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Development of novel DIF-1 derivatives that selectively suppress innate immune responses

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ABSTRACT

The multiple pharmacological activities of differentiation-inducing factor-1 (DIF-1) of the cellular slime mold *Dictyostelium discoideum* led us to examine the use of DIF-1 as a 'drug template' to develop promising seed compounds for drug discovery. DIF-1 and its derivatives were synthesized and evaluated for their regulatory activities in innate immune responses. We found two new derivatives (**4d** and **5e**) with highly selective inhibitory activities against production of the antimicrobial peptide attacin in *Drosophila* S2 cells and against production of interleukin-2 in Jurkat cells.

1. Introduction

The cellular slime mold *Dictyostelium discoideum* is an excellent model organism for the study of cellular and developmental biology.[1] Differentiation-inducing factor-1 (DIF-1) (**1c**) was previously identified as a signal molecule that induces stalk cell differentiation in *Dictyostelium discoideum* in the presence of cAMP.[2–4] In our previous studies, we showed that DIF-1 (**1c**) possesses multiple pharmacological activities in mammalian cells, such as anti-leukemic activity, acceleration of differentiation,[5–7] and promotion of glucose consumption.[8] Some DIF-1 (**1c**) derivatives showed novel pharmacological activities. Specifically, DIF-3 (**2**) suppressed the infection rate and growth of *Trypanosoma cruzi*,[9] and Br-DIF-1 (**3**) accelerated the differentiation of P19CL6 cells into spontaneously beating cardiomyocyte-like cells (Fig. 1).[10] These results suggest that DIF-1 (**1c**) may be considered as a promising 'drug template' that may be used to develop seed compounds with multiple pharmacological activities.

Innate immunity is the front line of self defense against microbial infections.[11] Innate immune regulators have potential as therapeutic medications for sepsis or opportunistic infective diseases. Recently, we screened for innate immune regulators by using transgenic *Drosophila*.[12–14] Because of the utility of DIF-1 (**1c**) as a drug template, we decided to evaluate the effects of DIF-1 (**1c**) and its derivatives on the innate immune system. We found that the novel derivatives **4d** and **5e** selectively inhibited production of the antimicrobial peptide attacin in *Drosophila* S2 cells. These compounds also selectively suppressed interleukin-2 (IL-2) production in Jurkat cells.[15]

2. Results

2.1. Effects of DIF-1 and benzene derivatives on the innate immunity of *Drosophila*

Using *att-luc* assay, we investigated the effect of DIF-1 (**1c**) on the innate immune system of *Drosophila*.[16] In this assay, we used transgenic *Drosophila* S2 cells carrying the *att-luc* reporter gene, which was activated by addition of peptidoglycan (PGN).[17] As shown in Figure 2A, DIF-1 (**1c**) inhibited attacin production in S2 cells in a concentration-dependent manner (IC_{50} 4.6 μM). However, because it also inhibited the proliferation of S2 cells (CC_{50} 22.5 μM), its selectivity to inhibition of attacin production was low, having a selective index (SI) of 4.9 (Table 1).

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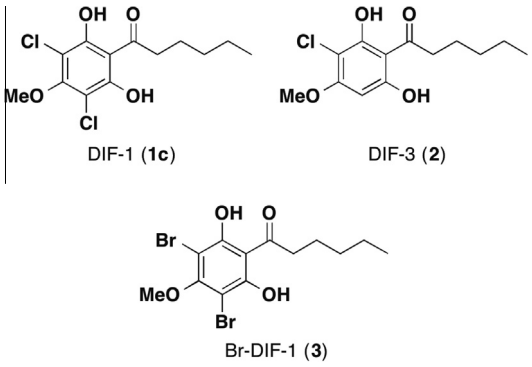


Figure 1. Structures of DIF-1 (**1c**), DIF-3 (**2**), and Br-DIF-1 (**3**).

