NOTE

3,6,7-tri-*epi*-invictolide, a diastereomer of queen recognition pheromone, and its analog from a marine-derived actinomycete

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Microorganisms are capable of producing a broad spectrum of secondary metabolites, and over the past five decades, the prokaryotic actinomycetes have been the most fruitful source of antibiotics, yielding 65–70% of all discovered antibiotics.¹ Therefore, screening of microorganisms for the production of new antibiotics continues to be an important approach in modern drug discovery programs. On the other hand, it is known that actinomycetes produce secondary metabolites of uncertain function, which led us to infer that the component analysis of culture solution can be the most efficient way to search for the undiscovered compounds of uncertain function. During our research on analyzing secondary metabolites from marine microorganisms,² we explored the metabolites from the culture solution of actinomycete strain NPS887 with characteristic peaks on LC-TOF-MS and spots on TLC (0.2 mm, Silica gel 60 F₂₅₄; Merck, Darmstadt, Germany) over time. Isolation and structure elucidation of the TLC positive metabolites resulted in the identification of two novel aliphatic δ -lactone compounds (**1**, **2**, Figure 1a). Surprisingly, the planar structure of **1** was the same as that of invictolide, queen recognition pheromone of the red imported fire ant. Invictolide, exhibiting pheromone activity in both levorotatory and the racemic forms, was isolated from the red imported fire ant queens, *Solenopsis invicta* (Buren). Its relative stereochemistry was proposed by Rocca *et al.*,³ and its absolute stereochemistry was established to have (3*R*,5*R*,6*S*,7*R*)-configuration by Mori's group.⁴ Having a great interest on the stereochemistry and biosynthetic pathway of δ -lactone compounds (**1**, **2**), microbially derived invictolide diastereomer and its analog, we focus our attention on the analysis of stereochemistry and biosynthetic origin. This report describes the isolation, structural elucidation and biosynthetic origin of tri-*epi*-invictolide and its analog.

The producing strain NPS887 was isolated from marine sediments at a depth of 28 m in the Uranouchi Bay, Kochi, Japan. The 16S rDNA sequence of the NPS887 strain (1409 base pairs) was deposited in the DDBJ Genbank (DDBJ accession number AB601427). This strain

shared 98% 16S rDNA sequence identity with *Nocardiopsis tangguensis* strain HBUM 174826. Strain NPS887 was cultured in 500 ml baffled shake flasks containing 100 ml of the production modified KG medium containing 0.8% glucose (Wako Pure Chemical Industries, Osaka, Japan), 0.8% maltose monohydrate (Wako Pure Chemical Industries), 0.8% soluble starch (Wako Pure Chemical Industries), 1.5% soytone (Becton, Dickinson and Company, Sparks, MD, USA), 0.2% yeast extract (Becton, Dickinson and Company) and 1.8% artificial seawater (Nihon Pharmaceutical, Tokyo, Japan) while shaking at 220 r.p.m., 28.5 °C for 8 days. At the end of the fermentation period, the whole culture broth (1800 ml) was extracted with equal amount of EtOAc. The EtOAc extract, which showed many spots on TLC by anisaldehyde reagent, was subjected to two chromatographic purification steps, silica gel chromatography (5 × 17 cm, Silica Gel 60; Kanto Chemical, Tokyo, Japan) with chloroform and partitioned TLC chromatography (0.5 mm, Silica gel 60 F₂₅₄; Merck) with chloroform, to afford pure samples of 3, 6, 7-tri-*epi*-invictolide (**1**, 69.1 mg) and its analog (**2**, 6.5 mg), with *R_f* values in CHCl₃ of 0.55 and 0.62, respectively.

Compounds **1** and **2** were obtained as colorless oil ($[\alpha]_D^{25} +108.6$ for **1** and $+72.0$ for **2**, *c* 0.45 in CHCl₃), and their molecular formulas were determined by HR-ESI-MS to be C₁₂H₂₂O₂ (obsd [M+H]⁺ at *m/z* 199.1673, calcd 199.1700) for **1** and C₁₅H₂₈O₂ (obsd [M+H]⁺ at *m/z* 241.2124, calcd 241.2169) for **2**.

