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PROMOTING EFFECTS ON HEPATOCYTE GROWTH FACTOR PRODUCTION OF DAPHNANE DITERPENOIDS FROM *DAPHNE ODORA*

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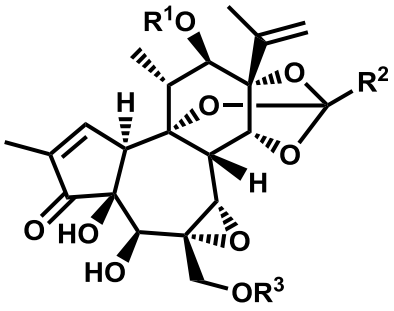
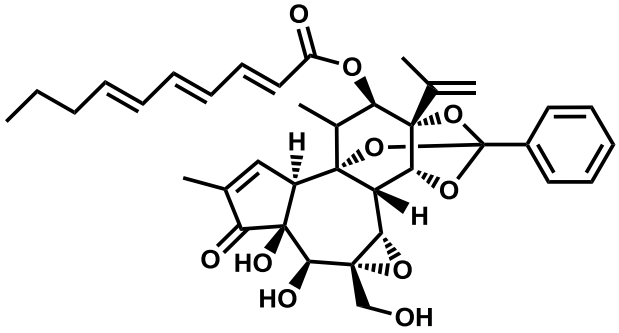
Abstract – Five daphnane diterpenoids, gnidilatidin (**1**), gnidilatidin 20-palmitate (**2**), yuanhuajine (**3**), odoratrin (**4**), and synaptolepis factor K4 (**5**), isolated from *Daphne odora* were used to evaluate the promoting effect on hepatocyte growth factor (HGF) production in normal human dermal fibroblasts (NHDF). All the tested compounds except for **2** increased the amount of HGF production significantly. According to the structure-activity relationships of compounds **1**-**5** and the derivative **6**, it was revealed that a hydroxy group at C-20 was essential for expression of HGF production promoting activity.

Daphnane diterpenoids, which usually have a 5/7/6-tricyclic ring system, are characteristically contained in Thymelaeaceae and Euphorbiaceae.[1] About 80 daphnane diterpenoids have been isolated from these plants.[1] They have various bioactivities such as cytotoxic and anti-HIV effects.[2-4] During our recent research, it was found that five daphnane diterpenoids including two new compounds isolated from *Thymelaea hirsuta* (Thymelaeaceae) showed antimelanogenic effect for the first time.[5] On the other hand, it was demonstrated that the secretion of hepatocyte growth factor (HGF) by human skin fibroblasts had been markedly stimulated by the phorbol ester, phorbol 12-myristate 13-acetate (PMA) and it has been used as an HGF production inducer.[6] HGF is a cytokine initially isolated as a potent growth factor for rat hepatocytes in primary culture.[7,8] Moreover it expresses multiple functions including mitogenic, angiogenetic, morphogenic, motogenic, and anti-apoptotic activities through c-Met receptor.[7-9] Since

daphnane diterpenoids have the same basic structure as that of PMA, it was expected that they also possess the promoting effect on HGF production. We selected *Daphne odora* to isolate daphnane diterpenoids because its roots are known to contain these compounds and it can be easily obtained in Japan. In this paper, we describe the promoting effect on HGF production of daphnane diterpenoids from *Daphne odora* and the structure-activity relationship on the effect of those compounds.

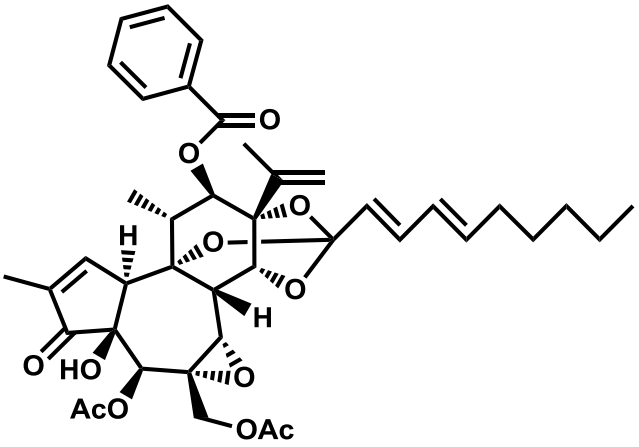
Five daphnane diterpenoids, gnidilatidin (**1**), gnidilatidin 20-palmitate (**2**), yuanhuajine (**3**), odoratrin (**4**), and synaptolepis factor K4 (**5**) (Figure 1), isolated from *Daphne odora* were used to evaluate the promoting effect on HGF production in normal human dermal fibroblast (NHDF) (Figure 2).



