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Novel anticancer agents, kayeassamins A and B from the flower of *Kayea assamica* of Myanmar

Nwet Nwet Win [a], Suresh Awale [a], Hiroyasu Esumi [b], Yasuhiro Tezuka [a], Shigetoshi Kadota [a,*]

[a] Institute of Natural Medicine, University of Toyama, 2630-Sugitani, Toyama 930-0194, Japan
[b] National Cancer Center Hospital East, 6-5-1, Kashiwanoha, Kashiwa, Chiba 277-8577, Japan

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ABSTRACT

The $CHCl_3$-soluble fraction of 70% EtOH extract of the flower of *Kayea assamica* completely killed human pancreatic PANC-1 cancer cells preferentially under nutrient-deprived conditions at 1 μg/mL. Bioassay-guided fractionation and isolation afforded two novel compounds, kayeassamins A (**1**) and B (**2**). Their structures were elucidated using extensive spectroscopic methods and the modified Mosher method. Each compound showed 100% preferential cytotoxicity (PC_{100}) against PANC-1 cells under nutrient-deprived conditions at 1 μM. Furthermore, both compounds inhibited the migration of PANC-1 cells in the wound closure assay.

Pancreatic cancer is the fifth leading cause of cancer death with a median survival of <6 months and a relative 5-year survival rate of 5.5%. In 2005, it was estimated that 22,926 men and women died of pancreatic cancer in Japan.[1,2] It is largely resistant to conventional forms of treatment. Therefore, the development of effective adjunct strategies is urgently necessary. Gemcitabine currently represents the standard chemotherapeutic drug for metastatic and advanced disease, but it only leads to a modest improvement in quality of life and survival.[3] Pancreatic cancer cells have marked tolerance to nutrient deprivation that enables them to survive for prolong period of time. Thus, despite poor angiogenesis, the ability of cancer cells tolerance to nutrient starvation (austerity) is another critical factor for tumor progression under hypovascular conditions. Hence, the agent that can eliminate the cancer cells tolerance to nutrient starvation (anti-austerity agent) was considered as a novel target in anticancer drug discovery.[4–7] Under this hypothesis, we have screened the medicinal plants used in Japanese Kampo medicine and Myanmar traditional medicine for their preferential cytotoxicity against human pancreatic cancer PANC-1 cells under nutrient-deprived conditions. This work led to the identification of arctigenin,[8] angelmarin,[9] panduratin A,[10,11] and nicolaioidesin B[10,11] as compounds having the activity to abolish cancer cells tolerance to nutrient starvation. In our continuing study, we recently discovered that the $CHCl_3$-soluble fraction of 70% EtOH extract of the flower of *Kayea assamica* King & Prain (Clusiaceae) collected in Myanmar exhibited 100% preferential cytotoxicity (PC_{100}) against PANC-1 cancer cells under nutrient-deprived conditions at 1 μg/mL. We thus carried out further bioassay-guided fractionation and isolation that led to the isolation of two novel alkylated coumarins named kayeassamins A (**1**) and B (**2**). Furthermore, wound closure assay[12] was performed to investigate the potency of these compounds against PANC-1 cells migration.

