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Overexpression of transcriptional regulator and tailoring enzyme leads to the discovery of anti-inflammatory meroterpenoids from marine-derived fungus *Alternaria alternata* JJY-32

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Four new ACTG-toxins like meroterpenoids tricycloalternarenes O-R were discovered from a marine-derived fungus *Alternaria alternata* JJY-32 by the overexpression of a LaeA-like global transcriptional regulator and a MpaB-like biosynthetic tailoring enzyme. Among those structures, tricycloalternarene O possesses a non-canonical cyclohexanone-pyran fused furan ring structure and tricycloalternarene R contains a truncated isoprenoid terminal. The structures were elucidated based on comprehensive 1D and 2D NMR data and assisted by ECD calculations. Tricycloalternarenes O-R showed potent anti-inflammatory activity in TLR4-transfected microphage cells (RAW264.7).

KEYWORDS

Alternaria alternata, meroterpenoid, transcriptional regulator, tailoring enzyme, anti-inflammatory

Introduction

Marine Microorganisms are remarkable producers of therapeutic compounds that significantly improve human health and well-being. However, the speed of new bioactive compound discovery has been slowing down due to the consistent employment of conventional cultivation and isolation procedure. Recently, benefiting from the

development of genome sequencing, genome mining, and gene transfer and expressing technologies, a number of new approaches have been developed to alleviate this challenge (Albarano et al., 2020; Chu et al., 2020). Among those methods, the overexpression of the secondary metabolism regulator (Bayram and Braus, 2012; Gacek and Strauss, 2012) or critical biosynthetic genes (Shin et al., 2012; Li et al., 2016) has been demonstrated as an effective tool for triggering the expression of the inert biosynthetic pathway and providing diversified secondary metabolites.

The genus *Alternaria* produces a broad diversity of secondary metabolites with a large range of bioactivities (Bräse et al., 2009; Lou et al., 2013; Lou et al., 2013; Abdessemed et al., 2021; Wang et al., 2022). ACTG toxins are a family of meroterpenoids mainly discovered from *Alternaria alternata* and *Alternaria citri*. As a group of phytotoxins, the skeleton of ACTG toxins particularly contains a terpenoid part derived from a farnesyl unit and a non-terpenoid part which is usually present in the form of six-membered cyclic α , β -unsaturated ketone (Kono et al., 1986; Liebermann et al., 1997; Liebermann et al., 2000; Logrieco et al., 2009; Tsuge et al., 2013; Wang et al., 2013; Shen et al., 2018). ACTG toxin derivatives usually possess the ability to bind membrane proteins thus affecting the permeability of the membrane structure and acting on signaling receptors to exert a variety of biological activities such as antibacterial, antiparasitic, antitumor, and anti-inflammatory effects (Matsumoto et al., 1992; Horiuchi et al., 2006; Gozari et al., 2021).

During our previous work in searching bioactive secondary metabolites from marine-derived fungi, the strain *A. alternata* JYJ-32 was obtained from a sea sponge sample collected from the South China Sea. One Strain Many Compounds (OSMAC) strategy guided chemical studies on JYJ-32 have provided a series of ACTG toxin derivatives that showed promising anti-inflammatory activity (Yang et al., 2013; Zhang et al., 2013; Wang et al., 2020). In a recent work, with the intent to obtain diversified ACTG toxin structures, we sequenced the genome and processed genome mining on JYJ-32. As a result, one gene analog of LaeA, a common global regulation gene of secondary metabolism in filamentous fungi (Kosalková et al., 2009; Jiang et al., 2016; Yu et al., 2019; Wang et al., 2020), and one analog of MpaB (Zhang et al., 2019), a terpenoid-trimming enzyme in mycophenolic acid biosynthesis, are screened out for the potential to modulate ACTG toxin production based on phylogenetic analysis (Figure S1 and Figure S2). The overexpression of the above genes successfully altered the metabolic profile of *A. alternata* and further chemical investigation afforded four new ACTG toxin derivatives with anti-inflammatory activity. Herein, we will describe the details of metabolic profile modulation through the overexpression of aLaeA and aMpaB, the isolation and characterization of new ACTG toxins, as well as bioactivity evaluation of obtained structures.

Results and discussion

Metabolic profile modulation by overexpression of aLaeA and aMpaB

A group of transcriptional regulators such as VeA, HogA, CmrA, and LaeA has been discovered for their ability in modulating growth morphology, asexual development, and mycotoxin production in *A. alternata* strains (Fetzner et al., 2014; Estiarte et al., 2016; Igbalajobi et al., 2019; Gao et al., 2022). We sequenced JYJ-32 followed by genome mining using bioinformatics tools of LocalBLAST and InterPro analysis. A homologue of LaeA (named aLaeA) with a length of 1583 bp encoding 301 amino acids and one homolog of MpaB (named aMpaB) with a length of 2133 bp encoding 710 amino acids were identified from the genome of JYJ-32 using LaeA (BAP58881.1) and MpaB (AJG44380.1) as references. Further protein sequence alignment and phylogenetic analysis revealed the S-adenosyl-L-methionine-dependent methyltransferase feature for aLaeA and the oxygenase feature for MpaB, which indicated their potent modulation functions in secondary metabolites production (Figure S1 and Figure S2). The genes aLaeA and aMpaB were PCR amplified from genomic DNA and inserted into the pHyg vector to generate OE-alaeA and OE-aMpaB (Figures S3 and S4). The recombinant vectors were transformed into wild-type JYJ-32 to give the engineered strains JYJ32-OE-alaeA and JYJ32-OE-aMpaB.

