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In vitro stability and in vivo anti-inflammatory efficacy of synthetic jasmonates

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ABSTRACT

A chlorinated methyl jasmonate analog (**J7**) was elaborated as an in vitro anti-inflammatory lead. However, its in vitro efficacy profile was not reproduced in a subsequent in vivo evaluation, presumably due to its rapid enzymatic hydrolysis in a biological system. In an attempt to improve the metabolic stability of the lead **J7** by replacement of its labile methyl ester with reasonable ester groups, several analogs resistant to enzymatic hydrolysis were synthesized. In vivo evaluation of the stability-improved analogs showed that these compounds displayed higher efficacy than the lead **J7**, suggesting that these new jasmonate analogs may serve as potential anti-inflammatory leads.

1. Introduction

In our continuing study on the potential anti-inflammatory leads derived from the red alga *Gracilaria verrucosa*, methyl jasmonate (**1**) was employed as a prostaglandin-like template for structural modification.[1,2] Introduction of an unsaturation to the cyclopentanone ring of **1** yielded a highly electrophilic enone functionality (**2**), and led to a significant enhancement of in vitro activity.[2] Methyl 4,5-didehydrojasmonate (**2**) exhibited in vitro anti-inflammatory potency comparable to that of the most potent natural anti-inflammatory prostaglandin 15d-$\Delta^{12,14}$-PGJ_2.[2] This type of jasmonate analog inhibited the production of pro-inflammatory mediators [nitric oxide (NO), interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α] through down-regulation of NF-κB activity in LPS-activated murine macrophage (RAW264.7) cells.[3] α-Halogenation of the enone function of **2** enhanced its in vitro potency even further.[2] Subsequently, one of the α-haloenone analogs, methyl 5-chloro-4,5-didehydrojasmonate (**J7**) was subjected to in vivo trial, employing the carrageenan-induced paw edema assay. However, the in vitro activity profile of **J7** was not reproduced in the in vivo study. Though **J7** displayed still significant activity, it was not as effective as diclofenac or indomethacin (Fig. 1; in vivo dosage regimen of **J7** was determined as 50 mg/kg, based on the in vitro potency and cytotoxicity to RAW264.7 cells, and standard dosage regimen was used for diclofenac and indomethacin).[2]

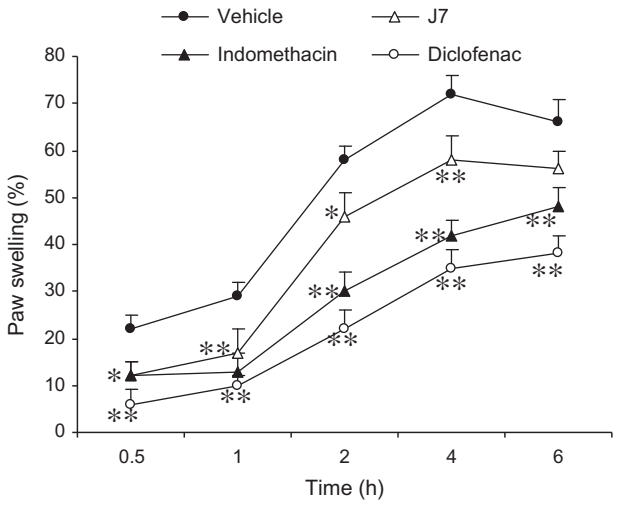

Figure 1. Effect of single dose administration of **J7**, indomethacin, or diclofenac (50, 5 and 25 mg/kg, respectively, intraperitoneal injection) on carrageenan-induced swelling of the rat hind paw. The dosage regimen of **J7** was determined on the basis of in vitro potency and cytotoxicity to RAW264.7 cells, and standard dosage regimen was used for diclofenac and indomethacin. Each value represents the mean ± S.D. from 8 to 10 rats per group. Asterisks indicate significant differences from the control group ($^{*}p$ <0.05, $^{**}p$ <0.01).

However, in an in vitro assessment using the same dosage regimen as that used for in vivo evaluation, **J7** was more effective than

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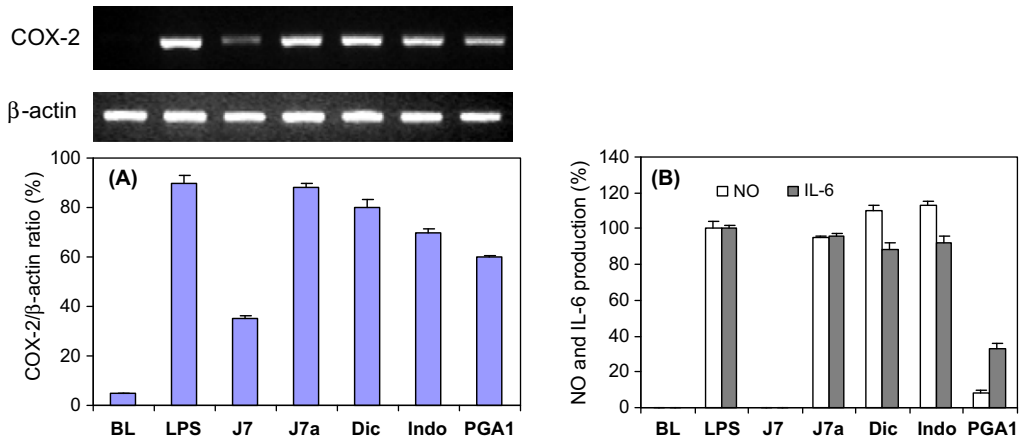


Figure 2. Inhibitory effects of test samples on the mRNA expression of COX-2 and the production of NO and IL-6. (A) RAW264.7 cells (1.5×10^5 cells/mL) were stimulated with LPS (1 μg/mL) alone or with test samples (the same dosage regimen as that of in vivo: **J7** 10 μM; **J7a** 10 μM; indomethacin 1 μM; diclofenac 5 μM; PGA_1 10 μM) for 20 h (BL, blank). Total RNA was isolated in an RNase-free environment and the COX-2 mRNA expression was determined by RT-PCR. (B) RAW264.7 cells (1.5×10^5 cells/mL) were stimulated with LPS (1 μg/mL) alone or with test samples at a concentration of 10 μM. Nitric oxide production was determined using the Griess reagent method. The production of IL-6 was determined by ELISA. The data represent the mean ± SD of triplicate experiments.