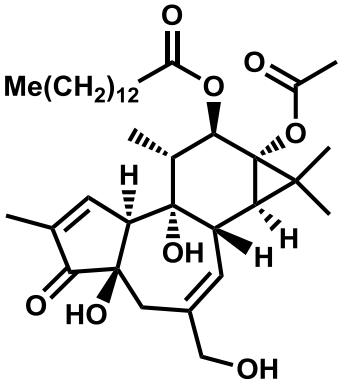


1 R^1= Bz, R^2= $(CH{=}CH)_2C_5H_{11}$, R^3= H
2 R^1= Bz, R^2= $(CH{=}CH)_2C_5H_{11}$, R^3= $COC_{15}H_{31}$
3 R^1= Bz, R^2= $(CH{=}CH)_3C_3H_7$, R^3=H
5 R^1= Ac, R^2= $(CH{=}CH)_2C_5H_{11}$, R^3=H

4



6



PMA

Figure 1. Structures of **1**-**6**

All the tested compounds promoted the HGF production except for **2**, while these compounds showed toxicity in their higher concentration. The promoting activity of **5** on HGF production is more potent than those of compounds **1**, **3**, and **4**, suggesting that the smaller functional group at C-12 in daphnane diterpenoids may be important for the activity. In addition, there was a considerable difference in the amount of HGF production between **1** and **2**. Compound **1** at 15.6-1000 nM increased the amount of HGF

production by 150-315%, while **2** showed weak activity. Their chemical structures are different only in substructure at the C-20. Compound **1** has a hydroxyl group, while **2** has an aliphatic ester at C-20. It was assumed that the hydroxyl group at C-20 is essential for expression of HGF production promoting activity, or the aliphatic acid at C-20 reduces the activity.

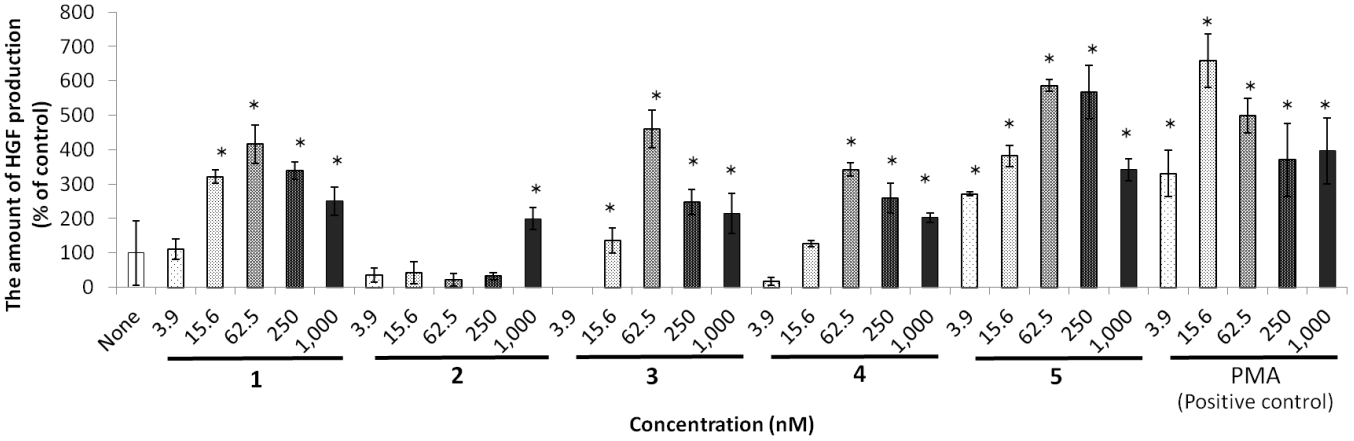


Figure 2. Effects of daphnane diterpenoids **1**-**5** on HGF production by NHDF.
The cells were seeded at a density of 5.0×10^3 cells/well and treated with various concentrations of the samples for 24 h. The amount of HGF in the supernatant was determined by ELISA. PMA was used as a positive control. Results represent means ±SD (n=4). *Statistically significant (P<0.05) difference between treatments.

