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ORIGINAL ARTICLE

Trehangelins A, B and C, novel photo-oxidative hemolysis inhibitors produced by an endophytic actinomycete, *Polymorphospora rubra* K07-0510

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Three new natural products, designated trehangelins A, B and C, were isolated by solvent extraction, silica gel and octadecylsilyl silica gel column chromatographies and subsequent preparative HPLC from the cultured broth of an endophytic actinomycete strain, *Polymorphospora rubra* K07-0510. The trehangelins consisted of a trehalose moiety and two angelic acid moieties. Trehangelins A (IC_{50} value, 0.1 mg ml^{-1}) and C (IC_{50} value, 0.4 mg ml^{-1}), with symmetric structures, showed potent inhibitory activity against hemolysis of red blood cells induced by light-activated pheophorbide *a*. However, trehangelin B, with an asymmetric structure, displayed only a slight inhibition (IC_{50} value, 1.0 mg ml^{-1}).
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Keywords: chemical screening; endophytic actinomycete; hemolysis inhibitor; *Polymorphospora rubra*; trehangelins

INTRODUCTION

We have isolated many rare actinomycete strains from the roots of plants over the past few years and discovered a new genus and several new species of actinomycetes.[1–5] To enable the discovery of new compounds from the cultured broth of the endophytic actinomycete isolates, we have exploited biological screening programs, looking for antibacterial, antifungal, cytotoxic and antiprotozoal activities, as well as a chemical screening approach using LC/UV and a LC/MS/MS system. During the course of chemical screening, new compounds possessing antitrypanosomal activity, namely spoxazomicins A–C, were discovered using Dragendorff's reagent from the cultured broth of *Streptosporangium oxazolinicum* K07-0460^T.[6] Further research using LC/UV and LC/MS has identified compounds with unique UV and MS spectra, isolated from the cultured broth of an endophytic actinomycete strain, *Polymorphospora rubra* K07-0510. The new compounds, designated trehangelins A (**1**), B (**2**) and C (**3**), were isolated and the structures were elucidated (Figure 1). The trehangelins showed potent inhibitory activity against hemolysis of red blood cells (RBCs) induced by light-activated pheophorbide *a*.

This report details the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties, structural elucidation and some biological activities of the compounds **1**, **2** and **3**.

RESULTS

Taxonomy of the producing strain, *Polymorphospora rubra* K07-0510

Strain K07-0510 was isolated from the roots of a plant, a variety of orchid, collected on Iriomote Island, Okinawa, Japan. The strain grew well on yeast extract–malt extract agar, inorganic salts–starch agar and tyrosine agar but did not grow on oatmeal agar, glucose–nitrate agar or glucose–peptone agar. The colony color was reddish orange. Aerial mycelia were produced on GPM agar (glucose 1.0%, peptone 0.5%, meat extract 0.5%, NaCl 0.3%, agar 1.2%, pH 7.0) of the test media, and the aerial mass color was white. Short spore chains were formed, and the spores with the smooth surface were cylindrical in shape, 0.7 ~ 1.1 μm long × 0.5 ~ 1.0 μm wide in size (Figure 2). No soluble pigment was produced. Whole-cell hydrolysates contained *meso*-DAP (diaminopimelic acid). The predominant menaquinones of the strain were MK-10 (H_6) and MK-9 (H_6), whereas MK-10 (H_8), MK-10 (H_4), MK-9 (H_8) and MK-9 (H_4) were detected as minor menaquinones. The 16S rRNA gene sequence (1200 nucleotides) was determined and analyzed using the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net/). The result indicated that the strain K07-0510 is closest to *Polymorphospora rubra* TT97-42^T (AB223089, similarity; 99.9%), which is the only valid species in the genus *Polymorphospora*.[7] On the basis of the morphological and cultural

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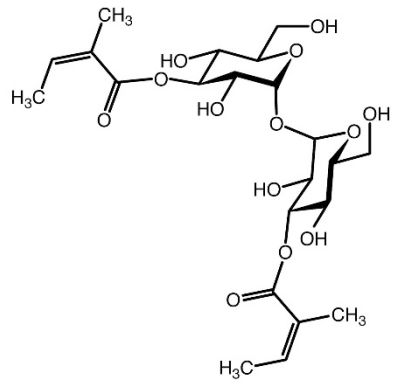
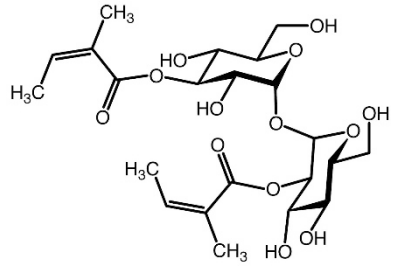
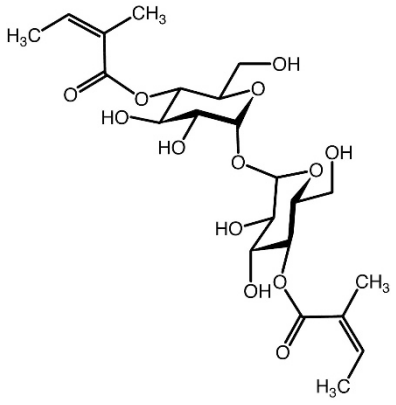


Trehangelin A (**1**)



Trehangelin B (**2**)



Trehangelin C (**3**)

Figure 1 Structures of trehangelins A, B and C isolated from a cultured broth of strain K07-0510.

