CHEMISTRY & BIODIVERSITY

Accepted Article

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To be cited as: *Chem. Biodiversity* 10.1002/cbdv.201900531

Link to VoR: http://dx.doi.org/10.1002/cbdv.201900531

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Synthesis of new lathyrane diterpenoid derivatives from *Euphorbia lathyris* and evaluation of their anti-inflammatory activities

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10.1002/cbdv.201900531

ABSTRACT: Euphorbia factor L_3, a lathyrane diterpenoid extracted from *Euphorbia lathyris*, was found to display good anti-inflammatory activity with very low cytotoxicity. To find more potent anti-inflammatory drugs, two series of Euphorbia factor L_3 derivatives with fatty and aromatic acids were designed and synthesized. Among them, compound **5n** exhibited most potent inhibition on LPS-induced NO production in RAW264.7 cells with no obvious cytotoxicity. To determine the key characteristics of Euphorbia factor L_3 derivatives that contribute to anti-inflammatory activity, we conducted a structure–activity relationship study of these compounds.

Keywords: *Euphorbia lathyris*, lathyrane diterpenoid, anti-inflammatory activity.

Introduction

Inflammation is a host generated protective immune response evolved to protect the host in response to acute trauma or pathogens or their PAMPs by containing the damage or removing/killing the pathogen responsible for infection.[1] Severe and systemic acute inflammation may result in pathology, organ failure and death as seen during sepsis.[2] At present, clinical methods for treating inflammation include anti-inflammatory steroids, non-selective non-steroidal anti-inflammatory drugs (NSAID), COX-2 selective inhibitors and biological therapy, all of which have several unavoidable serious adverse reactions.[3] In this context, it is highly desirable to find more anti-inflammatory agents with better effect and safety.

Natural products extracted from plants, microbes, and animals have remarkable structural diversity and biological characteristics, providing researchers with more possibilities to develop novel drugs for disease therapeutics.[4] In the kingdom of natural products, terpenoids have emerged as one of the most important families because of their distinct biological activities and drug-like properties.[5, 6] In China and other countries, there are many kinds of terpenoids have been found with good anti-inflammatory activity and some of which were used clinically for treating inflammation (**Fig. 1**).[7-12] Finding new anti-inflammatory drugs from terpenoids is therefore a good strategy.[13]

