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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Design, synthesis and biological evaluation of alantolactone derivatives as potential anti-inflammatory agents

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Abstract

While screening for potential anti-inflammatory natural product scaffolds, alantolactone (**1**) was found to attenuate LPS-induced pro-inflammatory mediators in RAW264.7 macrophage cells. In this regard, a series of 17 novel thiol derivatives of **1** has been synthesized and evaluated as inhibitors of pro-inflammatory mediators viz. NO (nitric oxide), IL-6 (interleukin-6), and TNF-α (tumor necrosis factor-α) in LPS-treated RAW264.7 macrophage cells (In vitro) and female Balb/C mice (in vivo). In vitro, the best inhibition potencies were obtained with compounds **3**, **4**, **6**, and **18**, which were selected for further in vivo testing. The results of in vivo studies revealed that compounds **3**, **6**, and **18** are comparable to that of the parent molecule **1** on the inhibition of TNF-α and IL-6 release, whereas compound **4** was identified as the most potent inhibitor of cytokines IL-6 (69.49%) and TNF-α (66.12%) as compared with **1** at dose 10 mg/kg. Taken together, our results suggest that thiol analogs of **1** have therapeutic potential and could be further explored for potential anti-inflammatory activity.

Keywords *Inula racemosa* · Alantolactone · Thiol derivatives · Anti-inflammatory activities

Introduction

Alantolactone, a sesquiterpene lactone, occurs as one of the major constituents of *Inula helenium* and *Inula racemosa* (Family: Asteraceae) (Gao et al. 2017; Seca et al. 2014). Pharmacological studies as reported in the literature revealed that alantolactone mainly exhibits cytotoxic and antitumor-promoting activities (Maryam et al. 2017; Wang et al. 2017). Other than its antitumor activity, alantolactone was found to have anti-bacterial (Cantrell et al. 1999), anti-fungal (Picman and Schneider 1993), hepatoprotective (Yuliya et al. 2009), antineoplastic (Zhao et al. 2015), antihypertensive, anthelmintic, and antioxidant activities (Seo et al. 2008). Furthermore, alantolactone and their analogs were also known to possess anti-inflammatory properties on various cell types by activating intracellular processes such as inhibiting chemokine production and STAT1 phosphorylation (Lim et al. 2015); suppressing inducible nitric oxide synthase and cyclooxygenase-2 expression (Chun et al. 2012); and inhibiting TNF-α-induced activation of NF-κB and MAPK pathways (Chun et al. 2012).

In our continuing efforts on searching for natural product scaffolds with potent anti-inflammatory activities, (Gupta et al. 2018; Nalli et al. 2016, 2017), alantolactone (**1**) was found to inhibit LPS-induced NO, IL-6, and TNF-α release in RAW264.7 macrophage cells with 76.0%, 81.5%, and 71.2% at 10 μM concentration, respectively. This biological significance has prompted us to generate semi-synthetic analogs of **1** as inhibitors of pro-inflammatory mediators, viz. NO (nitric oxide), IL-6 (interleukin-6), and TNF-α (tumor necrosis factor-α), both in vitro and in vivo using LPS-treated RAW264.7 macrophage cells and female

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Balb/C mice, respectively. As sulfur functionalities enhance the potencies of lead compounds, a series of 17 thiol derivatives of **1** was synthesized. To the best of our knowledge, anti-inflammatory properties of thiol derivatives of **1** against TNF-α, IL-6, and NO have not yet been reported. Herein, synthesis of thiol derivatives of alantolactone as NO, IL-6, and TNF-α inhibitors is described.

