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Melophlins A and B, Novel Tetramic Acids Reversing the Phenotype of *ras*-Transformed Cells, from the Marine Sponge *Melophlus sarassinorum*

Shunji Aoki,[a] Kouichi Higuchi,[a] Ying Ye,[a] Rachmaniar Satari[b] and Motomasa Kobayashi[a,*]

[a]*Graduate School of Pharmaceutical Sciences, Osaka University, Yamada-oka 1-6, Suita, Osaka 565-0871, Japan*
[b]*Research and Development Centre for Oceanology, LIPI, JL. Pasir Putih I, Ancol Timur, Jakarta 11048, Indonesia*

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Abstract—Two novel tetramic acids named melophlins A (**1**) and B (**2**) were isolated from the marine sponge *Melophlus sarassinorum*, and the absolute stereostructures were elucidated on the basis of chemical and physicochemical evidence. Melophrins A (**1**) and B (**2**) induced reversion of the tumorous phenotype of *ras*-transformed NIH3T3 cells to normal at the concentration of 5 $\mu g\ mL^{-1}$.

Introduction

Most anti-cancer agents applied in clinical treatment attack not only tumor cells but also normal cells. These anti-cancer agents cause many serious undesirable side effects to cancer patients. So, ideal anti-cancer agents, which would act specifically against the tumor cell, have been sought for successful cancer chemotherapy. *Ras* oncogenes have an important role in cell growth and differentiation,[1] and so a substance, which reverses the transformed phenotype caused by *ras* oncogene, has high potential as a new type of anti-cancer agent. As a part of our continuing studies in search of new bioactive substances from marine organisms,[2] we focused on a search for selective compounds which would effectively reverse the *ras*-transformed phenotype to normal and have isolated novel mono-*O*-alkyl-diglycosyl glycerols named myrmekiosides A and B from a marine sponge of *Myrmekioderma* sp.[3] In the course of our study, we further isolated two novel tetramic acids named melophlins A (**1**) and B (**2**) from the Indonesian marine sponge *Melophlus sarassinorum*. In this paper, we describe the structure elucidation of melophlins A (**1**) and B (**2**).

Results and Discussion

The MeOH extract of the titled marine sponge, which was collected at Spermonde Islands, Ujung Pandang, Indonesia, showed cytotoxic activity against HL60 cells. The MeOH extract was partitioned into AcOEt–water mixture to obtain the AcOEt-soluble portion. The water phase was further partitioned with *n*-BuOH to obtain the *n*-BuOH-soluble portion. The AcOEt-soluble portion having cytotoxicity against HL60 cells also reversed the phenotype of *ras*-transformed NIH3T3 cells to normal. Then, the AcOEt-soluble portion was subjected to bioassay-guided separation. Repeated column chromatography (SiO_2 and ODS) and HPLC (SiO_2 and ODS) of the AcOEt-soluble portion furnished melophlins A (**1**) and B (**2**) as the major active components. Furthermore, we investigated the chemical constituents of the *n*-BuOH-soluble portion and isolated sarasinoside A_1 and its prosapogenol (=sarasinoside A_1-pro-2), which were norlanostane-triterpenoidal oligoglycosides isolated from the Palauan marine sponge *Asteropus sarasinosum*.[4]

Melophlin A (**1**) was obtained as a colorless oil. The FAB MS of **1** gave a quasi-molecular ion peak at *m/z* 352 $(M+H)^+$ and the molecular formula was determined as $C_{21}H_{37}NO_3$ by HR-FAB MS. The IR spectrum of **1** showed an absorption band due to amide (3339, 1624 cm^{-1}) and ketone (1717 cm^{-1}). The ^1H and ^{13}C NMR spectra of **1** indicated that **1** was an inseparable tautomeric mixture; the signals assignable to the major component were then analyzed. The ^{13}C NMR spectrum of **1** showed the characteristic carbon signals assignable to amide (δc 173.5), ketone (δc 191.3) and enol (δc 187.6, 101.6). The ^1H NMR spectrum of **1** revealed the presence of an *N*-methyl group (δ 3.00, 3H, s) and a long alkyl chain (δ 0.85, 3H, t, J=6.7 Hz; δ 1.20–1.35, ca. 22H, m). The UV spectrum of **1** showed the absorption maxima (284 nm; ϵ=15000, 244 nm; ϵ=9000) shifted to longer wavelength, which indicated the conjugation between amide, ketone and enol. All