Strains JYJ32-OE-alaeA and JYJ32-OE-aMpaB were fermented separately in 10 L PDB medium and the High-Performance Liquid Chromatography (HPLC) analysis of the crude extracts showed altered metabolic profiles for both engineered strains with new peaks presenting at 23.6 min for JYJ32-OE-alaeA and at 21.6, 26.8 and 27.6 min for JYJ32-OE-aMpaB (Figure 1A), which indicated the capacity for new secondary metabolites production.

Compound isolation and structure elucidation

The EtOAc extracts of strain JYJ32-OE-alaeA (10.0 g) and JYJ32-OE-aMpaB (15.0 g) were subjected to vacuum-liquid chromatography (VLC), Medium Pressure Liquid Chromatography (MPLC) column chromatography and HPLC semi-preparative column chromatography for purification. Compound 1 was obtained from the crude extract of JYJ32-OE-alaeA, and compounds 2-6 were isolated from the extract of JYJ32-OE-aMpaB.

Compound 1 was obtained as a colorless oil with the $[M + H]^+$ ion at m/z 363.2186 in HRESIMS indicative of a molecular formula of $C_{21}H_{30}O_5$, requiring seven double bond equivalents (DBEs). The 1H NMR and ^{13}C NMR spectroscopic data of

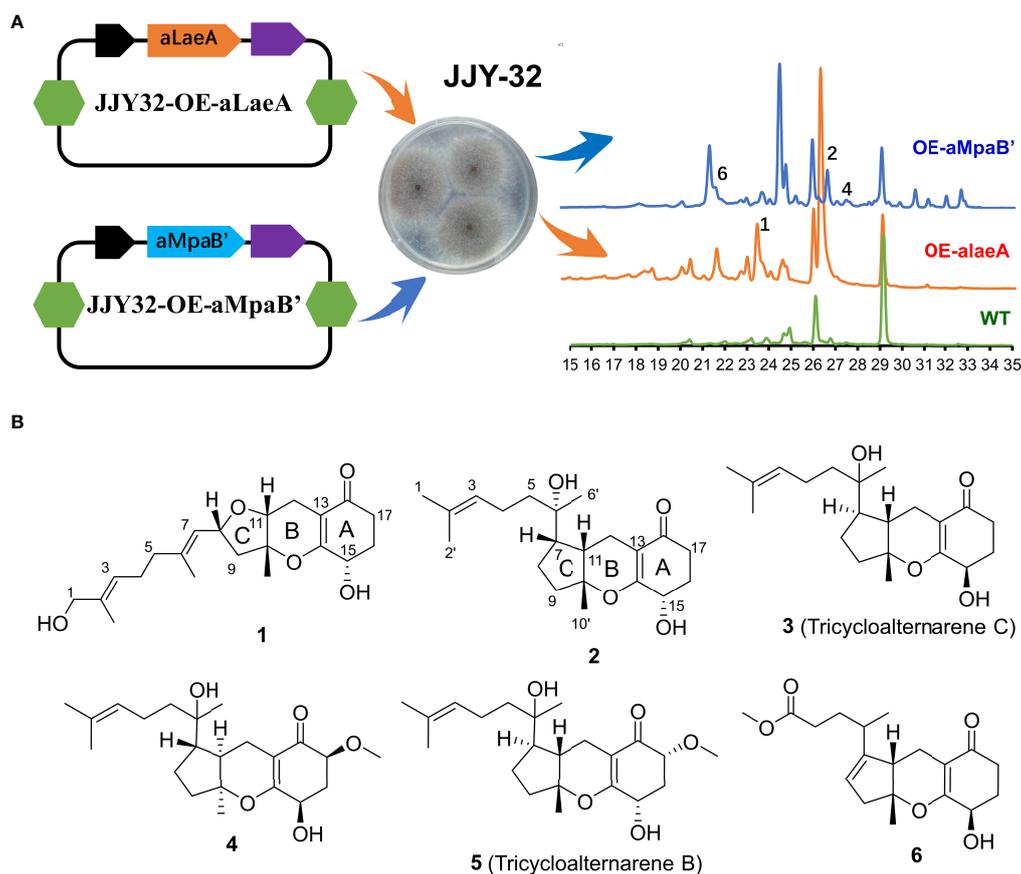


FIGURE 1

The overexpression of aLaeA and aMpaB in *A. alternata* JJY-32. (A) Heterologous expression of aLaeA and aMpaB altered the metabolic profile of JJY-32 and led to the production of compounds 1-2, 4, and 6 by HPLC (mobile phase: 5% methanol, 5 min; 100% methanol, 35 min); (B) Structure of compounds obtained from cultures of JJY32-OE-aLaeA and JJY32-OE-aMpaB.

compound **1** displayed three methyls, seven methylenes with one attached to oxygen, three oxygen-attached sp^3 methines, two sp^2 methines, and six quaternary carbons including one conjugated carbonyl carbon, four sp^2 quaternary carbons, and one oxygenated sp^3 quaternary carbon (Tables 1 and 2). Above information counted for four DBEs, which indicated a tricyclic structure for **1**. The 1H - 1H COSY correlations of H-3/H-4/H-5, H-7/H-8/H-9, H-11/H-12, and H-15/H-16/H-17, together with the HMBC correlations from H_3 -2' to C-1, C-2 and C-3, from H_3 -6' to C-5, C-6 and C-7, from H_3 -10' to C-9, C-10 and C-11, from H_2 -12 to C-13, C-14 and C-18, from H_2 -15 to C-9, C-10, and from H_2 -17 to C-18 established the cyclohexanone-sesquiterpenoid skeleton as shown in Figure 2A. Based on the formula and the chemical shifts of C-10 (δ_C 84.3)/C-14 (δ_C 166.4), and C-8 (δ_C 70.3)/C-11 (δ_C 76.4), C-10 and C-8 were respectively linked to C-14 and C-11 via an ether bond to form the rest rings (Rings B and C), thus completing the planar structure of compound **1** (Figure 2A), which was the first example of the ACTG-toxin-like compounds with a

