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BRIEF COMMUNICATION

Umezawamides, new bioactive polycyclic tetramate macrolactams isolated from a combined-culture of *Umezawaea* sp. and mycolic acid-containing bacterium

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Abstract

New polycyclic tetramate macrolactams, Umezawamides A (**1**) and B (**2**) were isolated from a combined-culture of *Umezawaea* sp. RD066910 and mycolic-acid containing bacterium *Tsukamurella pulmonis* TP-B0596. Their planar structures and partial stereochemistries were determined based on the spectroscopic analysis, MMFF conformational search, and ECD calculations. Umezawamides are the first secondary metabolites isolated from the genus *Umezawaea* and they exhibited cytotoxicities to P388 murine leukemia cells. Furthermore, umezawamide A (**1**) showed growth inhibitory activity against *Candida albicans*.

Actinomycetes have been regarded as one of the most promising resource for drug seeds and other bioactive compounds. Although *Streptomyces* species are major producer of actinomycetical antibiotics, some rare-actinomycete species (= non-*Streptomyces*) also served as producers of bioactive secondary metabolites [1–3]. In fact, recent actinobacterial genome analyses showed that not only *Streptomycetales* but also certain orders of rare-actinomycetes, including *Pseudonocardiales, Streptosporangiales*, and *Micromonosporales*, are gifted with natural product biosynthetic gene clusters (NPGCs) [4]. Furthermore, it was also reported that there are few overlaps of NPGC contents among phylogenetically distant actinobacteria [4]. Thus, rare-actinomycetes have the significant potential to produce novel bioactive compounds, while the large parts of their NPGCs are not expressed under standard culture conditions. Co-culture is one of the most important method for activating cryptic NPGCs present in bacterial strains [5, 6].

The genus *Umezawaea*, belonging to *Pseudonocardiales*, was recently proposed by Labeda and Kroppenstedt based on the 16S rRNA analysis [7]. Thus far, there were a small number of researches on strain identification, phenotype, and chemo-taxonomies of the genus *Umezawaea* [7, 8], while any secondary metabolites have not been reported from them. In this study, we focused on the metabolic profile of *Umezawaea* sp. RD066910 (purchased from National Institute of Technology and Evaluation, NITE) and isolated two new bioactive polycyclic tetramate macrolactams (PTMs), named umezawamides A (**1**) and B (**2**). To activate the secondary metabolite production in the producing strain, we applied co-culture strategy using mycolic-acid containing bacterium (MACB). We previously reported that a co-culture with MACB efficiently induces secondary metabolite production in broad-spectrum of actinomycetes, and named the method "combined-culture" [9–12]. Intriguingly, the production of **1** and **2** was only observed when *Umezawaea* sp. was combined-cultured with the MACB strain *Tsukamurella pulmonis* TP-B0596 [9]. Herein, we reported the isolation, structural elucidation, and biological evaluation of **1** and **2**.

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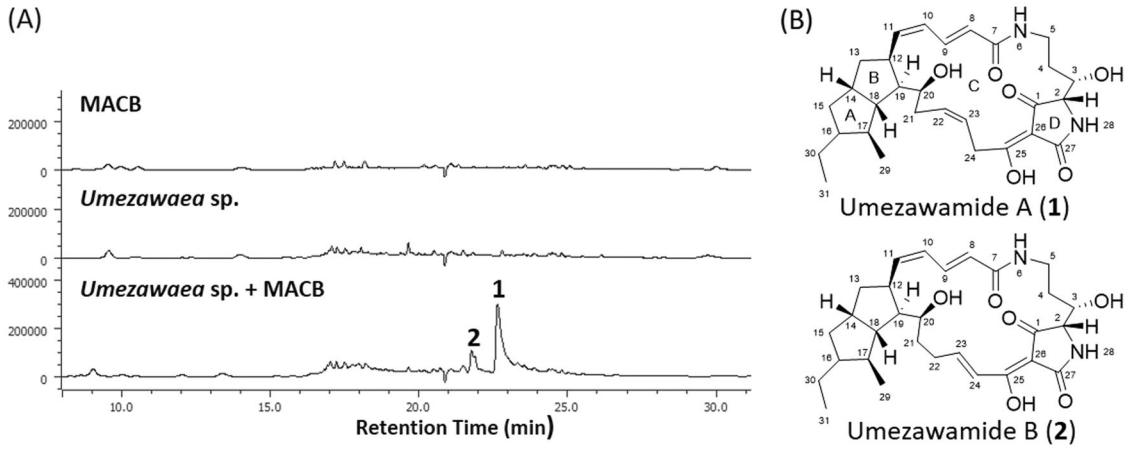