Kayeassamin A (**1**)[13] was obtained as pale yellow oil with $[\alpha]^{23}_{D}$ +65.61°. The molecular formula $C_{26}H_{34}O_6$ was established by HRE-IMS together with the ^1H and ^{13}C NMR data (Table 1). The IR spectrum exhibited the presence of hydroxyl group (3500 cm^{-1}), α,β-unsaturated lactone (1730 cm^{-1}), and chelated acyl group (1610 cm^{-1}). The UV spectrum of **1** showed the absorption maxima at 220 and 329 nm in EtOH. The ^1H NMR resonances of **1** displayed an oxygenated methine at δ_H 4.62 (H-1′), three olefinic methines at δ_H 5.05 (H-7″), 5.16 (H-2″), and 5.97 (H-3), six methylenes at δ_H 1.70 (H_2-3‴), 1.76 and 1.92 (H_2-2′), 1.96 (H_2-5″), 2.03 (H_2-6″), 3.00 (H_2-2‴), and 3.34 (H_2-1″), two aliphatic methyls overlapping at δ_H 1.00 (H_3-3′, H_3-4‴), three vinyl methyls at δ_H 1.56 (H_3-10″), 1.66 (H_3-9″), and 1.79 (H_3-4″), and a hydrogen-bonded hydroxyl proton at δ_H 14.33 (7-OH). On the other hand, the ^{13}C NMR spectrum of **1** exhibited 26 carbons including those for a ketone carbonyl carbon, a lactone carbonyl carbon, six aromatics, one oxygenated methine, three olefinic methines, six methylenes, five methyls, and three quaternary olefinic carbons. These data were similar to those of theraphin A,[14] an isolate from the same species. Detailed analysis of COSY and HMBC correlations (Fig. 1A) showed that **1** has the same 5,7-dihydroxycoumarin core as theraphin A.

* Corresponding author. Tel.: +81 76 434 7625; fax: +81 76 434 5059.
E-mail address: kadota@inm.u-toyama.ac.jp (S. Kadota).

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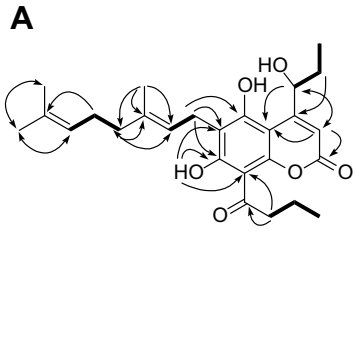
Table 1
^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data of **1** and **2** in $CDCl_3$

Position	**1**		Position	**2**	
	δ_H	δ_C		δ_H	δ_C
2		160.9	2		160.3
3	5.97 (1H, s)	108.7	3	6.07 (1H, s)	108.9
4		157.0	4		156.5
4a		100.7	4a		100.6
5		157.6	5		156.3
6		114.8	6		104.1
7		166.8	7		166.6
8		103.9	8		114.3
8a		156.2	8a		157.6
1′	4.62 (1H, t, 7.3)	78.3	1′	4.71 (1H, t, 7.1)	77.9
2′	1.76, 1.92 (each 1H, m)	27.5	2′	1.76, 1.93 (each 1H, m)	27.8
3′	1.00[a] (3H, t, 7.3)	10.5	3′	1.02[a] (3H, t, 6.8)	10.5
1″	3.34 (2H, m)	21.9	1″		205.4
2″	5.16 (1H, t, 6.8)	121.2	2″	3.09 (2H, m)	46.8
3″		136.6	3″	1.73 (2H, m)	18.1
4″	1.79 (3H, s)	16.2	4″	1.02[a] (3H, t, 6.8)	13.8
5″	1.96 (2H, m)	39.8	1‴	3.39 (2H, m)	21.9
6″	2.03 (2H, m)	26.7	2‴	5.18 (1H, t, 7.1)	121.1
7″	5.05 (1H, t, 6.8)	124.3	3‴		137.0
8″		131.3	4‴	1.79 (3H, m)	16.3
9″	1.66 (3H, s)	25.7	5‴	1.98 (2H, m)	39.8
10″	1.56 (3H, s)	17.7	6‴	2.04 (2H, m)	26.7
1‴		205.1	7‴	5.06 (1H, t, 6.6)	124.2
2‴	3.00 (2H, m)	46.8	8‴		131.4
3‴	1.70 (2H, m)	17.9	9‴	1.64 (3H, s)	25.7
4‴	1.00[a] (3H, t, 7.3)	13.8	10‴	1.57 (3H, s)	17.8
7-OH	14.33 (1H, s)		1′-OH	4.49 (1H, br s)	
			5-OH	10.81 (1H, br s)	
			7-OH	14.37 (1H, s)	

[a] Overlapping resonances within the same column.