cyclohexanone-pyran fused furan ring system. The NOESY correlations of H-1/H-3 and H-5/H-7 defined the *E* stereochemistry of the double bonds at C-2 and C-6. The NOESY correlations of H8/H10'/H11 assigned the 8*R**, 10*R**, 11*R** relative configuration. To facilitate computational processing and ECD spectra fitting (Nugroho and Morita, 2014), compound **1** was truncated as **1a-1d**, where the C-6 side chain was replaced by a methyl group. The ECD spectra of (8*R*, 10*R*, 11*R*, 15*R*)-**1a**, (8*R*, 10*R*, 11*R*, 15*S*)-**1b**, (8*S*, 10*S*, 11*S*, 15*S*)-**1c**, and (8*S*, 10*S*, 11*S*, 15*R*)-**1d** were produced using density functional theory (DFT) calculations performed at the B3LYP/6-31+G(d) level. The experimental spectra of compound **1** showed an identical trend with that of (8*R*, 10*R*, 11*R*, 15*R*)-**1a** (Figure S6 and Table S5), thus determining the absolute configuration at C-8, C-10, C-11, and C-15. Compound **1** was then named tricycloalternarene O.

Compound **2** was obtained as a colorless oil. The HRESIMS $[M + H]^+$ peak at m/z 349.2383 indicated a molecular formula of $C_{21}H_{32}O_4$. The 1H and ^{13}C NMR spectra indicated four methyls,

TABLE 1 ¹H NMR data for compounds 1-2, 4, and 6.

No.	1 ^a	2 ^b	4 ^b	6 ^b
1	4.0, s	1.64, s	1.63, s	3.53, s
2				
2'	1.35, s	1.57, s	1.54, s	
3	5.36, t (6.6)	5.08, t (10.0)	5.06, t (5.9)	1.80, m; 1.94, m
4	2.15, m	1.94, m	1.89, m	2.08, m; 2.18, m
5	2.06, m	1.31, t-like (8.3)	1.32, t-like (7.7)	1.88, m
6				0.91, d (7.7)
6'	1.65, s	1.15, s	1.00, s	
7	5.17, d (8.8)	2.09, m	1.9, m	
8	4.77, dt (8.8, 4.4)	1.86, m; 1.51, m	1.57, m	5.35, t
9	2.05, m; 2.34, m	1.72, m	1.87, m; 1.53, m	2.43, m; 2.50, m
10'	1.67, s	1.39, s	1.24, s	1.40, s
11	3.9, d (4.4)	1.65, m	2.04, m	2.78, m
12	2.24, m; 2.74, d (18.0)	2.49, m; 1.85, m	2.34, m; 2.09, m	2.03, m; 2.40, m
15	4.43, t (6.6)	4.11, t (6.0)	4.40, t (4.5)	4.02, dd (5.46, 9.9)
16	2.0, m; 2.29, m	2.01, m; 1.79, m	2.38, m; 1.65, m	1.51, m; 1.64, m
17	2.36, m; 2.60, dt (5.5, 17.4)	2.43, m; 2.17, m	3.72, dd (6.9, 3.2)	2.11, m; 2.46, m
17-OCH ₃			3.40, s	
6-OH		4.03, s		
15-OH		5.33, d (8.2)		

^a Recorded in CDCl₃, ^b Recorded in DMSO.

TABLE 2 ¹³C NMR data for compounds 1-2, 4, and 6.

No.	1 ^a	2 ^b	4 ^b	6 ^b
1	68.7	25.4	25.4	51.1
2	135.0	130.2	130.1	173.2
2'	19.1	17.4	17.4	
3	125.4	124.8	125.0	29.5
4	25.4	22.3	22.3	31.2
5	39.0	42.4	40.7	31.5
6	138.1	71.8	72.6	19.9
6'	16.4	25.3	24.3	
7	126.5	47.5	49.6	148.7
8	73.0	18.0	23.6	120.4
9	46.1	34.2	38.1	44.4
10	84.3	84.9	88.2	87.4
10'	13.7	27.3	22.6	23.0
11	76.4	40.6	41.0	46.0
12	19.1	16.3	18.8	14.7
13	106.1	108.9	105.2	106.6
14	166.4	168.4	170.3	170.8
15	66.0	64.6	64.2	64.6
16	28.9	29.6	36.9	29.3
17	33.5	32.7	77.5	32.2
18	197.1	196.0	195.9	195.8
17-OCH ₃			57.5	

^a Recorded in CDCl₃, ^b Recorded in DMSO.