Fig. 1 **a** HPLC profiles of MACB (*T. pulmonis*) pure culture (Top), *Umezawaea* sp. pure culture (middle), and combined-culture of both strains (bottom), monitored by UV absorption at 280 nm. **b** Chemical structures and absolute stereochemistry of **1** and **2** except for C-16 position

The producing strain *Umezawaea* sp. RD066910 and MACB strain *T. pulmonis* TP-B0596 were cultured on the ISP-2 agar plate at 30 °C for 1–2 weeks. Then, each strain was inoculated into 500-ml baffled flask containing V-22 medium [9] (100 ml), and cultured on a rotary shaker at 30 °C for 3 days (*Umezawaea*)/2 days (*Tsukamurella*). The seed cultures (*Umezawaea*: 3 ml, *Tsukamurella*: 0.3 ml) were simultaneously inoculated into 500-ml baffled flask containing 100 ml of A-3M medium [9], and we continued cultivation in the same condition for 5 days. The culture broth (10 ml) was collected and extracted with a 1:1 mixture of MeOH–$CHCl_3$ (10 ml) after lyophilization. The extract was concentrated under reduced pressure, dissolved with MeOH–$CHCl_3$ (0.5 ml), and subjected to HPLC analysis along with the crude extracts from pure culture as negative controls. As shown in Fig. 1a, the production of **1** and **2** was observed only when *Umezawaea* sp. was combined-cultured (Fig. 1a). To determine the structures of **1** and **2**, we collected the mycelium from combined-culture (2.0 L) by centrifugation. After lyophilizing, the mycelium was extracted with methanol (800 ml), and concentrated under reduced pressure. The crude extract (2.4 g) was subjected to flash silica-gel column chromatography (MeOH–$CHCl_3$ = 0:1, 1:9, 1:4, 1:1, and 1:0 (v/v)). The fourth fraction (MeOH–$CHCl_3$ = 1:1) was further purified by a reversed-phase HPLC equipped with YMC-Triart C18 column (10 × 250 mm, YMC Co. Ltd.) to yield 7.8 mg of **1** as a white powder. Although we obtained lesser amount of **2** from the mycelium extract, we found that **1** was immediately and quantitatively converted to **2** in d_6-DMSO solution (Fig. S26). For structural elucidation and biological evaluation of **2**, the half quantity of **1** was incubated in d_6-DMSO at room temperature for 3 days and converted to **2**.

Initially, we conducted the structural elucidation of **2** because it was more stable than **1** in the all NMR solvents that we tested. The molecular formula of **2** was deduced to be $C_{29}H_{40}N_2O_6$ based on the HRTOFMS data (observed $[M-H]^-$ at *m/z* 511.2828), indicating 11 degrees of unsaturation. ^{13}C NMR data also showed 29 resonances, including four carbonyl/enol carbons (δ_C 193.4, 176.2, 174.3, 167.2), and six olefinic carbons (δ_C 150.1-121.1), explaining the 7 degrees of unsaturation, and **2** has a tetracyclic structure (Table 1). In addition, ^1H NMR spectra in d_6-DMSO showed the presence of three exchangeable protons (Table 1) corresponding to two amide NHs (δ_H 8.88, 8.15), and one hydroxyl group (δ_H 5.03). ^1H, ^{13}C, HSQC, and DQF-COSY analyses of **2** clearly indicated the presence of three isolated spin systems (Fig. 2a, fragments I–III), while the connections between C2/C3 and C16/C17 could not be confirmed because of signal overlaps. To clarify the uncovered connections, we performed HMBC analysis of **2** (Fig. 2a). Initially, HMBC correlations of H2/C3, H2/C4, and H29/C16 clearly indicated connections between C2/C3 and C16/C17, which were not confirmed by DQF-COSY analysis. HMBC analysis of **2** also revealed the position of four carbonyl/enol carbons (C1, C7, C25, and C27). HMBC correlations of H5/C7, H6/C7, H8/C7, and H9/C7 constructed unsaturated amide moiety from N6 to C9, and correlations of H23/C25, H24/C25, and H24/C26 established C–C connections among C24/C25/C26. Furthermore, the presence of tetramic acid ring moiety (D-ring in Fig. 1a) was inferred from key HMBC correlations (H2/C1, H2/C27, and H28/C1) and comparison of chemical shifts with known PTMs [13–15]. Finally, a hydroxyl group should be attached to C20 position to satisfy the molecular formula and chemical shifts at C20 position (δ_H/δ_C 3.33/73.5) although the corresponding -OH signal was not observed in ^1H NMR of **2**. We also assigned double bond geometries of **2** to be 8*E*, 10*Z*, 23*E* based on the vicinal