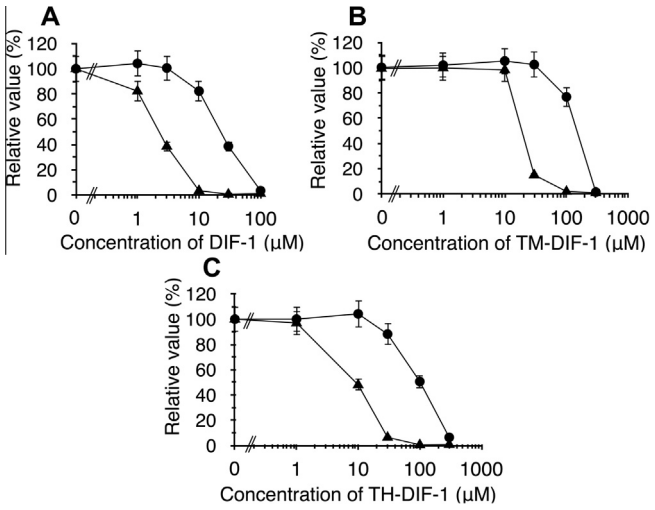


Figure 2. Effects of DIF-1 (**1c**) (A), TM-DIF-1 (**4c**) (B), and TH-DIF-1 (**5c**) (C) on peptidoglycan (PGN)-induced attacin production (*att-luc* assay) in S2 cells and cell viability. Cells were incubated for 8 h with PGN at the indicated concentrations of DIF-1 derivatives and then assayed for luciferase production (black triangles). Cell viability (black circles) was determined by incubating cells with the indicated concentrations of DIF-1 derivatives for 24 h. Results are presented as mean values (±SD) of four independent experiments ($n = 4$).

Table 1
Suppressive effects of DIF-1 derivatives on innate immunity

Compound	Immunosuppressive effect IC_{50}[a] (μM)	Cytotoxicity CC_{50}[b] (μM)	Selective index[c]
DIF-1 (**1c**)	4.6	22.5	4.9
DIF-3 (**2**)	4.6	8.7	1.9
Br-DIF-1 (**3**)	3.7	12.9	3.5
	7		
TM-DIF-1 (**4c**)	18.9	148	7.8
TH-DIF-1 (**5c**)	9.9	102	10.3
6	58.6	>100	>1.7
7	3.8	13.8	3.6

[a] IC_{50} value is the concentration of compound that suppressed attacin production to 50% of the control (peptidoglycan only) level. It was determined by assessing the effects of various concentrations of the compound.

[b] CC_{50} value is the concentration of compound that suppressed the proliferation of S2 cells to 50% of the control (DMSO) level. It was determined by assessing the effects of various concentrations of the compound.

[c] Selective index (SI) is the CC_{50}/IC_{50} ratio.

To discover more inhibitors with high selectivity, we evaluated the derivatives with varied substituents on the benzene ring in DIF-1 (**1c**) (Scheme 1). DIF-3 (**2**), Br-DIF-1 (**3**), TM-DIF-1 (**4c**), and TH-DIF-1 (**5c**), were synthesized according to our previous work.[7] We could obtain the deoxo-derivative **6** by reductive removal of the carbonyl group of DIF-1 (**1c**) using triethylsilane in trifluoroacetic acid.[18] Compound **7**, which has a methyl group instead of a methoxy group, was synthesized from orcinol instead of 5-methoxyresorcinol through the same synthetic route for DIF-1 (**1c**).

Evaluation of the activities of the aforementioned compounds showed that DIF-3 (**2**), Br-DIF-1 (**3**), and **7** inhibited both attacin production and S2 cell proliferation, resulting in low SI values (Table 1). Derivative **6** showed weak inhibitory activity, indicating the role of the carbonyl group in the inhibition. Although TM-DIF-1 (**4c**) and TH-DIF-1 (**5c**) inhibited attacin production more weakly than did DIF-1 (**1c**), they almost did not influence the proliferation of S2 cells ($CC_{50} > 100$ μM). Therefore, these compounds show promise because of their high selectivity to inhibition of attacin production (having SI values of 7.8 and 10.3, respectively; Fig. 2B and C and Table 1).