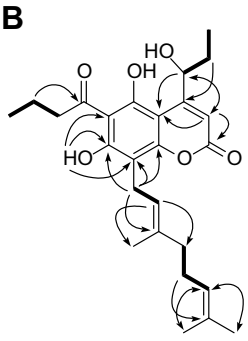
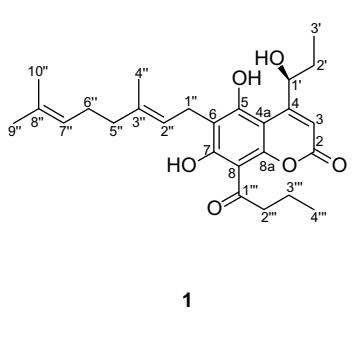


Figure 1. COSY (bold lines) and HMBC (^{1}H → ^{13}C) (arrows) correlations in **1** (A) and **2** (B).

However, they differed due to the presence of a geranyl group in place of an isoprenyl group at C-6 of theraphin A. In difference NOE experiment, irradiation of H_3-4″ gave enhancement of H-1″ (20%) suggesting the stereochemistry of geranyl side chain as *E*. Therefore, the planar structure of kayeassamin A (**1**) was established as 5,7-dihydroxy-4-(1-hydroxypropyl)-6-[(2*E*)-3,7-dimethyl-2,6-octadienyl]-8-(1-oxobutyl)-2*H*-benzopyran-2-one. The absolute configuration of **1** was determined by the modified Mosher method.[15,16] The MTPA esters of **1** were obtained by treating **1** with (*R*)- and (*S*)-MTPA chloride,[17] respectively, and their ^{1}H NMR resonances were assigned based on COSY correlations. The chemical shift differences ($\Delta_{SR} = \delta_S - \delta_R$) of the individual protons of **1a** and **1b** are shown in Figure 2A. In the ^{1}H NMR spectrum of the (*S*)-MPTA ester (**1a**), H_2-2′ and H_3-3′ appeared shielded, whereas H-1′ and H-3 were deshielded, in comparison to analogous data for (*R*)-MTPA ester (**1b**), suggesting the absolute configuration at C-1′ to be *S*.



Kayeassamin B (**2**)[18] indicated the same molecular formula as **1** by HREIMS. The ^{1}H and ^{13}C NMR data of **2** (Table 1) were also similar to those of **1**. The COSY and HMBC correlations (Fig. 1B) indicated that **2** also has an oxobutyl and a geranyl substituent as in **1**. The significant difference was the appearance of the additional hydroxyl proton at δ_H 10.81 (5-OH), a characteristic signal of 5,7-dihydroxy-6-acylcoumarin,[19] which was further supported by the bathochromic shift observed after addition of alkali in UV spectrum.[18] Thus, oxobutyl unit was determined to be at C-6, which was confirmed by the HMBC correlations of the hydroxyl proton at δ_H 14.37 (7-OH) with C-6, C-7, and C-8. The HMBC correlations of H-1′ with C-3 and C-4a, of H-2′ with C-4, and of H-1‴ with C-7, C-8, and C-8a indicated the hydroxypropyl group to be at C-4 and the geranyl unit at C-8.

Based on the abovementioned evidences, the planar structure of kayeassamin B (**2**) was established as 5,7-dihydroxy-4-(1-hydroxypropyl)-6-(1-oxobutyl)-8-[(2*E*)-3,7-dimethyl-2,6-octadienyl]-2*H*-benzopyran-2-one. The absolute configuration of C-1′ in **2** was also determined to be *S* (Fig. 2B) by the modified Mosher method.[16,17,20]

Both compounds were tested for their in vitro preferential cytotoxicity against human pancreatic cancer cell line, PANC-1.[21] Each compound exhibited 100% preferential cytotoxicity (PC_{100}) against PANC-1 cells under nutrient-deprived medium (NDM) at 1 μM, which was comparable to that of the positive control, arctigenin (PC_{100} 1 μM).[8] The selectivity index of both compounds **1** and **2** in normal nutrient-rich medium (DMEM) and nutrient-deprived medium (NDM) was observed to be 256.[22] Furthermore, as shown in Figure 3A and B, 0–24 h exposure to 1–16 μM of **1** and **2** killed PANC-1 cells in concentration- and time-dependent manners in NDM. It is notable that PC_{100} for both compounds was observed after 12 h exposure of **1** or **2** at 1 μM. The potency of preferential cytotoxicity was further enhanced when exposed at 4 and 16 μM of the tested compounds that showed total cell death within 9 and 6 h, respectively. In addition, both **1** and **2** induced apoptosis-like morphological change to PANC-1 cells within 24 h of treatment (Fig. 4).