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* Corresponding author. Tel.: +81-6-6879-8215; fax: +81-6-6879-8219; e-mail: kobayasi@phs.osaka-u.ac.jp

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Table 1. ^{1}H and ^{13}C NMR data for melophlins A (**1**) and B (**2**) (500 MHz in $CDCl_3$)

No.	Melophlin A (**1**)			No.	Melophlin B (**2**)		
	δc	δ (mult., *J* (Hz))	HMBC		δc	δ (mult., *J* (Hz))	HMBC
2	173.5	–		2	172.8	–	
3	101.6	–		3	100.4	–	
4	191.3	–		4	194.6	–	
5	57.7	3.69 (2H, s)	C2, C4	5	62.7	3.68 (q, 7.3)	C2, C4
6	187.6	–		6	14.8	1.35 (3H, d, 7.3)	C5
7	32.6	2.79 (2H, t, 7.3)	C6, C8	7	187.8	–	
8	26.0	1.65 (2H, qui.-like, 7.3)	C7, C9	8	32.5	2.81 (2H, m)	C3, C7, C9
9–19	28.0–31.9	1.20–1.35 (m)		9	26.9	1.65 (2H, m)	C7
20	22.7	1.25 (m)		10	32.7	1.38 (m)	
21	14.1	0.85 (3H, t, 6.7)	C20	11	36.8	1.27 (m) 1.11 (m)	C12
22	28.0	3.00 (3H, s)	C2, C5	12–16	29.4–31.7	1.25–1.27 (m)	
				17	22.6	1.24 (m)	
				18	14.0	0.88 (3H, t, 6.7)	C17
				19	19.6	0.84 (3H, d, 6.8)	C10, C11
				20	26.3	2.97 (3H, s)	C2, C5

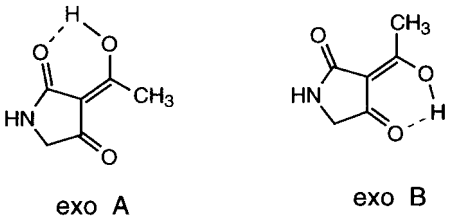
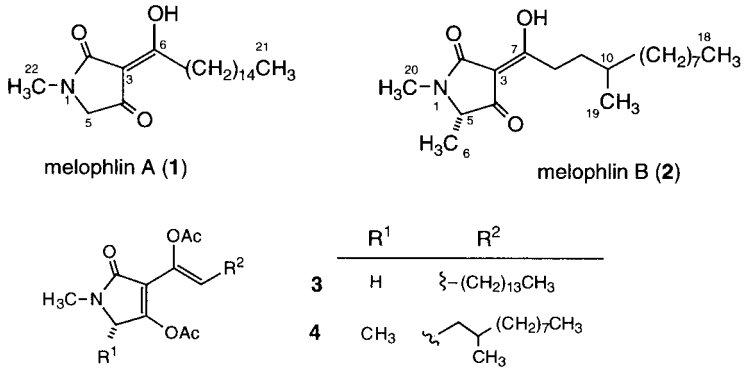


Figure 1. Two tautomers of tetramic acid.

