemiquinone moiety.

The configuration at C3″ in **3** was expected to be (*S*)-form by taking into account its structural and biosynthetic similarity with **1** and **2**. It was experimentally determined with chiral GC-technique after liberation of (*S*)-methyl 3-hydroxybutanate by basic methanolysis. Injection of the crude reaction mixture to GC–MS with Rt-γDEXsa™ (RESTEC) and monitored with the desmethylated ion (*m*/*z*=103) to afford the signal at 30.8 min. This signal was verified with the commercial (*S*)-enantiomer as shown in Fig. 6. On the other hand, the (*R*)-enantiomer appeared at 31.9 min under the same conditions. The enantiomeric mixture provided two peaks with sufficient separation and reproducibility. The chirality of C3″ was thus deduced to be (*S*)-form, which was identical to those of other neomacrophorins. These would support our previous ECD discussions for 2″,3″-moiety of **1** and **2**.

2.4. Biological properties

Neomacrophorins I (**1**), II (**2**), and III (**3**) inhibited the hyphal growth of *Cochliobolus miyabeanus* at 1.0, 10, and 10 μg/mL, respectively. Microscopic observation revealed these are due to hyphal branching. Neomacrophorin I (**1**) inhibited human colon adenocarcinoma (COLO 201) cell proliferation (IC_{50}: 46 μg/mL), while **2** and **3** did not show this activity. (IC_{50}: >100 μg/mL).

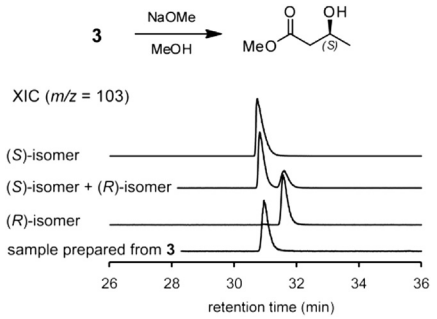

Fig. 6. Chiral GC analysis of methyl 3-hydroxybutanoates from **3** monitored with *m*/*z*=103 ($[M-(CH_3)]^+$) [conditions: Rt-βDEXsa (RESTEC, 0.25 mm×30 m), 40–230 °C (2 °C/min), injection: 230 °C].

3. Conclusion

We disclosed neomacrophorins I (**1**), II (**2**), and III (**3**) from *Trichoderma* sp. 1212-03. Their C1–C15 drimene+C1′–C7′ epoxyquinone moieties resemble those of macrophorins but possessing 3-α-hydroxy group. NMR and ECD studies suggested that these are diastereomeric forms of macrophorins for the 5′,6′-epoxide functionalities. Absolute configurations of C1′–C7′ epoxysemiquinone moiety in **2** and **3** were established by comparing their ECD spectra with those of related compounds. Configuration of their epoxyketone moieties accorded with inverse-octant rule by Snatzke.[26,27] Neomacrophorin III (**3**) was found to be 2″-deoxy form of **2**. Chiral GC–MS was effective in establishing the chirality of C3″. Antifungal assay disclosed that **1** not only induces hyphal branching of *C. miyabeanus*, but also shows cytotoxicity against human colorectal cancer COLO 201.

4. Experimental

4.1. General

Methyl (*S*)- and (*R*)-3-hydroxybutanoates were purchased from Sigma–Aldrich Corporation. Ultraviolet (UV) spectra were obtained by a HITACHI U-2010 spectrometer. Electronic circular dichroism (ECD) spectra were measured on a JASCO J-725 spectrometer with a micro-cell (2.0 mm cell-length). NMR spectra were recorded on a JEOL JNM-ECX500 spectrometer. Tetramethylsilane (TMS) was used as internal standard (0 ppm) in ^{1}H and ^{13}C spectra. Measurements of Electrospray-ionization mass (ESI) spectra were performed on a HITACHI NanoFrontier LD mass spectrometer. Gas chromatography-mass spectrometry (GC–MS) was performed with a Shimadzu Gas Chromatograph-Mass Spectrometer. In the antifungal assay, samples were observed with ×20 magnification using an Olympus CKX31 inverted microscope.

4.2. Fungus

Trichoderma sp. 1212-03 was isolated from a fruit body of *Daedaleopsis tricolor* in the Shirakami Natural Science Park of Hirosaki University, Aomori Prefecture Japan in 2012 and identified based on the sequences of the internal transcribed spacer (ITS) region and the D1/D2 region of the large subunit of the ribosomal RNA gene as well as morphological characteristics. The fungus was deposited at Biological Resource Center of National Institute of Technology and Evaluation, Japan (ID: NBRC 110000).[28]

4.3. Calculations

Calculations were performed with Spartan 10 (Wavefunction, Irvine, CA) using PC (operating system: Windows7 Professional, CPU: AMD Phenom(tm)×4 970 processor 3.50 GHz, RAM 32 GB). **Model X** was subjected to conformational analyses with AM1 by rotating C3–O, C9–C11, and C11–C6′ linkages to provide 18 tentative stable conformers. These were further optimized with density functional EDF2 employing 6-31G* basis set. Chemical shift calculations were performed also with EDF2/6-31G* based on the optimized conformations. Theoretical chemical shifts were obtained by considering Boltzmann distribution. Similar calculations were also performed for its C5′,C6′-diastereomer.