2.2. Synthesis of TM-DIF-1 and TH-DIF-1 derivatives and their effects on *Drosophila* innate immunity

To investigate in detail the structure–activity relationships for TM-DIF-1 (**4c**) and TH-DIF-1 (**5c**), we synthesized their derivatives, which have varied acyl chain lengths, and examined their effects on the innate immune response. Derivatives **4a**, **4b**, **4d**, **4e**, **5a**, **5e**, and **5f**, which, respectively, have 4, 5, 7, 8, 4, 8, and 9 carbons in the acyl chain, were synthesized from their corresponding acyl chlorides according to the synthetic routes for TM-DIF-1 (**4c**) and TH-DIF-1 (**5c**) (Scheme 1). Their inhibitory activities on the innate immune response suggest that derivatives with longer acyl chains tend to show higher activity and high selectivity to innate immunity (Table 2). However, the selectivities of **4e** and **5f** (respectively, having eight and nine carbons in the acyl chain) were relatively low. Overall, **4d** (SI 8.3) and **5e** (SI 23.9) showed promise.

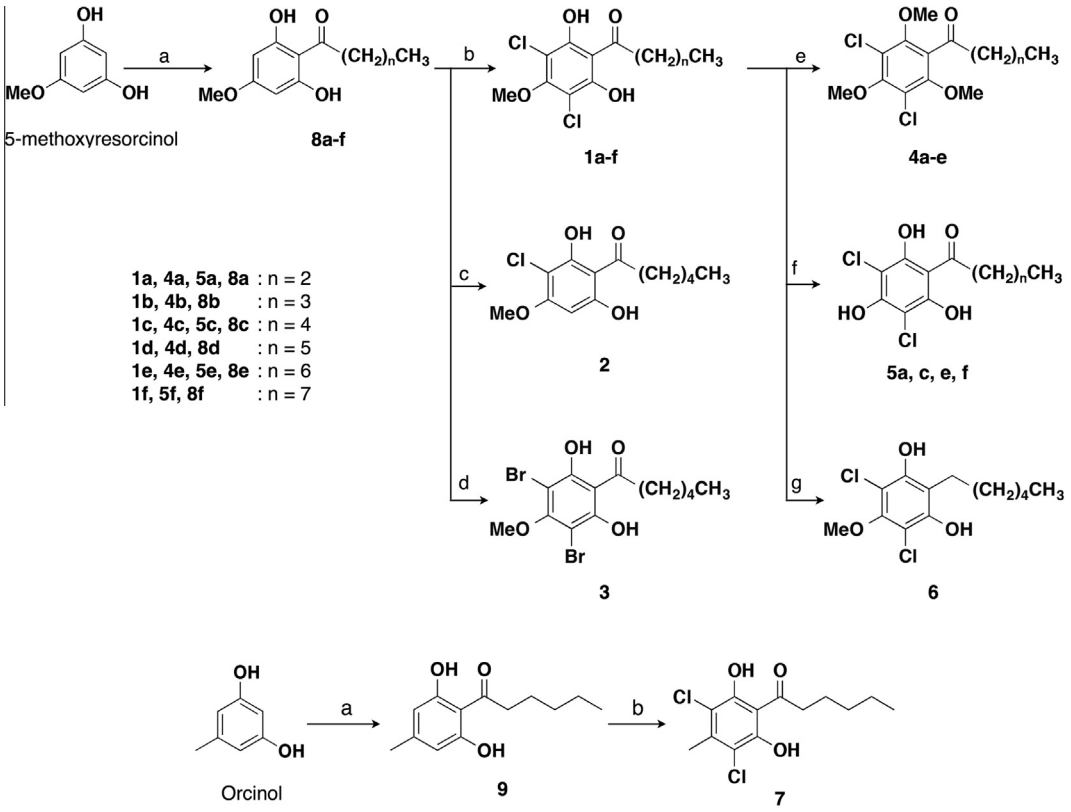
The acyl chain length of the most selective inhibitors of attacin production among TM-DIF-1 (**4c**) and TH-DIF-1 (**5c**) derivatives is related to lipophilicity, which influences their cell-membrane permeability. As TM-DIF-1 (**4c**) derivatives possess three methoxy groups, they are more liposoluble than are TH-DIF-1 (**5c**) derivatives, which have three hydroxyl groups. The former derivatives thus have greater lipophilicity for permeation of cell membranes with shorter acyl chains.

