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Inhibition of TNF-α-Induced Inflammation by Sesquiterpene Lactones from *Saussurea lappa* and Semi-Synthetic Analogues

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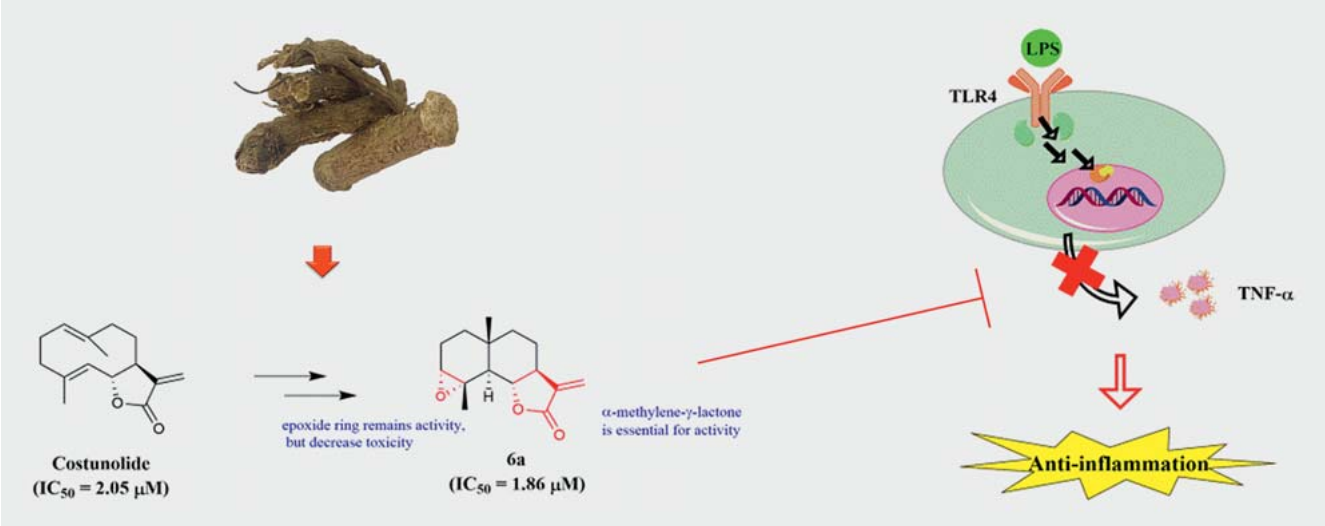
ABSTRACT

We investigated the tumor necrosis factor-alpha (TNF-α) inhibitory activity of sesquiterpenes from *Saussurea lappa* root extracts. According to the hexane and EtOAc extracts showing significant activity with IC_{50} values of 0.5 and 1.0 µg/mL, respectively, chromatographic fractionation of the extracts was performed and led to the isolation of 10 sesquiterpenes (**1**–**10**). Costunolide (**1**), a major compound, and dehydrocostus lactone (**4**) exhibited high efficiency in decreasing TNF-α levels, with IC_{50} values of 2.05 and 2.06 µM, respectively. In addition, sesquiterpene analogues were synthesized to establish their structure-activity relationship (SAR) profile. Among the semi-synthetic analogues, compounds **6a** and **16** showed the most potent activity with IC_{50} values of 1.84 and 1.97 µM, respectively. More importantly, compound **6a** showed less tox-



icity than costunolide and **16**. These results provided the first SAR profile of sesquiterpene lactones and indicated that the α-methylene-γ-lactone moiety plays a crucial role in TNF-α inhibition. Additionally, the epoxide derivative **6a** might represent a lead compound for further anti-TNF-α therapies, owing to its potent activity and reduced toxicity.

Introduction

Tumor necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine, is an important mediator of inflammatory responses [1]. It is mainly released by activated immune cells, such as macrophages and monocytes [2]. The excessive secretion of TNF-α has been implicated in a diverse range of infectious and inflammatory diseases, particularly Crohn's disease and rheumatoid arthritis [3]. The inhibition of this cytokine is thus considered an essential approach in the development of therapeutic agents. At present, three drugs licensed as TNF-α blocking agents–infliximab, adalimumab, and etanercept–are available for treatment of rheumatoid arthritis and other inflammatory diseases [4]. Although efficacious TNF-α inhibitors have already been developed, the challenge remains for researchers to identify more effective TNF-α inhibitors with reduced toxicity to treat various acute and chronic inflammatory diseases.