4.4. Isolation

Trichoderma sp. 1212-03 was cultured in potato–dextrose medium (200 mL in 500 mL baffled Erlenmeyer flask×5) on a rotary shaker (110 rpm) at 26 °C for 14 days. After filtration *in suction*, the filtrate was extracted with EtOAc (300 mL×4) and the organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give crude extract (235 mg). That was purified with silica gel column chromatography. The fraction eluted with acetone/$CHCl_3$ (10:90) was recovered and concentrated to give a residue (80.3 mg). Further silica gel chromatography [AcOEt/hexane=30:70, then 50:50] gave neomacrophorin I (**1**, 14.3 mg), II (**2**, 39.5 mg), III (**3**, 7.2 mg), and trichodermin (20.4 mg). The ^{1}H NMR spectra and the specific rotation value of trichodermin ($[\alpha]_D^{25}$ −11 (*c* 0.87, $CHCl_3$)) showed good accordance with the data in literature ($[\alpha]_D^{20}$ −10.2).[6,29]

*4.4.1. Neomacrophorin I (**1**).* The ^{1}H and ^{13}C NMR data are shown in Fig. 1. $[\alpha]_D^{25}$ −53 (*c* 0.58, MeOH), IR (film) 3440, 1745, 1687 cm^{-1}. UV (8.0×10^{-5} mol/L, CH_3CN, ε) λ 218 (8800), 260 (sh, 2800), ECD (8.0×10^{-5} mol/L, CH_3CN) $\Delta\varepsilon$ −0.64 (386 nm), +2.9 (261 nm), −2.2 (238 nm), −0.16 (228 nm), −5.04 (214 nm), −2.8 (204 nm), ESIMS *m*/*z* 477.2493 (calcd for $C_{26}H_{37}O_8$, M+H: 477.2488), HMBC: H-1α→C-2, C-10, H-1β→C-2, C-10, H-2α→C-1, C-3, H-2β→C-1, H-3→C-2, C-4, C-14, H-5→C-10, C-15, H-6β→C-5, C-7, H-7α→C-8, H-7β→C-5, C-6, C-8, C-9, C-12, H-9→C-8, C-10, C-11, C-6′, H-11→C-10, C-1′, C-6′, each H-12 (4.37, 5.81 ppm)→C-7, C-8, C-9, C-10, H_3-13→C-3, C-4, C-5, C-14, H_3-14→C-4, C-5, C-13, H_3-15→C-1, C-5, C-9, C-10, H-2′→C-11, C-6′, C-3′, C-7′, H-5′→C-11, C-3′, C-4′, C-7′, H-7′ (4.89 ppm)→C-2′, C-3′, C-4′ C-1″, H-7′ (5.11 ppm)→C-2′, C-4′, C-1″, H-2″→C-1″, C-3″, C-4″, H-3″→C–C1″, C2″, C4″, H_3–C4″→C-2″, C-3″, NOE: H-1β↔H_3–15, H-2β↔H–3, H-2β↔H_3–14, H-2α↔H–3, H-3↔H_3-13, H-3↔H_3-15, H-5↔H_3-13↔H_3-14, H-6α↔H_3-13, H-6β↔H_3-14, H-6β↔H_3-15, H-7α↔H9, H-7β↔H12 (4.81 ppm), H-9↔H-5′, H-11↔H-12 (4.37 ppm), H-12 (4.37 ppm)↔H5′, H-2′↔H-7′ (5.11 ppm), H-2″↔H-4″, H-3″↔H-4″.