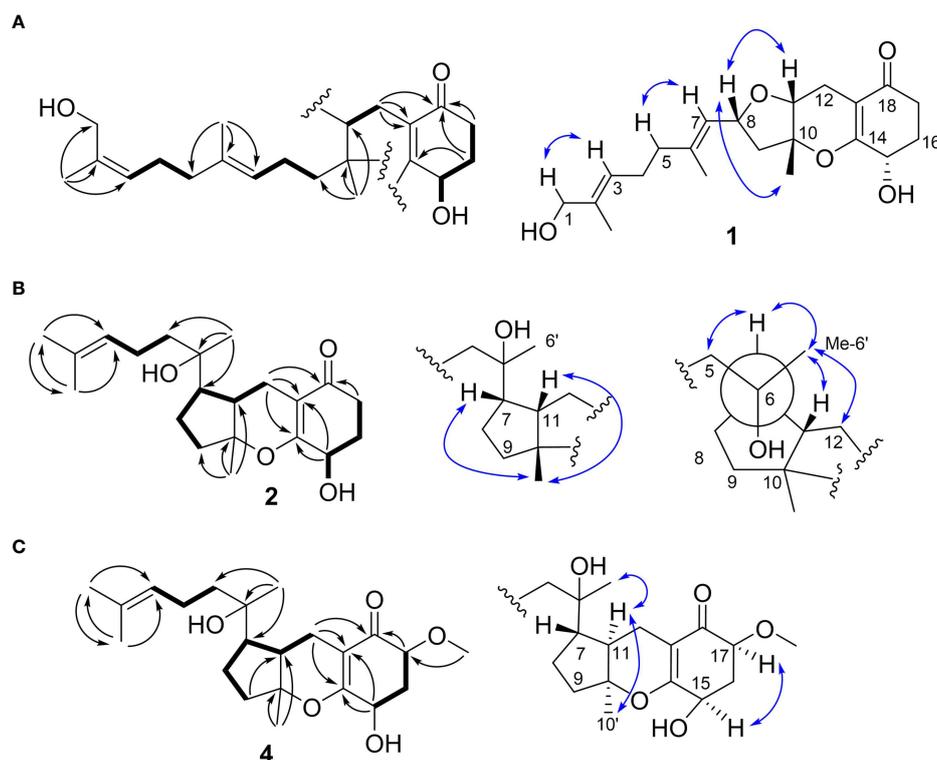


FIGURE 2
Key 2D correlations of compounds 1 (A), 2 (B) and 4 (C).

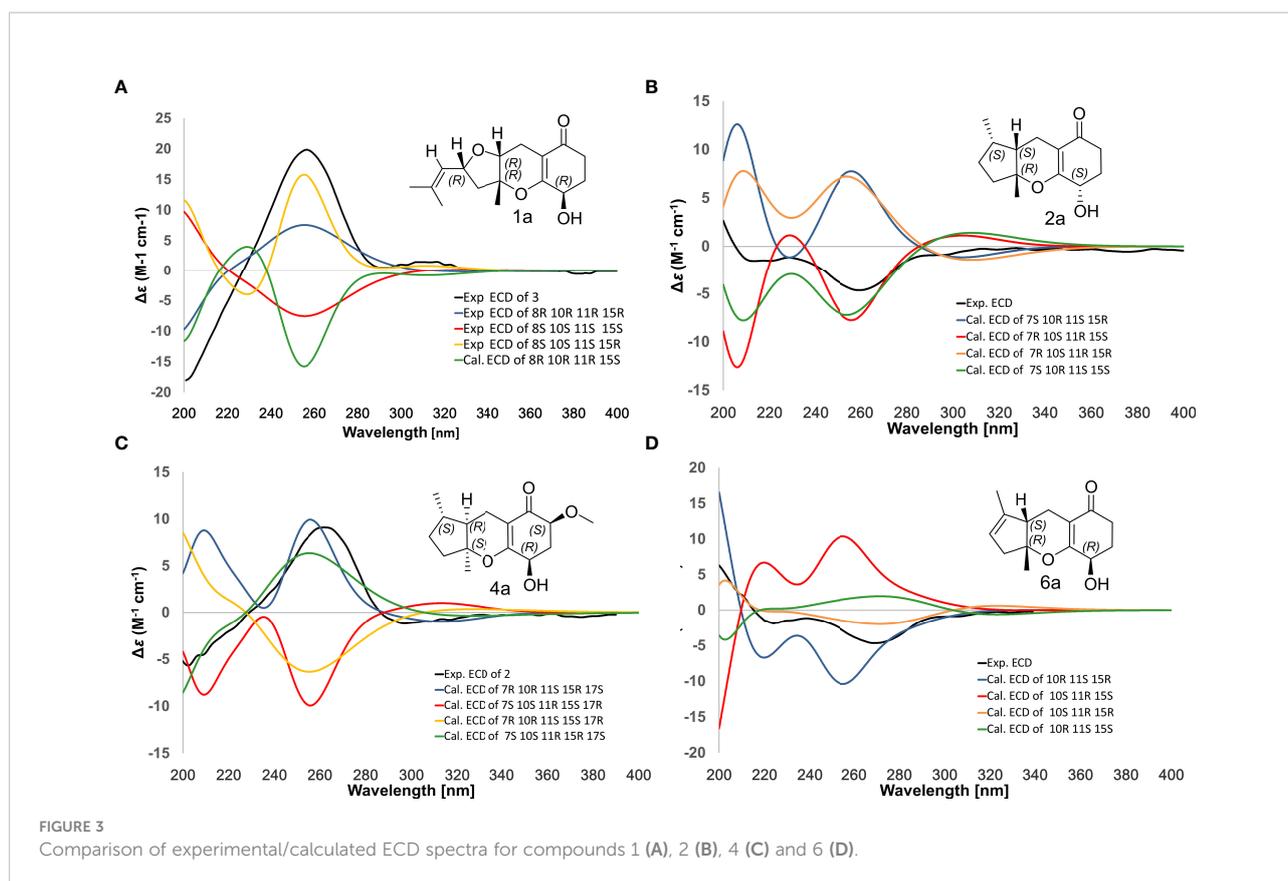
seven sp^3 methylenes, three sp^3 methines, one olefinic methine, two oxygenated sp^3 quaternary carbons, and four sp^2 quaternary carbons including a conjugated carbonyl carbon (Tables 1 and 2). The above information showed considerable resemblance to that of tricycloalternarene C (3), an ACTG toxin derivative isolated from JJY-32 by our previous study (Zhang et al., 2013). The COSY correlations of OH-15/H-15/H-16/H-17, H-9/H-8/H-7/H-11/H-12, and H-3/H-4/H-5, together with the HMBC correlations from OH-15 to C-13 and C-14, from H₂-17 to C-18, from H-1 and H-2' to C-3, from H₃-6' to C-5, C-6, and C-7, from H₃-10' to C-9, C-10, and C-11, and from H₂-12 to C-13, C-14, and C-18 confirmed that compound 2 possess the same planar structure as 3 (Figure 2B), while the different CD spectra (Zhang et al., 2013) indicated the different absolute configurations at the stereo-centers (Figure 3B).