The root of *Saussurea lappa* Clarke (Compositae), commonly known as costus or kuth root [5], is used in various Indian Ayurvedic and Chinese traditional formulations for the treatment of abdominal pain, distention, vomiting, allergy, and cancer [6, 7]. In Southeast Asia, it has also been used to reduce fever and headache and to treat diarrhea. In addition, the root extract has been used to relieve syphilis in Japan [8]. This plant was found to be rich in sesquiterpenes, particularly sesquiterpene lactones, of which costunolide is the main constituent. As the most abundant and bioactive component of *S. lappa* root, costunolide is reported to possess a broad range of biological activities including anti-cancer, anti-ulcer, anti-bacterial, anti-hepatitis B virus, and anti-inflammatory activities [9, 10]. However, few reports have examined the inhibition of TNF-α-induced inflammation by costunolide and other sesquiterpene lactones isolated from *S. lappa*. Therefore, this study was designed to investigate the anti-inflammatory activity of isolated secondary metabolites from *S. lappa* root against TNF-α inhibition. A number of semi-synthetic derivatives of sesquiterpenes were also prepared and their activity was evaluated to clarify the structure-activity relationship (SAR) of these compounds.

Results and Discussion

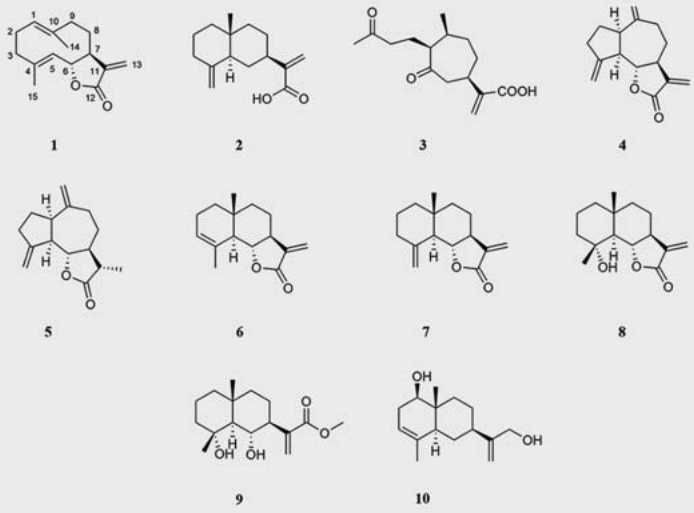
The anti-inflammatory effects of hexane, EtOAc, and MeOH extracts of *S. lappa* root were evaluated by measuring their potential to inhibit TNF-α production in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells. Both the hexane and EtOAc extracts exhibited promising activity with IC_{50} values of 0.5 and 1.0 µg/mL, respectively, while the MeOH extract did not show any significant activity. Moreover, an MTT assay indicated that cell viability was not affected by treatment with these extracts (**Fig. 1S**, Supporting Information). These results indicate that the hexane and EtOAc extracts of *S. lappa* root potently inhibited LPS-induced TNF-α release without affecting the viability of RAW264.7 cells.

To isolate the active components, the hexane and EtOAc extracts were subjected to silica gel and Sephadex LH-20 column chromatography followed by C-18 reversed-phase HPLC to obtain 10 sesquiterpenes (**1**–**10**) (▶ **Fig. 1**), together with one triterpene, betulinic acid. The major metabolite (**1**), obtained on a gram scale, was identified as costunolide from its spectroscopic data and by comparison with data reported in the literature. Similarly, the structures of compounds **2**–**10** were characterized by spectroscopic techniques and confirmed by comparing their 1D NMR data with those reported previously [11–17].

To examine whether the isolated sesquiterpenes affected the production of TNF-α in LPS-stimulated macrophage RAW264.7 cells, the cells were treated with the compounds at different concentrations in the presence of LPS and the levels of TNF-α in the culture supernatant were measured by ELISA kit. TNF-α level significantly increased in cells treated with LPS in comparison with that in untreated control cells, as shown in ▶ **Table 1**. Among the tested compounds, costunolide (**1**) and dehydrocostus lactone (**4**) exhibited the most potent activity, with IC_{50} values of 2.05 and 2.06 µM, respectively. The activity of α- and β-cyclocostunolide (**6** and **7**) was lower than that of **1** and **4**, with IC_{50} values of 5.35 and 15.34 µM, respectively. The remaining compounds showed IC_{50} values of over 20 µM, and were therefore deemed inactive. Based on these results, the existence of an α-methylene-γ-lactone moiety in the structure was considered crucial for activity, as described previously [18, 19]. Additionally, although costunolide (**1**) showed strong inhibitory activity against TNF-α production, cell viability, analyzed by MTT assay, markedly decreased (by up to 50 % at 10 µM), as shown in ▶ **Fig. 2**.