*4.4.2. Neomacrophorin II (**2**).* The ^{1}H and ^{13}C NMR data are shown in Fig. 1. $[\alpha]_D^{25}$ −33 (*c* 0.24, MeOH), IR (film) 3440, 1733, 1683 cm^{-1}. UV (8.0×10^{-5} mol/L, CH_3CN, ε) λ 284 (sh, 1000), ECD (8.3×10^{-5} mol/L, CH_3CN) $\Delta\varepsilon$ −3.2 (338 nm), −0.36 (282 nm), −5.4 (243 nm), +4.3 (215 nm), +2.0 (200 nm), ESIMS *m*/*z* 479.2645 (calcd for $C_{26}H_{39}O_8$, M+H: 479.2645), HMBC: H-1α→C-2, C-10, H-1β→C-2, C-10, H-2α→C-1, C-3, H-2β→C-1, H-3→C-2, C-4, C-14, H-5→C-10, C-15, H-6β→C-5, C-7, H-7α→C-8, H-7β→C-5, C-6, C-8, C-9, C-12, H-9→C-8, C-10, C-11, C-6′, H-11→C-10, C-1′, C-6′, each H-12 (4.48, 4.81 ppm)→C-7, C-8, C-9, C-10, H_3-13→C-3, C-4, C-5, C-14, H_3-14→C-4, C-5, C-13, H_3-15→C-1, C-5, C-9, C-10, H-2′→C-6′, C-3′, C-4′, H-4′→C-1′, C-2′, C-3′, C-5′, C-6′, C-7′, H-5′→C-11, C-3′, C-4′, C-6′, H-7′ (4.76 ppm)→C-2′, C-3′, C-4′ C-1″, H-7′ (5.07 ppm)→C-2′,,C-3′, C-4′, C-1″, H-2″→C-1″, C-3″, C-4″, H-3″→C-1″, C-2″, C-4″, H_3-4″→C-2″, C-3″, NOE: H-1β↔H_3-15, H-2β↔H-3, H-2β↔H_3-14, H-2α↔H-3, H-3↔H_3-13, H-3↔H_3-15, H-5↔H_3-13↔H_3-14, H-6α↔H_3-13, H-6β↔H_3-14, H-6β↔H_3-15, H-7α↔H9, H-7β↔H12 (4.81 ppm), H-9↔H-5′, H-11↔H-12 (4.48 ppm), H-12 (4.48 ppm)↔H5′, H-2′↔H-7′ (5.07 ppm), H-4′↔H–5′, H-2″↔H-4″, H-3″↔H-4″.

*4.4.3. Neomacrophorin III (**3**).* The ^{1}H and ^{13}C NMR data are shown in Fig. 1. $[\alpha]_D^{25}$ −150 (*c* 0.5.8, $CHCl_3$), IR (film) 3340, 2965, 1727, 1677 cm^{-1}. UV (8.0×10^{-5} mol/L, CH_3CN, ε) λ 239 (sh, 6000), ECD (8.0×10^{-5} mol/L, CH_3CN) $\Delta\varepsilon$ −3.2 (339 nm), −0.19 (286 nm), −6.1 (243 nm), +8.23 (215 nm), +1.54 (200 nm), ESIMS *m*/*z* 467.2723 (calcd for $C_{26}H_{39}O_7$, M+H: 463.2696), HMBC: H-1α→C-2 and C-10, H-1β→C-2, C-10, H-2α→C-1, C-3, H-2β→C-1, H-3→C-2, C-4, C-14, H-5→C-10, C-15, H-6β→C-5, C-7, H-7α→C-8, H-7β→C-5, C-6, C-8, C-9, C-12, H-9→C-8, C-10, C-11, C-6′, H-11→C-10, C-1′, C-5′, C-6′, each H-12 (4.43, 4.83 ppm)→C-7, C-8, C-9, C-10, H_3-13→C-3, C-4, C-5, C-14, H_3-14→C-4, C-5, C-13, H_3-15→C-1, C-5, C-9, C-10, H-2′→C-6′, C-3′, C-7′, H-4′→C-3′, C-5′, C-6′, H-5′→C-11, C-3′, C-4′, C-6′, H-7′ (4.80 ppm)→C-2′, C-3′, C-4′ C-1″, H-7′ (5.07 ppm)→C-2′,,C-3′, C-4′, C-1″, H-2″(2.50 ppm)→C-1″, C-3″, C-4″, H-2″(2.57 ppm)→C-1″, C-3″, C-4″, H-3″→C-1″, C-2″, C-4″, H_3-4″→C-2″, C-3″.

4.5. Benzoylation of (1)

A solution of **1** (5.0 mg, 11 μmol) in CH_2Cl_2 (500 μL) and pyridine (8.0 μL) was stirred with benzoyl chloride (6.0 mg, 43 μmol) at room temperature for 100 min. Et_2O (2.0 mL) and saturated aqueous $CuSO_4$ solution (1.0 mL) were added and the resulting mixture was stirred vigorously for 1 min. After aqueous solution was removed by pipetting, $MgSO_4$ was added. The mixture was filtered and concentrated in vacuo. Purification with silica gel column chromatography (AcOEt/benzene=20:80) gave dibenzoate **1a** (3.0 mg) and monobenzoate **1b** (3.0 mg) both as oil. The amounts of **1a** and **1b** were estimated based on UV absorption (ε 15,000, 230 nm) for a benzoyl group.