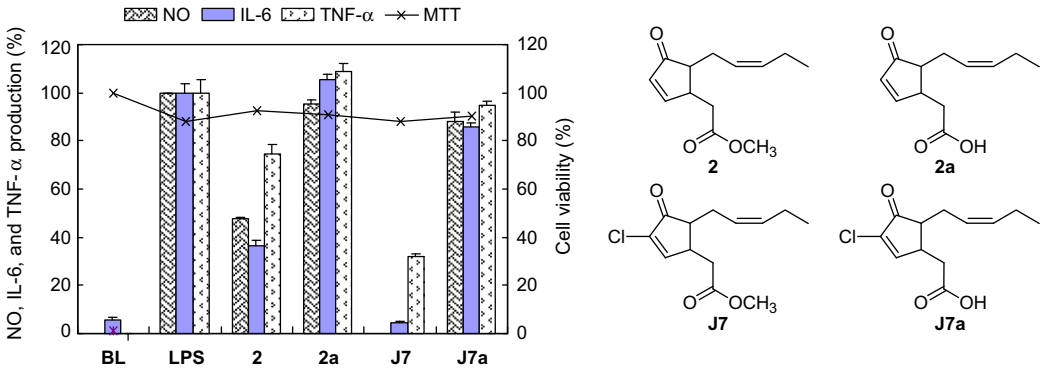


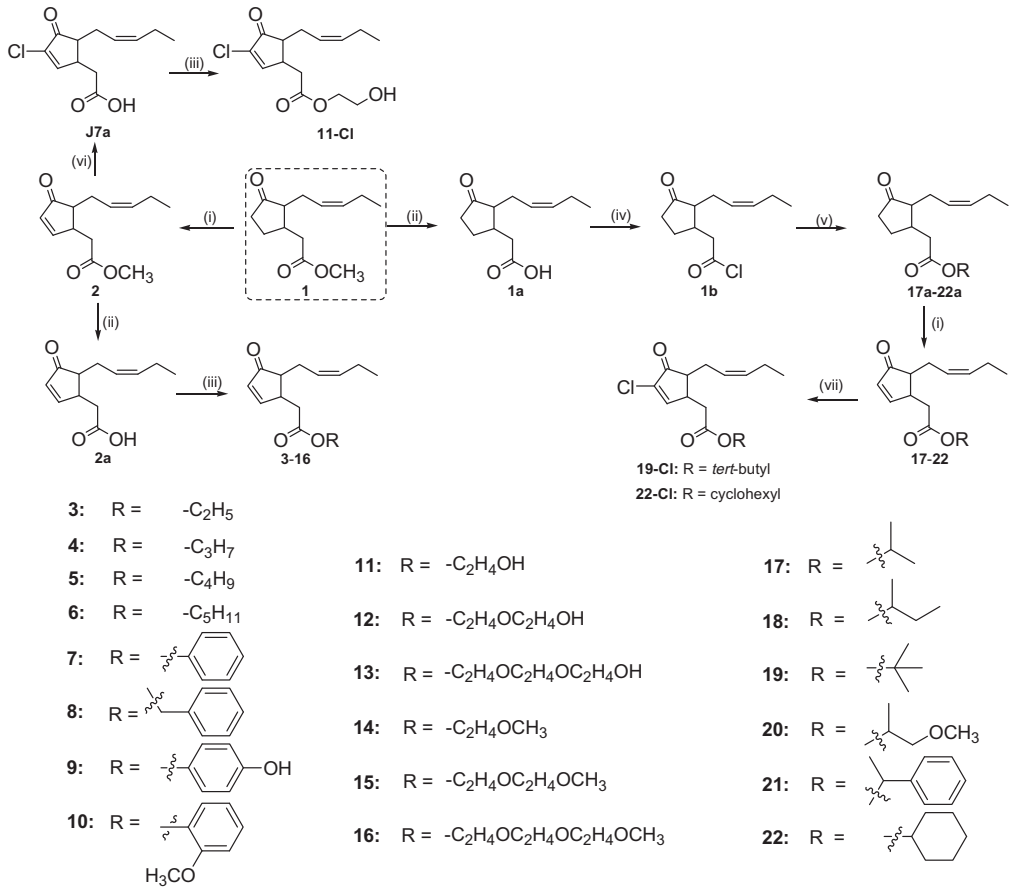
Figure 3. Inhibitory effects of jasmonate analogs (**2** and **J7**) and their corresponding acids (**2a** and **J7a**) on the production of NO, IL-6, and TNF-α in LPS-activated RAW264.7 cells (data edited from our previous publication[2]). RAW264.7 cells (1.5×10^5 cells/mL) were stimulated with LPS (1 μg/mL) alone or with test samples at concentrations of 12.5 μM for 24 h (BL, blank). Nitric oxide production was determined using the Griess reagent method, and the production of pro-inflammatory cytokines (IL-6 and TNF-α) was determined by ELISA. Cell viability was determined using the MTT method. The data represent the mean ± SD of triplicate experiments.

diclofenac, indomethacin, or prostaglandin A_1 (PGA_1) in inhibiting COX-2 (diclofenac and indomethacin are typical COX-2 inhibitors, Fig. 2A). The lead **J7** also showed more strong suppression of NO and IL-6 than diclofenac, indomethacin, and PGA_1 at the equal concentration of 10 μM (Fig. 2B).

The discrepancy between the relative in vitro and in vivo efficacies of **J7** might be due to a quick degradation of **J7** to an inactive free acid form (**J7a**) catalyzed by esterases in biological systems. This was supported by the observation that leads **2** and **J7** lost their in vitro potency after hydrolysis to their corresponding acids **2a** and **J7a**, respectively (Fig. 3). The loss of activity via hydrolysis to the corresponding acid has been encountered in many bioactive esters,[4–7] leading to low efficacy in in vivo trials.[8,9] Therefore, bioactive molecules of ester forms are usually subjected to structural modification to improve their metabolic stability.[4–7] One of the strategies is to replace the metabolically labile ester moiety with appropriately designed bioisosteres (such as amides, isoxazolones, isoxazoles, or oxazolones) that would defy enzymatic degradation while retaining the desired potency of the parent esters.[10,11] An alternative approach is to modify the polarity (lipophilicity/hydrophilicity) and/or the steric properties (e.g., steric hindrance) of the ester moiety.[12,13] In our previous study, several amide derivatives of **2** were prepared as bioisoteres. However, this modification led to complete loss of in vitro activity, suggesting that bioisosteric replacement of the ester bond with an amide bond is not suitable for this type of compound.[2] Therefore, modifications of the metabolically labile methyl ester moiety of compound **2** to linear alkyl or aryl (**3–10**), hydroxyalkyl (**11–13**), methoxyalkyl (**14–16**), and branched alkyl (**17–22**) esters were attempted to modulate their lipophilicity/hydrophilicity and steric hindrance. The stability-improved analogs were then subjected to subsequent α-chlorination of the enone function to enhance their potency.[14] In this article, the synthesis, structure–activity/stability relationship, and anti-inflammatory activity of new jasmonate analogs will be presented and discussed.

2. Results and discussion

The synthesis of jasmonate derivatives is outlined in Scheme 1. Esters **3–16** were prepared from methyl jasmonate (**1**) in three steps. Transformation of compound **1** into methyl 4,5-didehydrojasmonate (**2**) was performed in 1 step, utilizing the palladium(II) acetate/allyl diethyl phosphate dehydrogenation system.[15] Hydrolysis of compound **2** by LiOH in aqueous THF yielded the corresponding acid (**2a**), which was further coupled with various alcohols in EtOAc using a very efficient coupling agent 2-(1*H*-benzotriazole-1-yl)-1,2,3,3-tetramethyluronium tetrafluoroborate (TBTU)[16] to generate esters **3–16**. On the other hand, esters **17–22**



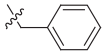
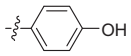
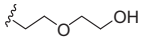
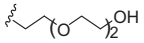
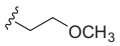
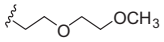
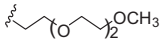
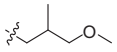
Scheme 1. Synthesis of jasmonate analogs. Reagents and conditions: (i) $Pd(OAc)_2$, diethyl allyl phosphate, sodium carbonate, DMF, 80 °C, 48 h; (ii) LiOH, aqueous THF, rt, 12 h; (iii) ROH, 2-(1*H*-benzotriazole-1-yl)-1,2,3,3-tetramethyluronium tetrafluoroborate (TBTU), TEA, EtOAc, RT, 12 h; (iv) oxalyl chloride, dichloromethane, 40 °C, 1 h; (v) ROH, TEA, DMAP, dichloromethane, RT, 12 h; (vi) H_2O_2, 10% KOH, MeOH, −10 °C, 12 h, and then HCl/acetic acid, $CHCl_3$, RT, 12 h (vii) H_2O_2, 10% KOH, MeOH, −10 °C, 12 h, and then $CeCl_3$, H_2O/MeOH (1:3), reflux, 3 h.

