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Diaporine, a Novel Endophyte-derived Regulator of Macrophage Differentiation

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Diaporine (1), an unprecedented symmetric polyketide, was characterized from the endophytic fungus. The structure was determined by extensive spectroscopic analyses. Diaporine can inhibit significantly the differentiation of macrophage and have potential to induce its conversion from M2 to M1 phenotype, in addition to its regulation of the TLR4-MAPK signal pathway and PPARγ activity.

Macrophages are key cells in the immune response to foreign invaders. The phenotype of macrophages has been generally defined into two separate polarization states, M1 and M2.[1,2] The M1 macrophages are characterized by an enhanced production of pro-inflammatory cytokines, a lowered level of anti-inflammatory IL-10, and the generation of reactive oxygen species such as NO. The function of M1 macrophages includes mainly Th1 responses and killings of intracellular pathogens and tumors. The M2 macrophages have low expression of pro-inflammatory cytokines, but generate high levels of anti-inflammatory cytokines IL-10. Overall, M2 macrophages are believed to participate in the blockade of inflammatory responses, Th2 responses, promotion of tissue repair and tumor promotion.[1] There are many types of cell in tumor microenvironment. Among them, tumor associated macrophages (TAMs) are acknowledged as the most abundant leukocytes in melanoma lesions (constituting up to 30% of tumor cells).[3] TAMs have been increasingly characterized as M2 macrophages within many kinds of tumors, and the regulation of M1/M2 polarization in TAMs may provide new strategies for anti-tumor therapies.[4,5]

As part of our ongoing search for bioactive secondary metabolites from microbes from special niche,[6] we focused on *Diaporthe*sp. IFB-3lp-10, an endophytic fungus isolated from the healthy mangrove leaves of *Rhizophora stylosa*. Previous chemical investigation on this strain has led to the isolation of nine new sesquiterpenoids.[6a] Continued attempts aimed at the characterization of TAM-phenotype regulator(s) have led to the present discovery of an unprecedented symmetrical polyketide we have named diaporine (1). As desired, compound 1 was shown to turn TAMs from M2 to M1 phenotypes in cellular and animal (ectopic breast cancer) models. Moreover, we demonstrated that TLR4-MAPK signal pathway and PPARγ activity regulation were involved in this phenotype change process.

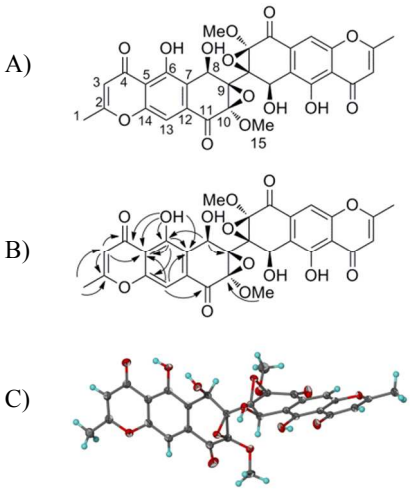


Figure 1. Symmetrical structure (A), Key HMBC correlations (B) and X-ray crystal structure (C) of diaporine (**1**).

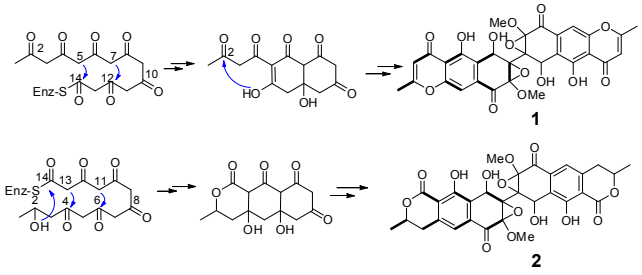
Compound **1**,[7] afforded as a yellow crystal, was evidenced to have a molecular formula of $C_{30}H_{22}O_{14}$ (20 indices of hydrogen deficiency) from the Na^+-liganded molecular ion at *m/z* 629.0893 ($C_{30}H_{22}O_{14}Na$ requires 629.0902) in its high-resolution electrospray ionization mass spectrometry (HR-ESI-MS). However, the ^{13}C NMR spectrum of **1** displayed only a total of 15 discrete carbon resonance lines (Table 1), indicative of its molecular symmetry (Figure 1A). In its HMBC spectrum (Figure1B), two- and three-bond correlations of H-1 with C-2 and C-3, H-3 with C-5, C-4 and C-2, 6-OH with C-4, C-7, C-8 and C-9, and H-13 with C-5 and C-7 revealed the presence of a 6-hydroxyl, 2-methyl chromone moiety in conjunction with the shift magnitude of these proton and carbon resonances. Furthermore, the HMBC correlations of aromatic proton H-13 with a conjugated ketone C-11, of the oxygenated methine proton H-8 with C-6, C-7 and C-12 indicated two more substituents in chromone moiety. The remaining two unsaturated indices in the monomer motif could be rationalized by two rings. The HMBC correlations of H-8/C-9 and

H-15/C-10 together with the remaining one oxygen atom suggested the presence of cyclohexanone and epoxide rings. The confirmation of the proposal and determination of its absolute configuration were accomplished by the single-crystal X-ray crystallographic analysis of **1** using the anomalous scattering of copper Kα emission (Figure 1C).