The IR spectroscopy spectrum (KBr) showed characteristic absorptions at 2966, 2935, 2874, 1747, 1458, 1382, 1345, 1284, 1204, 1164, 1143, 1120, 1090 and 989 cm⁻¹ for **1**, and 2958, 2927, 2871, 1725, 1468, 1385, 1332, 1304, 1279, 1245, 1207, 1165, 1133, 1094, 1071 and 994 cm⁻¹ for **2**, suggesting the presence of an aliphatic ester. These physicochemical properties and ¹H, ¹³C NMR spectra of **1** and **2** were similar to those of tri-substituted δ -lactone compounds. The ¹H and ¹³C NMR spectral data for **1** and **2** are listed in Table 1.

The ¹³C NMR spectrum of **1** showed signals for 12 carbons, whereas ¹H NMR and edited gs-HSQC⁵ spectral data indicated the presence of

Figure 1 (a) Structure of tri-*epi*-invictolide and its analog. (b) Key correlations COSY (bold line) and HMBC (arrow, proton to carbon) of **1** and **2**. (c) NOE observation for **1** and **2** (hollow arrowhead).

four methyl groups, three methylene carbons, four methine carbons and a quaternary carbon. One of the methine carbons was observed in low magnetic field at δ 83.0, and the others in high magnetic field at δ 28.0, 32.2 and 34.0. Detailed analysis of 2D NMR spectral data, COSY, edited gs-HSQC and gHMBC obtained from **1**, observed only one spin system, allowed an aliphatic acid or ester as shown in Figure 1b. The ^1H – ^1H correlations observed in the COSY, together with the spin coupling constants of geminal protons, revealed the presence of a sterically distorted structure such as ring-shaped. In the HMBC spectrum, the strong correlation observed from the methine proton 3-H (δ_{H} 1.89), methyl proton 3-Me (δ_{H} 1.07) and geminal methylene proton 4-H₂ (δ_{H} 0.58 and 1.68) to quaternary carbon C-2 (δ_{C} 175.1), which suggested C-2 was located next to C-3. Finally, making a comprehensive assessment of chemical analysis data as chemical shifts of C-2, C-3, and C-6, and molecular formula revealed the δ -lactone compound, same planar structure as invictolide.

The planar structure of **2** was also established as C-3 isoprenyl (C-1'–C-4') analog of **1** by 2D NMR correlations, a bunch of correlations from 3-H (δ_{H} 2.15) march up to 3'-H₃ (δ_{H} 0.84) and 4'-H₃ (0.82) on COSY, and HMBC correlations from 2'-H to C-3 (δ_{C} 34.9), C-1' (δ_{C} 40.5), C-3' (δ_{C} 23.2) and C-4' (δ_{C} 22.2).

The relative configurations of the stereocenters in **1** and **2** were assigned on the basis of 2D NOESY experiments. The 2D NOESY NMR experiments for **1** and **2** showed three key correlations between 3-H/6-H, 6-H/7-Me and 7-Me/5-H. These data allowed the methine protons 3-H, 6-H and methyl group 7-Me to be placed on the same face of the δ -lactone ring and the methyl groups 3-Me and 5-Me to be placed on the opposite face. Based on these data, **1** and **2** were assigned as diastereomers of invictolide and its analog, with the relative stereochemistry shown in Figure 1c.

The absolute stereochemistries of **1** and **2** were determined using the modified Mosher's method,⁶ and/or comparing $[\alpha]_{\text{D}}^{25}$ values between **1**, **2** and reported (+ or –) invictolide. Two-step chemical conversion produced a hydroxyl group at C-6, and subsequent esterification with (S)- and (R)-MTPA-Cl afforded the (R)- and (S)-MTPA esters **1a** and **1b**, respectively, as shown in Figure 2a. Analysis of ^1H NMR chemical