2.3. Effects of compounds 4d and 5e on IL-2 production in Jurkat cells

Compounds that are effective against *Drosophila* innate immunity may regulate mammal immunity[12–14] because of the prominent conservation of innate immunity between *Drosophila* and mammals.[19,20] In our previous study,[21] TM-DIF-1 (**4c**) and TH-DIF-1 (**5c**) showed inhibitory activity against IL-2 production[15] in Jurkat cells. Thus, we evaluated the inhibitory activity of **4d** and **5e** against IL-2 production. Concanavalin A (ConA) was added to a culture of Jurkat cells, in which the compounds had been added previously.[22,23] Compounds **4d** and **5e** showed selective inhibition against ConA-induced IL-2 production without suppressing cell proliferation (Fig. 3). Although **4d** showed weaker activity than did **5e** in the *att-luc* assay (IC_{50} values of 16.8 μM and 3.7 μM, respectively), they showed very similar inhibitory activities against IL-2 production at the same concentration (5 μM).

3. Conclusion

We synthesized and evaluated the effects of DIF-1 (**1c**) derivatives on the production of attacin in *Drosophila* S2 cells to test their



Scheme 1. General procedure for the synthesis of DIF-1 derivatives. Reagents and conditions: (a) acyl chloride, $AlCl_3$, CH_2Cl_2 (44–57%), rt; (b) SO_2Cl_2 (2.2 equiv), $CHCl_3$–EtOH, rt (74–93%); (c) SO_2Cl_2 (1.1 equiv), $CHCl_3$–EtOH, rt (85%); (d) Py·HBr_3, pyridine, rt (62%); (e) *p*-TsOMe, K_2CO_3, DMF, rt (80–95%); (f) BBr_3, CH_2Cl_2, −78 °C, then rt (40%); (g) Et_3SiH, CF_3COOH, rt (96%).

Table 2
Suppressive effects of TH-DIF-1 and TM-DIF-1 derivatives on innate immunity

Compound	Immunosuppressive effect IC_{50}[a] (μM)	Cytotoxicity CC_{50}[b] (μM)	Selective index[c]
4a	20.8	144	6.9
4b	23.5	149	6.3
TM-DIF-1 (**4c**)	18.9	148	7.8
	7		
4d	16.8	140	8.3
4e	15.3	51.4	3.4
5a	21.5	>100	>4.7
TH-DIF-1 (**5c**)	9.9	102	10.3
5e	3.7	88.5	23.9
5f	6.5	82.8	12.7

[a] IC_{50} value is the concentration of compound that suppressed attacin production to 50% of the control (peptidoglycan only) level. It was determined by assessing the effects of various concentrations of the compound.

[b] CC_{50} value is the concentration of compound that suppressed the proliferation of S2 cells to 50% of the control (DMSO) level. It was determined by assessing the effects of various concentrations of the compound.

[c] Selective index (SI) is the CC_{50}/IC_{50} ratio.

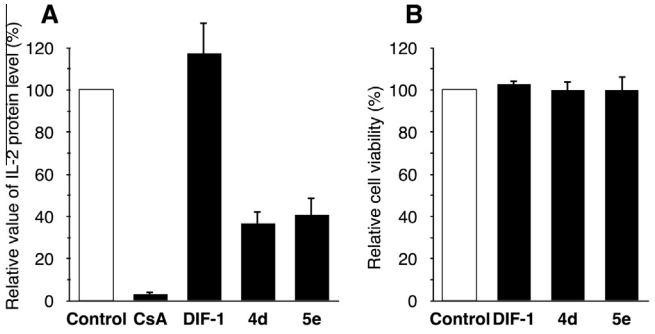


Figure 3. (A) Effects of derivatives **4d** and **5e** on concanavalin A (ConA)-induced interleukin-2 (IL-2) production in Jurkat cells. Cells were pre-incubated for 30 min with 0.1% DMSO (control), 1 μM cyclosporine A (CsA), or 5 μM of each DIF-1 derivative. After addition of ConA, cells were further incubated for 12 h and assayed for IL-2 protein production. (B) Cell viability of Jurkat cells. Cells were pre-incubated for 30 min with 0.1% DMSO (control) or 5 μM of each DIF-1 derivative. Results are presented as mean values (±SD) of three independent experiments ($n = 3$).