The NOESY correlation between H₃-10' and H-11 revealed the *cis* fusion between rings B and C, which was inconsistent with ACTG toxin structures from literature reports (Figure 3). NOESY correlation between H₃-10' and H-7 further suggested the 7*S*^{*}, 10*R*^{*}, 11*S*^{*} relative configuration. The electronic circular dichroism (ECD) spectra calculation method was adopted to solve the absolute configuration. By comparing the experimental ECD spectrum with the four output curves (7*S*, 10*R*, 11*S*, 15*S*; 7*R*, 10*S*, 11*R*, 15*S*; 7*S*, 10*R*, 11*S*, 15*R*; 7*R*, 10*S*, 11*R*, 15*R*) from

DFT computational process, the absolute configuration of 2 was determined as 7*S*, 10*R*, 11*S*, 15*S*.

The configuration at C-6 was tentatively proposed based on a detailed conformational analysis (Figure 2B). In the 2D NOESY spectrum, consistent correlations were observed from H₃-6' (δ_H 1.15) to H-11 (δ_H 1.65), H₂-12 (δ_H 2.49 and 1.85) and H-7 (δ_H 2.09), and from H-7 (δ_H 2.09) to H₂-5 (δ_H 1.31). However, there was no apparent NOESY correlation between H-7 and 6-OH (δ_H 4.03), and between H₃-6' and H₂-8 (δ_H 1.86 and 1.51). The above data indicated the 6*S* configuration. Compound 2 was named tricycloalternarene P.

Compound 4, named tricycloalternarene Q, was also obtained as a colorless oil. The molecular composition was determined to be C₂₂H₃₄O₅ by HRESIMS and 1D NMR data (Tables 1 and 2). Careful comparison of its NMR spectroscopic data with that of 2 revealed the absence of a methylene and the presence of an extra methine and a methoxyl group. The HMBC correlation from 17-OCH₃ to C-17 attached the methoxyl groups to C-17. Further 2D NMR data of 4 completed the same planar structure as tricycloalternarene B (5), an ACTG toxin derivative previously isolated from JJY-32 (Zhang et al., 2013) (Figure 1B). The NOESY correlations between H₃-10' and H-11, H-11 and H₃-6' indicated the 7*S*^{*}, 10*S*^{*}, 11*R*^{*} relative configuration; the NOESY correlations from H-15 to H-17



indicated the 15R*, 17S* relative configuration (Figure 2C). As evidenced from the ECD fitting experiments using the truncated structure **4a** as a model structure, the absolute configuration of compound **4** was determined as 7S, 10S, 11R, 15R, 17S.

Compound **6**, trivially named tricycloalternarene R, exhibited $[M + H]^+$ ion at m/z 335.1801 accompanied by $[M + Na]^+$ ion at m/z 357.1681 in HRESIMS, indicating a molecular formula of $C_{19}H_{26}O_5$. The 1H NMR spectrum (Table 1) showed one methyl doublet at δ_H 0.91 ($J = 7.68$ Hz, H-6'), one methyl singlet at δ_H 1.40 (H-10'), one methoxy singlet at δ_H 3.53 (CH₃O-3), one broad double doublet at δ_H 4.02 ($J = 9.91, 5.46$ Hz, H-15) attributable to an oxymethine and one broad singlet at δ_H 5.35 (H-8) due to an olefinic proton. The ^{13}C NMR spectrum (Table 2) along with DEPT and HSQC data demonstrated the presence of three methyls, six methylenes, four methines and six unprotonated carbons. The above data showed considerable similarity with that of nortricycloalternarene, except for the upfield shift of the chemical shifts for CH-15 (δ_H 4.02 vs δ_H 4.38; δ_C 64.6 vs δ_C 66.6), indicating the same planar structure as nortricycloalternarene (Shi et al., 2018). However, the CD spectrum of **6** showed the opposite trend at around 280–300 nm compared with nortricycloalternarene, which indicated that **6** was an isomer of nortricycloalternarene. Finally, on considering the conserved co-facial orientation of H-11 and H₃-10', and by comparing the experimental ECD spectrum with the calculated curves presenting the four possible configurations

(Figure 3D), the absolute configuration at C-10, C-11, and C-15 was proposed to be 10R, 11S, 15R.

Anti-inflammatory activity evaluation

Toll-like receptors (TLR) associated inflammatory responses usually involve the over-activation of macrophages, which may cause damage to the host by excessive release of various inflammatory mediators such as NO and PGE₂, and pro-inflammatory cytokines such as iNOS, COX-2, and IL-6 (Lee et al., 1992; Surh et al., 2001; Lin and Karin, 2007). During this process, NF- κ B acts as a key transcription factor complex controlling the expression of cell survival genes as well as those pro-inflammatory cytokines and enzymes (Brown et al., 2007).