shift differences ($\Delta\delta_{\text{S-R}}$) between **1a** and **1b** revealed that the absolute stereochemistry of C-6 is *R* (Figure 2b). This result supports the assignment of the absolute stereochemistry at C-3 and C-7 as *S*, whereas C-5, C-6 were assigned as *R*. Specific optical rotation of levorotatory invictolide with pheromone activity was established -101° ($c=0.450$, CHCl_3) by total synthesis study. The $[\alpha]_{\text{D}}^{25}$ values of the enantiomer, dextrorotatory form, without activity were also established $+101^\circ$ ($c=0.450$, CHCl_3). Result of the modified Mosher's method and $[\alpha]_{\text{D}}^{25}$ values (**1**: $+108.6$, **2**: $+72.0$, $c=0.45$, CHCl_3) indicate that **1** and **2** have same dextrorotatory form (3*S*,5*R*,6*R*,7*S*) configuration.

The biosynthetic origins of **1** and **2** were investigated on the basis of NMR data of ^{13}C -enriched samples obtained by feeding experiment with [^{13}C] sodium propionate in the culture of a marine-derived actinomycete strain NPS887. The condition of feeding experiment for [^{13}C] sodium propionate was as follows. The NPS887 strain was cultured in 100 ml volumes of modified KG medium with 0.1% [^{13}C] sodium propionate while shaking at 220 r.p.m., 28.5°C for 8 days. At the end of the fermentation period, the culture solution was centrifuged (2320 g for 10 min), the supernatant solution was partitioned with EtOAc, and EtOAc extracts were purified by preparative TLC with CHCl_3 . Thus [^{13}C]-**1** (3.2 mg) and **2** (2.0 mg) were obtained.

Assignments of ^{13}C NMR signals and isotope incorporation results of **1** and **2** are presented in Figure 3. The ^{13}C NMR spectrum (C_6D_6) of **1** derived from [^{13}C] sodium propionate showed the significant enrichment of four carbons (C-2, C-4, C-6 and C-8). Likewise, **2** also took in [^{13}C] sodium propionate. These incorporation patterns might suggest that **1** and **2** can be constructed by the C3 units derived from propionate or methylmalonate. Further biosynthetic studies using other stable isotope-labeled precursors and different culture conditions are now in progress.

The compound **1** was evaluated for activity in the *Aspergillus niger* ATCC 8740, *Bacillus subtilis* ATCC 43223, *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 10536, *Mycobacterium phlei* ATCC 11758, *Pseudomonas aeruginosa* ATCC 9027, *Saccharomyces cerevisiae* ATCC 9763, *Salmonella typhimurium* ATCC 13311, methicillin-resistant *Staphylococcus aureus* ATCC33591 and *Trichophyton rubrum*