Table 1 NMR data of 1 and 2 in d_4-methanol

	Umezawamide A (**1**)		Umezawamide B (**2**)	
	δ_H (multi., J in Hz)	δ_C, type of carbon	δ_H (multi., J in Hz)	δ_C, type of carbon
1	–	192.8, C[a]	–	193.4, C
2	3.93 (s)	68.8, CH[a]	4.01 (d, 2.0)	68.4, CH
3	3.97 (m)	69.7, CH	4.05 (ddd, 2.0, 2.0, 8.0)	69.8, CH
4	1.73 (m)	30.2, CH_2	1.53 (m)	30.9, CH_2
	1.40 (m)		1.43 (m)	
5	3.52 (m)	36.5, CH_2	3.61 (ddd, 3.5, 3.5, 13.0)	36.3, CH_2
	2.93 (m)		2.88 (dd, 13.0, 13.0)	
6 (NH)	7.45 (m)[a]	–	8.15 (dd, 6.0, 6.0)[c]	–
7	–	167.7, C	–	167.2, C
8	5.95 (d, 15.0)	124.5, CH	5.99 (d, 15.0)	124.3, CH
9	7.42 (dd, 11.5, 15.0)	134.7, CH	7.42 (dd, 12.0, 15.0)	134.3, CH
10	6.13 (dd, 11.5, 11.5)	125.8, CH	6.15 (dd, 12.0, 12.0)	125.1, CH
11	5.86 (dd, 11.5, 11.5)	140.5, CH	5.86 (dd, 12.0, 12.0)	140.0, CH
12	3.34 (m)	43.8, CH	3.40 (m)	44.7, CH
13	1.75 (m)	40.6, CH_2	1.73 (dd, 8.0, 13.0)	40.2, CH_2
	1.57 (m)		1.62 (m)	
14	2.64 (m)	41.9, CH	2.72 (m)	42.1, CH
15	2.09 (m)	40.2, CH_2	2.14 (m)	40.0, CH_2
	0.86 (m)		0.92 (m)	
16	1.34 (m)	53.5, CH	1.45 (m)	53.9, CH
17	1.30 (m)	48.0, CH[b]	1.37 (m)	47.5, CH[b]
18	2.11 (m)	55.8, CH	2.12 (m)	56.4, CH
19	1.93 (m)	56.1, CH	1.90 (ddd, 6.0, 9.5, 9.5)	57.1, CH
20	3.46 (ddd, 4.5, 4.5, 9.0)	72.4, CH	3.33 (m)	73.5, CH
21	2.26 (m)	33.8, CH_2	1.52 (m)	35.1, CH_2
22	5.81 (m)	129.0, CH	2.56 (m)	29.5, CH_2
			2.10 (m)	
23	5.54 (d, 11.0)	121.7, CH	7.23 (m)	150.1, CH
24	4.00 (m)[a]	32.3, CH_2[a]	6.96 (d, 16.0)	121.1, CH_2
	3.31 (m)[a]			
25	–	186.4, C[a]	–	174.3, C
26	–	100.5, C[a]	–	99.5, C
27	–	177.1, C[a]	–	176.2, C
28 (NH)	7.87 (brs)[a]	-	8.88 (s)[c]	–
29	1.08 (d, 6.0)	17.7, CH_3	1.13 (d, 6.5)	17.7, CH_3
30	1.64 (m)	26.2, CH_2	1.67 (m)	26.3, CH_2
	1.08 (m)		1.13 (m)	
31	0.93 (dd, 7.5, 7.5)	11.7, CH_3	0.92 (dd, 7.5, 7.5)	11.6, CH_3
3-OH	–	–	5.03 (brs)[c]	-

[a]Obserbed in d_6-acetone

[b]Overlapped with solvent signal and deduced from HMQC spectrum

[c]Obserbed in d_6-DMSO

coupling constants (^3J(H8, H9) = 15.0 Hz, ^3J(H10, H11) = 12.0 Hz, ^3J(H23, H24) = 16.0 Hz).