regulatory effects on innate immunity. We found two novel derivatives, **4d** and **5e**, which selectively suppressed attacin production with only weak inhibition of the proliferation of S2 cells. Compound **4d** possesses a benzene ring with three methoxy groups and an acyl chain with seven carbons, whereas **5e** possesses three phenolic hydroxyl groups and an acyl chain with eight carbons. As **4d** and **5e** selectively suppressed IL-2 production in Jurkat cells, they are also effective in the human immune system. Our results suggest that **4d** and **5e** are promising candidates for development into novel immunosuppressive drugs.

4. Experiment section

4.1. General methods

Analytical TLC was performed on silica gel 60 F_{254} (Merck). Column chromatography was carried out on silica gel 60 (70–230 mesh, Merck). NMR spectra were recorded on JEOL JNM ECA-600 and AL-400. Chemical shifts for ^1H and ^{13}C NMR were given in parts per million (δ) relative to tetramethylsilane (δ_H 0.00) and residual solvent signals (δ_C 77.0) as internal standards. Mass spectra were measured on JEOL JMS-700 and JMS-DX303.

HPLC was carried on LC-908 (Japan Analytical Industry Co. Ltd), using the column YMC-GPC-T2000 (ϕ 20 mm × 600 mm) (YMC Co. Ltd).

4.2. Synthesis of DIF-1 (1c), DIF-3 (2) and their derivatives

DIF-1 (**1c**) and DIF-3 (**2**) were synthesized according to previous study.[7] Sulfuryl chloride (64.0 mg, 0.474 mmol) and ethanol (40 μL) were added to a solution of **8c** (51.4 mg, 0.216 mmol) in chloroform (2.5 mL) at room temperature. After being stirred for 1 h, the mixture was evaporated. The residue was chromatographed over silica gel eluted by hexane–ethyl acetate (9:1) to give DIF-1 (**1c**) (61.6 mg, 0.201 mmol (yield 93%)). In a similar procedure, DIF-3 (**2**) (85%) was synthesized from **8c** with 1.1 equiv of sulfuryl chloride. These compounds have been characterized before.[7]

In the similar procedure, compounds **1a** (yield 87%), **1b** (78%), **1d** (83%), **1e** (74%) and **1f** (81%) were prepared from **8a**, **8b**, **8d**, **8e** and **8f**, respectively. **1a**, **1b**, **1d** and **1e** have been characterized before.[7] Data for **1f**: Colorless amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.34 (2H, br s), 3.98 (3H, s), 3.13 (2H, t, J = 7.3 Hz), 1.70 (2H, quin, J = 7.3 Hz), 1.23–1.40 (10H, m), 0.89 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.4, 166.5, 163.9 (2C), 105.2, 94.3 (2C), 61.4, 44.1, 31.8, 29.4, 29.2, 29.0, 24.9, 22.7, 14.3; EIMS m/z 352 $[M+4]^+$, 350 $[M+2]^+$, 348 $[M]^+$, 330, 263, 235 (base); HREIMS m/z 348.0886 $[M]^+$ (348.0895 calculated for $C_{16}H_{22}O_4{}^{35}Cl_2$).

4.3. Synthesis of TM-DIF-1 (4c) and its derivatives

TM-DIF-1 (**4c**) was synthesized according to previous study.[7] Potassium carbonate (20.8 mg, 0.750 mmol) and methyl *p*-toluenesulfonate (70.0 mg, 0.461 mmol) were added to a solution of DIF-1 (**1c**) (70.5 mg, 0.230 mmol) in acetone (2 mL) at room temperature. After being stirred for 16 h, the mixture was poured into water (30 mL) and extracted with ethyl acetate (30 mL) three times. The organic layer was washed with brine, dried over anhydrous sodium sulfate and evaporated. The residue was chromatographed over silica gel eluted by hexane–ethyl acetate (19:1) to give TM-DIF-1 (**4c**) (73.2 mg, 0.218 mmol (yield 95%)). This compound has been characterized before.[7]