In order to clarify which hypothesis is reasonable, **1** was acetylated by Ac_2O/pyridine to give gnidilatidin diacetate (**6**) and its promoting effect on HGF production was evaluated. Figure 3 shows the promoting effects of **1**, **2**, and **6** on HGF production. Compound **1** at 3.9-1000 nM increased the amount of HGF production by 95-590%, while **6** and **2** only at 1000 nM increased it by 193 and 258%, respectively, compared with that of the none treated cells. In short, HGF production promoting activities of **2** and **6** were weaker than that of **1**. It was concluded that the hydroxy group at C-20 is essential for HGF production promoting activity. All the tested daphnane diterpenoids except for **2** and PMA, used as a positive control, have a hydroxy group at C-20. They showed the stronger activity than **2**. This also supports the importance of the hydroxy group at C-20.

It is supposed that PMA promotes HGF production through activating protein kinase C (PKC) because other PKC activators were also effective, whereas inactive 4α-phorbol 12,13-didecanoate (4α-PDD) exerted no effect.[6] There is a possibility that daphnane diterpenoids also promote HGF production through activating PKC because gnidicin, one of the daphnae diterpenoids which promoted HGF production (data not shown), has been reported to activate PKC α, β1, γ.[1]

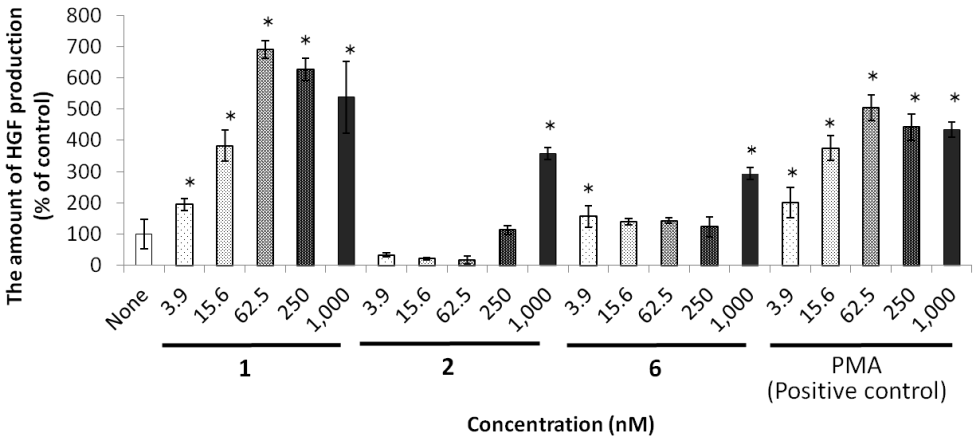


Figure 3. Effects of daphnane diterpenoids **1**, **2**, and the derivative **6** on HGF production by NHDF. The cells were seeded at a density of 5.0×10^3 cells/well and treated with various concentrations of the samples for 24 h. The amount of HGF in the supernatant was determined by ELISA. PMA was used as a positive control. Results represent means ±SD (n=4). *Statistically significant (P<0.05) difference between treatments.

EXPERIMENTAL

Plant and Isolation of Daphnane Diterpenoids.

Daphne odora Thunb. (Thymelaeaceae) were purchased from Okubobussan Co., Ltd. (Japan). Dried roots of *D. odora* (665 g) were extracted with MeOH (2.5 L) and evaporated to dryness *in vacuo* at 30 ℃. Then the residue (115 g) was partitioned with EtOAc (500 mL x 3) and H_2O (500 mL). The H_2O-soluble portion was further partitioned with *n*-BuOH (500 mL x 3) and H_2O (400 mL). The EtOAc-soluble portion (30.6 g) was partitioned with *n*-Hexane (500 mL x 3) and MeOH (500 mL). The MeOH-soluble portion (10.6 g) was subjected to silica gel column chromatography (ϕ 4.0 x 33 cm, $CHCl_3$/MeOH, 98:2 →0:100). A fraction (226 mg) eluted with $CHCl_3$/MeOH (90:10) was applied to a ODS column chromatography (ϕ 1.1 x 32 cm, MeOH/H_2O, 50:50→100:0) and the fraction (6.8 mg) containing daphnane diterpenoids judging from the ^{1}H NMR spectrum was further separated by reversed-phase HPLC [Inertsil ODS-3, ϕ1.0 x 25 cm, GL Sciences, USA, flow rate 2.0 mL/min; solvent MeCN/H_2O (4:1)] to yield synaptolepis factor K4 (**5**) (0.4 mg, t_R 23.5 min), odoratrin (**4**) (1.0 mg, t_R 25.9 min), yuanhuajine (**3**) (1.5 mg, t_R 27.9 min), and gnidilatidin (**1**) (1.2 mg, t_R 32.8 min). Moreover, the *n*-BuOH-soluble portion was subjected to silica gel column chromatography (ϕ 4.0 x 35 cm, $CHCl_3$/MeOH, 95:5→0:100). A fraction (337 mg) eluted with $CHCl_3$/MeOH (90:10) was applied to a Sep-Pak SiO2 cartridge (Waters; Hexane/EtOH, 90:10→$CHCl_3$/MeOH, 0:100) and the fraction (66.8 mg) containing daphnane diterpenoids judging from the ^{1}H NMR spectrum was further separated by reversed-phase HPLC [Inertsil ODS-3, ϕ1.0 x 25 cm, GL Sciences, USA, flow rate 2.0 mL/min; MeCN isocratic] to yield gnidilatidin 20-palmitate (**2**) (6.2 mg, t_R 114.3 min). The isolated compounds were