were synthesized from methyl jasmonate (**1**) via four steps. The reactive acyl chloride (**1b**) was prepared by treatment of acid (**1a**) with oxalyl chloride in dichloromethane. The intermediate esters **17a–22a** were consequently obtained by coupling the acyl chloride (**1b**) with various alcohols in the presence of triethylamine and 4-dimethylaminopyridine in dichloromethane. Finally, transformation of the intermediate esters **17a–22a** into their corresponding target esters **17–22** was performed using the palladium(II) acetate/allyl diethyl phosphate dehydrogenation system as described for the synthesis of compound **2**. An α-chloroenone derivative (**11-Cl**) was synthesized by treatment of the acid (**J7a**), which was formed by treatment of the epoxide of **2** with HCl in acetic acid,[2] with ethylene glycol in EtOAc using TBTU as described for the synthesis of compounds **3–16** from **2a**. On the other hand, compounds **19-Cl** and **22-Cl** were directly synthesized from the esters **19** and **22**, respectively, by treating their corresponding epoxides with cerium(III) chloride.[17]

All synthesized esters (**2–22**) were evaluated for their in vitro metabolic stability using porcine liver esterase (PLE). The esters were subjected to hydrolysis by incubating with PLE under physiological conditions (phosphate buffer, pH 7.4, and 37 °C).[4–7] At regular intervals, the reaction mixture was withdrawn and analyzed by RP-HPLC to quantify the amount of the intact ester and its corresponding acid. The in vitro anti-inflammatory activity of the analogs were evaluated by monitoring their inhibitory effects on the production of NO in LPS-stimulated RAW264.7 cells. In addition to the EC_{50} values of NO inhibition, the toxicity (TC_{50}) of these esters to the RAW264.7 cells was also measured to estimate their in vitro safety (TC_{50}/EC_{50}). The in vitro metabolic stability (ratio of esters/acids), biological activity (EC_{50} and TC_{50}), and calculated log*P* value were employed as criteria for further lead selection (Table 1).

As shown in Table 1, the in vitro metabolic stability was strongly dependent on both the lipophilicity/hydrophilicity and the steric hindrance of the ester moiety. Linear alkyl and aryl esters (**3–10**) with the most lipophilic properties were highly susceptible to enzymatic hydrolysis compared to other esters. At 30 min of incubation with PLE, the peaks of these esters (**3–7**, **9**, and **10**) were mostly replaced by the peaks of their corresponding acids. Among them only benzyl ester (**8**) showed better stability than the methyl ester (**2**) with a twice higher stability at 30 min (Table 1). These results suggested that extension of the linear alkyl chain (increment of log*P* value) or replacement of the methyl moiety by other aryl groups is detrimental to their metabolic stability. In contrast, the metabolic stability of the hydroxy alkyl esters (**11–13**) and the methoxy alkyl esters (**14–16**) was improved. In particular, the improvement was notable for hydroxy alkyl esters (**11–13**), suggesting that the more hydrophilic they are, the more resistant to enzymatic hydrolysis. Elongation of the ethylene glycol or ethylene glycol methyl ether moieties led to increased stability (stability order: **13** > **12** > **11** > **16**, **15** > **14**) (Table 1). Branched or cyclic alkyl esters (**17–22**) were much more stable than linear alkyl/aryl esters (**3–10**), even though they exhibited similar lipophilicities, suggesting that bulkiness around the ester bond suppresses enzymatic hydrolysis. Among them, the *tert*-butyl ester (**19**) was the most stable. The strong resistance to enzymatic

Table 1
In vitro metabolic stability, biological activity, and the structure–activity/stability relationships of jasmonate derivatives[a]

	R=	Log*P*		Bulkiness	Stability			EC_{50}		TC_{50}	TC_{50}/EC_{50}
					30 min	60 min					
2	$-CH_3$	2.0 ± 0.33		None	0.1 ± 0.06	0		19		140	7
3	$-C_2H_5$	2.53 ± 0.33	↑	≈	0	ND	↓	20	≈	146	7
4	$-C_3H_7$	3.06 ± 0.33	↑↑	≈	0	ND	↓	23	≈	35	1
5	$-C_4H_9$	3.59 ± 0.33	↑↑	≈	0	ND	↓	23	≈	31	1
6	$-C_5H_{10}$	4.12 ± 0.33	↑↑↑	≈	0	ND	↓	25	≈	37	1
7		3.37 ± 0.32	↑↑	≈	0	ND	↓	33	↓	158	5
8		3.75 ± 0.34	↑↑	≈	0.2 ± 0.04	0	≈	20	≈	31	1
9		2.55 ± 0.34	↑	≈	0	ND	↓	23	≈	49	2
10		3.20 ± 0.34	↑↑	↑	0	ND	↓	32	↓	161	5
11		1.17 ± 0.45	↓	≈	1.7 ± 0.15	0.7	↑↑	24	≈	161	7
12		1.02 ± 0.48	↓↓	≈	5.9 ± 0.78	3.1	↑↑↑	36	↓	166	5
13		0.66 ± 0.54	↓↓↓	≈	7.6 ± 0.65	3.5	↑↑↑	42	↓↓	162	4
14		1.74 ± 0.45	~	≈	0.1 ± 0.04	0	≈	27	↓	160	6
15		1.38 ± 0.52	↓	≈	0.3 ± 0.08	0	↑	33	↓	161	5
16		1.02 ± 0.58	↓	≈	0.3 ± 0.07	0	↑	35	↓	163	5
17		2.87 ± 0.33	↑	↑	0.5 ± 0.1	0.1	↑	20	≈	58	3
18		3.17 ± 0.34	↑↑	↑	0.4 ± 0.09	0.1	↑	22	≈	53	2
19		3.22 ± 0.34	↑↑	↑↑	11.2 ± 1.2	9.8	↑↑↑↑	20	≈	33	1
20		2.08 ± 0.33	~	↑	0.5 ± 0.15	0.2	↑	23	≈	161	7
21		4.1 ± 0.34	↑↑↑	↑	1.4 ± 0.25	0.4	↑↑	18	≈	31	2
22		4.06 ± 0.33	↑↑↑	↑	4.1 ± 0.46	1.1	↑↑↑	22	≈	31	2

[a] In vitro metabolic stability was examined by the esterase-catalyzed hydrolysis assay. Data are the ratio of intact esters and their corresponding acids at 30 and 60 min expressed as the mean ± SD of two separate experiments; ND: not determined. In vitro anti-inflammatory activity was gauged by inhibitory activity on NO production in LPS-activated RAW264.7 cells. EC_{50} and TC_{50} are expressed as μM. Log*P* values were calculated using ACD/ChemSketch 12.0 software. Definition of 'bulkiness' is found in Ref. 21. Comparison of log*P*, bulkiness, stability, and activity between 2 and other esters is shown by symbols: ↑ (increased), ↓ (decreased), ≈ (comparable).

hydrolysis of the *tert*-butyl ester **19** might be due to its inability to enter the hydrolytic enzyme pocket caused by steric hindrance.[12,13] The cyclohexyl ester (**22**) also displayed highly improved stability compared to the branched alkyl esters **17** and **18**.