Table 1 NMR spectral data for 1 and 2 in benzene-*d*₆

1						2					
C/H n	δ_C , mult ^{a,b}	δ_H , mult (<i>J</i> in Hz; integration) ^{c,d}	COSY	HMBC	NOESY	C/H n	δ_C , mult ^{a,b}	δ_H , mult (<i>J</i> in Hz; integration) ^{c,d}	COSY	HMBC	NOESY
1						1					
2	175.1, qC					2	174.4, qC				
3	32.2, CH	1.89, ddq, (8.9, 6.8, 6.5; 1H)	4a/b, 3-Me	2, 4, 5	6	3	34.9, CH	2.15, dddd (8.8, 7.0, 6.8, 6.3; 1H)	1', 4a/b	2, 4	6
3-Me	16.4, CH ₃	1.07, d (6.5; 3H)	3	2, 3, 4							
4 ^{a,b}	35.8, CH ₂	0.58, ddd (13.5, 8.5, 6.8; 1H)	3, 4b, 5	2, 3, 5, 3-Me		4 ^{a,b}	33.9, CH ₂	0.61, ddd (13.6, 8.5, 6.8; 1H)	3, 5, 4b	2, 3, 5, 5-Me	
		1.68, ddd (13.5, 9.7, 8.9; 1H)	3, 4a, 5	2, 3, 5, 3-Me				1.83, ddd (13.6, 9.9, 8.8; 1H)	3, 5, 4x	2	
5	28.0, CH	1.59, dddd (9.7, 8.5, 2.7, 6.8; 1H)	4a/b, 6, 5-Me	3, 4	7-Me	5	27.9, CH	1.66, dddd (9.9, 8.5, 2.5, 6.8; 1H)	4, 6		7-Me
5-Me	14.8, CH ₃	0.55, d (6.8; 3H)	5	4, 3, 6		5-Me	14.9, CH ₃	0.63, d (6.8; 3H)	5	4, 5, 6	
6	83.0, CH	3.34, dd (10.4, 2.7; 1H)	5, 7	7, 7-Me,	3, 7-Me	6	82.6, CH	3.44, dd (10.1, 2.5; 1H)	5, 7	7, 7-Me	3, 7-Me
7	34.0, CH	1.62, ddq (10.4, 3.5, 7.0; 1H)	6, 8a, 7-Me			7	33.8, CH	1.63, ddq (10.1, 3.3, 6.8; 1H)	6, 8a, 7-Me		
7-Me	14.9, CH ₃	0.60, d (7.0; 3H)	7	6, 7, 8	5, 6, 9	7-Me	14.6, CH ₃	0.56, d (6.8; 3H)	7	6, 7, 8	5, 6, 9
8 ^{a,b}	35.9, CH ₂	1.01, dddd (13.4, 5.0, 4.9, 3.5; 1H)	7, 8b, 9a/b	7, 9, 10		8 ^{a,b}	35.7, CH ₂	0.98, dddd (13.4, 4.9, 4.8, 3.3; 1H)	7, 8b, 9a/b	7, 9, 10	
		1.84, dd (13.4, 8.9; 1H)	8a, 9a	7, 9, 10				1.86, dd (13.4, 8.9; 1H)	8a, 9a	7, 9, 10	
9 ^{a,b}	20.3, CH ₂	1.18, dddd (13.1, 8.9, 4.9, 7.3; 1H)	8a/b, 9b, 10	8, 10		9 ^{a,b}	20.1, CH ₂	1.17, dddd (13.1, 8.9, 4.8, 7.2; 1H)	8a/b, 9b, 10	10	
		1.35, ddq (13.1, 5.0, 7.3; 1H)	8a, 9a, 10	8, 10				1.35, ddq (13.1, 4.9, 7.2; 1H)	8a, 9a, 10	10	
10	14.7, CH ₃	0.90, t (7.3; 3H)	9a/b			10	14.5, CH ₃	0.88, d (7.2; 3H)	9a, 9b	8, 9	
						1 ^{a,b}	40.5, CH ₂	1.11, ddd (13.9, 7.9, 5.1; 1H)	3, 2', 1 β	2, 3, 4, 2', 3', 4'	
								1.92, ddd (13.9, 6.4, 8.0; 1H)	3, 2', 1 α	2, 3, 4, 2', 3', 4'	
						2'	25.4, CH	1.70, dddd (8.0, 6.8, 6.4, 5.1; 1H)	1', 3', 4'	3, 1', 3', 4'	
						3'	23.2, CH ₃	0.84, d (6.8; 3H)	2'	1', 2', 4'	
						4'	22.2, CH ₃	0.82, d (6.4; 3H)	2'	1', 2', 3'	

^a100 MHz.^bAssignments by edited gs-HSQC experiments.^c400 MHz.^dCoupling constants of multiple line were obtained by double homodecoupling experiments.

Figure 2 (a) Chemical conversion for Mosher ester analysis (b) $\Delta\delta_{S-R}$ values for the Mosher esters.

Figure 3 ^{13}C -labeling patterns of **1** and **2** resulting from feeding experiments with ^{13}C -labeled propionate. (a) ^{13}C -labeled **1**. (b) Non-labeled **1**. (c) ^{13}C -labeled **2**. (d) Non-labeled **2**.

ATCC 10218 microbial assays at concentrations that range from 50 to $0.39\ \mu\text{M}$. Unfortunately, no significant responses were noted at the concentrations tested. Additionally, the *in vitro* cytotoxic activity of **1** and **2** were assessed against HeLa cells (Human epithelial carcinoma cell line), and they showed no cytotoxicity in the HeLa cells exposed at $100\ \mu\text{M}$ for 48 h.