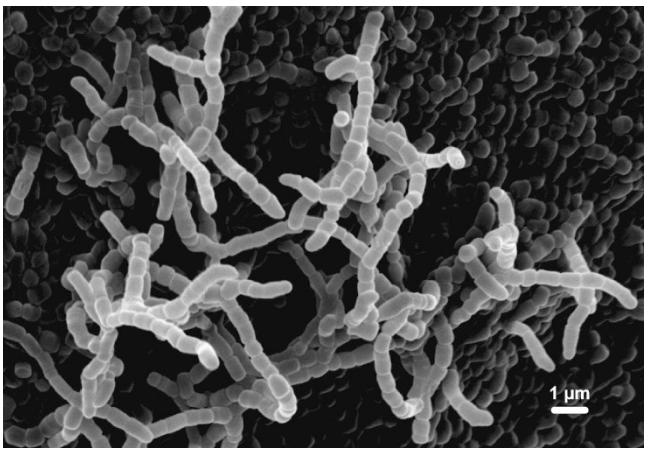
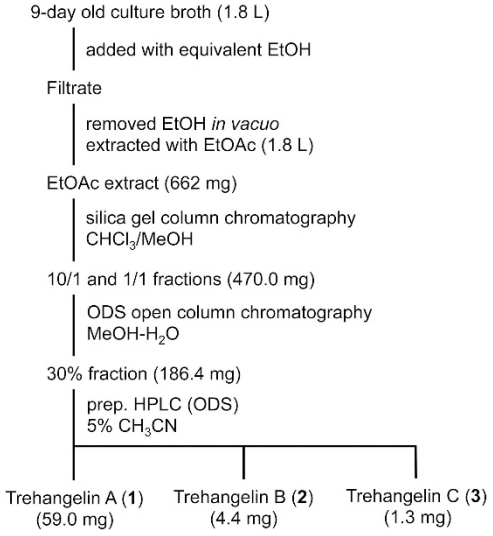


Figure 2 Scanning electron micrograph of strain K07-0510 grown on GPM agar at 27 °C for 2 weeks.



Scheme 1 Purification of trehangelins A (**1**), B (**2**) and C (**3**).

properties and 16S rRNA gene sequence similarity, strain K07-0510 is identified as *P. rubra*.

Isolation of the bioactive compounds

The procedure for isolation of compounds **1**, **2** and **3** is summarized in Scheme 1. The 9-day old cultured broth (1.8 l) was added to an equal amount of ethanol and then filtered. The filtrate was concentrated under reduced pressure to remove ethanol and the aqueous solution was extracted three times with ethyl acetate (total volume of 1.8 l). The organic layer was concentrated under reduced pressure to yield a crude material (662 mg). The ethyl acetate extract was applied to silica gel column chromatography that was eluted with each 500 ml of a mixture of $CHCl_3$–MeOH (100:0, 100:1, 50:1, 10:1, 1:1, 0:100) in this order. The $CHCl_3$–MeOH (10:1 and 1:1) fractions (470.0 mg) were applied to an octadecylsilyl silica gel (ODS) column chromatography, which was eluted with 100 ml each of 20, 30 and 100% MeOH. Trehangelins B (**2**, r.t. = 16 min, 4.4 mg), C (**3**, r.t. = 17 min, 1.3 mg) and A (**1**, r.t. = 18 min, 59.0 mg) were isolated, each as a yellowish white powder, by HPLC (Inertsil ODS-4, 10 i.d. × 250 mm) with 5% CH_3CN aq. (flow rate, 5.0 ml min^{-1}; detection, UV 254 nm) from the crude mixture of 30% MeOH eluate in ODS column chromatography (186.4 mg).

Physico-chemical properties of trehangelins A (1), B (2) and C (3)

The physico-chemical properties of compounds **1**, **2** and **3** are summarized in Table 1. They are readily soluble in H_2O and MeOH. Compounds **1**, **2** and **3** showed absorption maxima at 216–218 nm in UV spectra. The IR absorption at 1700 cm^{-1} suggested the presence of carbonyl groups. The similarity in physico-chemical properties strongly suggested that these three compounds are structurally related.