*4.5.1. Physical data of **1a**.* ^{1}H NMR ($CDCl_3$) δ 0.74, 0.84, 0.97 (each 3H, s), 1.55 (3H, d, *J*=6.5 Hz), 1.96 (2H, m), 2.24 (1H, dd, *J*=2.4, 16.2 Hz), 2.30 (1H, dd, *J*=10.6, 16.2 Hz), 2.39 (1H, ddd, *J*=2.4, 4.2, 13.0 Hz), 3.43 (1H, s), 3.44 (1H, t, *J*=2.8 Hz), 4.32 (1H, br s), 4.79 (1H, br s), 4.88 (1H, dd, *J*=1.9, 16.5 Hz), 4.98 (1H, dd, *J*=1.9, 16.5 Hz), 5.49 (1H, d, *J*=3.4 Hz), 5.77 (1H, dq, *J*=3.4, 6.5 Hz), 6.54 (1H, t, *J*=1.9 Hz), 7.45, 7.50 (each 2H, t, *J*=7.8 Hz), 7.58, 7.63 (each 1H, t, *J*=7.8 Hz), 8.02, 8.14 (each 2H, dd, *J*=1.0, 7.8 Hz). ESIMS (rel int. %) *m*/*z*=707.2814 (10, $C_{40}H_{44}NaO_{10}$ $[M+Na]^+$: 707.2832), 702.3248 (100, calcd for $C_{40}H_{48}NO_{10}$ $[M+NH_4]^+$: 702.3278), 667.2872 (10, $C_{40}H_{43}O_9$ $[M-OH]^+$: 667.2907).

*4.5.2. Physical data of **1b**.* ^{1}H NMR ($CDCl_3$) δ 0.74, 0.84, 0.97 (each 3H, s), 1.40 (3H, d, *J*=6.6 Hz), 1.92 (1H, ddd, *J*=2.1, 4.2, 14.1 Hz), 2.00 (2H, m), 2.28 (1H, dd, *J*=2.6, 16.1 Hz), 2.33 (1H, dd, *J*=10.4, 16.1 Hz), 2.40 (1H, ddd, *J*=2.0, 3.7, 13.0 Hz), 3.44 (1H, t, *J*=2.7 Hz), 3.50 (1H, s), 4.35 (1H, br s), 4.46 (1H, br dq, *J*=3.2, 6.6 Hz), 4.80 (1H, br s), 4.90 (1H, dd, *J*=1.9, 16.6 Hz), 5.14 (1H, dd, *J*=1.9, 16.6 Hz), 5.28 (1H, d, *J*=3.2 Hz), 6.25 (1H, t, *J*=1.9 Hz), 7.49 (2H, t, *J*=7.8 Hz), 7.63 (1H, t, *J*=7.8 Hz), 8.11 (2H, dd, *J*=1.0, 7.8 Hz). ESIMS (rel int. %) *m*/*z*=563.2624 (30, $C_{33}H_{39}O_8$ $[M-OH^-]^+$: 563.2645), 598.2991 (100, $C_{33}H_{44}NO_9$ $[M+NH_4]^+$: 598.3016), 603.2562 (10, $C_{33}H_{40}NaO_9$ $[M+Na]^+$: 603.2570).

4.6. Chiral investigation of the C1″–C4″ moiety of 3

A solution of **3** (0.5 mg) in MeOH was stirred with NaOMe (0.2 mg) at room temperature. After consumption of the starting **3** based on TLC analysis, acetic acid (1.0 μL) was added to neutralize the solution. The mixture was sampled (0.1 mL) and diluted with MeOH, which was subjected to gas chromatography equipped with

Rt-γDEXsa® (RESTEC, 0.25 mm×30 m, conditions: 40–230 °C (2 °C/min), injection temperature: 230 °C). The signal was monitored with an MS detector.

4.7. Inhibition assay against *C. miyabeanus*

A series of suspension of spores of *C. miyabeanus* and 0.1% sucrose containing 0.01, 0.1, 10, 100, 1000 μg/mL of samples in Petri dish were prepared and incubated at 25 °C for 24 h. These were observed under the microscope.

4.8. Proliferation assay

The effect of compound **1**, **2**, and **3** on human colon adenocarcinoma (COLO 201) cell proliferation was measured by WST-1 assay.[30] COLO 201 cells were cultured in RPMI medium (5×10^3 cells/well) containing compounds **1**, **2**, and **3** in 96-well tissue culture plates with 0.01, 0.1, 10, 100, 1000 μg/mL concentrations. After 24 h, 10 μL of WST-1 reagent was added to each well and the absorbance measured at 450 nm using a titer-plate reader.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2013.12.087.

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