(R)-MTPA ester (**1a**) ^1H -NMR (400 MHz, CDCl_3): δ 0.78 (3H, d, $J=7.2\ \text{Hz}$, 10- H_3), 0.85 (3H, d, $J=6.8\ \text{Hz}$, 7-Me), 0.86 (3H, d,

$J=6.8\ \text{Hz}$, 5-Me), 0.91 (3H, d, $J=6.8\ \text{Hz}$, 3-Me), 0.97 (1H, m, 4- H_b), 0.99 (1H, m, 8- H_b), 1.10 (1H, m, 9- H_b), 1.20 (1H, m, 8- H_a), 1.26 (1H, m, 4- H_a), 1.36 (1H, m, 9- H_a), 1.77 (1H, m, 7-H), 1.91 (1H, m, 3-H), 1.93 (1H, m, 5-H), 3.80 (1H, dd, $J=11.0, 4.4\ \text{Hz}$, 2- H_b), 3.91 (1H, dd, $J=11.0, 4.4\ \text{Hz}$, 2- H_a), 4.88 (1H, dd, $J=8.4, 3.2\ \text{Hz}$, 6-H), 1.20 (9H, s, OPiv-Me $_3$), 7.40–7.59 (5H, m, MTPA-Ph), 3.53 (3H, s, MTPA-OMe).

(S)-MTPA ester (**1b**) ^1H -NMR (400 MHz, CDCl_3): δ 0.80 (3H, d, $J=7.2\ \text{Hz}$, 10- H_3), 0.84 (3H, d, $J=6.8\ \text{Hz}$, 5-Me), 0.87 (3H, d,

$J=6.8$ Hz, 7-Me), 0.90 (3H, d, $J=7.8$ Hz, 3-Me), 0.95 (1H, m, 4-H_b), 0.99 (1H, m, 8-H_b), 1.12 (1H, m, 8-H_b), 1.25 (1H, m, 8-H_a), 1.26 (1H, m, 4-H_a), 1.38 (1H, m, 9-H_a), 1.79 (1H, m, 7-H), 1.89 (1H, m, 3-H), 1.92 (1H, m, 5-H), 3.79 (1H, dd, $J=11.0, 4.4$ Hz, 2-H_b), 3.91 (1H, dd, $J=11.0, 4.4$ Hz, 2-H_a), 4.88 (1H, dd, $J=8.4, 3.2$ Hz, 6-H), 1.21 (9H, s, OPiv-Me₃), 7.40–7.60 (5H, MTPA-Ph), 3.54 (3H, s, MTPA-OMe).

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- 1 Berdy, J. Bioactive Microbial Metabolites. *J. Antibiot.* **58**, 1–26 (2005).
- 2 Sato, S. *et al.* Indoxamycins A–F. Cytotoxic tricyclic polypropionates from a marine-derived actinomycete. *J. Org. Chem.* **74**, 5502–5509 (2009).
- 3 Rocca, J. R., Tumlinson, J. H., Glancey, M. B. & Lofgren, C. S. Synthesis and stereochemistry of tetrahydro-3,5-dimethyl-6-(1-methylbutyl)-2H-pyran-2-one, a component of the queen recognition pheromone of *Solenopsis invicta*. *Tetrahedron Lett.* **24**, 1893–1896 (1983).
- 4 Mori, K. & Nakazono, Y. Synthesis of both the enantiomers invictolide, a pheromone component of the red imported fire ant. *Tetrahedron* **42**, 6459–6464 (1986).
- 5 Willker, W., Leibfritz, D., Kerssebaum, R. & Bermel, W. Gradient selection in inverse heteronuclear correlation spectroscopy. *Magn. Reson. Chem.* **31**, 287–292 (1993).
- 6 Otani, I., Kusumi, T., Kashman, Y. & Kakisawa, H. High-field FT NMR application of Mosher's method. The absolute configurations of marine terpenoids. *J. Am. Chem. Soc.* **113**, 4092–4096 (1991).