In order to improve the activity and reduce the toxicity of the compounds, some structural modification was performed and the relationship between the activity and structure was studied. Since several previous studies have revealed that α-methylene-γ-lactone is a crucial building block of many natural products exhibiting diverse biological activities including anti-inflammatory activity [20, 21], it was considered important to retain this structural moiety. To verify whether an α-methylene-γ-lactone moiety was required for inhibition of TNF-α production, three derivatives, **11**, **12**, and **13**, were prepared by methoxylation, basic hydrolysis, and epoxidation of costunolide (**1**) [22], respectively (▶ **Fig. 3**), and their activity was determined. As shown in ▶ **Table 2**, both **11** and **12**, which had no α-methylene-γ-lactone moiety in their structure, did not show any detectable activity even at the highest concentration tested (50 µM), whereas compound **13** showed active TNF-α inhibition. This result confirmed that the α-methylene-γ-lactone moiety was essential for the anti-inflammatory effect on TNF-α secretion in activated macrophages.

As the main component, costunolide (**1**), a germacranolide-type sesquiterpene, was used as the starting material for synthesis. The 10-membered ring of this type of compound has been shown to be highly prone to cyclization to a fused 6,6-bicyclic ring under



▶ **Fig. 1** Isolated compounds from *S. lappa* root.

acidic condition, providing a eudesmanolide-type sesquiterpene. As little is known about their anti-inflammatory properties, a series of eudesmanolides was thus synthesized, starting from costunolide (**1**), and their ability to inhibit TNF-α secretion in LPS-activated macrophages was examined. The synthetic approach utilized to prepare the desired analogues is described in ▶ **Fig. 4**. First, the treatment of costunolide (**1**) with *m*-chloroperoxybenzoic acid (*m*-CPBA), followed by $BF_3.OEt_2$, led to the formation of eudesmanolides **14** and **15,** bearing a hydroxyl (-OH) group at the C-1 position. Subsequently, alcohol **14** was subjected to oxidation with pyridinium dichromate (PDC) and acetylation with Ac_2O to afford the acetylated derivative **14a** and related ketone **14b**. In the same manner, compound **15** was converted to the acetylated analogue **15a** and related ketone **15b**. As expected, epoxidation of **14** with *m*-CPBA provided the epoxide **14c** as a single stereoisomer, while that of the C-4 exomethylene of **15** produced two epoxide isomers, **15c** and **15d**. The relative stereochemistry of compounds **14c, 15c**, and **15d** was deduced from their NOESY spectra.

To increase the quantity of α- and β-cyclocostunolide (**6** and **7**) for further synthesis, costunolide (**1**) was treated with a catalytic amount of *p*-toluenesulfonic acid in dichloromethane to yield compounds **6** and **7**, as expected. Additionally, the γ-cyclocostunolide **16** was obtained as a minor product (2% yield). Similarly, compounds **6** and **7** were subsequently subjected to epoxidation with *m*-CPBA to produce the epoxides **6a, 7a**, and **7b**, as shown in ▶ **Fig. 5**.

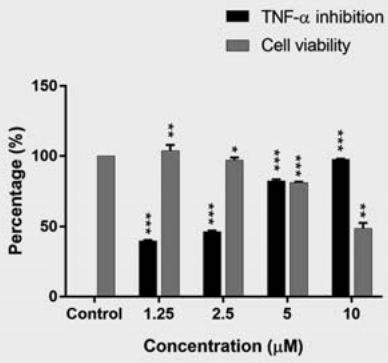
All semi-synthetic eudesmanolides were further assessed for their anti-inflammatory effects on TNF-α levels in LPS-stimulated RAW264.7 macrophage cells. As can be seen in ▶ **Table 2**, most of the compounds affected the secretion of TNF-α to a statistically significant extent. The substituent group at C-1 had a significant effect on anti-inflammatory activity; the existence of the hydroxyl, ketone, and ester moieties decreased the activity. Compounds **6a** and **16** showed the most potent inhibitory activity among all compounds, comparable to the parent compound, costunolide (**1**), which was greater than that of the standard drug, indomethacin. Interestingly, compound **6a** did not show any significant cytotoxicity at the concentrations tested (▶ **Fig. 6**) and was markedly less toxic than costunolide, whereas the cytotoxicity of **16** was comparable to that of costunolide (**Fig. 2S**, Supporting Information). These results indicated that eudesmanolide **6a** might represent an interesting lead compound for the development of anti-TNF-α therapies. Moreover, to the best of our knowledge, this is the first report to study and develop an early SAR profile of eudesmanolide sesquiterpene lactone on TNF-α inhibition, as summarized in ▶ **Fig. 7**.