In terms of biological activity, all the lipophilic esters including linear and branched alkyl or aryl analogs (**3–10** and **17–22**) with log*P* >2.0, were almost equipotent to the methyl ester **2** ($EC_{50} \approx$ 20 μM), suggesting that the methyl ester could be replaced by any ester group without losing biological potency provided that the log*P* value stays higher than that of **2** (log*P* 2.0). However, their cytotoxicity was rather variable with the safety index (TC_{50}/EC_{50}) ranging from 1 to 7. On the other hand, alkoxy esters, including

Table 2
In vitro anti-inflammatory activity of α-chlorinated jasmonate derivatives[a]

Esters	log*P*	EC_{50}	TC_{50}	TC_{50}/EC_{50}
J7	2.40 ± 0.33	0.77	11.32	14.7
11-Cl	1.57 ± 0.45	0.58	6.25	10.8
19-Cl	3.62 ± 0.34	0.83	11.52	13.9
22-Cl	4.45 ± 0.33	0.65	9.69	14.9

[a] In vitro anti-inflammatory activity was gauged by inhibitory activity on NO production in LPS-activated RAW264.7 cells. Data are expressed in EC_{50} and TC_{50} (μM) as the mean ± SD of triplicate experiments. The log*P* values were calculated using ACD/ChemSketch 12.0 software.

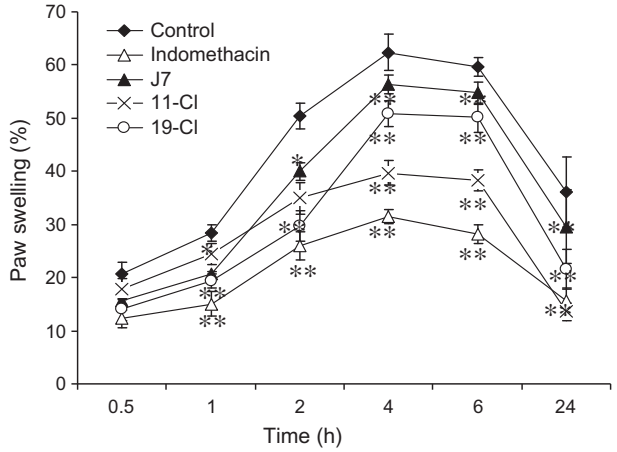


Figure 4. Effect of single dose administration by an intraperitoneal injection of **J7** (50 mg/kg), **11-Cl** (50 mg/kg), **19-Cl** (50 mg/kg), or indomethacin (20 mg/kg) on carrageenan-induced swelling of the rat hind paw. Each value represents the mean ± S.D. ($n = 5$). Asterisks indicate significant differences from the control group ($^{*}p < 0.05$, $^{**}p < 0.01$).

hydroxy alkyl esters (**11–13**) and methoxy alkyl counterparts (**14–16**), showed lower potency than **2**, but their cytotoxicity was almost constant, with a higher safety index than other esters. A clear structure–activity/stability relationship was found for this type of alkoxy esters. Elongation of the ethylene glycol or ethylene glycol methyl moieties improved stability, but with loss of potency. Among them, the most hydrophilic and stable ester (**13**) (log*P* 0.66) was only half as potent as the methyl ester (**2**) and the hydroxyethyl ester (**11**), suggesting that enhancing the metabolic stability by increasing hydrophilicity is a matter of compromise. The binding site of metabolizing enzymes is generally lipophilic in nature. Hence, these enzymes more readily accept and metabolize lipophilic molecules. Lipophilicity also modulate the binding of a ligand to the biological target. In our study, by introducing reasonably polar (**11**) or non-polar bulky (**19** and **22**) groups, the metabolic stability of the molecule was improved without losing in vitro potency. The structure–activity/stability relationship of the jasmonate analogs is illustrated in Table 1.

Taken together, by replacing the methyl ester of compound **2** with other moieties possessing different lipophilic/hydrophilic and steric properties, the hydroxy alkyl ester **11**, branched alkyl ester **19**, and cycloalkyl ester **22** showed improved metabolic stability without losing potency. Thereafter, these stable esters (**11**, **19**, and **22**) were transformed to final α-chlorinated derivatives (**11-Cl**, **19-Cl**, and **22-Cl**, respectively)[14] and their inhibitory effects on the production of NO were evaluated. These α-chloroenone analogs exhibited strong in vitro potency, which was comparable to that of the lead **J7** (Table 2). Compounds **11-Cl** and **19-Cl**, having a lower and higher log*P* value than **J7**, respectively, were selected for in vivo evaluation using the carrageenan-induced paw edema model.[18] The maximal effect of carrageenan was produced at 4 h after injection (Fig. 4). The stability-improved analogs **11-Cl** and **19-Cl** displayed higher in vivo efficacy than the methyl jasmonate **J7**, suggesting stability-dependent in vivo efficacy. However, the most stable ester **19-Cl** exhibited lower efficacy than **11-Cl**, suggesting that not only metabolic stability but also other pharmacokinetic parameters are important factors for their in vivo efficacy. When compared to indomethacin (20 mg/kg), these compounds exhibited lower efficacy at early time points (before 6 h). However, up to 24 h, the rate of resolution of edema by the test jasmonates seemed to gradually increase and was comparable to indomethacin (Fig. 4). Compound **11-Cl** (50 mg/kg) displayed higher suppression of the paw edema (62.4%) than indomethacin (57.1%) at 24 h. Different pro-inflammatory mediators (e.g., histamine, cytokines, NO, *i*NOS, COX-2, and prostaglandins) are involved in two different inflammatory phases, which include an early (before 6 h) and a sustained phase of paw edema.[19] During the accelerating phase (1–6 h), COX-2 and the production of inflammatory prostaglandins play a predominant role, whereas NO production by *i*NOS is involved in the maintenance of the inflammatory response at the later phase.[21] Indomethacin is a typical non-selective COX-2 inhibitor; whereas, jasmonate analogs suppress the production and gene expression of several pro-inflammatory mediators (NO, COX-2, IL-1β, IL-6, and TNF-α).[3] Therefore, it is possible that the strong inhibition of edema before 6 h by indomethacin was correlated with its primary inhibition of COX-2. On the other hand, the higher inhibition rate of the jasmonate analogs at the later phase might be due to their principal suppression of NO, *i*NOS, and cytokines.

3. Conclusion

This study was focused on the optimization of metabolic stability of the anti-inflammatory lead **J7** to improve its in vivo efficacy. Replacement of the methyl ester with a sterically bulky or hydrophilic esters produced stability-improved analogs **11-Cl** and **19-Cl.** These analogs showed higher in vivo efficacy than **J7** in a carrageenan-induced paw edema model. The anti-inflammatory effect of **11-Cl** was comparable to that of indomethacin, and the efficacy was sustained. This study offers a reasonable starting point for the further optimization of jasmonate analogs as potential anti-inflammatory agents.

4. Experimental section

4.1. General

The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova 400 spectrometer. Chemical shifts were reported with reference to the respective residual solvent or deuterated solvent peaks (δ_H 7.24 and δ_C 77.0 for $CDCl_3$). The FABMS data were obtained on a JEOL JMS SX-102A spectrometer. HPLC was performed on a YMC ODS-H80 column (250 × 10 mm, 4 μm, 80 Å) and a C18-5E Shodex packed column (250 × 10 mm, 5 μm, 100 Å) using a Shodex RI-71 detector. Quantitative analysis for metabolic stability was performed using Gilson 321 and 322 pumps/UV-8000 systems with a YMC ODS-H80 column (250 × 4.6 mm, 4 μm, 80 Å). All chemical reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

4.2. Synthesis of jasmonate analogs

4.2.1. Synthesis of methyl 4,5-didehydrojasmonate (2)

Allyl diethyl phosphate (2.0 equiv), sodium carbonate (2.4 equiv), and palladium(II) acetate (0.12 equiv) were added to a stirred solution of methyl jasmonate **1** (1 equiv) in DMF (dimethylformamide). The mixture was stirred at 80 °C for 48 h, and after cooling, the reaction mixture was diluted with H_2O and extracted with EtOAc (three times). The combined organic phase was washed with water and brine. After being dried over $MgSO_4$, the organic layer was concentrated. The resulting residue was purified by reversed-phase HPLC (YMC ODS-H80) eluting with 65% aqueous CH_3CN to yield compound **2**; a colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.60 (1H, dd, $J = 6.0$, 2.8 Hz, H-4), 6.17 (1H, dd, $J = 6.0$, 2.4 Hz, H-5), 5.46 (1H, m, H-10), 5.24 (1H, m, H-9), 3.70 (3H, s, OCH_3), 2.99 (1H, m, H-3), 2.57 (1H, dd, $J = 15.6$, 6.8 Hz, H-2b), 2.51 (1H, m, H-8b), 2.45 (1H, dd, $J = 15.6$, 8.4 Hz, H-2a), 2.29 (1H, m, H-8a), 2.09–2.01 (3H, m, H-7 and H-11), 0.94 (3H, t, $J = 8.0$ Hz, H-12); FABMS *m/z* 223 $[M+H]^{+}$.