Structure elucidation of compound 1

The molecular formula of **1** was elucidated by ESI-MS to be $C_{22}H_{34}O_{13}$, requiring six degrees of unsaturation. The ^{1}H and ^{13}C

NMR spectra data measured in CD_3OD of **1** are listed in Table 2. The ^{13}C NMR and HSQC spectra indicated only 11 carbons, half the expected number, implying a symmetric structure, which were classified into one ester carbonyl carbon (δ_C 169.6), one sp^2 quaternary carbon, one sp^2 methine carbon, five oxygenated sp^3 methine carbons, including one sp^3 anomeric carbon (δ_C 95.1), one oxygenated sp^3 methylene carbon and two olefinic methyl carbons. In ^1H–^1H COSY experiments, the spin systems were from H-1 to H-5 and from 8-CH_3 to 9-CH_3 (Figure 3). The HMBC correlations from H-1 (δ_H 5.21) to C-3 (δ_C 76.5) and C-5 (δ_C 73.9), from H-2 (δ_H 3.71) to C-4 (δ_C 70.0) and from H-3 (δ_H 5.48) to C-5 indicated the linkage between C-1 and C-5 through an oxygen atom to form a tetrahydropyran ring. The HMBC correlations from H_2-6 (δ_H 3.73 and 3.80) to C-4 and from H-4 (δ_H 3.56) to C-6 (δ_C 62.2) indicated that the oxygenated sp^3 methylene carbon in 6-position was attached to C-5 to form a carbohydrate moiety. A small vicinal coupling constant was observed between H-1 and H-2 ($J = 3.6$ Hz), and large coupling constants were observed between H-2 and H-3 ($J = 10.0$ Hz), between H-3 and H-4 ($J = 9.6$ Hz) and between H-4 and H-5 ($J = 10.0$ Hz). Moreover, the ROESY correlations were observed for H-2/H-4 and H-3/H-5 (Figure 4). These results indicated that the carbohydrate moiety of **1** is a α-glucopyranose. The HMBC correlations from H-1 (δ_H 5.21) to C-1′ (δ_C 95.1) indicated the linkage between C-1 and C-1′ of two glucopyranose units through an oxygen atom to form an α, α-1,1-glycoside bond, the same as trehalose. This was supported by characteristic IR absorption at 850 cm^{-1} and chemical shift of anomeric carbon (δ_C 95.1).[8]

The HMBC correlations from H-9 (δ_H 6.11) to 8-CH_3 (δ_C 20.8), from 8-CH_3 (δ_H 1.95) to C-7 (δ_C 169.9), C-8 (δ_C 129.6) and C-9 (δ_C 138.2) and from 9-CH_3 (δ_H 2.01) to C-8 and C-9 (Figure 3), together with the ROESY correlations for H-9/8-CH_3 (Figure 4) indicated that **1** had an angelic acid (*Z*-2-methyl-2-butenoic acid) moiety. Finally, the HMBC correlation from H-3 to C-7 indicated linkage between C-3 and C-7 through an oxygen atom to form an ester bond. Therefore, the structure of **1** was elucidated as a 3,3′-diangeloyl trehalose, and **1** was designated as trehangelin A (Figure 1).

Table 1 Physico-chemical properties of trehangelins A (1), B (2) and C (3)

	1	*2*	*3*
Appearance	White powder	White powder	White powder
Molecular formula	$C_{22}H_{34}O_{13}$	$C_{22}H_{34}O_{13}$	$C_{22}H_{34}O_{13}$
Molecular weight	506	506	506
ESI-MS (m/z)			
Calcd	507.2078 (for $C_{22}H_{35}O_{13}$)	507.2078 (for $C_{22}H_{35}O_{13}$)	507.2078 (for $C_{22}H_{35}O_{13}$)
Found	507.2087 $[M+H]^+$	507.2074 $[M+H]^+$	507.2074 $[M+H]^+$
UV λ_{max}(MeOH) nm	216	218	218
IR ν_{max}(KBr) cm^{-1}	2360, 1699, 1649, 1458, 1385, 1240, 1153, 1038, 997, 851	2382, 1701, 1649, 1458, 1383, 1243, 1151, 1047, 1001, 855	2378, 1701, 1649, 1455, 1385, 1236, 1153, 1045, 999, 858
$[\alpha]_D^{25}$(MeOH)	167.1° (*c* 0.1)	13.5° (*c* 0.1)	11.4° (*c* 0.1)
Solubility			
Soluble	H_2O, MeOH	H_2O, MeOH	H_2O, MeOH
Insoluble	Hexane, $CHCl_3$	Hexane, $CHCl_3$	Hexane, $CHCl_3$

Table 2 ^1H-NMR and ^{13}C-NMR data of trehangelins A (1), B (2) and C (3) in CD_3OD (400 MHz)