In this work, we studied the effect of sesquiterpenes isolated from *S. lappa* root on the inhibition of TNF-α secretion. Costunolide (**1**), the major component, exhibited potent activity; however, it was shown to be cytotoxic in RAW264.7 macrophage cells. As the α-methylene-γ-lactone moiety has been shown to play a

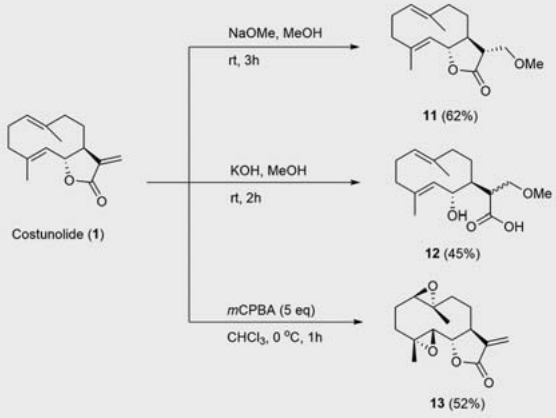
▶ **Table 1** Effect of isolated compounds from *S. lappa* root on TNF-α production.

Compounds	IC_{50}[a] (μM)	CI[b] (95%, n = 3)
1	2.05	[1.52–2.61]
2	> 50	
3	> 50	
4	2.06	[1.97–2.16]
5	32.49	[29.70–35.33]
6	5.35	[5.06–5.67]
7	15.34	[15.29–17.83]
8	48.63	[32.39–95.10]
9	21.52	[19.24–24.22]
10	> 50	
Indomethacin[c]	121.4	[108.1–142.3]

[a] IC_{50}: 50% inhibition concentration; [b] CI: confidence interval; [c] positive control



▶ **Fig. 2** Effect of costunolide (**1**) on TNF-α secretion and cell viability in LPS-activated RAW264.7 cells. Data are expressed as mean ± SEM (n = 3). *p < 0.033, **p < 0.002, ***p < 0.001 vs. control



▶ **Fig. 3** Synthesis of compounds **11**–**13**.

crucial role in TNF-α inhibition, the eudesmanolide-type sesquiterpenes were semi-synthesized from costunolide and their activity was determined. The epoxide **6a** exhibited the most promising activity, comparable to that of costunolide, the parent compound, but with less toxicity. Therefore, eudesmanolide **6a** might represent a lead compound as a therapeutic target of TNF-α inhibition. The effect of *S. lappa* crude extracts on TNF-α inhibition, the cell viability of γ-cyclocostunolide, and all mass (*m*/*z*) and 1D NMR data are available as Supporting Information

Materials and Methods

General experimental procedures

NMR spectra were measured in chloroform-*d* and acetone-d_6 using JEOL EC600 M Hz NMR. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) spectra were determined using a Shimadzu AXIMA-Resonance spectrometer. Preparative HPLC (Shimadzu LC-6AD) was performed using an Inertsil ODS-3 column (20 mm Φ × 250 mm; GL Sciences) and

▶ **Table 2** Effect of semi-synthetic analogues on TNF-α production.

Compounds	IC_{50}[a] (μM)	CI[b] (95 %, n = 3)
11	> 50	
12	> 50	
13	8.83	[7.19–11.19]
14	6.64	[6.24–7.06]
14a	8.20	[6.72–10.15]
14b	20.41	[20.03–20.82]
14c	8.26	[7.74–8.79]
15	5.20	[4.79–5.62]
15a	21.87	[17.52–29.10]
15b	13.50	[13.33–13.68]
15c	12.56	[11.74–13.56]
15d	15.50	[14.80–16.26]
16	1.97	[1.75–2.18]
6a	1.84	[1.58–2.10]
7a	18.93	[17.47 to 20.72]
7b	> 50	

[a] IC_{50}: 50 % inhibition concentration; [b] CI: confidence interval

semi-preparative HPLC was performed using an Inertsil ODS-3 column (10 mm Φ × 250 mm; GL Sciences). Silica gel (BW-200, Chromatotex) and Sephadex LH-20 (18–111 μm, GE Healthcare) were used for open-column chromatography. Analytical thin layer chromatography was performed on pre-coated silica gel 60 F254 glass plates (Merck).