4.2.2. Synthesis of compounds 3–16

Lithium hydroxide (LiOH) (2 equiv) was added to a solution of compound **2** (1 equiv) in aqueous THF, and the reaction mixture was stirred at room temperature for 12 h. The reaction mixture was then poured into water, and this was followed by extraction with diethyl ether. After removal of diethyl ether, the water layer was neutralized with 1 N HCl, and the suspension solution was extracted with EtOAc (three times). The combined organic layer was washed with brine, dried over Na_2SO_4 and concentrated to yield the acid **2a**, which was used without purification for the next step. 2-(1*H*-Benzotriazole-1-yl)-1,2,3,3-tetramethyluronium tetrafluoroborate (TBTU, 1 equiv) was added to a solution of compound **2a** and triethylamine (TEA, 2 equiv) in EtOAc. After 10 min of stirring at room temperature, alcohols (2 equiv) were separately added and the reaction mixture was stirred for 12 h. The mixture was washed with water, dried, and concentrated to give a residue that was purified by reversed-phase HPLC (YMC ODS-H80) and eluted with 80% aqueous CH_3CN to yield the target esters **3–16** (yields of 65–75%).

4.2.2.1. Ethyl 4,5-didehydrojasmonate (3). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.62 (1H, dd, J = 5.6, 2.4 Hz, H-4), 6.15 (1H, dd, J = 5.6, 2.4 Hz, H-5), 5.46 (1H, m, H-10), 5.24 (1H, m, H-9), 4.15 (2H, m, –O*CH*$_2$CH$_3$), 2.99 (1H, m, H-3), 2.57 (1H, dd, J = 15.6, 6.8 Hz, H-2b), 2.53–2.49 (1H, m, H-8b), 2.45 (1H, dd, J = 15.6, 8.4 Hz, H-2a), 2.29 (1H, m, H-8a), 2.06 (3H, m, H-7 and H-11), 1.25 (3H, t, J = 8.0 Hz, –OCH$_2$*CH*$_3$), 0.94 (3H, t, J = 7.2 Hz, H-12); FABMS m/z 237 $[M+H]^+$.

4.2.2.2. Propyl 4,5-didehydrojasmonate (4). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.61 (1H, dd, J = 5.6, 2.4 Hz, H-4), 6.18 (1H, dd, J = 5.6, 2.4 Hz, H-5), 5.45 (1H, m, H-10), 5.22 (1H, m, H-9), 4.07 (2H, m, –O*CH*$_2$CH$_2$CH$_3$), 2.97 (1H, m, H-3), 2.57 (1H, dd, J = 15.6, 6.8 Hz, H-2b), 2.51–2.49 (1H, m, H-8b), 2.47 (1H, dd, J = 15.6, 8.4 Hz, H-2a), 2.30 (1H, m, H-8a), 2.05 (3H, m, H-7 and H-11), 1.65 (2H, m, –OCH$_2$*CH*$_2$CH$_3$), 0.93 (6H, m, –OCH$_2$CH$_2$*CH*$_3$ and H-12); FABMS m/z 251 $[M+H]^+$.

4.2.2.3. Butyl 4,5-didehydrojasmonate (5). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.60 (1H, dd, J = 5.6, 2.4 Hz, H-4), 6.16 (1H, dd, J = 6.0, 2.4 Hz, H-5), 5.44 (1H, m, H-10), 5.23 (1H, m, H-9), 4.08 (2H, m, –O*CH*$_2$CH$_2$CH$_2$CH$_3$), 2.97 (1H, m, H-3), 2.56 (1H, dd, J = 15.6, 6.4 Hz, H-2b), 2.54–2.50 (1H, m, H-8b), 2.46 (1H, dd, J = 15.6, 8.4 Hz, H-2a), 2.28 (1H, m, H-8a), 2.08 (3H, m, H-7 and H-11), 1.60 (2H, m, –OCH$_2$*CH*$_2$CH$_2$CH$_3$), 1.35 (2H, m, –OCH$_2$CH$_2$*CH*$_2$CH$_3$), 0.93 (6H, m, –OCH$_2$ CH$_2$CH$_2$*CH*$_3$ and H-12); FABMS m/z 265 $[M+H]^+$.

4.2.2.4. Pentyl 4,5-didehydrojasmonate (6). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.63 (1H, dd, J = 5.6, 2.4 Hz, H-4), 6.15 (1H, dd, J = 5.6, 2.4 Hz, H-5), 5.45 (1H, m, H-10), 5.21 (1H, m, H-9), 4.08 (2H, m, –O*CH*$_2$CH$_2$CH$_2$ CH$_2$CH$_3$), 3.01 (1H, m, H-3), 2.56 (1H, dd, J = 15.6, 6.4 Hz, H-2b), 2.55–2.50 (1H, m, H-8b), 2.43 (1H, dd, J = 15.6, 8.4 Hz, H-2a), 2.31 (1H, m, H-8a), 2.04 (3H, m, H-7 and H-11), 1.60 (2H, m, –OCH$_2$*CH*$_2$CH$_2$ CH$_2$CH$_3$), 1.30 (4H, m, –OCH$_2$*CH*$_2$*CH*$_2$CH$_3$), 0.92 (6H, m, –OCH$_2$CH$_2$ CH$_2$CH$_2$*CH*$_3$ and H-12); FABMS m/z 279 $[M+H]^+$.

4.2.2.5. Phenyl 4,5-didehydrojasmonate (7). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.70 (1H, dd, J = 5.6, 2.8 Hz, H-4), 7.37-7.07 (5H, m, –OC$_6$H$_5$), 6.22 (1H, dd, J = 5.6, 2.4 Hz, H-5), 5.44 (1H, m, H-10), 5.26 (1H, m, H-9), 3.11 (1H, m, H-3), 2.84 (1H, dd, J = 16.0, 6.4 Hz, H-2b), 2.70 (1H, dd, J = 15.6, 8.0 Hz, H-2a), 2.60–2.53 (1H, m, H-8b), 2.38–2.31 (1H, m, H-8a), 2.18 (1H, m, H-7), 2.06 (2H, m, H-11), 0.91 (3H, t, J = 7.6 Hz, H-12); FABMS m/z 285 $[M+H]^+$.