Pancreatic cancer has also a high metastatic rate. Thus, in order to explore the inhibition potency of **1** and **2** on the migration ability of PANC-1 cells, we further performed wound closure assay according to the procedure by Chung et al.[12,23] In this assay, a wound was induced with a 200 μL pipette tip on a confluent culture of cells. Wound closure was then allowed to precede from 0 to 72 h in the presence or absence of kayeassamins A (**1**) and B (**2**). The 0, 48, and 72 h time points are shown in Figure 5. The gap of PANC-1 cells in control had totally closed at 48 h. However, the cell migration to induced wounds has not been affected even after 72 h exposure with 1 μM of both tested compounds. Thus, **1** and **2** not only exhibited preferential cytotoxicity under nutrient-deprived medium (NDM), but also inhibited migration of PANC-1 cancer cells in nutrient-rich medium.

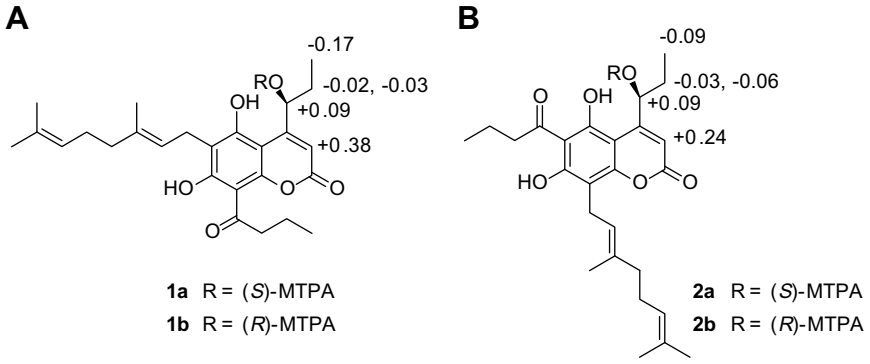


Figure 2. Difference in the Δ_{SR} ($\delta_S - \delta_R$) values for the (*S*)- and (*R*)-MTPA esters of **1** (A) and **2** (B) in $CDCl_3$.

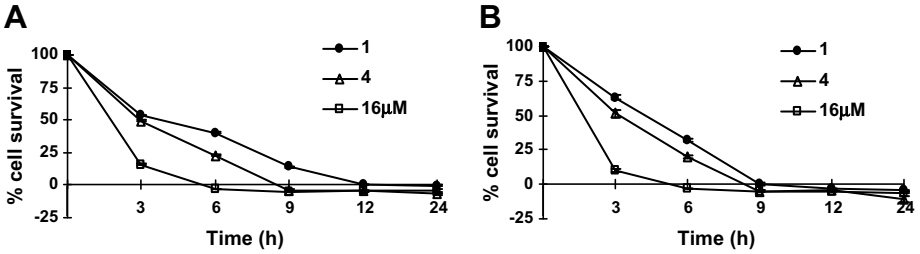


Figure 3. Survival of human pancreatic cancer PANC-1 cells under nutrient-deprived conditions within 0–24 h by 1–16 μM of **1** (A) and **2** (B). Data are means ± SEM, $n = 3$.