Results and discussion

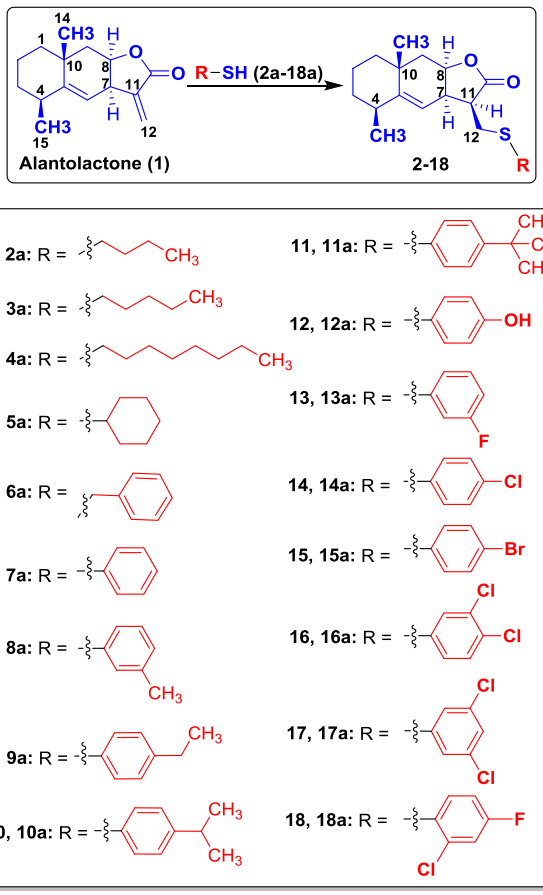
The hexane extract of whole plant *Inula racemosa* was chromatographed on silica gel column to afford a mixture of two isomeric sesquiterpene lactones, alantolactone, and isoalantolactone (Helenin). But, preparative-scale separation of alantolactone and isoalantolactone from a mixture is difficult and need repeated chromatography on silica gel coloumn. Therefore, we purified alantolactone by applying a method described by Ma et al. (Ma et al. 2013). As compound **1** showed 76.0%, 81.5%, and 71.2% inhibition of NO, IL-6, and TNF-α, respectively (Table 1), 17 thiol derivatives (**2**–**18**) were synthesized using Michael addition reaction. Briefly, alantolactone in methanol was treated with corresponding thiols (**2a**–**18a**) in the presences of base (triethylamine). The reaction was conducted at 0–5 °C for the first 15 min and then at room temperature for another 3 h (Scheme 1). The reactants used were included two aliphatic thiols with various chain lengths, 15 aromatic thiols having different substituents at ortho, meta, para positions, and one alicyclic thiol (cyclohexylthiol) (Fig. 1). All the analogs (**2**–**18**) were obtained as a single diastereomer with an overall yield of 90%. The structure including newly formed stereo center at C-11 of the analog **7** (R = –C_6H_5) was established by mass, 1D and 2D NMR data. The HRESIMS data ($[M + Na]^+$ *m/z* 365.1537) established the molecular formula as $C_{21}H_{26}O_2S$. The ^1H and ^{13}C NMR spectra of **7** showed the signals for phenyl group [δ_H 7.44–7.39 (m, 2H), 7.36–7.30 (m, 2H), 7.24 (ddd, $J = 7.3$, 3.9, 1.2 Hz, 1H); δ_C 134.9, 129.84 (×2), 129.24 (×2), 126.77]. Above data suggested that the presence of thiophenol group in the compound **7**. The position of the thiophenol group at at C-12 (δ 30.21) was ascertained by the HMBC correlations between H-12a (δ 3.52) and H-12b (δ 2.92–2.79) with C-1′ (δ 134.9), C-13 (δ 176.52), C-7 (δ 37.30), and C-11 (δ 45.27). The relative configuration of **7** was defined through NOE data (Fig. 1). The NOE correlations of H-8 (δ 4.81–4.59)/H-7 (δ 3.29–3.14)/H-11 (δ 2.98) suggested that H-8, H-7, and H-11 are co-facial and α-oriented, and thus β-orientation was assigned to the thiol group. As **2**–**6** and **8**–**18** were also showing similar optical rotation values as **7** ($[\alpha]_D^{25} + 126$ (c

Table 1 Percentage (%) inhibition of compounds **1**–**18** against NO, IL6, and TNF-α in LPS (1 μg/mL) administered RAW 246.7 cells