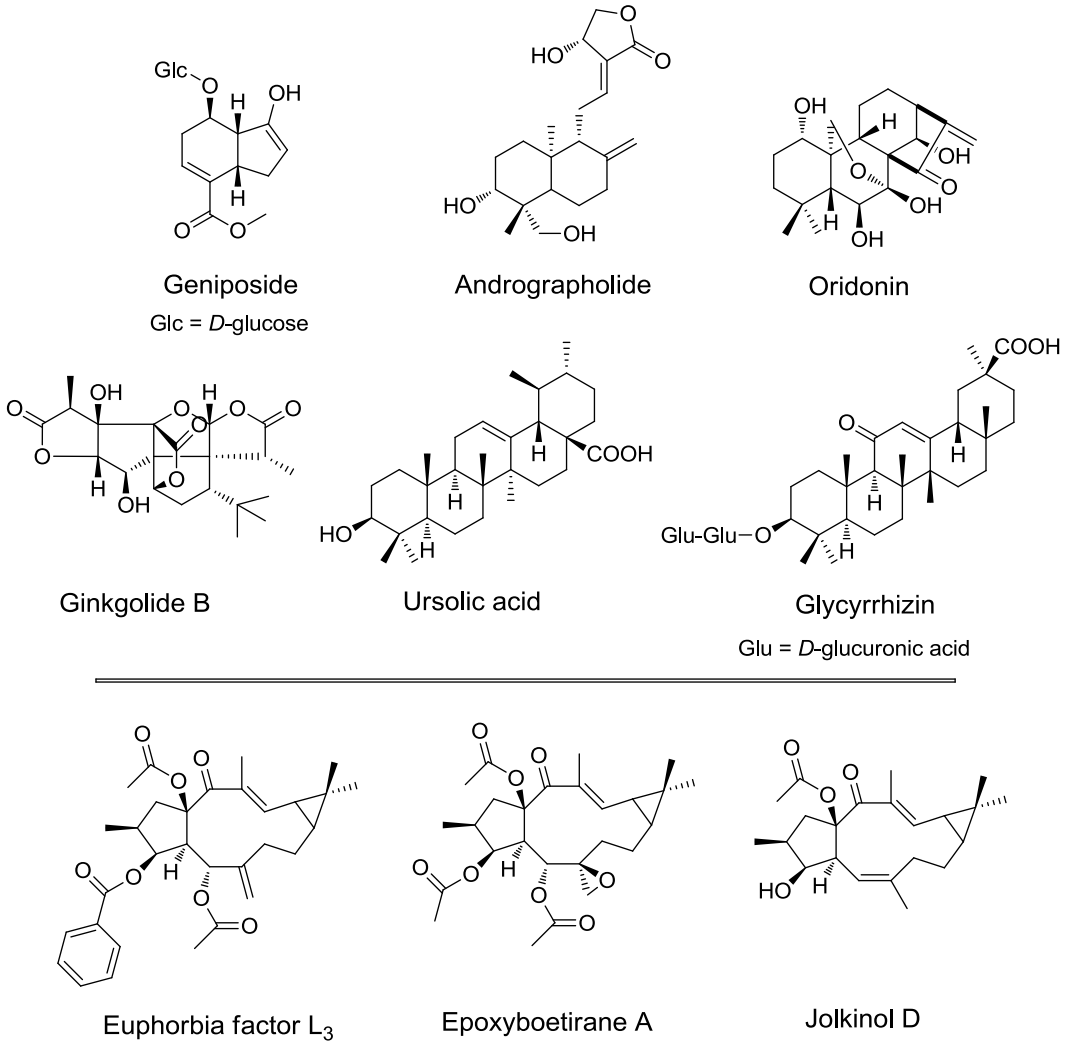


Fig.1. Structures of some terpenoids with good anti-inflammatory activity and some lathyrane diterpenoids.

The seeds of *Euphorbia lathyris* are utilized as traditional Chinese medicine for treating terminal schistosomiasis, ascities, hydropsy, and snakebites.[14-16] It is an abundant source of special diterpenoids with various macrocyclic and polycyclic skeletons including jatrophane, ningenane, daphnane, tigliane, and lathyrane.[17, 18] Some of these diterpenoids possess various biological activities, such as anticancer,[19, 20] multidrug resistance (MDR) reversal,[21, 22] antiviral,[23] and antifungal effects.[24] It's reported that the esterified and Michael addition derivatives of Euphorbia factor L_3,[25] Epoxyboetirane A,[26] and Jolkinol D exhibited more potent MDR-modulating

effects through inhibiting P-glycoprotein.[27, 28]