4.2.2.6. Benzyl 4,5-didehydrojasmonate (8). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.58 (1H, dd, J = 5.6, 2.4 Hz, H-4), 7.34 (5H, m, –OCH$_2$*C*$_6$*H*$_5$), 6.14 (1H, dd, J = 5.6, 2.4 Hz, H-5), 5.43 (1H, m, H-10), 5.26 (1H, m, H-9), 5.13 (2H, s, –O*CH*$_2$C$_6$H$_5$), 3.01 (1H, m, H-3), 2.60 (1H, dd, J = 16.0, 6.8 Hz, H-2b), 2.51 (2H, m, H-8b and H-2a), 2.27 (1H, m, H-8a), 2.09–1.98 (3H, m, H-7 and H-11), 0.93 (3H, t, J = 7.6 Hz, H-12); FABMS m/z 299 $[M+H]^+$.

4.2.2.7. 4-Hydroxyphenyl 4,5-didehydrojasmonate (9). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.68 (1H, dd, J = 5.6, 2.4 Hz, H-4), 6.93–6.79 (4H, m, –OC$_6$H$_4$OH), 6.15 (1H, dd, J = 6.0, 2.4 Hz, H-5), 5.45 (1H, m, H-10), 5.25 (1H, m, H-9), 3.01 (1H, m, H-3), 2.80 (1H, dd, J = 15.6, 6.4 Hz, H-2b), 2.67 (1H, dd, J = 15.6, 8.4 Hz, H-2a), 2.56 (1H, m, H-8b), 2.34 (1H, m, H-8a), 2.18 (1H, m, H-7), 2.05 (2H, m, H-11), 0.94 (3H, t, J = 7.6 Hz, H-12); FABMS m/z 301 $[M+H]^+$.

4.2.2.8. 2-Methoxyphenyl 4,5-didehydrojasmonate (10). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.74 (1H, dd, J = 6.0, 2.8 Hz, H-4), 7.20–6.95 (4H, m, –OC$_6$*H*$_5$OCH$_3$), 6.20 (1H, dd, J = 6.0, 2.4 Hz, H-5), 5.47 (1H, m, H-10), 5.29 (1H, m, H-9), 3.80 (3H, m, –OC$_6$H$_5$O*CH*$_3$), 3.11 (1H, m, H-3), 2.86 (1H, dd, J = 15.6, 6.8 Hz, H-2b), 2.69 (1H, dd, J = 15.6, 8.4 Hz, H-2a), 2.58 (1H, m, H-8b), 2.36 (1H, m, H-8a), 2.21 (1H, m, H-7), 2.06 (2H, m, H-11), 0.93 (3H, t, J = 7.2 Hz, H-12); FABMS m/z 315 $[M+H]^+$.

4.2.2.9. 2-Hydroxyethyl 4,5-didehydrojasmonate (11). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.62 (1H, dd, J = 5.6, 2.4 Hz, H-4), 6.16 (1H, dd, J = 5.6, 2.4 Hz, H-5), 5.46 (1H, m, H-10), 5.22 (1H, m, H-9), 4.27 (2H, t, J = 4.4 Hz, –*CH*$_2$CH$_2$OH), 3.82 (2H, t, J = 4.4 Hz, –CH$_2$*CH*$_2$OH), 2.99 (1H, m, H-3), 2.62 (1H, dd, J = 16.0, 6.8 Hz, H-2b), 2.54–2.46 (2H, m, H-2a and H-8b), 2.27 (1H, m, H-8a), 2.10–2.00 (3H, m, H-7 and H-11), 0.94 (3H, t, J = 7.6 Hz, H-12); FABMS m/z 253 $[M+H]^+$.

4.2.2.10. 2-(2-Hydroxyethoxy)ethyl 4,5-didehydrojasmonate (12). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.62 (1H, dd, J = 6.0, 2.8 Hz, H-4), 6.16 (1H, dd, J = 6.0, 2.4 Hz, H-5), 5.46 (1H, m, H-10), 5.26 (1H, m, H-9), 4.25 (2H, t, J = 4.4 Hz, –*CH*$_2$CH$_2$OCH$_2$CH$_2$OH), 3.72 (2H, t, J = 4.4 Hz, –CH$_2$*CH*$_2$OCH$_2$CH$_2$OH), 3.68 (2H, t, J = 4.8 Hz, –CH$_2$CH$_2$O*CH*$_2$CH$_2$OH), 3.57 (2H, t, J = 4.8 Hz, –CH$_2$CH$_2$OCH$_2$*CH*$_2$OH), 2.98 (1H, m, H-3), 2.61 (1H, dd, J = 16.0, 6.8 Hz, H-2b), 2.54–2.46 (2H, m, H-2a and H-8b), 2.30 (1H, m, H-8a), 2.13 (1H, m, H-7), 2.06 (2H, m, H-11), 0.93 (3H, t, J = 7.6 Hz, H-12); FABMS m/z 297 $[M+H]^+$.

4.2.2.11. 2-[2-(2-Hydroxyethoxy)ethoxy]ethyl 4,5-didehydrojasmonate (13). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.62 (1H, dd, J = 5.6, 2.4 Hz, H-4), 6.16 (1H, dd, J = 5.6, 2.4 Hz, H-5), 5.45 (1H, m, H-10), 5.22 (1H, m, H-9), 4.25 (2H, t, J = 4.0 Hz, –*CH*$_2$CH$_2$OCH$_2$CH$_2$OCH$_2$CH$_2$OH), 3.71–3.57 (10H, m, –CH$_2$*CH*$_2$O*CH*$_2$-*CH*$_2$O*CH*$_2$*CH*$_2$OH), 2.98 (1H, m, H-3), 2.60 (1H, dd, J = 16.0, 6.8 Hz, H-2b), 2.53–2.44 (2H, m, H-2a and H-8b), 2.30 (1H, m, H-8a), 2.08 (1H, m, H-7), 2.03 (2H, m, H-11), 0.92 (3H, t, J = 8.0 Hz, H-12); FABMS m/z 341 $[M+H]^+$.

4.2.2.12. 2-Methoxyethyl 4,5-didehydrojasmonate (14). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.62 (1H, dd, J = 6.0, 2.8 Hz, H-4), 6.16 (1H, dd, J = 6.0, 2.4 Hz, H-5), 5.46 (1H, m, H-10), 5.22 (1H, m, H-9), 4.27 (2H, t, J = 4.4 Hz, –*CH*$_2$CH$_2$OCH$_3$), 3.57 (2H, t, J = 4.4 Hz, –CH$_2$*CH*$_2$OCH$_3$), 3.36 (3H, s, –CH$_2$CH$_2$O*CH*$_3$), 2.99 (1H, m, H-3), 2.61 (1H, dd, J = 15.6, 6.4 Hz, H-2b), 2.54–2.44 (2H, m, H-2a and H-8b), 2.29 (1H, m, H-8a), 2.10–2.00 (3H, m, H-7 and H-11), 0.93 (3H, t, J = 7.2 Hz, H-12); FABMS m/z 267 $[M+H]^+$.

4.2.2.13. 2-(2-Methoxyethoxy)ethyl 4,5-didehydrojasmonate (15). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.62 (1H, dd, J = 5.6, 2.4 Hz, H-4), 6.16 (1H, dd, J = 6.0, 2.4 Hz, H-5), 5.46 (1H, m, H-10), 5.26 (1H, m, H-9), 4.25 (2H, t, J = 4.4 Hz, –CH_2CH$_2$OCH$_2$CH$_2$OCH$_3$), 3.72 (2H, t, J = 4.4 Hz, –CH$_2$CH$_2$OCH$_2$-CH$_2$OCH$_3$), 3.68 (2H, t, J = 4.8 Hz, –CH$_2$CH$_2$OCH$_2$CH$_2$OCH$_3$), 3.57 (2H, t, J = 4.8 Hz, –CH$_2$CH$_2$OCH$_2$CH$_2$OCH$_3$), 3.36 (3H, s, –CH$_2$CH$_2$OCH$_2$CH$_2$OCH$_3$), 2.98 (1H, m, H-3), 2.61 (1H, dd, J = 16.0, 6.8 Hz, H-2b), 2.54–2.46 (2H, m, H-2a and H-8b), 2.30 (1H, m, H-8a), 2.13 (1H, m, H-7), 2.06 (2H, m, H-11), 0.93 (3H, t, J = 7.2 Hz, H-12); FABMS m/z 311 [M+H]$^+$.