Position	*Trehangelin A (**1**)*		*Trehangelin B (**2**)*		*Trehangelin C (**3**)*	
	δ_C	δ_H (int., mult., *J* in Hz)	δ_C	δ_H (int., mult., *J* in Hz)	δ_C	δ_H (int., mult., *J* in Hz)
1	95.1	5.21 (1H, d, 3.6)	95.6	5.16 (1H, d, 3.6)	95.1	5.20 (1H, d, 4.0)
2	71.6	3.71 (1H, dd, 3.6, 10.0)	71.5	3.68 (1H, dd, 3.6, 10.0)	73.4	3.61 (1H, dd, 4.0, 10.0)
3	76.5	5.48 (1H, dd, 9.6, 10.0)	76.4	5.30 (1H, dd, 8.8, 10.0)	72.3	4.02 (1H, dd, 9.6, 10.0)
4	70.0	3.56 (1H, dd, 9.6, 10.0)	69.0	3.64 (1H, dd, 8.8, 10.0)	72.4	4.91 (1H, dd, 9.6, 10.0)
5	73.9	3.92 (1H, ddd, 2.4, 4.8, 10.0)	74.0	3.59 (1H, m)	72.0	4.07 (1H, ddd, 2.4, 4.8, 10.0)
6	62.2	3.73 (1H, m)	61.6	3.60 (1H, m)	62.3	3.51 (1H, m)
		3.80 (1H, dd, 2.4, 12.0)		3.67 (1H, dd, 2.4, 12.0)		3.59 (1H, dd, 2.4, 12.4)
7	169.6		169.6		168.8	
8	129.6		129.6		129.0	
9	138.2	6.11 (1H, qq, 1.2, 7.2)	138.5	6.12 (1H, qq, 1.6, 7.2)	139.5	6.15 (1H, qq, 1.2, 7.2)
8-Me	20.8	1.95 (3H, dq, 1.2, 1.2)	20.8	1.94 (3H, dq, 1.6, 1.6)	20.7	1.91 (3H, dq, 1.2, 1.2)
9-Me	16.0	2.01 (3H, qd, 1.2 , 7.2)	16.0	2.00 (3H, qd, 1.6, 7.2)	16.1	1.99 (3H, qd, 1.2, 7.2)
1′			92.8	5.37 (1H, d, 4.0)		
2′			74.3	4.76 (1H, dd, 4.0, 10.0)		
3′			72.0	4.13 (1H, dd, 8.8, 10.0)		
4′			72.1	3.44 (1H, dd, 8.8, 10.0)		
5′			73.9	3.93 (1H, ddd, 2.4, 5.2, 10.0)		
6′			62.5	3.72 (1H, dd, 5.2, 12.0)		
				3.82 (dd, 2.4, 12.0)		
7′			168.8			
8′			128.5			
9′			141.2	6.22 (1H, qq, 1.6, 7.2)		
8′-Me			20.9	1.96 (3H, dq, 1.6, 1.6)		
9′-Me			16.5	2.08 (3H, qd, 1.6, 7.2)		

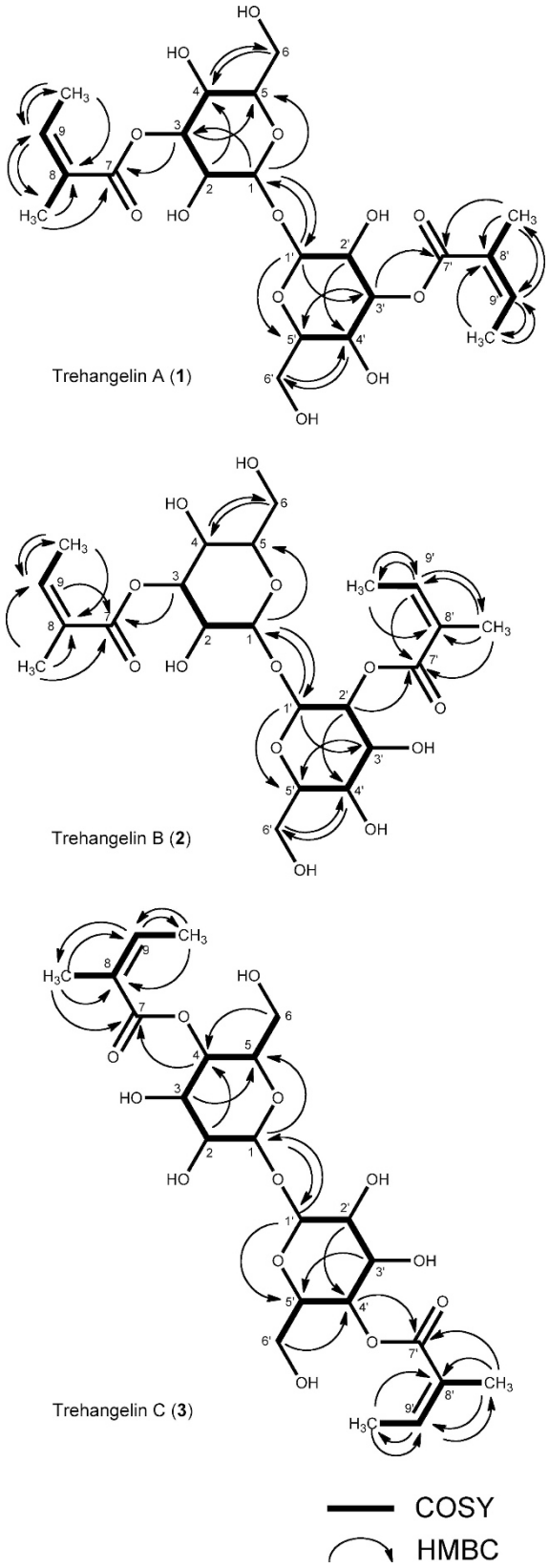


Figure 3 COSY and HMBC correlations of trehangelins A (**1**), B (**2**) and C (**3**).