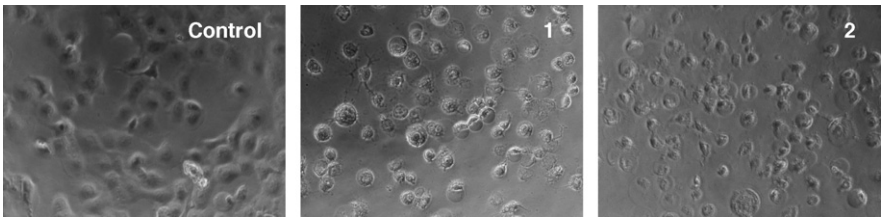


Figure 4. Morphological change of human pancreatic cancer PANC-1 cells under nutrient-deprived medium (NDM) after exposure with 1 μM of **1** or **2**.

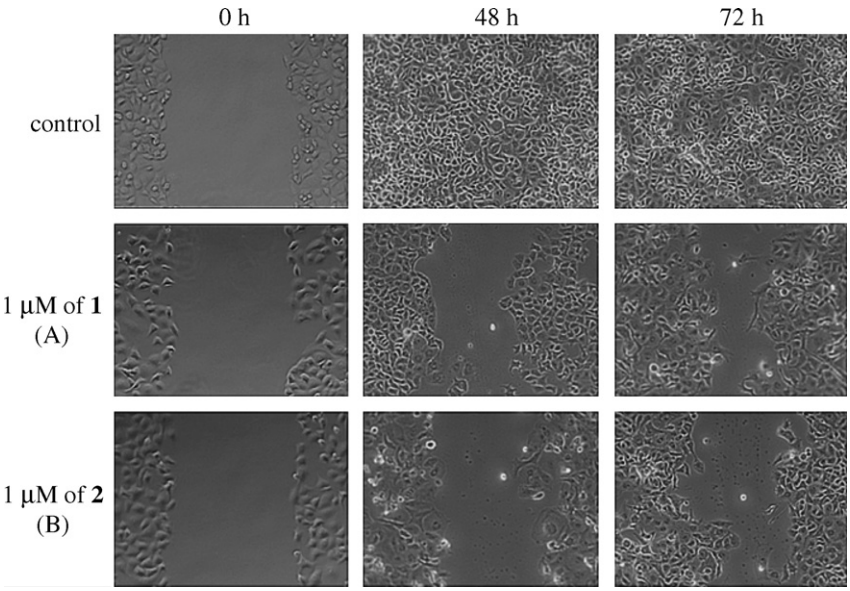


Figure 5. Effect of 1 μM of **1** (A) and **2** (B) on the closing rate of induced wound in human pancreatic PANC-1 cells under DMEM.

This suggests that both compounds are not only potent anti-austerity agents but also effective inhibitors against migration of PANC-1 cells and might be useful to inhibit the metastatic process of pancreatic cancer.

In conclusion, we have identified novel compounds, kayeassamins A (**1**) and B (**2**), from the flower of *K. assamica* of Myanmar as potent preferential cytotoxic agents which might be of potential therapeutic use for the pancreatic cancer treatment in the future. Further studies on their mechanism of action and in vivo anti-tumor activity as well as detailed phytochemical investigation are underway and will be reported in due time.