The new structures **1-2**, **4**, and **6** were subjected to cytotoxicity tests using K562 and RAW264 cancer cells, and no cytotoxicity was observed for all the above compounds. Then **1-2**, **4**, and **6** were evaluated for the anti-inflammatory effects using TLR4-transfected microphage cells (RAW264.7) and human embryonic kidney 293T cells. First, the inhibitive activity of nitric oxide (NO) and prostaglandin E₂ (PGE₂) production was tested in LPS-activated RAW264.7 cells. As shown in Table 3, the compounds showed moderate inhibition

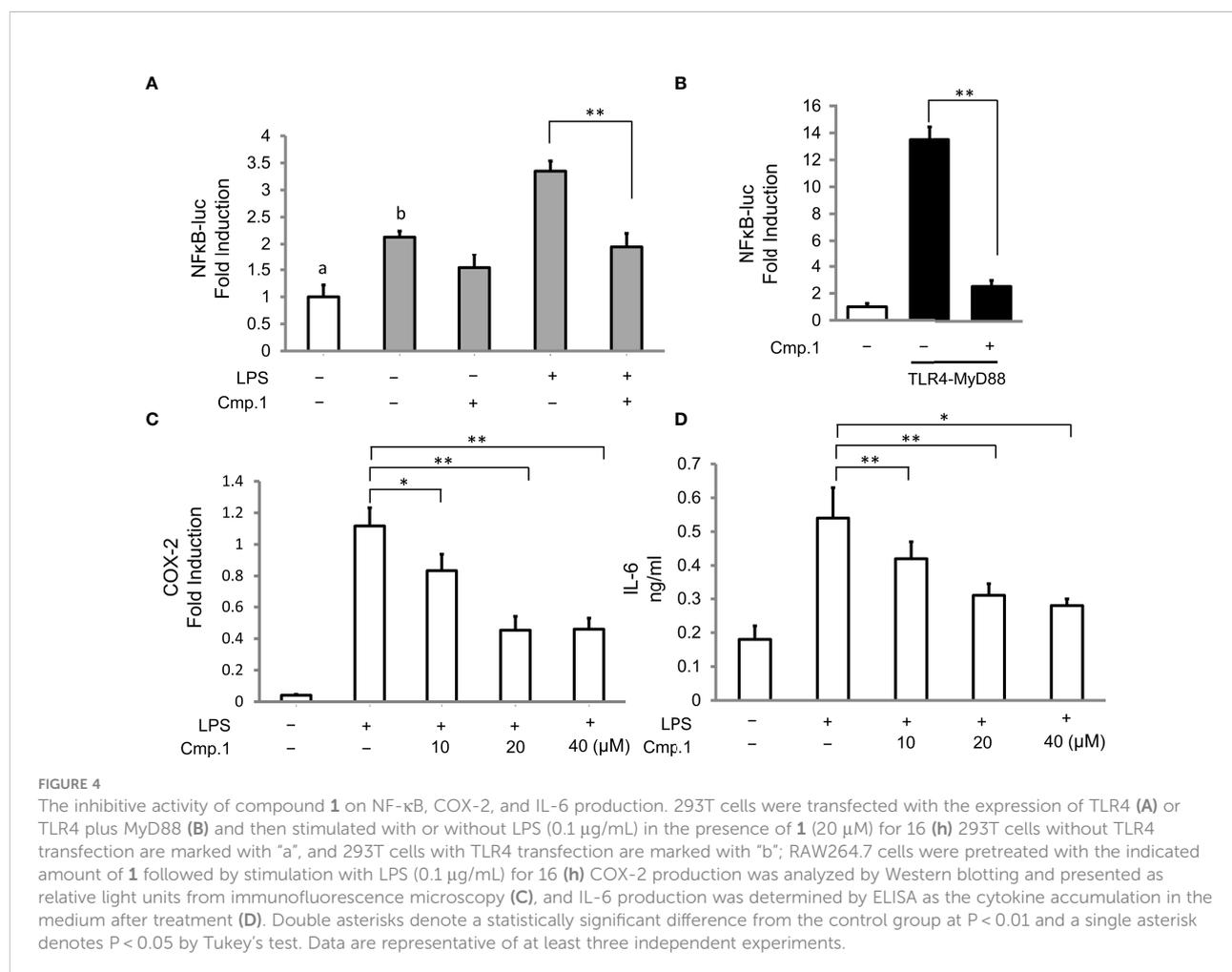
of NO production with IC_{50} values ranging from 17.5 to 30.6 μ M and of PGE_2 production with IC_{50} values ranging from 14.5 to 25.2 μ M (curcumin as a positive control with an IC_{50} value of 5.2 μ M), while compounds **1** and **6**, whose isopentenyl terminal are oxidized, were revealed to be more potent than compounds **2** and **4** (Table 3).

To determine whether the decrease in the production of NO and PGE_2 mediated by ACTG-toxins was due to the suppressed activity of upstream signaling molecules, compound **1** was selected to examine the inhibitive effects on LPS-induced

expression of the key regulator NF- κ B and the pro-inflammatory cytokines of iNOS COX-2 and IL-6 in 293T cells. The NF- κ B luciferase activity induced by the overexpression of TLR4 together with its driving adaptor molecule MyD88 (Pasare and Medzhitov, 2004) was suppressed under the presence of **1** (20 μ M) (Figures 4A, B). The expression of iNOS and COX-2, two enzymes directly related to the production of NO and PGE_2 , were also found to be dose-dependent and inhibited by **1** with concentrations from 10 to 40 μ M (Figure 4C and Table 4). In addition, the LPS-

TABLE 3 Inhibitive activity on NO and PGE_2 production for compounds 1-2, 4, and 6.