	% Inhibition of nitric oxide			% Inhibition of IL-6			% Inhibition of TNF-α		
C. code	10 μM	5 μM	1 μM	10 μM	5 μM	1 μM	10 μM	5 μM	1 μM
1	**76.09 ± 0.6**	52.65 ± 0.7	31.79 ± 0.9	**81.54 ± 0.1**	44.2 ± 0.5	15.94 ± 0.9	**71.23 ± 0.3**	46.59 ± 0.2	29.40 ± 0.5
2	57.98 ± 0.4	22.51 ± 0.9	1.39 ± 0.9	69.09 ± 0.5	40.51 ± 0.2	19.67 ± 1.1	62.47 ± 0.4	38.31 ± 0.5	16.35 ± 0.8
3	65.74 ± 04	28.75 ± 1.8	24.94 ± 1.1	**86.48 ± 0.1**	66.53 ± 0.1	19.52 ± 0.0	**77.61 ± 0.6**	51.25 ± 0.4	33.17 ± 0.5
4	**87.97 ± 0.5**	72.29 ± 0.9	34.08 ± 1.6	**84.75 ± 0.1**	48.32 ± 0.1	11.99 ± 0.1	65.76 ± 0.2	50.3 ± 0.4	36.53 ± 0.6
5	26.01 ± 1.6	19.16 ± 1.8	13.98 ± 1.0	54.63 ± 0.7	47.16 ± 0.7	22.47 ± 0.2	30.39 ± 0.5	27.57 ± 0.2	15.75 ± 0.3
6	**87.05 ± 0.5**	68.18 ± 1.3	33.77 ± 0.7	52.05 ± 0.2	41.76 ± 0.7	27.66 ± 0.5	69.51 ± 0.8	32.22 ± 0.3	12.23 ± 0.3
7	27.53 ± 1.6	22.96 ± 2.3	10.94 ± 0.9	28.15 ± 0.8	14.32 ± 1.6	4.64 ± 0.1	30.48 ± 0.7	27.58 ± 0.5	15.85 ± 0.7
8	15.35 ± 0.6	10.48 ± 0.7	5.30 ± 1.1	50.90 ± 0.6	38.07 ± 0.1	8.15 ± 0.4	38.05 ± 0.4	16.30 ± 0.3	11.19 ± 0.7
9	27.68 ± 0.9	22.29 ± 0.5	17.79 ± 0.4	57.13 ± 0.6	28.14 ± 0.9	23.06 ± 0.4	50.38 ± 0.6	35.93 ± 0.9	17.67 ± 0.7
10	37.27 ± 02	31.64 ± 1.3	21.18 ± 0.7	64.43 ± 0.2	34.81 ± 0.8	16.16 ± 0.8	33.40 ± 0.8	24.49 ± 0.5	17.82 ± 1.1
11	31.95 ± 1.2	28.90 ± 0.9	26.47 ± 0.4	58.35 ± 0.4	31.74 ± 0.9	12.75 ± 0.5	37.29 ± 0.7	30.05 ± 0.2	23.79 ± 0.2
12	69.24 ± 0.9	55.85 ± 1.3	17.64 ± 0.2	26.93 ± 0.7	12.72 ± 0.8	2.72 ± 0.4	18.67 ± 0.9	11.65 ± 0.5	4.86 ± 0.5
13	50.98 ± 2.0	29.81 ± 0.9	22.96 ± 1.6	56.04 ± 0.1	24.25 ± 0.2	9.36 ± 0.9	55.99 ± 1.0	45.38 ± 0.6	14.37 ± 0.9
14	27.09 ± 0.5	14.13 ± 1.6	12.76 ± 0.9	65.75 ± 0.4	45.9 ± 0.8	20.04 ± 0.6	28.74 ± 0.6	18.38 ± 1.0	4.19 ± 0.5
15	19.77 ± 0.6	15.66 ± 1.4	8.96 ± 0.9	34.64 ± 0.7	28.61 ± 0.5	21.02 ± 0.6	33.14 ± 0.4	24 ± 0.6	19.16 ± 0.2
16	49.45 ± 1.0	33.93 ± 0.9	24.94 ± 0.2	43.55 ± 0.8	37.41 ± 0.4	13.79 ± 0.4	66.19 ± 0.5	56.12 ± 0.4	26.17 ± 0.6
17	47.78 ± 0.7	24.18 ± 1.8	4.7 ± 1.1	34.44 ± 1.0	24.93 ± 0.5	13.38 ± 0.6	24.09 ± 0.6	17.62 ± 0.8	8.61 ± 1
18	22.81 ± 0.7	7.74 ± 1.6	1.96 ± 0.2	54.54 ± 0.7	28.66 ± 0.5	15.7 ± 1	**76.68 ± 0.3**	48.57 ± 0.4	20.67 ± 0.3
L-NIL 100 μM		77.16 ± 0.9		–			–		
Dexa (1 μM)		–		78.30 ± 0.7			64.81 ± 0.8		

L-name (nω-nitro-l-arginine methyl ester hydrochloride) is used as positive control for NO inhibition. Dexamethasone (1 μM) is used as positive control for TNF-α and IL6. All the results were represented as ± standard deviation ($n = 3$)

The table significance values are in bold



Scheme 1 Reagents and conditions: alantolactone (1.2 mmol), triethylamine (0.5 mL), corresponding thiol (**2a–18a**) (1.1 mmol), MeOH (10 mL), first at 0–5 °C for 15 min after at RT for 3 h

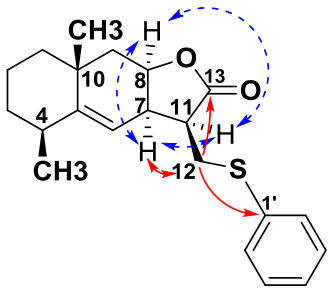


Fig. 1 Key HMBC (⟷) and NOESY (⟷) correlations of **7**

0.1, $CHCl_3$)), though not conclusive, the stereochemistry of thiol unit in **2–6** and **8–18** were also assigned to β-orientation.

Effect of new derivatives on production of NO, IL-6, and TNF-α

Above series of 17 new thiol derivatives of alantolactone were screened for their anti-inflammatory activity against NO, IL-6, and TNF-α in LPS-induced RAW 246.7 cell line.