Acknowledgments

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13. **Kayeassamin A (1):** pale yellow oil; $[\alpha]^{23}_{D}$ +65.61° (*c* 1.34, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 220 (4.16), 329 (4.37); UV (EtOH + 0.1 N KOH) λ_{max} (log ε) 213 (4.79), 329 (4.18); IR ($CHCl_3$) ν_{max} 3500, 1730, 1610, 1420, 1380, 1210, 1030, 925, 790, 720 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HREIMS *m/z* 442.2343 $[M]^+$ (calcd for $C_{26}H_{34}O_6$, 442.2355).
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17. Preparation of the (*S*)- and (*R*)-MTPA esters of **1**: Two equal portions of **1** (each 2.5 mg) were dissolved in pyridine (250 μL) and (*R*)-MTPA-Cl (5 μL) and (*S*)-MTPA-Cl (5 μL) were added, respectively. The reaction mixtures were maintained at room temperature for 12 h. The reaction products were then purified by normal phase pTLC with hexane–EtOAc (3:1) afforded 1 mg each of **1a** and **1b**.**1a**: ^1H NMR ($CDCl_3$, 400 MHz): δ 0.87 (H_3-3′), 1.75; 1.99 (H_2-2′), 6.22 (H-3), 6.80 (H-1′); EIMS *m/z* 658 $([M]^+)$.**1b**: ^1H NMR ($CDCl_3$, 400 MHz): δ 1.04 (H_3-3′), 1.77; 2.02 (H_2-2′), 5.84 (H-3), 6.71 (H-1′); EIMS *m/z* 658 $([M]^+)$.
18. **Kayeassamin B (2):** pale yellow oil; $[\alpha]^{23}_{D}$ +15.0° (*c* 0.2, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 221 (4.77), 256 (4.32), 326 (4.81); UV (EtOH + 0.1 N KOH) λ_{max} (log ε) 215 (4.82), 380 (4.28); IR ($CHCl_3$) ν_{max} 3500, 1720, 1610, 1420, 1380, 1220, 1050, 930, 790, 720 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HREIMS *m/z* 442.2366 $[M]^+$ (calcd for $C_{26}H_{34}O_6$, 442.2355).
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20. Preparation of the (*S*)- and (*R*)-MTPA esters of **2**: Two equal portions of **2** (each 2.1 mg) were dissolved in pyridine (250 μL) and (*R*)-MTPA-Cl (5 μL) and (*S*)-MTPA-Cl (5 μL) were added, respectively. The reaction mixtures were maintained at room temperature for 12 h. The reaction products were then purified by normal phase pTLC with hexane–EtOAc (3:1) afforded 0.5 mg each of **2a** and **2b**.**2a**: ^1H NMR ($CDCl_3$, 400 MHz): δ 0.87 (H_3-3′), 1.72; 1.93 (H_2-2′), 6.21 (H-3), 6.81 (H-1′); EIMS *m/z* 658 $([M]^+)$.**2b**: ^1H NMR ($CDCl_3$, 400 MHz): δ 0.96 (H_3-3′), 1.75; 1.99 (H_2-2′), 5.97 (H-3), 6.72 (H-1′); EIMS *m/z* 658 $([M]^+)$.
21. The selectivity index (SI) was calculated as PC_{100} in DMEM/PC_{100} in NDM.
22. PANC-1 cells were seeded in 96-well plates (2×10^4 cells per well) and incubated in fresh Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceuticals; Tokyo, Japan) at 37 °C under 5% CO_2 and 95% air for 24 h. NDM was prepared following the procedure described by Izuishi et al.[4] After the cells were washed with PBS (Nissui Pharmaceuticals), the medium was changed to either DMEM or NDM, and serial dilutions of the test samples were added. For general preferential cytotoxicity assay, the cells were incubated for 24 h. For the concentration- and time-dependent experiments, the cells were incubated for 3, 6, 9, 12, and 24 h, respectively, after addition of the samples. Morphological changes of PANC-1 cells were photographed under 20× magnification using phase-contrast microscopy (Olympus D-340L/C-840L Digital Camera, Tokyo, Japan). Then, the cells were washed with PBS, and 100 μL of DMEM containing 10% WST-8 cell counting kit (Dojindo, Kumamoto, Japan) solution was added to the wells. After 2 h incubation, the absorbance at 450 nm was measured. Cell viability was calculated from the mean values of data from three wells by using the following equation:(%) Cell viability = $[\{Abs_{(test\ sample)} - Abs_{(blank)}\}/\{Abs_{(control)} - Abs_{(blank)}\}] \times 100$.
23. PANC-1 cells were seeded in 24-well plates (2×10^5 cells per well) and incubated in fresh DMEM at 37 °C under 5% CO_2 and 95% air for 24 h. After 24 h incubation, the wound was induced by scrapping the confluent monolayers with a micropipette tip, washed with PBS, and examined on films under a phase-contrast microscope. The test sample (300 μL) at a concentration of 1 μM, which was dissolved in DMSO and prepared in DMEM, was then added into the well. The closure rate of induced wound was compared between compound-treated and control (compound-untreated) groups. Photographs were taken under 100× magnification using phase-contrast microscopy at 0, 48, and 72 h, respectively.