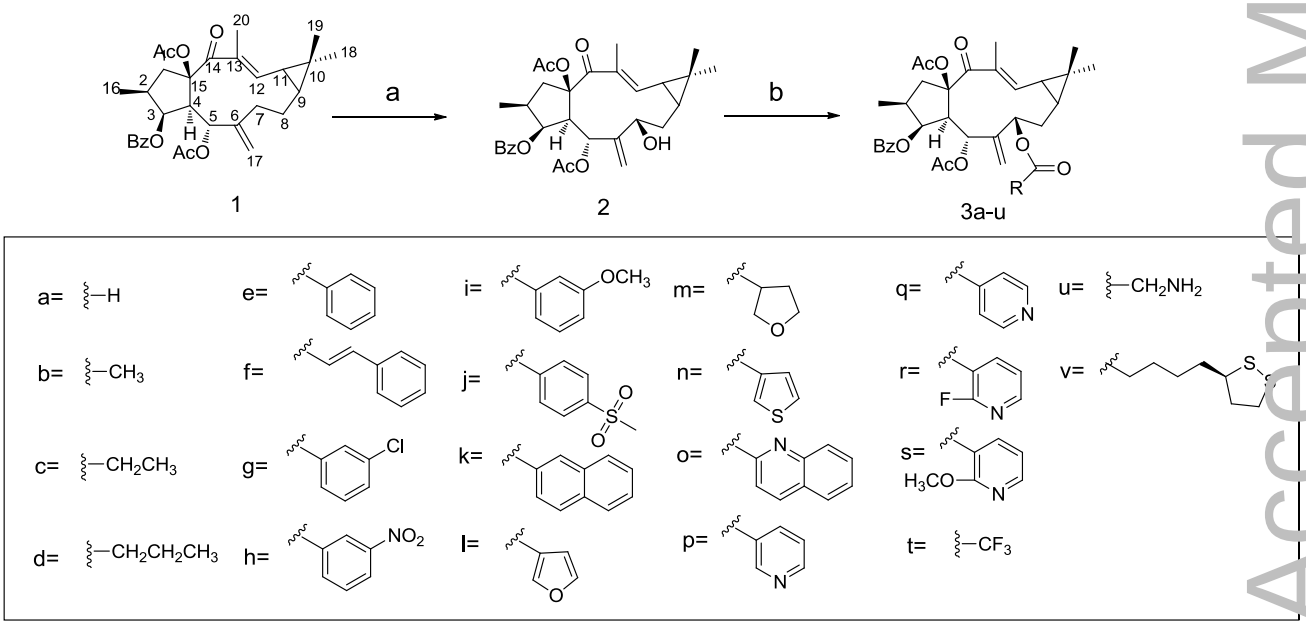
In our group's previous work,[29] Euphorbia factors L_1, L_3 and some new lathyrane diterpenoids were found to display good inhibition on NO production induced by LPS in RAW264.7 macrophage cells with very low cytotoxicity. The mechanism of these compounds was related to decreasing the production of inflammatory factors and also reducing the expression of iNOS and NF-κB and the phosphorylation of IκBα. It is the first time to report the anti-inflammatory activity and mechanism of lathyranes. In order to investigate the influence of the kind of acids on the anti-inflammatory efficacy of Euphorbia factor L_3 derivatives, and to find more potent anti-inflammatory drugs with low toxicity, herein, we design and synthesize two series of Euphorbia factor L_3 derivatives with fatty and aromatic acids. The inhibitory activity on LPS-induced NO production in RAW264.7 macrophage of these derivatives was investigated as well.

Results and discussion

Chemistry

In our previous work,[29] we have found that Euphorbia factor L_3 displayed good inhibition on NO production induced by LPS in RAW264.7 macrophage cells. When a hydroxyl was added to C-7 position (compound **2** in **Scheme 1**), the inhibition activity could be improved. Meanwhile, the inhibition activity of compounds could be changed when the hydroxyl group was esterified with different acids. To investigate the influence of the kind of acids on anti-inflammatory efficacy of compound **2**, we designed and synthesized series **1** (**Scheme 1**). Since there is difficult to get enough

compound **2** from plants, at first, Euphorbia factor L_3 was oxidized by Selenium dioxide (SeO_2) and tert-butyl hydroperoxide (TBHP) to yield compound **2**. As described in some previous work,[30-33] this reaction could achieve stereo-selectivity. In our study, this reaction is stereo-selectivity, but the yield is relatively low (15%), which might be related to steric hindrance of compound **1**. The stereostructure of C-7 hydroxyl was determined by NOESY experiment. Then we use fatty acids, substituted benzoic acids, cinnamic acid and heterocyclic acids for its esterification. Our previous study has shown that the inhibition activity of compound **2** could be improved significantly when the hydroxyl group was esterified with nicotinic acid,[29] so we also used isonicotinic acid and substituted nicotinic acid for esterification. In addition, α-ipoic acid and N-Boc-glycine are also used for esterification.



Scheme 1. Synthetic route of series **1**. Reagents and conditions: (a) SeO_2, TBHP, AcOH/DCM, 30 ℃, 48 h; (b) EDCI, DMAP, DIPEA, DCM, rt, 8-10 h.

To investigate the influence of the kind of acids on anti-inflammatory efficacy of Euphorbia factor L_3, we designed and synthesized series **2** (**Scheme 2**). It has been reported that C-5 hydroxyl can be esterified, but C-3 and C-15 hydroxyls can't under