Table 1. ^{13}C and ^{1}H NMR data of **1** ($CDCl_3$)

No.	δ_C	δ_H (mult.)	No.	δ_C	δ_H (mult.)
1	20.9	2.46 (s)	10	82.7	
2	169.5		11	187.8	
3	109.5	6.20 (s)	12	133.2	
4	183.2		13	105.9	7.57 (s)
5	112.8		14	155.6	
6	160.1		15	57.0	3.81 (s)
7	121.6		6-OH		13.65 (s)
8	62.4	5.46 (s)	8-OH		4.53 (brs)
9	67.9				

Compound **1** looks similar with floccosin (**2**) (Scheme 1), a pigment from the pathogenic fungus *Epidermophyton floccosum*.[8] Biosyntheses of **1** and **2** start both from the heptaketide backbone formation, but are followed by strikingly different cyclizations (Scheme 1). The backbone of **2** can be partially reduced at C2 and cyclized via O2-C14, C4-C14, and C6-C11, a typical F-mode cyclization catalyzed by fungal polyketide synthase (PKS).[9] However, to assemble **1**, the heptaketide intermediate cyclizes between C7 and C12 under a S-mode cyclization pattern found in filamentous bacterial PKS, together with C5-C14, and O14-C2 cyclization.[9] Thus, it is likely the rarer S-mode folding of the fungal PKS that results in the novel framework of **1**.



Scheme 1. Proposed biosynthetic pathway for **1** *vs*.**2**.

TAMs, closely related with the development of tumor,[10] are a mixture of M1 and M2 macrophages with the M1 phenotype constituting a smaller part but repressing the tumor. Most TAMs are M2 phenotype, which can promote the invasion and migration of tumor.[11] We are motivated to establish a TAM-phenotype conversion model to test whether compound **1** could turn TAMs from M2 ("bad") to M1 ("good") phenotypes. Accordingly, F10 melanoma cell supernatant and RAW264.7 were co-cultured for 24 h with 10μM **1** added during the course, and the concentration of **1** was determined by the high efficiency and low toxicity in the preliminary experiment (Figure S1). The results showed that **1** could significantly reduce the M2 markers including arginase activity and IL-10 (Figures 2A, 2C and 2D), with the M1 marker (release of NO) boosted (Figure 2B). Furthermore, we examined the function of **1** in an animal model. Briefly, 10^6 4T1 breast cancer cells were subcutaneously injected into mice mammary fat pads. Once the tumor (approximately 50 mm^3) appeared, the mice were randomly divided into three groups, and each group consisted of 8 animals received either 1) vehicle as a control, 2) compound **1** at 0.2 mg/kg (BW, bodyweight), which was determined by the highest efficiency in the preliminary experiment (Figure S2) or 3) anti-tumor drug paclitaxel at 20 mg/kg (BW), administered daily for 12 days. Then the primary tumor-associated macrophages sorting from tumor mass were harvested to detect macrophages phenotype. As expected, this *in vivo* assay also demonstrated that TAMs treated with **1** could increase the expression of M1-indicating marker CCR7 but reduce that of M2-signifying marker CD206 (Figure 2E). In conclusion, diaporine (**1**) could convert the phenotype of TAMs from M2 to M1, which might be able to inhibit the tumor propagation.