the proton and carbon signals of **1** were assigned by 2D NMR (COSY, HMQC) analysis (Table 1), and the chemical structure of **1** having a tetramic acid moiety was constructed by the detailed analysis of the HMBC spectrum of **1**. On the basis of NMR analysis, tetramic acid (=3-acylpyrrolidine-2,4-dione) has been elucidated to exist mainly as two tautomers (exo A form and exo B form) (Fig. 1) in the ratio of ca. 9:1.[5–7] The ^{1}H and ^{13}C NMR spectra of **1** also showed that **1** exists as a mixture of two tautomers assignable to exo A form and exo B form in a similar ratio (Table 2). The assignment was based on the evidence that a signal of the hydrogen-bonded carbonyl carbon in the ^{13}C NMR spectrum has been observed in lower field than that of the corresponding normal carbonyl carbon.[8] The chemical structure of **1** was further confirmed by acetylation. Thus, **1** was treated with Ac_2O in pyridine to furnish diacetate **3** as a major product. The IR spectrum of **3** showed an absorption band due to amide carbonyl at 1697 cm^{-1}, which was observed at 1624 cm^{-1} in the case of **1** (Scheme 1).

The EI MS of melophlin B (**2**) showed a molecular ion peak at *m/z* 323 (M^+) and the molecular formula was determined as $C_{19}H_{33}NO_3$ by HR-EI MS. The IR and UV spectra of **2** were very similar to those of **1**. The ^{1}H and ^{13}C NMR spectra of **2** also showed closely similar signals with those of **1** except for two additional secondary methyl signals (δ1.35, 3H, d, *J*=7.3 Hz, δc14.8 and δ0.84, 3H, d, *J*=6.8 Hz, δc19.6). All the proton and carbon signals of the major tautomer in **2** were assigned on the basis of 2D NMR (COSY, HMQC) analysis of **2** (Table 1). The HMBC analysis of **2** disclosed the plane structure of **2**, in which additional secondary methyl groups were attached at C-5 in the ring part and C-10 in the long chain part. To determine the absolute configuration at C-5 in **2**, **2** was treated with $NaIO_4$ and $KMnO_4$ followed by aq HCl to furnish *N*-methylalanine. Then, the obtained *N*-methylalanine was derivatized with (1-fluoro-2,4-dinitrophenyl-5)-l-leucinamide (advanced Marfey's reagent, l-FDLA) prepared from 1,5-difluoro-2,4-dinitrobenzene (FFDNB) and l-leucinamide.[9,10] Next, the resulting derivative was subjected to HPLC analysis, and the absolute configuration at C-5 in **2** was determined as *S* by comparison with an authentic sample. This result was also supported by the characteristic CD maxima[11] [234 nm ($\Delta\epsilon=-1.1$), 264 nm (+0.9), 296 nm (−1.4)] of **2**. Consequently, the chemical structure of



Scheme 1.

Table 2. ^{1}H and ^{13}C NMR data for tautomers of melophlins A (**1**) and B (**2**) (500 MHz in $CDCl_3$)

No.	Melophlin A (**1**)				No.	Melophlin B (**2**)			
	δc		δ			δc		δ	
	Exo A	Exo B	Exo A	Exo B		Exo A	Exo B	Exo A	Exo B
2	173.5	167.0			2	172.8	166.5		
3	101.6	105.0			3	100.4	104.0		
4	191.3	197.5			4	194.6	201.0		
5	57.7	54.6	3.69	3.82	5	62.7	59.5	3.68	3.83
6	187.6	190.6			7	187.8	191.0		
22	28.0	26.5	3.00	2.98	20	26.3	26.1	2.97	2.94

melophlin B (**2**) was elucidated to be **2** (the absolute configuration at C-10 was not determined).

Melophlins A (**1**) and B (**2**) showed moderate cytotoxic activity against HL60 cells at 0.2 and 0.4 μg mL^{-1} concentration, respectively, and both compounds reversed the morphology of H-*ras* transformed NIH3T3 fibroblasts to normal at 5 μg mL^{-1} concentration.[12] Furthermore, **1** and **2** were found to arrest NIH3T3 cells in the G_1 phase of the cell cycle at 1 μg mL^{-1} concentration. Melophlins A (**1**) and B (**2**) might act on the components of the *ras*-mediated signal transduction pathway. So far, FR901228[13] (histone deacetylase inhibitor) and lovastatin[14] (HMG-CoA reductase and protein geranylgeranylation inhibitor) have been found to reverse the transformed phenotype of *ras*-transformants to normal and also cause G_1 arrest in the cell cycle. Further mechanistic study is under way.