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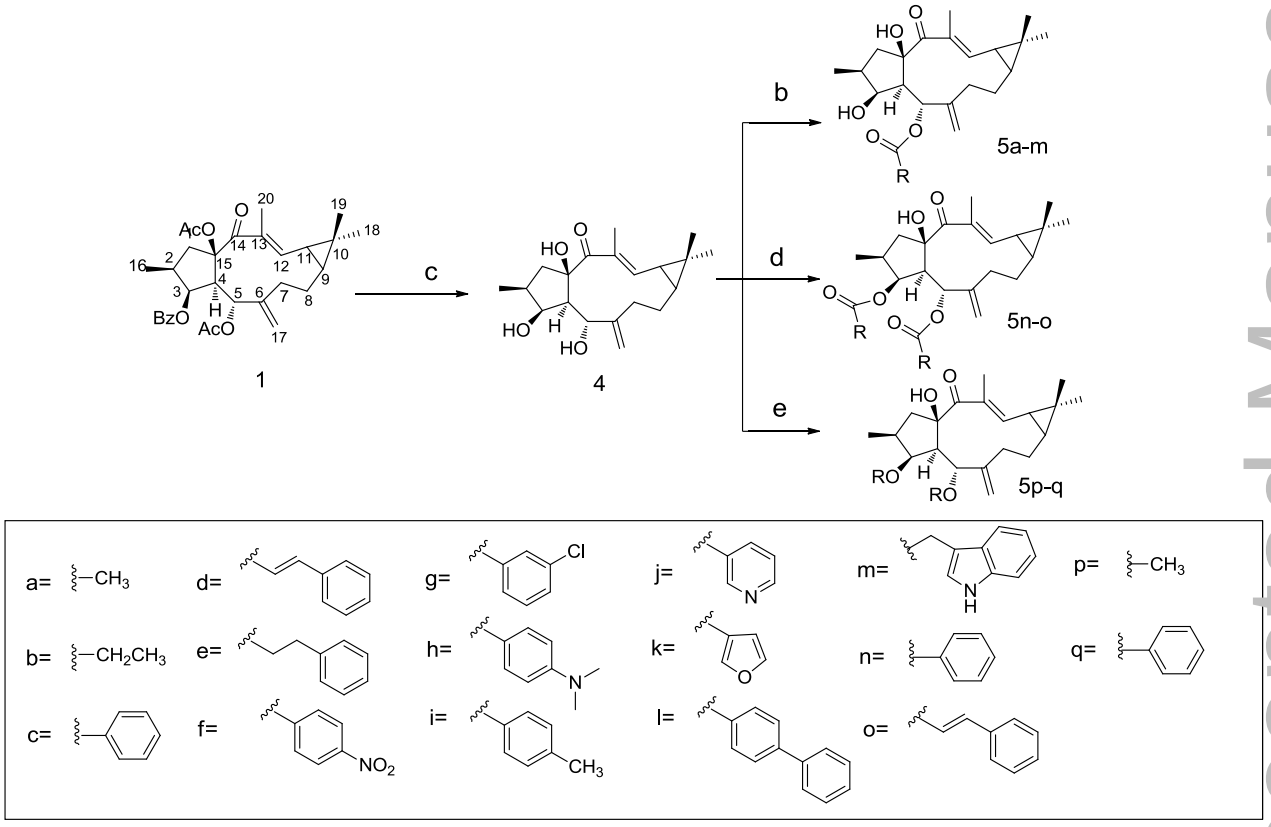
the condition of EDCI and DMAP. But C-3 hydroxyl could be acylated by corresponding acid chloride.[25] Alkaline hydrolysis of Euphorbia factor L_3 afforded lathyrol **4** with three free hydroxyl groups, followed by the esterification of C-5 hydroxyl with fatty acids, substituted benzoic acids, cinnamic acid and heterocyclic acids to yield the final products **5a-m**. We also synthesized compounds **5n-o** and **5p-q** to investigate the influence of C-3 hydroxyl and its etherification on anti-inflammatory efficacy.



Scheme 2. Synthetic route of series **2**. Reagents and conditions: (c) KOH, MeOH, rt, 6 h; (d) corresponding acid chloride, TEA, DCM, rt/40 ℃, 10 h; (e) $(CH_3)_2SO_4$/BnBr, NaH, DMF, 0 ℃, 0.5 h.