Next, we sought to decide the chemical structure of **1**. HRTOFMS data indicated that **1** has the same molecular formula to **2** (observed $[M-H]^-$ at *m/z* 511.2826), and the NMR spectra of **1** were similar to those of **2**, expect for the absence of resonances corresponding to C22-C24 positions (Table 1). On the other hand, the ^{1}H, ^{13}C, COSY, and HMQC spectra of **1** in d_4-methanol showed the signals of isolated double bond (δ_H/δ_C 5.81/129.0, 5.54/122.0) at C22/C23 position. Although the ^{1}H and ^{13}C signals for C24 methylene were absent in d_4-methanol, due to proton exchange, we confirmed the presence of C24 methylene based on the NMR data measured in d_6-aceton (δ_H/δ_C 4.00 and 3.31/32.3), and concluded that **1** is the isomer of **2** whose double bond position is shifted. In d_6-DMSO solution, the double bond between C22 and C23 in **1** should be easily isomerized into the C23/C24 olefin conjugated with tetramic acid moiety to yield **2**. We also determined the geometries of three C-C double bonds to be 8*E*, 10*Z*, 22*Z* based on the vicinal coupling constants (^3J(H8, H9) = 15.0 Hz, ^3J(H10, H11) = 11.5 Hz, ^3J(H22, H23) = 11.0 Hz).

We also performed NOESY analysis of **2** to speculate the relative configuration of the umezawamides (Fig. 2b). The NOESY correlations of H12/H19, and H17/H19 strongly suggested that H12, H17, and H19 are located on the same face of 5,5-bicyclic system (A/B ring system in Fig. 1b). On the other hand, H11, H14, H18, and Me29 were deduced to be located on the opposite face against H12, H17, and H19 based on the NOESY correlations of H11/H14, H14/H18, and H18/Me29, and large vicinal coupling constant between H11 and H12 (^3J(H11, H12) = 12.0 Hz). Therefore, except for the C16 position, the relative stereochemistries of 5,5-bicyclic system (A/B ring system in Fig. 1b) were determined to be 12*S**, 14*R**, 17*S**, 18*S**, 19*R**. Besides, as described by Shugeng Cao et al. [13], the small vicinal coupling constants between H2 and H3 (^3J(H2, H3) = 2.0 Hz) strongly suggested the relative stereochemistry of **2** to be (2*S**, 3*S**).