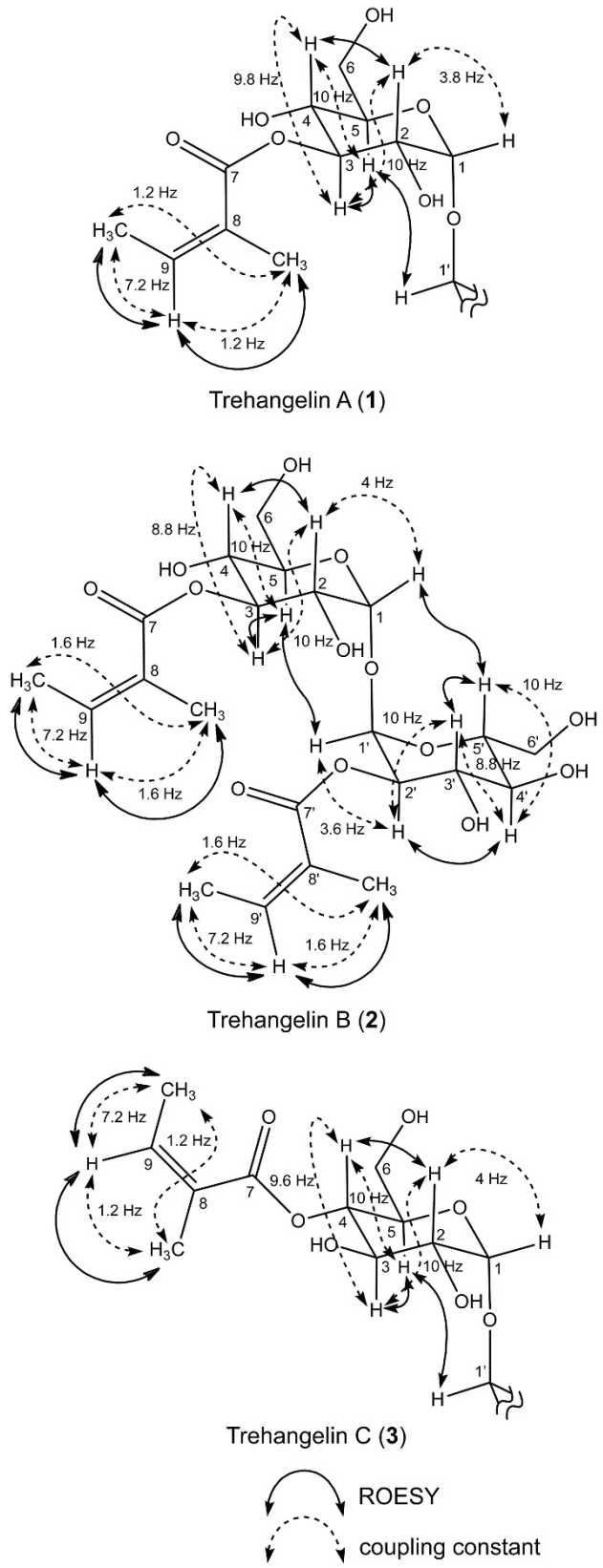


Figure 4 Relative configuration of trehangelins A (**1**), B (**2**) and C (**3**).

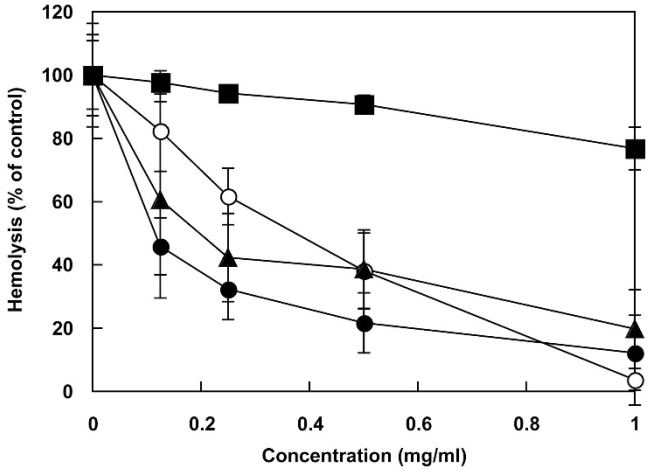


Figure 5 Inhibitory effects of trehangelins A, B and C on hemolysis of red blood cells by light-activated pheophorbide *a*. ●, trehangelin A; ■, trehangelin B; ▲, trehangelin C; ○, ascorbic acid.