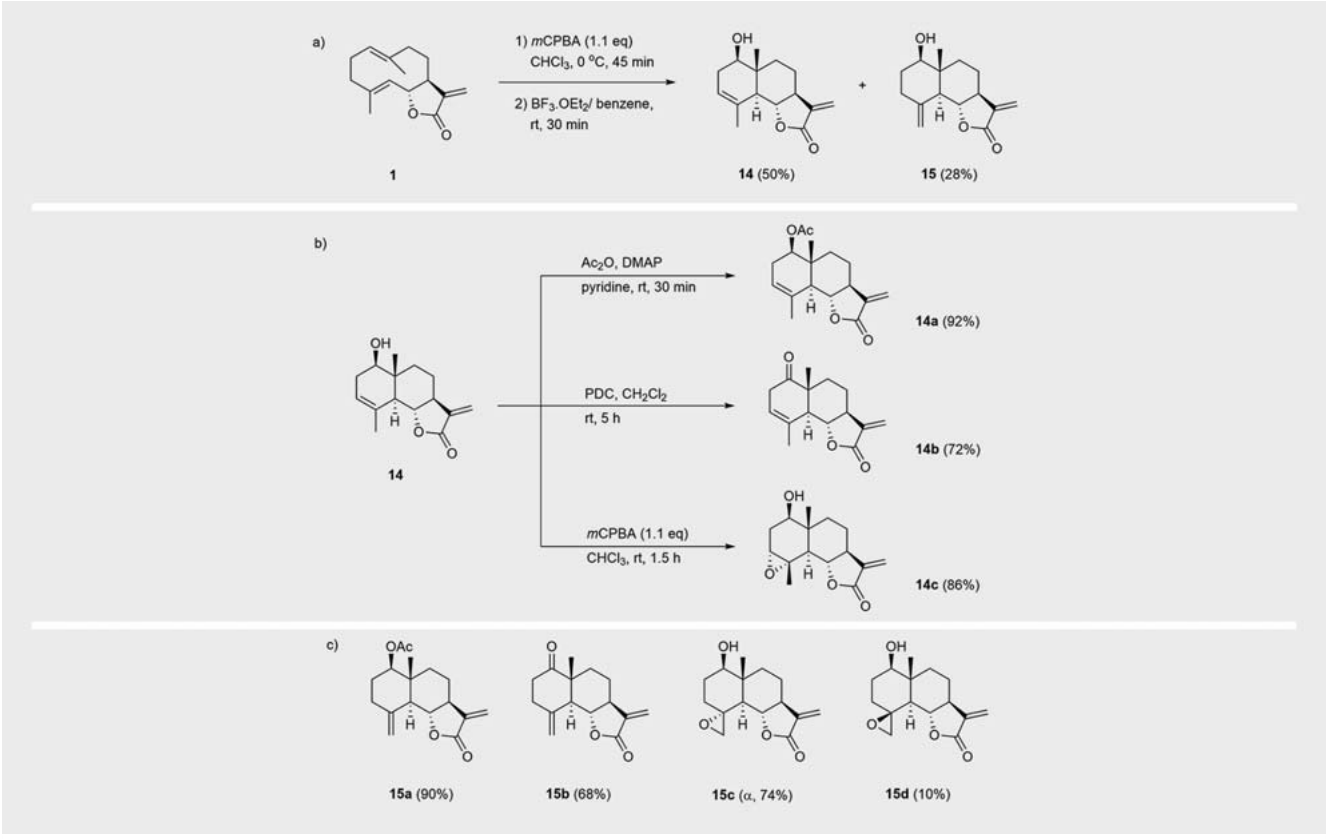
Plant material

The roots of *S. lappa* (5 kg) were purchased from an herbal medicine store (Marma-Samunprai) at Mae Sod, Tak province, Thailand, in August 2015. Voucher specimen was designated with the code CUCHEM2015-006 and is deposited at Department of Chemistry, Faculty of Science, Chulalongkorn University.