In the similar procedure, compounds **4a** (yield 90%), **4b** (88%), **4d** (83%) and **4e** (80%) were prepared from **1a**, **1b**, **1d** and **1e**, respectively. Data for **4a**: Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.92 (3H, s), 3.83 (6H, s), 2.75 (2H, t, J = 7.6 Hz), 1.71 (2H, sext, J = 7.4 Hz), 0.98 (3H, t, J = 7.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.5, 154.5, 151.9 (2C), 129.2, 119.57 (2C), 62.7 (2C), 60.9, 46.8, 16.9, 13.5; EIMS m/z 310 $[M+4]^+$, 308 $[M+2]^+$, 306 $[M]^+$, 263 (base); HREIMS m/z 306.0420 $[M]^+$ (306.0426 calculated for $C_{13}H_{16}O_4{}^{35}Cl_2$). Data for **4b**: Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.92 (3H, s), 3.83 (6H, s), 2.77 (2H, t, J = 7.4 Hz), 1.66 (2H, quin, J = 7.8 Hz), 1.38 (2H, sext, J = 7.2 Hz), 0.93 (3H, t, J = 7.7 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.6, 154.4, 151.9 (2C), 129.2, 119.7 (2C), 62.7 (2C), 60.9, 44.7, 25.4, 22.1, 13.8; EIMS m/z 324 $[M+4]^+$, 322 $[M+2]^+$, 320 $[M]^+$, 263 (base); HREIMS m/z 306.0570 $[M]^+$ (320.0582 calculated for $C_{14}H_{18}O_4{}^{35}Cl_2$). Data for **4d**: Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.92 (3H, s), 3.82 (6H, s), 2.76 (2H, t, J = 7.4 Hz), 1.67 (2H, quin, J = 7.0 Hz), 1.25–1.39 (6H, m), 0.89 (3H, t, J = 6.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.6, 154.4, 151.9 (2C), 129.2, 119.7 (2C), 62.7 (2C), 60.9, 45.0, 31.5, 28.7, 23.3, 22.5, 14.0; EIMS m/z 352 $[M+4]^+$, 350 $[M+2]^+$, 348 $[M]^+$, 263 (base); HREIMS m/z 348.0912 $[M]^+$ (348.0896 calculated for $C_{16}H_{22}O_4{}^{35}Cl_2$). Data for **4e**: Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.92 (3H, s), 3.82 (6H, s), 2.76 (2H, t, J = 7.7 Hz), 1.67 (2H, quin, J = 7.2 Hz), 1.24–1.37 (8H, m), 0.88 (3H, t, J = 7.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.6, 154.4, 151.9 (2C), 129.2, 119.7 (2C), 62.7 (2C), 60.9, 45.0, 31.7, 29.0 (2C), 23.3, 22.6, 14.0; EIMS m/z 366 $[M+4]^+$, 364 $[M+2]^+$, 362 $[M]^+$, 263 (base); HREIMS m/z 362.1040 $[M]^+$ (362.1050 calculated for $C_{17}H_{24}O_4{}^{35}Cl_2$).

4.4. Synthesis of TH-DIF-1 (5c) and its derivatives

1 M Solution of boron tribromide in dichloromethane (520 μL, 0.520 mmol) was added to a solution of DIF-1 (**1c**) (20.0 mg, 0.065 mmol) in dichloromethane (2 mL) at 0 °C. After being stirred for 16 h at 0 °C, the mixture was poured into water (30 mL) and extracted with ethyl acetate (30 mL) three times. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and evaporated. The residue was chromatographed over silica gel eluted by hexane–ethyl acetate (9:1) to give TH-DIF-1 (**5c**) (7.5 mg, 0.026 mmol (yield 40%)). This compound has been characterized before.[7]