Inhibitory effect on LPS-induced nitric oxide (NO) production and SAR study

As an important inflammatory mediator, NO is over produced and secreted out of mouse macrophages in response to bacterial lipopolysaccharide (LPS).[34] The

anti-inflammatory activity of these synthesized compounds was evaluated by detecting the secretion level of NO in the medium of RAW264.7 cells after LPS stimulated, using dexamethasone as positive control. Among the tested compounds, series **2** displayed a better inhibitory activity than series **1**. Compounds **5n** and **5o** showed the most remarkable inhibition against NO production (IC_{50} values: 3.93 ± 1.04 and 3.70 ± 1.01 μM, respectively), being about twofold more potent than dexamethasone (**Table 1**).

In series **1**, when C-7-hydroxyl was esterified, many derivatives exhibited weaker NO production inhibitory activity than compound **2**. So the preliminary SARs showed that the C-7-hydroxyl is important for maintaining the anti-inflammatory activity of compound **2**. Curiously, when C-7-hydroxyl was esterified with nicotinic acid, isonicotinic acid and glycine, the derivatives exhibited comparable or even better inhibitory activity than compound **2** (*i.e.* compounds **3p, q, u**). But when C-7-hydroxyl was esterified with substituted nicotinic acid, the derivatives exhibited poor inhibitory activity (*i.e.* compounds **3r-s**). So the above results suggested that the anti-inflammatory activity of compound **2** couldn't be enhanced by esterification.

In series **2**, lathyrol **4** didn't exhibit NO production inhibitory activity. When the C-5-hydroxyl was esterified, many derivatives exhibited better inhibitory activity than **4**. The preliminary SARs showed that the esterification of C-5-hydroxyl is important for improving the anti-inflammatory activity of **4**. It could be concluded that the compounds with aromatic groups were more potent than compounds with aliphatic substituents. Meanwhile, the substituents of the benzene ring or changing benzene

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ring into heterocyclic ring could weaken the inhibitory activity of these compounds (*i.e.* compounds **5f-m**). And the electron donating group of the benzene ring could weaken the inhibitory activity more than the electron withdrawing group (*i.e.* compounds **5f-g** and **5h-i**). When C-3 and C-5 hydroxyl groups are simultaneously esterified, the activity of these compounds could be further enhanced (*i.e.* compounds **5n-o**), and the anti-inflammatory activity of etherification derivatives are weaker than esterification derivatives (*i.e.* compounds **5p-q**). It could be concluded that esterification is an effective way to improve the anti-inflammatory activity of **4**. Therefore, the above results would provide helpful information for the further structure modification of Euphorbia factor L_3.

Table 1. The IC_{50} values of the inhibition against NO production of all compounds.[a]

Compounds	NO IC_{50} (μM)	Compounds	NO IC_{50} (μM)
1 (Euphorbia factor L_3)	11.24±1.12	**3t**	>100
2	7.93±1.03	**3u**	7.05±1.04
3a	>50	**3v**	>100
3b	11.74±1.08	**4**	>100
3c	>50	**5a**	>50
3d	>50	**5b**	>50
3e	>50	**5c**	5.88±1.01
3f	>100	**5d**	6.57±1.07
3g	>100	**5e**	9.50±1.01
3h	21.37±2.42	**5f**	7.89±1.04
3i	23.69±2.37	**5g**	14.50±1.22
3j	>50	**5h**	21.55±1.72
3k	>100	**5i**	19.35±1.65
3l	>100	**5j**	8.86±1.22
3m	>100	**5k**	23.51±2.42
3n	>50	**5l**	30.53±2.65
3o	>100	**5m**	16.59±1.47
3p	5.72±1.04	**5n**	3.93±1.04
3q	5.03±1.13	**5o**	3.70±1.01
3r	>100	**5p**	42.26±3.52
3s	>100	**5q**	13.78±1.52

Dexamethasone[b]	8.36±1.32

[a] The results were showed as means ±SD of at least three independent experiments.

[b] Positive control.

Cell survival of RAW264.7

The cytotoxicity of compounds **5n** and **5o** were measured by MTT to eliminate its influence on the detection of NO production. The result showed that compound **5n** had no obvious effect on the cell survival at the concentration up to 100 μM (**Fig. 2**), revealing that the anti-inflammatory activity of compound **5n** is not produced by cytotoxicity. As the result, compound **5n** could be selected for further pharmacological study.