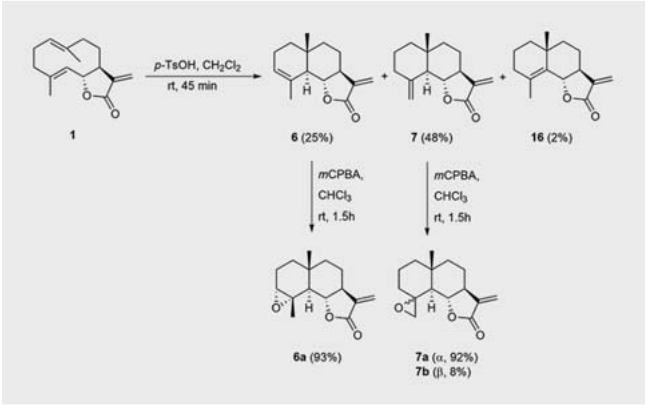
Extraction and isolation

Air-dried roots of *S. lappa* (5 kg) were powdered and extracted three times with MeOH at room temperature. The solvent was removed under reduced pressure to give the MeOH extract, which was suspended in H_2O (1 : 1 v/v) and successively partitioned with *n*-hexane and EtOAc to yield a hexane extract (70 g) and an EtOAc extract (30 g), respectively.

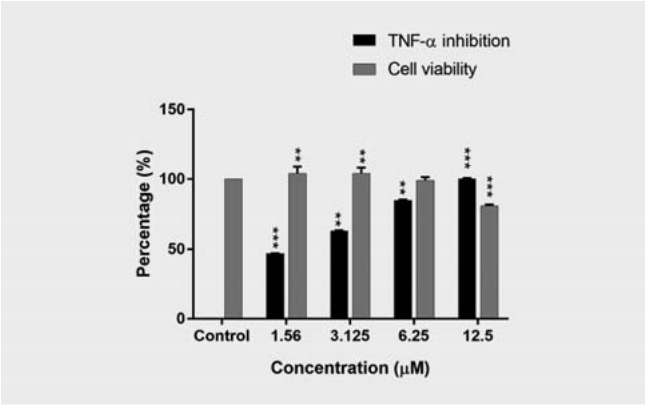
The hexane extract (70 g) was subjected to normal-phase silica gel column chromatography and eluted with gradient conditions of *n*-hexane/EtOAc (10 : 0–0 : 10) to give 16 fractions (Fr. 1–16). Fr. 8 was recrystallized from *n*-hexane/EtOAc (9 : 1) to give costunolide (**1**, 2.95 g), and the mother liquor was then subjected to silica gel column chromatography and further purified by preparation HPLC (Inertsil ODS-3, 20 mm Φ × 250 mm) with the gradient condition MeOH : H_2O (7 : 3–10 : 0 v/v) to yield costic acid (**2**, 11.7 mg), rupestonic acid G (**3**, 15.8 mg), dehydrocostus lactone (**4**, 60.7 mg), dehydrodihydrocostus lactone (**5**, 3.8 mg), and betulinic acid (14.2 mg).



▶ **Fig. 4** Synthesis of eudesmanolide sesquiterpenes: **a** compounds **14** and **15** from costunolide (**1**), **b** derivatives from **14**, **c** derivatives from **15**.



▶ **Fig. 5** Synthesis of eudesmanolide sesquiterpenes from costunolide (**1**).



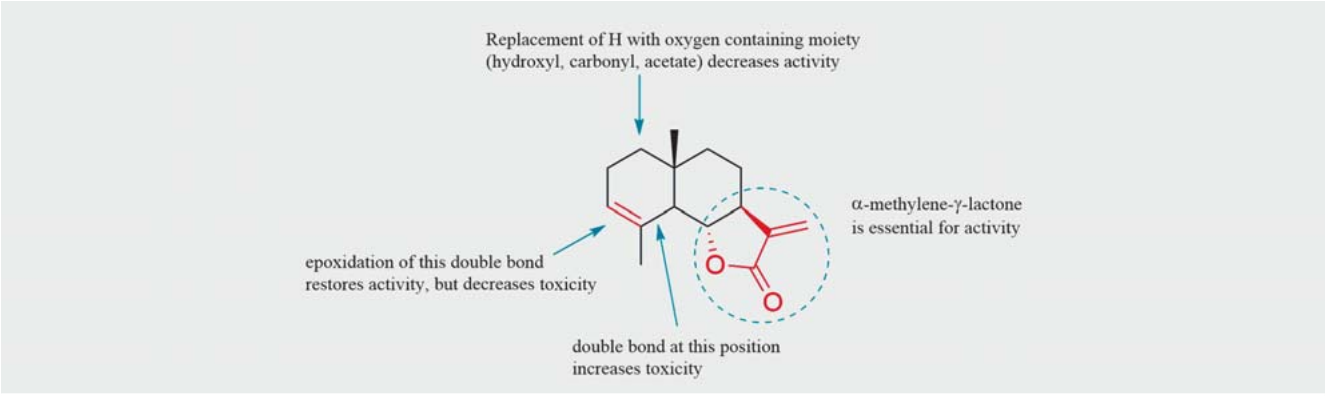
▶ **Fig. 6** Effect of eudesmanolide **6a** on TNF-α secretion in LPS-activated RAW264.7 cells and on cell viability. Data are expressed as mean ± SEM (n = 3). *p < 0.033, **p < 0.002, ***p < 0.001 vs. control.