Compound	IC_{50} (μ M)	
	NO	PGE_2
1	17.5 \pm 1.1	14.5 \pm 1.3
2	30.6 \pm 2.1	25.2 \pm 1.9
4	22.4 \pm 2.3	18.6 \pm 1.7
6	19.4 \pm 2.3	16.6 \pm 1.4



induced expression of IL-6 was also potently abated by compound **1** treatments (Figure 4D). The above results indicated the potent inhibitory effects of **1** on MyD88-dependent TLRs signaling pathways.

Conclusion

Four new ACTG-toxin-like meroterpenoids were discovered from a marine-sponge-associated fungus *Alternaria* sp. JYJ-32 using metabolism modulation methods involving overexpression of a metabolism regulator aLaeA and a biosynthetic tailoring enzyme aMpaB. Among those structures, compound **1** was the first example of ACTG-toxin-like compounds with a cyclohexanone-pyran fused furan ring structure, while compound **6** represented a non-canonical ACTG-toxin whose terpenoid part was truncated by losing three carbons, which responded to the modifying function of aMpaB. Meanwhile, compounds exhibited potent anti-inflammatory activity by interfering with MyD88-dependent TLRs signaling pathways in LPS-activated 293T cells. The above results showed overexpression of metabolism regulators and pathway-specific tailoring enzymes could be an efficient method to develop structural diversity of secondary metabolites produced in marine-derived *Alternaria* strains.

Experimental section

General experimental procedures

The specific rotations were measured on a JASCO P-1020 digital polarimeter. The UV spectra were recorded on a Beckman DU 640 spectrophotometer. The NMR spectra were obtained on an Agilent 500 MHz DD2 spectrometer with TMS as an internal standard. The ESIMS spectra were obtained on a Thermo Scientific LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) or a Micromass Q-TOF ULTIMA GLOBAL GAA076 LC Mass spectrometer (Waters Corporation). Semi-preparative HPLC was performed on an ODS column (HPLC (YMC-Pack ODS-A, 10 × 250 mm, 5 μm, 3 mL/min)) (YMC Co., Ltd.). MPLC was performed on a Bona-Agela CHEETAHTM HP100 (Beijing Agela Technologies Co., Ltd.).

Fungal material

The wild-type fungus *Alternaria alternata* JYJ-32 was isolated from a sponge-derived sample collected in Qionghai, Hainan (China). JYJ-32 was deposited at the Key Laboratory of Marine Drugs, the Ministry of Education of China, School of Medicine and Pharmacy, Ocean University of China, Qingdao, People's Republic of China.

Construction of the aLaeA and aMpaB expression vector

The integrative vector pHyg which mainly contains a constitutive promoter *gpdA*, ampicillin, and hygromycin-resistant genes was digested with restriction endonucleases *XmaI* and *XbaI* (New England Biolabs, NEB). The *LaeA* gene was amplified from the genomic DNA library of JYJ-32 using specific primers containing *KpnI* and *XbaI* restriction sites and the *MpaB* gene was amplified from the genomic DNA library of JYJ-32 using specific primers containing *XmaI* and *XbaI* restriction sites (Table S12) via PCR catalyzed by *TransStart[®] Fastpfu* DNA Polymerase (Transgen Biotech, Beijing, China). After digested with the corresponding endonucleases above mentioned, the *LaeA* gene and *MpaB* gene were placed downstream of the *gpdA* promoter within the pHyg vector to generate plasmids OE-*alaeA* and OE-*aMpaB* respectively (Figures S3 and S4). The constructed plasmids were transformed into *E. coli* DH10B competent cell for propagation and then transformed into wild-type JYJ-32 to give the engineered strains JYJ32-OE-*alaeA* and JYJ32-OE-*aMpaB*.

Transformation and Overexpression of aLaeA and aMpaB

The strain *Alternaria alternata* JYJ-32 was inoculated on PDA plates and cultured at 28 °C for 4 days to generate mycelium. Fresh mycelia were selected and added to 50 mL of MR medium (2% Sucrose, 1% KNO₃, 0.25% MgSO₄•6H₂O, 0.5% KH₂PO₄, 0.1% Yeast Extract, 0.6 M Mannitol and 0.5 M NaCl add water to a constant volume 1 L). They were cultured at 200 rpm at 28 °C for 36 hours, then transferred from the culture

TABLE 4 The inhibitive activity of compound **1** on COX-2 and iNOS production.

Loading sample(μM)	LPS Cmp.1	-	+	+	+	+
		-	-	10	20	40
Proteins expression (Fold)	iNOS	1.08	1.76	1.65	0.87	0.79
	COX-2	0.12	2.11	1.57	0.95	0.62

medium to a 50 mL centrifuge tube and centrifuged at 4000 rpm for 15 min. The supernatant was discarded and mycelium was collected, weighed, and cleaned twice by washing buffer (1 M NaCl and 10 mM CaCl₂). The resulting mycelium was then treated with osmotic buffer (10 mM Na₂HPO₄, pH 5.8, 20 mM CaCl₂, 1.2 M NaCl) containing a mixture of cell wall-degrading enzymes (4 mg ml⁻¹ driselase, 0.2 mg ml⁻¹ lyticase, 4 mg ml⁻¹ cellulase and 8 mg ml⁻¹ glusulase). Every 1 g mycelium was treated with 10 mL osmotic buffer.