4.2.2.14. 2-[2-(2-Methoxyethoxy)ethoxy]ethyl 4,5-didehydrojasmonate (16). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.63 (1H, dd, J = 5.6, 2.8 Hz, H-4), 6.16 (1H, dd, J = 5.6, 2.4 Hz, H-5), 5.45 (1H, m, H-10), 5.24 (1H, m, H-9), 4.24 (2H, t, J = 4.0 Hz, –CH_2CH$_2$OCH$_2$CH$_2$OCH$_2$CH$_2$OCH$_3$), 3.69–3.51 (10H, m, –CH$_2$CH$_2$OCH$_2$CH$_2$OCH$_2$CH$_2$OCH$_2$CH$_2$OCH$_3$), 3.36 (3H, s, –CH$_2$CH$_2$OCH$_2$CH$_2$OCH$_2$CH$_2$OCH$_3$), 2.99 (1H, m, H-3), 2.62 (1H, dd, J = 16.0, 6.8 Hz, H-2b), 2.54–2.44 (2H, m, H-2a and H-8b), 2.30 (1H, m, H-8a), 2.08–2.00 (3H, m, H-7 and H-11), 0.94 (3H, t, J = 8.0 Hz, H-12); FABMS m/z 315 [M+H]$^+$.

4.2.3. Synthesis of compounds 17–22

Acyl chloride **1b**, which was prepared by reaction of the acid **1a** with an excess amount of oxalyl chloride in dichloromethane at 40 °C for 1 h, was treated with different alcohols in the presence of TEA (1 equiv) and dimethylaminopyridine (0.1 equiv) in dichloromethane at RT for 12 h to yield the intermediate esters **17a–22a**. The esters **17a–22a** were consequently transformed into their corresponding target esters **17–22**, respectively by the same procedure used for the preparation of ester **2** from methyl jasmonate **1**. Purification of these target esters was performed using reversed-phase HPLC (YMC ODS-H80) and eluting with 80% aqueous CH_3CN (product yields of 50–65%).

4.2.3.1. Isopropyl 4,5-didehydrojasmonate (17). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.61 (1H, dd, J = 6.0, 2.4 Hz, H-4), 6.15 (1H, dd, J = 6.0, 2.4 Hz, H-5), 5.46 (1H, m, H-10), 5.25 (1H, m, H-9), 5.01 (1H, m, –OCH(CH$_3$)$_2$), 2.97 (1H, m, H-3), 2.56 (1H, dd, J = 15.6, 6.4 Hz, H-2b), 2.54–2.47 (1H, m, H-8b), 2.39 (1H, dd, J = 15.6, 8.4 Hz, H-2a), 2.29 (1H, m, H-8a), 2.09–2.00 (3H, m, H-7 and H-11), 1.22 (6H, d, J = 8.0 Hz, –OCH(CH$_3$)$_2$), 0.94 (3H, t, J = 7.6 Hz, H-12); FABMS m/z 251 [M+H]$^+$.

4.2.3.2. *sec*-Butyl 4,5-didehydrojasmonate (18). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.61 (1H, dd, J = 6.0, 2.8 Hz, H-4), 6.15 (1H, dd, J = 6.0, 2.4 Hz, H-5), 5.45 (1H, m, H-10), 5.24 (1H, m, H-9), 4.86 (1H, m, –OCH(CH$_3$)(C$_2$H$_5$)), 2.98 (1H, m, H-3), 2.59–2.26 (4H, m, H-2 and H-8), 2.09–2.01 (3H, m, H-7 and H-11), 1.56 (2H, m, –OCH(CH$_3$)(CH$_2$CH$_3$)), 1.22 (3H, d, J = 7.8 Hz, –OCH(CH$_3$)(C$_2$H$_5$)), 0.94 (3H, t, J = 8.0 Hz, H-12), 0.87 (3H, t, J = 7.6 Hz, –OCH(CH$_3$)(CH$_2$CH$_3$)); FABMS m/z 265 [M+H]$^+$.

4.2.3.3. *tert*-Butyl 4,5-didehydrojasmonate (19). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.61 (1H, dd, J = 5.6, 2.4 Hz, H-4), 6.15 (1H, dd, J = 6.0, 2.4 Hz, H-5), 5.45 (1H, m, H-10), 5.24 (1H, m, H-9), 2.95 (1H, m, H-3), 2.52–2.46 (2H, m, H-2b and H-8b), 2.37–2.31 (2H, m, H-2a and H-8a) 2.09–2.01 (3H, m, H-7 and H-11), 1.44 (9H, s, –O(CH$_3$)$_3$), 0.93 (3H, t, J = 7.2 Hz, H-12); FABMS m/z 265 [M+H]$^+$.

4.2.3.4. 1-Methyl-2-methoxyethyl 4,5-didehydrojasmonate (20). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.63 (1H, dd, J = 5.6, 2.8 Hz, H-4), 6.15 (1H, dd, J = 6.0, 2.4 Hz, H-5), 5.47 (1H, m, H-10), 5.24 (1H, m, H-9), 5.11 (1H, m, –CH(CH$_3$)CH$_2$OCH$_3$), 3.40 (2H, m, –CH(CH$_3$)CH$_2$OCH$_3$), 3.36 (3H, m, –CH(CH$_3$)CH$_2$OCH$_3$), 2.99 (1H, m, H-3), 2.63–2.39 (3H, m, H-2 and H-8b), 2.29 (1H, m, H-8a), 2.10–2.01 (3H, m, H-7 and H-11), 1.22 (3H, d, J = 8.4 Hz, –CH(CH$_3$)CH$_2$OCH$_3$), 0.94 (3H, t, J = 7.6 Hz, H-12); FABMS m/z 281 [M+H]$^+$.

4.2.3.5. 1-Methylbenzyl 4,5-didehydrojasmonate (21). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.61 (1H, dd, J = 6.0, 2.8 Hz, H-4), 7.30 (5H, m, –OCH(CH$_3$)C$_6$H$_5$), 6.18 (1H, dd, J = 6.0, 2.4 Hz, H-5), 5.89 (1H, m, –OCH(CH$_3$)C$_6$H$_5$), 5.45 (1H, m, H-10), 5.24 (1H, m, H-9), 2.98 (1H, m, H-3), 2.64–2.39 (3H, m, H-2 and H-8b), 2.26 (1H, m, H-8a), 2.08–1.97 (3H, m, H-7 and H-11), 1.54 (3H, d, J = 7.4 Hz –OCH(CH$_3$)C$_6$H$_5$), 0.92 (3H, t, J = 7.6 Hz, H-12); FABMS m/z 313 [M+H]$^+$.

4.2.3.6. Cyclohexyl 4,5-didehydrojasmonate (22). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.61 (1H, dd, J = 6.0, 2.8 Hz, H-4), 6.16 (1H, dd, J = 6.0, 2.4 Hz, H-5), 5.46 (1H, m, H-10), 5.26 (1H, m, H-9), 4.75 (1H, m, –OCHC$_5$H$_{10}$), 2.98 (1H, m, H-3), 2.54 (1H, dd, J = 15.2, 6.4 Hz, H-2b), 2.48 (1H, m, H-8b), 2.41 (1H, dd, J = 15.2, 8.2 Hz, H-2a), 2.30 (1H, m, H-8a), 2.10–2.02 (3H, m, H-7 and H-11), 1.84–1.33 (10H, m, –OCHC$_5$H$_{10}$), 0.94 (3H, t, J = 7.6 Hz, H-12); FABMS m/z 291 [M+H]$^+$.