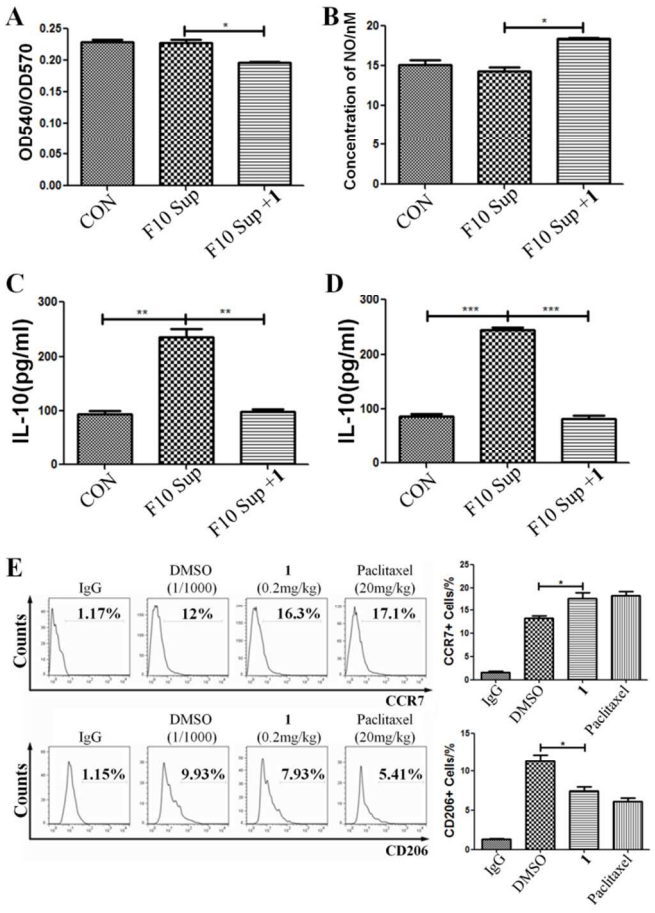


Figure 2. The phenotype of TAMs in vitro and in vivo. (A) F10 melanoma cell supernatant and RAW264.7 were co-cultured for 24h, 10μM **1** was added during the course, then the arginase activity was detected. (B) The supernatants from co-cultured cells were harvested and the release of NO were examined. Cells were co-cultured for 12h and 24h, then the release of IL-10 (C and D) were examined. (E) Drugs were administered daily for 12 days in animal models, then the animals were sacrificed and primary TAMs sorting from tumor mass were harvested to detect macrophages phenotype by Flow Cytometry Analysis. *P < 0.05, **P<0.01, ***P<0.001.

To investigate the genetic profile of phenotype conversion, the mRNA expression of inflammation-related genes were measured by RT-qPCR, and we found that most up-regulated genes were related to TLR4-MAPK signal pathway (Figure 3A), an important signal pathway involved in the regulation of pro-inflammation effect.[12] Moreover, the expression of p-ERK1/2 and p-JNK, which indicated the activation of MAPK signal pathway, was determined by western blot. As expected, both of p-ERK1/2 and p-JNK was increased by the addition of **1** (Figures 3B and 3C). The results suggested that diaporine (**1**) might alter the phenotype of macrophages from M2 to M1 through the TLR4-MAPK signal pathway.

Peroxisome proliferator activated receptor (PPARγ) belongs to the nuclear receptor superfamily. The activation of PPARγ plays an important role in the differentiation of M2 macrophages, and the phosphorylation of PPARγ on Ser112 resulted in the inactivation of PPARγ.[13] Consequently, we evaluated **1** for its presumable regulation on the expression and transcriptional activity of PPARγ. Although mRNA and protein expressions were elevated, the phosphorylation of PPARγ on Ser112 was stimulated by **1** (Figure 3D and 3E), which, consistent with the results above, led to the lower transcriptional activity of PPARγ (Figure 3F).

Heterogeneity of macrophages has long been comprehended, in part, is a result of the specialization of tissue macrophages in particular microenvironments.[14] Within tumor microenvironment, tumor-associated macrophages (TAMs) play substantial roles in tumor development. The M1/M2 activation of macrophages has been widely recognized during the past decade, and many studies have reported that TAMs resemble M2-polarized macrophages, which closely related to tumorigenesis, tumor invasiveness and then metastasis.[15-17] Thus, regulating the M1/M2 status in TAMs becomes one of the new strategies for the cancer therapy.

In the present work, we identified a small molecule, compound **1**, showing obvious function to switch the phenotype of TAMs from M2 to M1. Previous studies have reported that some small molecules could reduce tumor progression by altering the phenotype of TAMs.[18,19] Therefore, a novel cancer therapy by switching the phenotype of TAMs from M2 to M1 by drug is feasible. Since compound **1** has such a desired activity to realize the M2-to-M1 phenotype conversion of TAMs, we were curious about its mode of action. Through gene expression sort analysis, we found the genes majorly involved in TLR4-MAPK pathway such as TLR4, MyD88, TRAF6 and ERK1/2. Here clarified is that diaporine (**1**) could trigger the TLR4-MAPK pathway, an important signal pathway involved in inflammation and M1/M2 phenotype profiling of TAMs.[20,21]

Additionally, PPARγ is a key transcription factor involved in the acquisition of M2 phenotype, many drug are used to change the M1/M2 status of macrophages by altering the activity of PPARγ.[22,23] Given that fact, we detected the transcriptional activity of PPARγ in order to see whether they were affected by **1**. The results showed that **1** significantly inhibited the activity of PPARγ suggesting another advantage of **1** in inhibiting the formation of M2 macrophages.