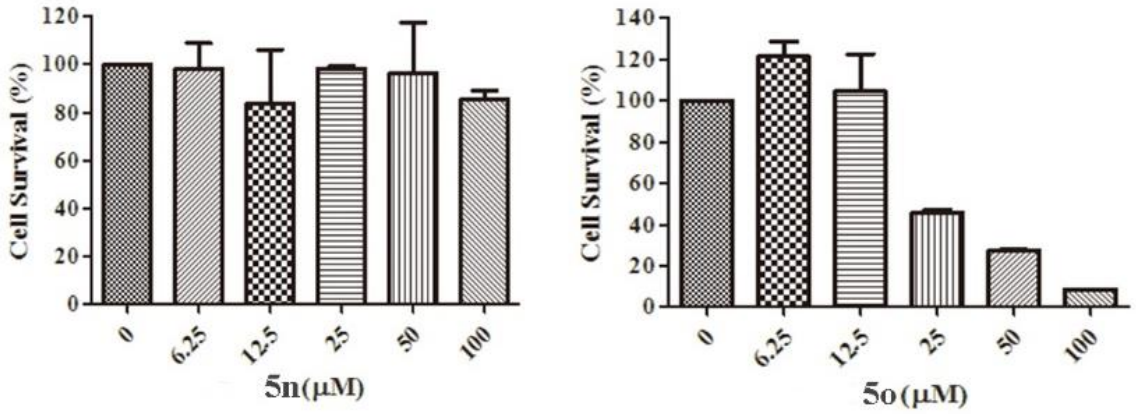

Fig. 2. The RAW264.7 cell viability after exposure to different concentrations of compounds **5n** and **5o** for 24 h.

Conclusion

Two series of Euphorbia factors L_3 derivatives with fatty and aromatic acids were designed and synthesized. About half of tested compounds showed obvious inhibitory effect on LPS-induced NO production in RAW264.7 macrophage. Among them, compound **5n** exhibited the most potent inhibition with IC_{50} of 3.38 ± 1.03 μM with no obvious cytotoxicity. The preliminary SARs showed that a hydroxyl at C-7 position is helpful to maintain the anti-inflammatory activity of Euphorbia factors L_3.

And the esterification of C-5-hydroxyl with aromatic acids is important for improving the anti-inflammatory activity of lathyrol scaffold. Therefore, these results are expected to be useful in the development of lathyrane diterpenoid derivatives or structurally related compounds as clinical trial candidates.

Experimental Section

Chemistry

All commercially available starting materials and solvents were reagent grade and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) on 0.25 mm silica gel plates (GF_{254}) and visualized under UV light or by heating after spraying with anisaldehyde-H_2SO_4 reagent. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV-400 spectrometer (Bruker Biospin, Fallanden, Switzerland). Chemical shifts are stated relative to TMS and expressed in δ values (ppm), with coupling constants reported in Hz. High resolution mass spectra (HRMS) of all derivatives were recorded on a Bruker micrOTOF focusII mass spectrometry by electrospray ionization (ESI). Optical rotations were recorded on a Autopol IV-T polarimeter (Rudolph Research Analytical). The physical characteristics, ^{1}H NMR, ^{13}C NMR, MS and specific optical rotation data for all intermediates and target compounds, were reported in Supporting Information.

Synthetic methods of all compounds

*Synthesis of compound **2***

Euphorbia factor L_3 (compound **1**, 1.0 g, 1.9 mmol) was dissolved in DCM (50 mL). Then acetic acid (5 mL), TBHP (733 mL, 5.7 mmol), and SeO_2 (107 mg, 0.96 mmol)

were added to the solution. The mixture was stirred for 48 h at 30 ℃. Upon completion, the organic layers was washed with brine, and saturated sodium bicarbonate solution, respectively, dried over anhydrous Na_2SO_4, and then filtered, evaporated, and purified by silica gel CC to obtain compound **2** as white solid (15% yield).

*Synthesis of compounds **3a-v***

EDCI (44 mg, 0.23 mmol) and acid molecules (0.23 mmol) were dissolved in DCM (3 mL). After 20 minutes, compound **2** (80 mg, 0.15 mmol), DMAP (catalytic amount) and DIPEA (49 μL, 0.3mmol) were added to the mixture. The mixture was stirred for about 8-10 h at room temprature. Upon completion, the organic layer was washed with 20% citric acid solution and brine, respectively, dried over anhydrous Na_2SO_4, filtered, evaporated, and purified by CC to afford compounds **3a-v**.