Experimental

Isolation from the marine sponge *Melophlus sarassinorum*

The dried marine sponge *Melophlus sarassinorum* (order: Astrophorida, family: Ancorinidae) (4 kg) collected in August, 1997 at Spermonde Islands, Ujung Pandang, Indonesia, was extracted with MeOH (10 L) at room temperature 3 times for 12 h each. The residue obtained by evaporation of the solvent under reduced pressure was partitioned into an AcOEt–water mixture (1:1), and the AcOEt layer was evaporated to give the AcOEt-soluble portion (101 g). The remaining water phase was further partitioned with an equal volume of *n*-BuOH, and the *n*-BuOH layer was evaporated to give the *n*-BuOH-soluble portion (114 g). The AcOEt-soluble portion (31 g) was subjected to SiO_2 column (eluted with *n*-hexane—AcOEt=1:1→$CHCl_3$–MeOH=10:1→MeOH) to give three fractions [fr. A (11 g), fr. B (10 g), fr. C (6 g)]. The fr. B was further separated by reversed-phase column (Cosmosil 75C_{18}-OPN, MeOH–H_2O=3:2→4:1→MeOH) to afford fr.B-1 (1.9 g) and fr. B-2 (6.1 g). Then, the fr. B-1 (387 mg) was separated by HPLC (Cosmosil 5C_{18}-AR, MeOH containing 0.1% TFA) to afford melophlin A (**1**, 19 mg, 1.0% from the AcOEt-soluble portion). The fr. B-2 (96 mg) was purified by HPLC (Cosmosil 5C_{18}-AR, CH_3CN–H_2O=10:1 containing 0.1% TFA; Cosmosil[5] SL, *n*-hexane–AcOEt=3:1) to give melophlin B (**2**, 37 mg, 2.4%). The *n*-BuOH-soluble portion (51 g) was subjected to SiO_2 column (eluted with *n*-hexane–AcOEt=40:1→10:1→5:1→2:1→AcOEt→acetone) and HPLC (Cosmosil 5C_{18}-AR, MeOH–H_2O=4:1) to furnish sarasinoside A_1 (2.3 g, 4.5% from the *n*-BuOH extract) and its hydrolysis product (sarasinoside A_1-pro-2, 1.8 g, 3.5%).

Melophlin A (1). IR cm^{-1} (KBr): 3339, 1717, 1624. UV λ max. (MeOH) nm (ε): 284 (15000), 244 (9000). Positive FAB MS: *m/z* 352 $(M+H)^+$. HR-FAB MS: *m/z* 352.2818 calcd for $C_{21}H_{38}NO_3$. Found: 352.2835. ^{1}H NMR (500 MHz in $CDCl_3$, δ) and ^{13}C NMR (125 MHz in $CDCl_3$, δc), as shown in Table 1.

Melophlin B (2). $[\alpha]_D$=−12.4 (*c*=1.2, MeOH). IR cm^{-1} (KBr): 1713, 1620. UV λ max. (MeOH) nm (ε): 285 (17000), 245 (13800). CD (MeOH); 234 nm (Δε=−1.1), 264 nm (+0.9), 296 nm (−1.4). EI MS: *m/z* 323 (M^+). HR-EI MS: *m/z* 323.2460 calcd for $C_{19}H_{33}NO_3$. Found: 323.2459. ^{1}H NMR (500 MHz in $CDCl_3$, δ) and ^{13}C NMR (125 MHz in $CDCl_3$, δc), as shown in Table 1.

Acetylation of melophlins A (1) and B (2) giving 3 and 4. Compound **1** (4.9 mg) was treated with Ac_2O (280 μL) in pyridine (500 μL) at room temperature for 10 min. The reaction mixture was concentrated under reduced pressure with toluene and the residue was purified by SiO_2 column (*n*-hexane–AcOEt) to furnish diacetate **3** (4.0 mg). Compound **2** (4.5 mg) was similarly treated with Ac_2O (270 μL) in pyridine (500 μL) to furnish diacetate **4** (4.2 mg).