In the similar procedure, compounds **5a** (yield 45%), **5e** (48%) and **5f** (40%) were prepared from **1a**, **1e** and **1f**, respectively. Data for **5a**: Yellow amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.50 (2H, br s), 6.49 (1H, br s), 3.10 (2H, t, J = 7.2 Hz), 1.59 (2H, sext, J = 7.3 Hz), 1.00 (3H, t, J = 7.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.0, 156.4, 153.0 (2C), 104.7 (2C), 99.8, 46.1, 17.7, 14.3; EIMS m/z 268 $[M+4]^+$, 266 $[M+2]^+$, 264 $[M]^+$, 221 (base); HREIMS m/z 263.9961 $[M]^+$ (263.9956 calculated for $C_{10}H_{10}O_4{}^{35}Cl_2$). Data for **5e**: Yellow amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.50 (2H, br s), 6.49 (1H, br s), 3.11 (2H, t, J = 7.3 Hz), 1.70 (2H, quin, J = 7.7 Hz), 1.25–1.40 (8H, m), 0.89 (3H, t, J = 7.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.2, 156.4, 153.0 (2C), 104.7 (2C), 99.8, 44.2, 31.7, 29.3, 29.1, 24.3, 22.6, 14.1; EIMS m/z 324 $[M+4]^+$, 322 $[M+2]^+$, 320 $[M]^+$, 302, 249, 221 (base); HREIMS m/z 320.0570 $[M]^+$ (320.0581 calculated for $C_{14}H_{18}O_4{}^{35}Cl_2$). Data for **5f**: Yellow amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.49 (2H, br s), 6.50 (1H, br s), 3.11 (2H, t, J = 7.3 Hz), 1.70 (2H, quin, J = 7.6 Hz), 1.21–1.40 (10H, m), 0.88 (3H, t, J = 7.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.2, 156.4, 153.0 (2C), 104.7 (2C), 99.8, 44.3, 31.9, 29.5, 29.3, 29.2, 24.2, 22.7, 14.1; EIMS m/z 338 $[M+4]^+$, 336 $[M+2]^+$, 334 $[M]^+$, 316, 249, 221 (base); HREIMS m/z 334.0744 $[M]^+$ (334.0737 calculated for $C_{15}H_{20}O_4{}^{35}Cl_2$).

4.5. Synthesis of 4,6-dichloro-2-hexyl-5-methoxybenzene-1,3-diol (6)

Triethylsilane (41 μL, 0.260 mmol) was added to a solution of DIF-1 (20.0 mg, 0.065 mmol) in trifluoroacetic acid (1 mL) at room temperature. After being stirred for 8 h, the mixture was evaporated. The residue was purified by GPC HPLC (column, YMC-GPC T-2000 (φ 20 mm × 600 mm, TMC Co., Ltd); solvent, ethyl acetate) to give **6** (18.2 mg, 0.063 mmol (yield 96%)). Data for **6**: Yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.65 (2H, br s), 3.90 (3H, s), 2.60 (2H, t, J = 7.9 Hz), 1.47 (2H, quin, J = 7.6 Hz), 1.19–1.32 (6H, m), 0.91 (3H, t, J = 6.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 149.7, 149.4 (2C), 113.6 (2C), 106.9, 60.8, 31.7, 29.3, 28.7, 24.3, 22.6, 14.1; EIMS m/z 296 $[M+4]^+$, 294 $[M+2]^+$, 292 $[M]^+$, 221 (base); HREIMS m/z 292.0617 $[M]^+$ (292.0632 calculated for $C_{13}H_{18}O_3{}^{35}Cl_2$).

4.6. Synthesis of 1-(2,6-dihydroxy-4-methylphenyl)hexan-1-one (9)

Aluminum chloride (215.7 mg, 1.62 mmol) was added to a solution of orcinol (100.0 mg, 0.81 mmol) in dichloromethane (5 mL) at room temperature. After 15 min, hexanoyl chloride (170 μL, 1.21 mmol) was added. The mixture was stirred for 3 h, poured into water (25 mL), and extracted with ethyl acetate (30 mL) three times. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and evaporated. The residue was