The absolute stereochemistry of the trehalose moiety of **1** was determined as described in the following. The trehalose purified from hydrolysate by the treatment of hydrazine hydrate was identified as D-(+)-trehalose (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside) by ^{1}H NMR spectra; δ_H 5.15 (d, 2H, *J* 4.0 Hz), δ_H 3.85-3.72 (m, 8H), δ_H 3.61 (dd, 2H, *J* 4.0, 10.0 Hz), and δ_H 3.41 (t, 2H, *J* 9.2 Hz), ESI-MS; calcd for $C_{12}H_{22}O_{11}Na$ $[M+Na]^+$ 365.1060, found $[M+Na]^+$ 365.1034, and optical rotation; $[\alpha]_D^{27} = +170.8$ ($c=1$, H_2O).[9]

Structure elucidation of compound 2

The molecular formula of **2** was elucidated by ESI-MS to be $C_{22}H_{34}O_{13}$ and identical to that of **1**. The ^{1}H and ^{13}C NMR spectra data measured in CD_3OD of **2** are listed in Table 2 and they were similar to those of **1**. The ^{13}C NMR and HSQC spectra showed 22 carbons, which indicated that the structure of **2** was asymmetric. Although the chemical shifts at H-2 (δ_H 3.68) and H-3 (δ_H 5.30) in **2** were almost the same as the chemical shifts at H-2 (δ_H 3.71) and H-3 (δ_H 5.48) in **1**, the chemical shifts of H-2′ (δ_H 4.76) and H-3′ (δ_H 4.13) in **2** were actually different from those of **1**. The HMBC correlations from H-3 (δ_H 5.30) to C-7 (δ_C 169.6) and H-2′ (δ_H 4.76) to C-7′ (δ_C 168.8) indicated linkage of C-3 and C-7 through an oxygen atom and C-2′ and C-7′ through an oxygen atom to form ester bonds (Figure 3). Therefore, the structure of **2** was elucidated as a 3,2′-diangeloyl trehalose, and **2** was designated as trehangelin B (Figure 1).

Structure elucidation of compound 3

The molecular formula of **3** was elucidated by ESI-MS to be $C_{22}H_{34}O_{13}$ and similar to that of **1**. The ^{1}H and ^{13}C NMR spectra data measured in CD_3OD of **3** are listed in Table 2 and they were similar to those of **1**. The ^{13}C NMR and HSQC spectra identified only 11 carbons, which indicated that the structure of **3** was symmetric. However, the H-3 (δ_H 5.48) and H-4 (δ_H 3.56) in **1** were shifted at H-3 (δ_H 4.02) and H-4 (δ_H 4.91) in **3**, respectively. The HMBC correlation from H-4 (δ_H 4.91) to C-7 (δ_C 168.8) indicated linkage of C-4 and C-7 through an oxygen atom to form an ester bond (Figure 3). Therefore, the structure of **3** was elucidated as a 4,4′-diangeloyl trehalose, and **3** was designated as trehangelin C (Figure 1).

Table 3 Cytotoxic activities of trehangelins A, B and C against several human cell lines

Cell lines	*Trehangelin A*	*Trehangelin B*	*Trehangelin C*
HEK-293FT	99.5±3.4	93.0±0.9	95.7±15.6
Panc-1	100.8±9.1	86.6±1.5	100.5±0.8
NCI-H1299	95.6±11.0	93.2±3.7	96.5±1.2
HT-29	106.3±38.4	98.9±5.6	97.7±2.3

Percentage of control treated with dimethyl sulfoxide.

Biological activities

The inhibitory effects of compounds **1**, **2** and **3** on the hemolysis of RBCs induced by light-activated pheophorbide *a* were evaluated. All three compounds had no effect on RBCs under dark at a final concentration of 1 mg ml^{-1}. However, as shown in Figure 5, compounds **1**, **2** and **3** showed inhibitory activity against the hemolysis in a concentration-dependent manner, and the IC_{50} values of compound **1**, **2** and **3** were estimated to be 0.1 mg ml^{-1} (0.2 mM), >1 mg ml^{-1} (>2 mM) and 0.4 mg ml^{-1} (0.8 mM), respectively. The compounds **1** and **3**, those with a symmetric structure, showed more potent inhibitory activities than that of ascorbic acid (IC_{50} value, 0.4 mg ml^{-1} (2.3 mM)), but compound **2**, which has an asymmetric structure, showed only slight inhibitory activity. On the other hand, no significant cytotoxic activities of compounds **1**, **2** and **3** against the several cell lines were observed even at 100 μg ml^{-1} (Table 3).

DISCUSSION

A endophytic rare actinomycete stain, *P. rubra* K07-0510, was isolated from roots of an orchid. This strain produced three new compounds **1**, **2** and **3**. All the three compounds consisted of a trehalose moiety and two angelic acid moieties.