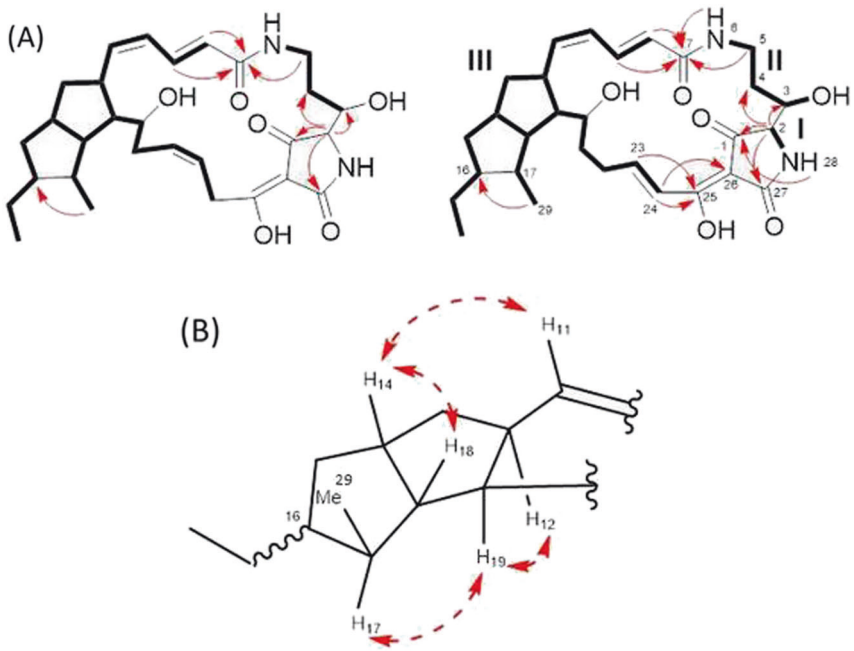

Fig. 2 **a** COSY (black bold bonds) and key HMBC (red arrows) correlations for **1** and **2** (**b**) Key NOESY correlations in A/B ring system (red dashed-arrows) for **2**

In order to make further assignment of relative stereochemistries of C-ring in **2**, we applied MMFF conformational search and theoretical electron circular dichroic (ECD) calculation. Initially, four possible stereoisomers of (2*S**, 19*S**, 20*S**), (2*S**, 19*S**, 20*R**), (2 *S**, 19 *R**, 20 *S**), and (2*S**, 19*R**, 20*R**)-**2** (as truncated structures shown in Fig. S1) were subjected to Merck Molecular Force Field (MMFF) conformational searches. The resulting low-energy conformers were compared to experimental NMR data (NOESY correlations and vicinal coupling constants), and the only (2*S**, 19*S**, 20*S**) and (2*S**, 19*R**, 20*R**)-**2** generated low-energy conformers without experimental contradictions (Figs. S2 and S3). To distinguish between two types of stereochemistries, the ECD spectrum of **2** in methanol was compared with the calculated ECD spectra of the truncate forms of (2*S*, 19*S*, 20*S*), (2*S*, 19*R*, 20*R*)-**2**, and their enantiomers (Figs. S2–S5). Finally, the only calculated ECD spectrum of (2*S*, 19*S*, 20*S*)-**2** showed good agreement with the experimental spectrum. Thus, the absolute configuration of C-ring was determined to be 2*S*, 3*S*, 12*S*, 19*S*, 20*S*, and the absolute configurations of **2** was assigned except for C-16 position. Given **2** was directly converted from **1** in d_6-DMSO, all the stereochemistries should be conserved between **1** and **2**.

Because the PTMs are a widely distributed class of natural product exhibiting various bioactivities, including cytotoxicity and antifungal activity [13–15], and we evaluated bioactivities of the isolated umezawamides. Based on the methyl-thiazole tetrazolium (MTT) assay [16], both **1** and **2** showed cytotoxicities against P388 murine leukemia cells with IC_{50}s of 3.7 and 4.8 μM, respectively. We also evaluated antimicrobial activities of **1** and **2** using agar diffusion assay. Umezawamide A (**1**) showed growth inhibitory activity against *Candida albicans* (zone of inhibition; 1.7 mm, at a concentration of 5 μg per disk (6 mm)), while **2** did not show the antifungal activity up to the concentration of 10 μg per disk (6 mm). Meanwhile, neither of **1** and **2** showed any activities against *Bacillus cereus*, methicillin-sensitive *Staphylococcus aureus* (MSSA), and *Escherichia coli* up to the concentration of 10 μg per disk (6 mm). These results indicated that the structure of macrocyclic moiety (C-ring in Fig. 1b) has an important role for cytotoxicities and antifungal activities of **1** and **2**.

Finally, we searched for a PTM biosynthetic gene cluster in a draft genome sequence of *T. pulmonis* TP-B0596 to identify the true producer of **1** and **2**. As a result, we could not find any gene clusters showing homology to known PTM biosynthetic genes [17, 18], indicating that *Umezawaea* sp. RD066910 is the true producer of **1** and **2**.

In conclusion, we identified two new bioactive PTMs, umezawamides A (**1**) and B (**2**), from the combined-culture between *Umezawaea* sp. RD066910 and MACB *T. pulmonis* TP-B0596. To our best knowledge, they are the first secondary metabolites isolated from the genus *Umezawaea*. Our study strongly illustrates the potential of rare-actinomycetes as a counterpart species of "combined-culture" aiming for natural product discovery.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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