*Synthesis of compound **4***

Compound **1** (5 g, 9.6 mmol) in 5% KOH/MeOH (100 mL) was stirred at room temperature for 6 h. Upon completion, the solvent was removed under reduced pressure, and the residue was extracted with DCM, washed with brine, dried with anhydrous Na_2SO_4, filtered, evaporated and purified by CC to afford compound **4** as white solid (82% yield).

*Synthesis of compounds **5a-m***

EDCI (44 mg, 0.23 mmol) and acid molecules (0.23 mmol) were dissolved in DCM (3 mL). After 20 minutes, compound **4** (40 mg, 0.15 mmol), DMAP (catalytic amount) and DIPEA (49 μL, 0.3mmol) were added to the mixture. The mixture was stirred for

about 8-10 h at room temprature. Upon completion, the organic layer was washed with 20% citric acid solution and brine, respectively, dried over anhydrous Na_2SO_4, filtered, evaporated, and purified by CC to afford compounds **5a-m**.

*Synthesis of compounds **5n-o***

To a 0 ℃ solution of **4** (120 mg, 0.36 mmol) and corresponding acid chloride (1.44 mmol) in dry DCM (4 mL) was added dropwise TEA (181 µL, 1.47 mmol) under argon atmosphere. The resulting mixture was stirred for 10 h at 0 ℃ to room temperature. Upon completion, the organic layer was washed with $NaHCO_3$ solution and brine, respectively, dried over anhydrous Na_2SO_4, filtered, evaporated, and purified by CC to afford compounds **5n-o**.

*Synthesis of compounds **5p-q***

To a 0 ℃ solution of **4** (120 mg, 0.36 mmol) and NaH (1.44 mmol) in dry DMF (2.5 mL) was added dropwise $(CH_3)_2SO_4$ (or BnBr) (1.47 mmol) under argon atmosphere. The resulting mixture was stirred for 0.5 h at 0 ℃. Upon completion, the organic layer was washed with NH_4Cl solution and brine, respectively, dried over anhydrous Na_2SO_4, filtered, evaporated, and purified by CC to afford compounds **5p-q**.

Biology

Cell cultures

RAW264.7 mouse macrophage cells were obtained from the American Type Culture Collection (ATCC, USA). The cells were cultured in high Glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Cell cultures were incubated in a

humidified tissue incubator containing 5% CO_2 at 37 ℃ according to standard tissue procedures.

Detection of NO production

Measurement of NO production in RAW264.7 mouse macrophages for all compounds was refered to our recent article.[35] The RAW264.7 cells seeded in 96-well plates were incubated with synthesized compounds for 3 h and then 1 μg/mL LPS was added for 24 h. NO content was measured using Griess reagent at 540 nm and the IC_{50} values of compounds were determined based on the inhibition rate (%) compared to vehicle control and model control. DMSO plus LPS or not was set as a model control or vehicle control.

Cytotoxicity test

RAW264.7 cells were incubated in serum-free medium with compound **5n** and **5o**. After incubation for 24 h, MTT (20 μL, 5 mg/mL) was added to each well and incubated with cells for 4 h. Then the culture medium was carefully removed and 150 μL dimethyl sulfoxide (DMSO) was added to dissolve the crystals by shaking for 10 min. The absorbance was determined at 490 nm. DMSO was also treated as a vehicle control. All of the experiments were performed in triplicate.

Author Contributions Statement

Xingzhou Li, Hua Li, and Lixia Chen designed the experiments. Wang Wang contributed to the synthesis and characterization of all compounds. Yanli Wu, Chen Li and Yueying Yang conducted the biological assays. Wang Wang and Lixia Chen prepared the manuscript. All the authors revised the final manuscript.

Acknowledgements

This work was financially supported by National Natural Science Foundation of China (NSFC) [NOs. 81773594, U1703111, U1803122, and 81773637], Program for Liaoning Innovation Talents in University (NO. LR2016002), Liaoning Province Natural Science Foundation (NO. 2019-MS-299), Liaoning Revitalization Talents Program (NO. XLYC1807182), and Shenyang Planning Project of Science and Technology (NO. 18-013-0-46).

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10.1002/cbdv.201900531