Diacetate 3. IR cm^{-1} (KBr): 1780, 1763, 1697. FAB MS: *m/z* 436 $(M+H)^+$. HR-FAB MS: *m/z* 436.3063 calcd for $C_{25}H_{42}NO_5$. Found: 436.3026. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 6.51 (t, *J*=7.3 Hz, H-7), 4.13 (2H, s, H-5), 2.93 (3H, s, H-22), 2.19 (3H, s, 4-OAc), 2.16 (3H, s, 6-OAc), 1.99 (m, H-8), 1.38 (m, H-9), 1.30 (m, H-19), 1.25 (m, H-20), 1.16–1.23 (18H, m, H-10-18), 0.81 (3H, t, *J*=6.7 Hz, H-21). ^{13}C NMR (125 MHz, $CDCl_3$, δc): 166.6 (C-2), 113.1 (C-3), 153.9 (C-4), 51.3 (C-5), 137.5 (C-6), 125.3 (C-7), 25.7 (C-8), 28.8–32.1 (C-9-19), 22.9 (C-20), 14.3 (C-21), 28.8 (C-22), 169.8, 22.8 (4-OAc), 168.8, 21.2 (6-OAc).

Diacetate 4. IR cm^{-1} (KBr); 1780, 1763, 1699. FAB MS: *m/z* 408 $(M+H)^+$. HR-FAB MS: *m/z* 408.2750 calcd for $C_{23}H_{38}NO_5$. Found: 408.2781. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 6.59 (t, *J*=7.3 Hz, H-8), 4.28 (q, *J*=7.3 Hz, H-5), 2.94 (3H, s, H-20), 2.27 (3H, s, 4-OAc), 2.20 (3H, s, 7-OAc), 2.04 (2H, m, H-9), 1.40 (m, H-10), 1.32 (m, H-11a), 1.11 (m, H-11b), 1.23–1.29 (15H, m, H-12-17

and H-6), 0.88 (3H, t, J=6.7 Hz, H-18), 0.83 (3H, d, J=6.7 Hz, H-19). ^{13}C NMR (125 MHz, $CDCl_3$ δc): 166.7 (C-2), 114.6 (C-3), 158.3 (C-4), 55.9 (C-5), 15.5 (C-6), 137.4 (C-7), 125.9 (C-8), 26.4 (C-9), 32.8 (C-10), 37.0 (C-11), 29.6-32.1 (C-12-16), 22.9 (C-17), 14.4 (C-18), 19.8 (C-19), 26.9 (C-20), 167.0, 20.7 (4-OAc), 168.6, 20.9 (7-OAc).

HPLC analysis of hydrolysate of melophlin B (2). Compound **2** (1.5 mg) was dissolved in a mixture of acetone and H_2O (1:1, 70 μL) and treated with $NaIO_4$ (6.0 mg) and $KMnO_4$ (0.2 mg) at 5°C for 2 h. The resulting mixture was treated with 2 N aq. HCl (60 μL) at 100°C for 5 h and neutralized with 1 M $NaHCO_3$ solution. FDLA[9,10] (0.4 mg in 380 μL of acetone), which was prepared from 1,5-difluoro-2,4-dinitrobenzene and l-leucinamide, was added to the reaction mixture and the whole was stirred at 37°C for 6 h. After quenching by 1N aq. HCl (76 μL), the reaction mixture was extracted with AcOEt, and the residue obtained after removal of AcOEt under reduced pressure was subjected to HPLC analysis. HPLC conditions: detection, UV (340 nm); column, Cosmosil $5C_{18}$-AR (10 mm∅×250 mm); mobile phase, CH_3CN–H_2O=2:3 containing 0.1% TFA; flow rate, 3.0 mL min^{-1}. Rt: (1-*N*-methyl-d-alanine-2,4-dinitrophenyl-5)-l-leucinamide=35.0 min, (1-*N*-methyl-l-alanine-2,4-dinitrophenyl-5)-l-leucinamide=38.0 min.