chromatographed over silica gel eluted by hexane–ethyl acetate (9:1) to give **9** (126.5 mg, 0.57 mmol (yield 70%)). Data for **9**: Colorless amorphous solid; ^{1}H NMR (400 MHz, acetone-d_6) δ 11.84 (2H, br s), 6.61 (2H, s), 3.06 (2H, t, J = 7.4 Hz), 2.34 (3H, s), 1.68 (2H, quin, J = 7.0 Hz), 1.30–1.35 (4H, m), 0.91 (3H, t, J = 6.9 Hz); ^{13}C NMR (100 MHz, acetone-d_6) δ 205.5, 162.9 (2C), 147.3, 105.5, 91.1 (2C), 44.4, 31.4, 23.6, 23.4, 18.9, 14.3; EIMS m/z 222 $[M]^+$, 204, 151 (base); HREIMS m/z 222.1245 $[M]^+$ (222.1256 calculated for $C_{13}H_{18}O_3$).

4.7. Synthesis of 1-(3,5-dichloro-2,6-dihydroxy-4-methylphenyl) hexan-1-one (7)

Sulfuryl chloride (31.0 mg, 0.23 mmol) and ethanol (20 μL) were added to a solution of **9** (25.0 mg, 0.11 mmol) in chloroform (1 mL) at room temperature. After being stirred for 1 h, the mixture was evaporated. The residue was chromatographed over silica gel eluted by hexane–ethyl acetate (9:1) to give **7** (26.1 mg, 0.09 mmol (yield 85%)). Data for **7**: Yellow amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.21 (2H, br s), 3.14 (2H, t, J = 7.7 Hz), 2.52 (3H, s), 1.71 (2H, quin, J = 7.0 Hz), 1.33–1.38 (4H, m), 0.92 (3H, t, J = 7.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.9, 154.7 (2C), 141.7, 113.4 (2C), 108.4, 44.6, 31.4, 23.9, 23.5, 18.9, 14.0; EIMS m/z 294 $[M+4]^+$, 292 $[M+2]^+$, 290 $[M]^+$, 272, 219 (base); HREIMS m/z 290.0458 $[M]^+$ (290.0476 calculated for $C_{13}H_{16}O_3{}^{35}Cl_2$).

4.8. Measurement of cytotoxicity and luciferase activity (*att-luc* assay)

Drosophila S2 cells and S2$^{att\text{-}luc}$ cells, harboring luciferase reporter gene driven by attacin promoter,[16] were cultured in Schneider's *Drosophila* medium (Gibco-BRL) supplemented with 10% FBS and 1% antibiotics/antimycotics at 25 °C. Cytotoxicity was measured using the colorimetric thiazoyl blue conversion assay using WST-8 solution (nacalai tesque) as described previously.[12] To determine the effect of compounds on attacin production, S2$^{att\text{-}luc}$ cells were pre-treated with compounds for 1.5 h and stimulated by 100 ng/mL peptidoglycan. At 8 h after stimulation, cells were lysed with Glo-lysis buffer (Promega), and luciferase activities were measured by One-Glo (Promega).

4.9. Assay for IL-2 production by Jurkat cells and determination of cell viability

Jurkat cells were pre-incubated for 30 min in 90 mm culture dishes filled with 10 mL RPMI (at 1×10^6 cells/mL) in the presence of the test compounds (5 μM) or 0.1% DMSO (vehicle). After the addition of ConA to a final concentration of 25 μg/mL, the cells were further incubated for 12 h. Aliquots of the culture media were collected, and the levels of IL-2 were assessed by using immunoassay kits (ENDOGEN, Rockford, IL). Briefly, 50 μL aliquots (in duplicate) of the culture media or standards for IL-2 from the kits were added to the wells of 96-well plates pre-coated with anti-human IL-2 antibody. After incubation with biotinylated antibodies to human IL-2 and then with streptavidin-horseradish peroxidase, color was developed and the levels of IL-2 were quantified by measuring the absorbance of each sample at 450 nm and 550 nm. The readings at 550 nm were subtracted from the readings at 450 nm. To determine cell viability, cells were incubated under the same conditions, and the percentage of viable cells compared with controls was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl tetrazolium bromide (MTT) assay according to standard procedures.

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