Trehalose was first found from an ergot of rye by Wiggers in 1832.[10] Trehalose is known to be one of the sources of energy in many living organisms and can be found in many organisms, including bacteria, fungi, insects, plants and invertebrates. Trehalose has protective activity against various natural stresses, such as dryness, freezing and osmopressure. Recently, it was reported that trehalose was capable of suppressing degradation of fatty acid by heat oxidation.[11] The protective effect of trehalose on heat oxidation of linoleic acid was demonstrated to be an interaction between the positions 3 and 6 of trehalose molecules and the positions 9, 10, 12 and 13 on olefin residue of the linoleic acid molecule, using NMR analysis. The results suggest that the interaction with trehalose stabilizes the olefin residue of linoleic acid against heat oxidation.

In our study, the inhibitory effects of compounds **1**, **2** and **3** on the hemolysis of RBCs induced by light-activated pheophorbide *a* were investigated. It is known that pheophorbide *a* activated with light leads to the production of a singlet oxygen and that cells suffer damage through oxidation of the cell membrane.[12,13] Compounds **1**, **2** and **3** demonstrated inhibitory activity against the hemolysis of RBCs induced by light-activated pheophorbide *a*. If this inhibitory effect of these compounds is the same as the inhibitory action of trehalose against the heat oxidation of linoleic acid, it is considered that the compound **1,** which contains two angelic acid moieties at 3 and 3′ positions of trehalose, should have decreased activity compared with compounds **2** or **3**, which have two angelic acid moieties at position 2, 3 or 4. However, compound **1** had more potent inhibitory activity than compounds **2** and **3**. Therefore, the inhibitory action of trehangelins on the hemolysis of RBCs induced by light-

activated pheophorbide *a* may occur via a different mechanism than the mode of action of trehalose on the heat oxidation of linoleic acid.

Furthermore, the symmetric compounds **1** and **3** showed more potent activities than the asymmetric compound **2**. Therefore, the positions of angelic acids bound to a trehalose molecule and the structural symmetry may be important for the hemolysis inhibition of RBCs induced by light-activated pheophorbide *a*.

Angelic acid is isolated from plants such as *Alkanna tinctoria*,[14] carrots[15] or *Euphorbia* species.[16] In addition, angelic acid esters included in Roman chamomile, a popular herb, are used as an aromatherapy essential oil. It is known that angelic acid esters are especially oriented towards mood and the psyche, with cephalic and psychotropic characteristics.[17] Thus, compounds **1**, **2** and **3**, consisting of trehalose and angelic acid, may have many other uses. In addition, we are particularly interested in knowing whether there is any physiological effects of these compounds produced by an endophytic actinomycete strain, *P. rubra* K07-0510, on the plants they are associated with. To our knowledge, this is the first report of the metabolites produced by the genus *Polymorphospora*.

METHODS

General experiments

All solvents (EtOAc, $CHCl_3$, EtOH, MeOH and CH_3CN) were purchased from Kanto Chemical (Tokyo, Japan). Silica gel and Pegasil ODS were purchased from Merck (Darmstadt, Germany) and Senshu Scientific Co. (Tokyo, Japan), respectively. The Inertsil ODS-4 column was obtained from GL Science Inc. (Tokyo, Japan).

NMR spectra were measured in CD_3OD using a Varian XL-400 spectrometer (Varian, Palo Alto, CA, USA). Chemical shifts were expressed in p.p.m. and were referenced to CD_3OD (3.31 p.p.m.) in the ^1H NMR spectra and to CD_3OD (49.0 p.p.m.) in the ^{13}C NMR spectra. ESI-MS spectra were measured on a QSTAR Elite Hybrid MS/MS spectrometer (AB Sciex Co., MA, USA). IR spectra (KBr) were taken on a Horiba FT-210 Fourier transform IR spectrometer (Horiba Ltd, Kyoto, Japan). UV spectra were measured with a Hitachi U-2810 spectrophotometer (Hitachi, Tokyo, Japan). Optical rotation was measured on a JASCO model DIP-181 polarimeter (JASCO Co., Tokyo, Japan).

Taxonomic studies

The International *Streptomyces* Project media recommended by Shirling and Gottlieb[18] and media recommended by Waksman[19] were used to investigate cultural characteristics. Cultures were observed after incubation for 3 weeks at 27 °C. The morphological characteristics were observed by scanning electron microscope (JSM-5600, JEOL, Tokyo, Japan) after incubation on GPM agar for 2 weeks at 27 °C and fixation with 4% osmium tetroxide vapor. Isomers of DAP in whole-cell hydrolysates were determined by TLC, following the standard methods of Becker *et al.*[20] and Hasegawa *et al.*[21] Menaquinones were extracted and purified by the method of Collins *et al.*,[22] then analyzed by LC/MS (JSM-T 100LP, JEOL) with a CAPCELL PAK C18 column (Shiseido, Tokyo, Japan) eluted with methanol/2-propanol (7:3). 16S rRNA gene sequence was amplified by PCR and sequenced on a DNA sequencer (Applied Biosystems 3130 Genetic Analyzer, Applied Biosystems, Foster, CA, USA) using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems), according to the manufacturer's instructions.