4.2.4. Synthesis of α-chloroenone jasmonate analogs 11-Cl, 19-Cl, and 22-Cl

The acid, **J7a**, which was obtained by treatment of the epoxide of **2** with HCl in acetic acid,[2] was coupled with ethylene glycol (2 equiv) in EtOAc using TBTU (1 equiv) and TEA (2 equiv), as described for the synthesis of esters **3–16** from **2a** in order to prepare compound **11-Cl**. On the other hand, compounds **19-Cl** and **22-Cl** were directly synthesized from the esters **19** and **22**, respectively, by treatment of their corresponding epoxides with $CeCl_3$ (1.2 equiv) in a mixture of H_2O/MeOH (1:3) under reflux for 3 h. After processing according to the standard method, the final products were purified by reversed-phase HPLC (YMC ODS-H80) and eluting with 80% aqueous CH_3CN.

4.2.4.1. 2-Hydroxyethyl 5-chloro-4,5-didehydrojasmonate (11-Cl). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.53 (1H, d, J = 2.8 Hz, H-4), 5.48 (1H, m, H-10), 5.22 (1H, m, H-9), 4.23 (2H, t, J = 4.4 Hz, –CH_2CH$_2$OH), 3.81 (2H, t, J = 4.4 Hz, –CH$_2$CH$_2$OH), 2.97 (1H, m, H-3), 2.65 (1H, dd, J = 16.0, 6.0 Hz, H-2b), 2.57–2.46 (2H, m, H-2a and H-8b), 2.34 (1H, m, H-8a), 2.23 (1H, m, H-7), 2.03 (2H, m, H-11), 0.93 (3H, t, J = 7.2 Hz, H-12); FABMS m/z 287 [M+H]$^+$.

4.2.4.2. *tert*-Butyl 5-chloro-4,5-didehydrojasmonate (19-Cl). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.51 (1H, d, J = 2.4 Hz, H-4), 5.46 (1H, m, H-10), 5.24 (1H, m, H-9), 2.92 (1H, m, H-3), 2.55–2.49 (2H, m, H-2b and H-8b), 2.38–2.32 (2H, m, H-2a and H-8a) 2.23 (1H, m, H-7), 2.04 (2H, m, H-11), 1.43 (9H, s, –O(CH$_3$)$_3$), 0.93 (3H, t, J = 7.6 Hz, H-12); FABMS m/z 299 [M+H]$^+$.

4.2.4.3. Cyclohexyl 5-chloro-4,5-didehydrojasmonate (22-Cl). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.53 (1H, d, J = 2.8 Hz, H-4), 5.45 (1H, m, H-10), 5.27 (1H, m, H-9), 4.76 (1H, m, –OCHC$_5$H$_{10}$), 2.98 (1H, m, H-3), 2.54 (1H, dd, J = 15.6, 6.8 Hz, H-2b), 2.48 (1H, m, H-8b), 2.41 (1H, dd, J = 15.6, 8.6 Hz, H-2a), 2.30 (1H, m, H-8a), 2.24 (1H, m, H-7), 2.05 (2H, m, H-11), 1.85–1.35 (10H, m, –OCHC$_5$H$_{10}$), 0.94 (3H, t, J = 7.2 Hz, H-12); FABMS m/z 325 [M+H]$^+$.

4.3. Calculation of log*P*

The calculation of the octanol–water partition coefficient log*P* was performed using ACD/ChemSketch 12.0 software (Advanced Chemistry Development, Inc., Toronto, ON, Canada)

4.4. In vitro stability assessment

The mixture of each ester (~0.5 mg) and 50 μL of porcine liver esterase (Sigma Chemical; Lot no. E2884-1KU: 1000 U/0.34 mL; 1 unit (1 U) of the enzyme hydrolyzes 1 μmol of an ester) in 0.01 M phosphate buffer solution (pH 7.4, 1 mL) and acetone (10 μL) was incubated at 37 °C. At regular intervals (30 and 60 min), the samples (100 μL) were withdrawn and directly injected into the Gilson 321 and 322 pumps/UV-8000 system with a YMC ODS-H80 column (250 × 4.6 mm, 4 μm, 80 Å) for analysis using 70% aq MeOH as the mobile phase, with a flow rate of 0.5 mL/min, and UV detection at 210 nm. The peak areas of intact esters and their hydrolyzed acids were analyzed using the Gilson Unipoint ProgramVersion 5.1.

4.5. In vitro anti-inflammatory assay

4.5.1. Cell culture

RAW 264.7 murine macrophage cells were purchased from ATCC (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) heat-activated fetal bovine serum, streptomycin (100 μg/mL), and penicillin (100 U/mL) at 37 °C in a 5% CO_2 incubator.

4.5.2. Cytotoxicity assay

The cytotoxic effects were evaluated using the MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) assay. Cells were seeded in 96-well plates and were treated with different conditions for 24 h. MTT solution was then added to each well, and the plate was incubated for 4 h. The culture supernatants were removed, and the formazan crystals in each well were dissolved in 200 μL of dimethyl sulfoxide (DMSO) for 30 min. The absorbances of the contents of each well were measured at 540 nm using VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA).

4.5.3. Nitrite assay

The production of NO was measured, as described previously by Ryu et al.,[20] using the Griess reagent (Sigma). Briefly, the RAW 264.7 cells were plated in 96-well plate and treated with LPS and the indicated compounds for 24 h. One hundred microliters of the supernatant was mixed with the same volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H_3PO_4) and this mixture was incubated for 10 min at room temperature (protected from light). The absorbance of the mixture at 540 nm was determined with VersaMax ELISA microplate reader and the results were compared to a calibration curve using sodium nitrite as the standard.

4.6. In vivo assay of anti-inflammatory effects

4.6.1. Animal

Male Sprague–Dawley (SD) rats (140–160 g, 5-weeks old) were purchased from Central Laboratory Animal Inc. (Seoul, Korea). Animals were housed under standard laboratory conditions with free access to food and water. The temperature was thermostatically regulated to 22 °C ± 2 °C, and a 12-h light/dark schedule was maintained. Prior to their use, they were allowed 1 week for acclimatization within the work area environment. All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee Guidelines of Seoul National University (SNU-201110-4).

4.6.2. Carrageenan-induced paw edema

The carrageenan-induced hind paw edema model in rats was used to assess anti-inflammatory activity.[21] Test compounds **J7**, **11-Cl**, and **19-Cl** (50 mg/kg) or indomethacin (25 mg/kg) dissolved in 5% cremophor and 5% ethanol in PBS were administered by intraperitoneal injection, and the solvent alone served as a vehicle control. Thirty minutes after the administration of the test compounds, vehicle, or indomethacin, paw edema was induced by subplantar injection of 0.1 mL of 1% freshly prepared carrageenan suspension in normal saline into the right hind paw of each rat. The left hind paw was injected with 0.1 mL of normal saline. The paw volume was measured before (0 h) and at intervals of 0.5, 1, 2, 4, 6, and 24 h after carrageenan injection using a plethysmometer (Ugo Basile, Comerio, Italy).

4.7. Statistics

All experiments were repeated at least 3 times. Data are presented as means ± SD for the indicated number of independently performed experiments. The statistical significances within a parameter were evaluated by one-way and multiple analysis of variation (ANOVA).

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Supplementary data

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