identified by NMR and ESIMS.[10-14]

Acetylation of gnidilatidin (1)

Acetic anhydride (0.1 mL) was added to a solution of gnidilatidin (**1**, 0.5 mg) in pyridine (0.1 mL). After stirring at room temperature for 21 h, the reaction mixture was concentrated under the reduced pressure to give gnidilatidin diacetate (**6**, 0.5 mg). **6**: colorless solid, ESIMS *m/z*: 733 $(M+H)^+$, HRESIMS *m/z*: 733.3215 $(M+H)^+$ (calcd for $C_{41}H_{49}O_{12}$, 733.3221), ^{1}H NMR ($CDCl_3$) δ:7.92 (2H, d, *J*= 7.0 Hz), 7.62 (1H, m), 7.50 (1H, s), 7.48 (2H, d, *J*= 7.0 Hz), 6.70 (1H, dd, *J*= 15.5, 11.0 Hz), 6.04, 5.88 (each 1H, m), 5.68 (1H, d, *J*= 15.5 Hz), 5.53, 5.17, 5.01 (each 1H, s), 4.92 (1H, d, *J*= 2.6 Hz), 4.79 (1H, d, *J*= 11.8 Hz), 3.96, 3.67 (each 1H, s), 3.63 (1H, d, *J*= 11.8 Hz), 3.58 (1H, s), 2.47 (1H, q, *J*= 7.2 Hz), 2.14 (3H, s), 2.11 (1H, m), 2.05 (3H, s), 1.87, 1.74 (each 1H, s), 1.39 (1H, m), 1.34 (1H, d, *J*= 7.3 Hz), 1.23 (2H, m), 0.89 (1H, m).

Cell Culture.

Normal human dermal fibroblasts (NHDF) were purchased from KURABO (Osaka, Japan) and grown in Dulbecco's modified Eagle's medium (DMEM) (SIGMA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo SCIENTIFIC, USA) at 37 ℃ in a humidified atmosphere of 5% CO_2 in air.

Determination of HGF Levels in Conditioned Media.

NHDF were trypsinized and suspended in the medium described above, were seeded in 96-well plate at a density of 5.0×10^3 cells/well. After culturing over night, the medium was replaced with the fresh medium or that containing test compounds diluted with the medium supplemented with 0.5% FBS. The conditioned medium was collected after incubation of the cells for 24 h. The sandwich ELISA for human HGF was performed at room temperature with a slight modification following the manufacturer's instructions of the biotinylated goat anti-human HGF antibody (R&D systems, Inc, USA). The conditioned medium or human HGF (SIGMA, USA) were added to an ELISA plate. The plate was coated with the mouse anti-human HGF antibody (R&D Systems, Inc, USA) overnight at 4 ℃ and blocked by adding PBS containing 1% bovine serum albumin (BSA) overnight in advance. After 2 h incubation, the wells were washed 3 times with PBS-Tween 20 (pH 7.4), and the biotinylated goat anti-human HGF antibody were added. After another 2 h incubation, the wells were washed 3 times, and streptavidin-horseradish peroxidase (HRP) conjugate (R&D Systems, Inc, USA) was added to each well. After 30 min incubation, the wells were washed 3 times, and substrate solution was added, and then, incubated for another 30 min. Stop solution was added, and the amount of HGF was determined

spectrophotometrically by measuring the absorbance at 450 nm. The standard curve for HGF was linear within the range of 0 to 5.0 ng HGF/mL. The data were expressed as percentages of the amount of HGF produced by the non treated cells.

The cells were disrupted with 0.5% Triton-X 100, and the amount of cellular protein was quantified using DC protein assay (Bio-Rad, USA). The amount of HGF production was expressed as pg per μg of cellular protein.

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