The EtOAc (30 g) extract was also subjected to normal-phase silica gel column chromatography (*n*-hexane/EtOAc, 10 : 0–0 : 10) to give 10 fractions (Fr. 1–10). Fr. 2 was separated over Sephadex LH-20 using MeOH and purified by semi-preparative HPLC (Inertsil ODS-3, 10 mm Φ × 250 mm, gradient condition, MeOH : H_2O [7 : 3–10 : 0 v/v]) to afford α-cyclocostunolide (**6**, 4.2 mg), β-cyclocostunolide (**7**, 12.3 mg), arbusculin A (**8**, 2.4 mg), arbusculin E methyl ester (**9**, 7.7 mg), and cnicothamnol (**10**, 2.3 mg).

Semi-synthesis

Methoxylation of costunolide (1)

A solution of costunolide (**1**, 30 mg, 0.129 mmol) in 0.1 % NaOMe-MeOH (5 mL) was stirred at room temperature for 3 h. Next, water was added and the mixture was extracted with EtOAc. After removal of the solvent, the crude mixture was purified by silica gel column chromatography (*n*-hexane : EtOAc, 8 : 2) to yield compound **11** (21.2 mg, 62 %).



▶ **Fig. 7** Summary of SAR of the eudesmanolide skeleton for anti-TNF-α activity.

Base hydrolysis of costunolide (1)

To a solution of costunolide (**1**, 30 mg, 0.129 mmol) in MeOH (5 mL), KOH (28.9 mg, 0.52 mmol) was added and the mixture was stirred at room temperature for 2 h. After neutralization with aqueous HCl (6 M), the reaction mixture was extracted with EtOAc and the solvent was removed under reduced pressure. Purification by silica gel column chromatography (*n*-hexane : EtOAc, 8 : 2) afforded lactone ring-opening product **12** (16.4 mg, 45 %).

Synthesis of santamarine (14) and reynosin (15)

According to a previously reported protocol [23], the epoxidation of costunolide (**1**, 300 mg, 1.29 mmol) with *m*-CPBA (333.9 mg, 1.94 mmol) in $CHCl_3$ (50 mL) was carried out at 0 °C for 45 min. The reaction mixture was poured into cold water and then extracted with EtOAc, washed with brine, and dried over Na_2SO_4. After removal of solvent, the residue was passed through a short silica gel column (*n*-hexane : EtOAc, 8 : 2) to give costunolide-1,10-epoxide. The product was further treated with $BF_3.OEt_2$ in benzene (20 mL) at room temperature. After 30 min, the reaction mixture was poured into cold water and extracted with EtOAc. The organic layer was successively washed with 5 % aqueous $NaHCO_3$ and brine and then dried over Na_2SO_4. The crude extract was purified by silica gel column chromatography to give santamarine (**14**, 160.3 mg, 50 %) and reynosin (**15**, 89.8 mg, 28 %), [22, 24].

General procedure for acetylation

To a solution of the starting alcohol (0.15 mmol) in pyridine (3 mL), an excess volume of Ac_2O (0.3 mL) and a catalytic amount of 4-dimethylaminopyridine were added at room temperature. After 30 min, the reaction was quenched with water, extracted with EtOAc, washed with brine, and dried over Na_2SO_4. After removal of solvent, the residue was purified by silica gel column chromatography (*n*-hexane : EtOAc, 8 : 2) to give the acetylated product.