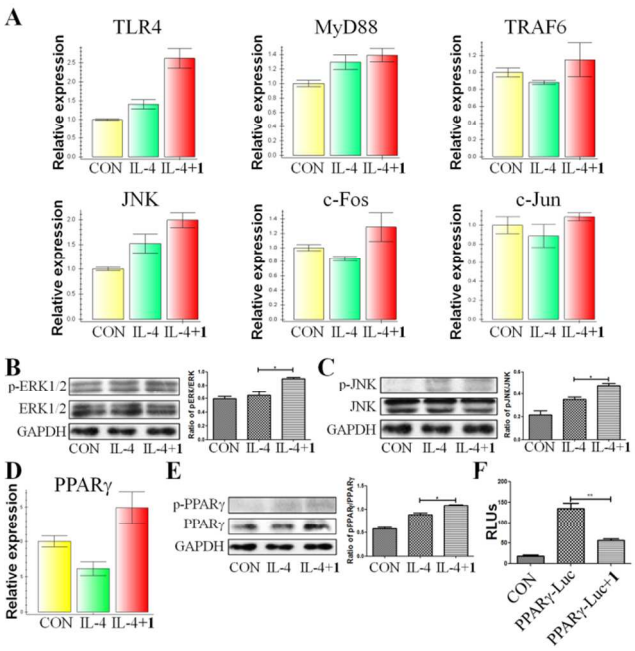


Figure 3. TLR4-MAPK signal pathway-related genes expressed by **1** treated M2 macrophages. (A) RAW 264.7 was treated with IL-4(10ng/mL) for 24h, 10 μM **1** was added during the course, the mRNA expression of TLR4-MAPK signal pathway-related genes were detected by RT-qPCR. Total protein was also extracted, ERK1/2, p-ERK1/2 (B) and JNK, p-JNK (C) were examined by western blot. mRNA (D) and protein (E) expression of PPARγ were detected by RT-qPCR and western blot respectively. (F) transcriptional activity of PPARγ was determined by luciferase reporter gene assay. All the results of western blot were quantified by ImageJ densitometry analysis. **P < 0.01.

Conclusions

In summary, the work discovered an unprecedented symmetric polyketide (**1**) with a novel framework from *Diaporthe* sp. Compound **1** had a unique function by switching the phenotype of TAMs from M2 to M1 in both breast-cancered animal and *in vitro* (cellular) models. Moreover, we found this specific function of **1** is mediated by the TLR4-MAPK pathway, and through its regulation of the PPARγ activity. In aggregation, a novel small molecule (**1**) has been characterized and verified to function in the polarization of tumor associated macrophages which might have a possibility for being used into the specific combinational strategy for cancer therapy.

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Notes and references

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7 Yellow crystalline solid, $[\alpha]_D^{28}$ = 175.4 (*c* = 0.55,CH_2Cl_2). UV (CH_2Cl_2): λ_{max} (log ε) = 244 (3.8), 261(3.8), 276 (3.8), 294 (4.0), 358(3.8), 376 (3.9) nm; IR (KBr): ν_{max}= 3568.5, 3549.7, 2961.8, 2921.9, 2851.0, 1706.4, 1649.5, 1261.2, 1011.1, 1029.2, 801.4 cm^{-1}; ^1H and ^{13}CNMR data see Table 1; HRESIMS *m/z* 629.0893 $[M+Na]^+$ (cald. for $C_{30}H_{22}O_{14}Na$ 629.0902); X-ray crystallography of **1**. $C_{30}H_{22}O_{14}\cdot 2CH_3OH$, *M* = 670.56, monoclinic, $P2_1$, a = 11.11080(7) Å, b = 14.8838(1) Å, c = 17.8512(1) Å, α = γ = 90.00°, β =90.9022(6)°, *V* = 2951.70(3) Å3, Z = 4, *Dx*= 1.509 g/cm^3, μ(Cu Kα) = 1.052 mm-1, F(000) = 1400;crystal dimensions: 0.30×0.25×0.20 mm^3. Independent reflections: 11150 (Rint = 0.0152). The final *R*1 values were 0.0270, *w*R2= 0.0730 [I >2σ(I)]. Flack parameter: 0.04(6).

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