At 30 °C and 100 rpm, the enzymolysis status was checked at 2.5 h, and the mycelium could be collected when the enzymolysis rate reached 70% under the microscope. The enzymolysis solution was passed through three layers of mirror paper, the filtrate was collected and centrifuged at 4000 rpm for 15 min, the supernatant was discarded, and the protoplast was precipitated. It was then rinsed gently with a small amount of STC (1.2 M sorbitol, 10 mM CaCl₂, 10 mM pH 7.5 Tris-HCl) twice and then added to the STC to re-hang.

The constructed plasmids were amplified by large extract, freeze-drying, and dissolved in 30 µl of STC and added into 100 mL protoplasts, mixed lightly, and left to rest on ice for 1 h. Next, 800 µL of PEG solution (60% PEG, 50 mM calcium chloride, and 50 mM pH 7.5 Tris-HCl) was added to the protoplasts, and let stand at room temperature for 30 min. The mixture was coated on a PSA (PDA medium with 1.2 M sorbitol and 100 µg/mL hygromycin) medium and cultured at 28 °C for 3 days until the single colony grew.

Fermentation and extraction

The engineered strains containing aLaeA and aMpaB genes were cultured under rotary shaker conditions at 180 rpm at 28°C in 500 mL Erlenmeyer flasks containing 200 mL of PDB. After 11 days of shaking, the supernatant and mycelium were separated by filtration through gauze. The supernatant part was extracted three times with ethyl acetate, and the mycelium part extracted with methanol three times, concentrated under reduced pressure to give an aqueous solution, and then extracted with ethyl acetate three times. All ethyl acetate solutions were combined and concentrated under reduced pressure to give an extraction.

Purification

The EtOAc extract (5.0 g) of JY32-OE-alaeA was separated by MPLC using a gradient elution of MeOH-H₂O (5%-100%) yielding five subfractions (fractions 1-5). Compound 1 (10.0 mg, t_R 23.6 min) was isolated from Fraction 3 by Sephadex LH-20 Column Chromatography and followed by semi-preparative

HPLC with a gradient elution of MeOH-H₂O (60%-100%). The EtOAc extract (8.0 g) of JY32-OE-aMpaB was separated by MPLC using a gradient elution of MeOH-H₂O (5%-100%) yielding eight subfractions (fractions 1-8). Guided by the retention time and ion peaks of UPLC-MS data, fractions 6 and 7 were selected. Fraction 6 was separated by semi-preparative HPLC eluting with MeOH-H₂O (50: 50) to furnish compounds 6 (9.0 mg, t_R 21.6 min) and 4 (5.0 mg, t_R 27.6 min). Fraction 7 was fractionated by semi-preparative HPLC with a gradient elution of MeOH-H₂O (60%-100%) to give compounds 2 (13.0 mg, t_R 26.8 min), 3 (5.0 mg, t_R 24.5 min) and 5 (15.0 mg, t_R 26.0 min), respectively.

Compound 1: Colorless oil; [α]_D²⁵ + 93.2 (c 1.0, MeOH); UV (MeOH) λ_{\max} (log ϵ) 250 (4.13); IR (KBr) ν_{\max} cm⁻¹ 1699, 1653, 1593, 1527; HRESIMS m/z 363.1958 [M+H]⁺, ¹H and ¹³C NMR data see [Table 1](#) and [2](#).

Compound 2: Colorless oil; [α]_D²⁵ - 21.4 (c 1.0, MeOH); UV (MeOH) λ_{\max} (log ϵ) 265 (4.10); IR (KBr) ν_{\max} cm⁻¹ 3850, 3746, 3671, 3647, 3564, 1699, 1649, 1539, 1508, 1457, 1034, 506 cm⁻¹; ¹H and ¹³C NMR see [Table 1](#) and [2](#); HRESIMS m/z 349.2379 [M+H]⁺ (calcd [M+H]⁺ 349.2383).

Compound 4: Colorless oil; [α]_D²⁵ + 54.3; (c 1.0, MeOH), UV (MeOH) λ_{\max} (log ϵ) 264 (4.07); IR (KBr) ν_{\max} cm⁻¹ 3850, 3673, 3615, 1747, 1649, 1540, 1521, 1458, 504, 420 cm⁻¹; ¹H and ¹³C NMR see [Table 1](#) and [2](#); HRESIMS m/z 401.2304 [M+Na]⁺ (calcd [M+ Na]⁺ 401.2304).

Compound 6: Colorless oil; [α]_D²⁰ + 97.3 (c 0.1, MeOH); IR (KBr)cm⁻¹: 1739, 1613, 1381, 1242, 1156, 977; HRESIMS m/z 335.2 [M+H]⁺, ¹H and ¹³C NMR data see [Table 1](#) and [2](#).

Assay of Cytotoxicity Inhibitory Activity: As previously reported ([Yang et al., 2013](#)).

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Materials](#). Further inquiries can be directed to the corresponding author.

Author contributions

GL and DLiu drafted the molecular biology work and natural product chemistry experiments; ZL contributed to the plasmid construction and bioinformatic analysis; JJ and XH performed the compounds isolation and structure elucidation; XZ performed the biological evaluation; QC, TZ and DLI contributed to the critical reading of the manuscript; GZ designed the study, supervised the laboratory work, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

After completion of the research, JJ was employed by Sino Silliker Testing Services Qingdao Co., Ltd.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.1015684/full#supplementary-material>

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