Effect on the morphology of H-*ras* transformed cells

Parental NIH3T3 cells and NIH3T3 cells transformed with human c-H-*ras* gene carrying a point mutation at codon 61 were used for the assay.[15] Both cells were cultured in DMEM supplemented with 0.44 mg mL^{-1} of glutamine, 50 μg mL^{-1} of kanamycin sulfate, and 10% newborn calf serum. Equal numbers of cells (2×10^3) were inoculated into each well of a 96-well plate with 90 μL of the culture medium, and a testing sample was added to each well as 10 μL of dimethylsulfoxide solution. After 48 h incubation (37°C, 5% CO_2), the morphology of cells was observed by phase-contrast microscope.

Cell cycle analysis

NIH3T3 Cells (4×10^4) were inoculated into each well of an 8-well plate with 1 mL of the culture medium, and a testing sample (1 μg mL^{-1}) was added to each well as 10 μL of dimethylsulfoxide solution. After 48 h incubation (37°C, 5% CO_2), the harvested cells with trypsin treatment were washed with PBS, and the nuclei in cells were stained with DNA-Prep Reagents Kit (Coulter Corporation, USA). The distribution of DNA content in the stained cell was analyzed by flow cytometry with the FACSCalibur system (Becton Dickinson Inc., USA).

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References

1. Duesberg, P. H. *Science* **1985**, *228*, 669–677.
2. Aoki, S.; Setiawan, A.; Yoshioka, Y.; Higuchi, K.; Fudetani, R.; Chen, Z.-S.; Sumizawa, T.; Akiyama, S.; Kobayashi, M. *Tetrahedron* **1999**, *55*, 13965–13972 (and preceding papers therein).
3. Aoki, S.; Higuchi, K.; Kato, A.; Murakami, N.; Kobayashi, M. *Tetrahedron* **1999**, *55*, 14865–14870.
4. Kobayashi, M.; Okamoto, Y.; Kitagawa, I. *Chem. Pharm. Bull.* **1991**, *39*, 2867–2877.
5. Steyn, P. S.; Wessels, P. L. *Tetrahedron Lett.* **1978**, *47*, 4707–4710.
6. Nolte, M. J.; Steyn, P. S.; Wessels, P. L. *J. Chem. Soc., Perkin Trans. 1* **1980**, 1057–1065.
7. Jones, R. C. F.; Begley, M. J.; Peterson, G. E.; Sumaria, S. *J. Chem. Soc., Perkin Trans. 1* **1990**, 1959–1968.
8. Stothers, J. B.; Lauterbur, P. C. *Can. J. Chem.* **1964**, *42*, 1563–1576.
9. Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591–596.
10. Fujii, K.; Ikai, Y.; Mayumi, T.; Oka, H.; Suzuki, M.; Harada, K. *Anal. Chem.* **1997**, *69*, 3346–3352.
11. Phillips, N. J.; Goodwin, J. T.; Fraiman, A.; Cole, R. J.; Lynn, D. G. *J. Am. Chem. Soc.* **1989**, *111*, 8223–8231.
12. Nakajima, H.; Kim, Y. B.; Terabo, H.; Yoshida, M.; Horinouchi, S. *Exp. Cell Res.* **1998**, *241*, 126–133.
13. Tsuchiya, K.; Kanbe, T.; Hori, M.; Uehara, Y.; Takahashi, Y.; Takeuchi, T. *Biol. Pharm. Bull.* **1993**, *16*, 908–911.
14. Vogt, A.; Qian, Y.; McGuire, T. F.; Hamilton, A. D.; Sebti, S. M. *Oncogene* **1996**, *13*, 1991–1999.
15. Sekiya, T.; Fushimi, M.; Hori, H.; Hirohashi, S.; Nishimura, S.; Sugimura, T. *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 4771–4775.