Fermentation

Strain K07-0510 was grown and maintained on agar slants consisting of 1.0% starch, 0.3% NZ amine, 0.1% yeast extract, 0.1% meat extract, 1.2% agar and 0.3% $CaCO_3$. A loop of spores of strain K07-0510 was inoculated into 100 ml of seed medium, consisting of 2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract and 0.4% $CaCO_3$ (adjusted to pH 7.0 before sterilization) in a 500-ml Erlenmeyer flask. The flask was incubated on a rotary shaker (210 r.p.m.) at 27 °C for 3 days. A 1-ml portion of the seed culture was transferred to 500-ml Erlenmeyer flasks (total 18 l), each containing 100 ml of production medium, consisting of 2.0% soluble starch, 0.5% glycerol, 1.0% defatted wheat germ, 0.3% Ehlrich meat extract from *Katsuwonus pelamis* (Kyokuto Pharmaceutical, Inc., Tokyo, Japan), 0.3% dry yeast, 0.3% $CaCO_3$ (adjusted to pH 7.0 before sterilization) and fermentation was carried out on a rotary shaker (210 r.p.m.) at 27 °C for 9 days.

Hydrazinolysis of trehangelin A (1)

Hydrazinolysis of the peripheral ester groups of compound **1** was performed for the determination of an absolute configuration of trehalose.[23] Compound **1** (30 mg) in MeOH (3 ml) was treated with hydrazine hydrate (3 ml) for 72 h. The reaction mixture was concentrated by evaporation under vacuum, and trehalose was separated by partition between H_2O and $CHCl_3$ (each 3 ml). Trehalose (16.5 mg) was obtained from the H_2O layer. Parts of trehalose were dissolved in D_2O for measurement of ^1H NMR spectroscopy, in H_2O for optical rotation and in MeOH for MS spectrometry.

Biological assays

The inhibitory activities of compounds **1**, **2** and **3** against photo-oxidative hemolysis of RBCs induced by light-activated pheophorbide *a* were measured *in vitro*. In addition, cytotoxic activities of compounds **1**, **2** and **3** were evaluated.

Hemolytic assay: The hemolysis of RBCs induced by light-activated pheophorbide *a* was measured by a modified method of light irradiation system as previously described.[12] Rabbit RBCs were obtained from Nippon Bio-Supply Center (Tokyo, Japan) and used within 7 days. The RBCs were washed three times with sterile phosphate-buffered saline (PBS) and made to a final concentration of 5% (v/v) in PBS. For the hemolysis inhibition assay, 20 µl aliquots of sample in PBS (at a final concentration of 0, 0.125, 0.25, 0.5, and 1 mg ml^{-1}) and the 100 µl of 5% (v/v) suspension of RBCs in PBS were added (in triplicate) to the wells of round-bottom 96-well microplates (Corning Inc., Corning, NY, USA). After incubation for 5 min at 37 °C, the reaction was initiated by the addition of 80 µl pheophorbide *a* (at a final concentration of 5 µM). The plate was illuminated for 30 min under a light from an Eye Lamp (PRS500W, Iwasaki Electric Co., Ltd, Tokyo, Japan) or placed in the dark. In addition, a clear plastic water container was put between the lamp and the plate with the RBCs to prevent any temperature increase. After illumination, the RBCs were centrifuged at 900 *g* for 10 min. A 100 µl of each supernatant was transferred to a flat-bottom microplate (Corning Inc.). Hemolysis was determined by measuring the absorbance at 570 nm, which is due to hemoglobin released from RBCs, using a microplate reader (model ELx 808, BioTek Instruments, Inc., Winooski, VT, USA).

Cell viability assay: Cell viabilities of compounds **1**, **2** and **3** were measured by Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) in four different cell lines, namely HEK-293FT (human embryonic kidney cell line), Panc-1 (human pancreatic cancer cell line), NCI-H1299 (human non-small cell lung carcinoma cell line) and HT-29 (human colon carcinoma cell line). Briefly, 2×10^4 cells were seeded in 96-well plates and cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries Ltd, Osaka, Japan) supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2. After culturing overnight, compounds **1**, **2** and **3** in dimethyl sulfoxide or dimethyl sulfoxide alone were added into each well (at final concentrations of 100 µg ml^{-1}). After 48 h of incubation at 37 °C, WST-8 solution was added to each well and incubated at 37 °C for 4 h.

After mixing the microplate, the cell viability was determined by measuring the absorbance at 450 nm. The absorbance of control cells (treated with 0.3% dimethyl sulfoxide) was considered as 100%. The rate of cell growth inhibition was calculated using the following formula: mean value of ((control group − treated group)/control group) × 100%. The effect of each concentration was determined in duplicate.

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