General procedure for oxidation with PDC

To a solution of the starting alcohol (0.15 mmol) in CH_2Cl_2 (10 mL) was added PDC (0.15 mmol). The reaction mixture was stirred for 5 h at room temperature and then filtered. The filtrate was concentrated under reduced pressure to yield the residue, which was further purified by silica gel column chromatography (*n*-hexane : EtOAc, 8 : 2) to give the ketone product.

Genera epoxidation with *m*-CPBA

A solution of the starting compound (0.15 mmol) in $CHCl_3$ (10 mL) was treated with *m*-CPBA (0.165 mmol) at room temperature. After stirring for 1.5 h, the reaction mixture was poured into cold water, and the organic phase was washed with 5 % aqueous $NaHCO_3$ and brine and dried over Na_2SO_4. The pure epoxidized product was obtained from silica gel column chromatography (*n*-hexane : EtOAc, 6 : 4).

Acid treatment of costunolide (1)

To a solution of costunolide (**1**, 0.86 mmol) in CH_2Cl_2 (30 mL), *p*-TsOH (10 mg) was added as a catalyzing agent, and the reaction mixture was stirred at room temperature for 45 min. The reaction was then quenched with the addition of 5 % aqueous $NaHCO_3$, extracted with EtOAc, washed with brine, and dried over Na_2SO_4. After filtration, the filtrate was concentrated and the residue was purified by silica gel column chromatography (*n*-hexane : EtOAc, 9 : 1) to afford three isomer products, α-, β-, and γ-cyclocostunolide as compound **6** (50.0 mg, 25 %), compound **7** (96.0 mg, 48 %), and compound **16** (γ-cyclocostunolide, 4.0 mg, 2 %).

Cell culture and biological assays

The murine macrophage RAW264.7 cell line was purchased from Riken BRC and cultured in Eagle's minimal essential medium (purchased from Wako). The medium was supplemented with 100 U/mL of penicillin, 100 U/mL of streptomycin, and 10 % FBS. The cells were seeded at a density of 5×10^4 cells/well in 24-well plates and then incubated overnight at 37 °C in humidified atmosphere of 5 % CO_2. The cells were pretreated for 2 h with test compounds at the indicated concentrations and DMSO as control and subsequently activated by LPS (1 µg/mL, purchased from Wako). After 24 h of incubation, the levels of TNF-α production were determined. The test compounds were dissolved in DMSO (stock solution) and diluted with medium to a final DMSO concentration of 0.2 %. Indomethacin (purchased from TCI, > 98.0 % by HPLC) was used as a standard drug.

Measurement of TNF-α production

In brief, cell-free supernatant was collected after 24 h of incubation with the stimuli and assayed for TNF-α. TNF-α levels were measured using a mouse TNF-α ELISA kit (Novex) for quantitative

determination using a monoclonal antibody specific for TNF-α coated onto 96-well plates. The absorbance was measured at 450 nm and a standard curve of 15.6–1,000 pg/mL was used to determine TNF-α levels.

Cell viability measurement by MTT assay

RAW264.7 cells were seeded in 24-well plates at a density of 5×10^4 cells/well and incubated overnight at 37 °C in a humidified atmosphere of 5 % CO_2. Subsequently, cells were treated with test compounds at the indicated concentrations and DMSO as control for 24 h. The test compounds were dissolved in DMSO (stock solution) and diluted with medium to a final DMSO concentration of 0.2 %. MTT solution (50 µL, 5 mg/mL in PBS) was added to each well and plates were incubated for an additional 4 h. Next, the medium was removed and 1.0 mL of isopropyl alcohol (containing 0.04 N HCl) was added to each well to dissolve the formazan crystals that formed, and 150 µL was transferred to a 96-well plate. The absorbance was measured at 590 nm using a microplate reader. Cell viability was calculated as a percentage of the viability of control cells treated with DMSO.

Statistical analysis

All experiments were performed in triplicate (n = 3) and data are expressed as mean values and standard deviation. Significance was analyzed using analysis of variance and Dunnett's multiple comparison test by GraphPad Prism (Version 7). A p-value of < 0.05 was considered statistically significance.

Supporting Information

The effect of *S. lappa* crude extracts on TNF-α inhibition, the cell viability of γ-cyclocostunolide, and all mass (*m*/*z*) and 1D NMR data are available as Supporting Information.

Acknowledgements

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Conflict of Interest

The authors have no conflicts of interest to declare.

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