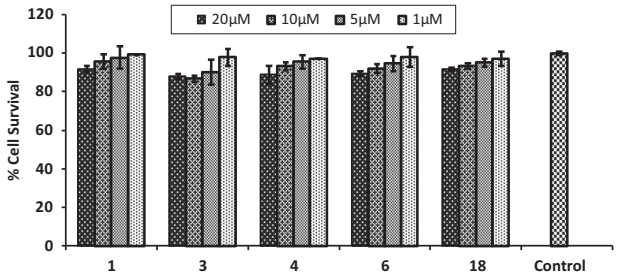


Fig. 2 Cytotoxicity of compounds **1, 3, 4, 6**, and **18**: RAW 264.7 cells were treated up to 20 μM concentrations with **1**, **3**, **4**, **6**, and **18** for 48 h, and percentage cell viability checked by MTT assay. The data represent mean ± SD ($n = 4$) of representative experiment

To evaluate inhibitory effects on the production of NO, IL-6, and TNF-α, Raw cells (1 lac/well) per well in 96-well plate were pretreated with various concentrations of derivatives for 1 h followed by stimulation with LPS (1 μg/mL) for 24 h. After 24 h cell culture supernatant was collected analyze for pro-inflammatory cytokines NO, IL-6, and TNF-α. The results are expressed in % inhibition with respect to control. The results are summarized in Table 1. Further, to assess the inhibition of cytokines and nitric oxide production exhibited was not due to toxicity to the cells, MTT [3-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide] toxicity assays were performed on RAW 264.7 cells. As shown in Fig. 2, None of these potent four molecules were seemed to alter the viability of RAW 264.7 cells up to 20 μM following 48 h incubation. All the analogs showed significant inhibition within non-cytotoxic concentration of 10 μM. Among 17 new compounds **4** (87.9%) and **6** (87.0%) showed stronger NO inhibition activities than the alantolactone (76.0%). Compounds **2**, **3**, **12**, **13**, **16**, and **17** showed moderate NO inhibition with percentage inhibition values of 57.9, 65.7, 69.2, 50.9, 49.4, and 47.7, respectively. However, compounds **5**, **7–11**, **14**, **15**, and **18** exhibited poor inhibitory activity. In the IL6 inhibitory assay, compounds **3** and **4** displayed inhibitory capacities with 86.4% and 84.7%, respectively, improving that of the alantolactone (81.5%). Compound **2**, **5**, **6**, **8–11**, **13**, and **14** possess moderate activity; whereas, compound **7**, **12**, and, **16–18** exhibited weak IL-6 inhibitory activity. In another experiment, compounds **3** and **18** showed the highest inhibitions against TNF-α of 77.6%, 76.6% which are more potent than alantolactone (71.2%). Others exhibited either moderate or weak activity than alantolactone (Table 1). On the basis of outcome from in vitro studies, we further explored the effect of active molecules in vivo to determine if the lead compounds identified from in vitro studies were able to replicate in animal models. The results of in vivo studies revealed that compounds **3**, **6**, and **18** are comparable to that of the parent molecule **1** on the inhibition of TNF-α and IL-6 release. Whereas the compound **4** was

Table 2 Percentage (%) inhibition of compounds 1, 3, 4, 6, and 18 against TNF-α and IL-6 in LPS (10 mg/kg) administered Balb/c mice

C. code	% Inhibition of TNF-α		% Inhibition of IL-6	
	Dose		Dose	
	5 mg/kg	10 mg/kg	5 mg/kg	10 mg/kg
1	40.41 ± 4.00	54.03 ± 3.07	42.98 ± 2.16	57.06 ± 1.83
3	31.72 ± 2.84	45.04 ± 3.08	37.43 ± 2.63	54.58 ± 2.19
4	49.09 ± 3.51	**66.12 ± 1.67**	51.19 ± 1.13	**69.49 ± .97**
6	23.62 ± 2.42	37.51 ± 1.34	31.59 ± 3.09	49.14 ± 2.21
18	14.50 ± 2.57	33.75 ± 1.08	16.02 ± 2.3	38.56 ± 1.56
Rolipram (2 mg/kg)	68.66 ± 2.01		73.86 ± 1.67	

Rolipram was used as positive control. The release of TNF-α and IL-6 in blood plasma level was estimated by ELISA and expressed as % inhibition calculated relative to LPS alone treated group. The results shown were mean ± SD ($n = 6$) of representative experiment

The table significance values are in bold

identified as the most potent inhibitor of cytokines IL-6 (69.49%) and TNF-α (66.12%) as compared with **1** at dose 10 mg/kg (Table 2). In Summary, herein we report a series of 17 new thiol derivatives of alantolactone and their anti-inflammatory activities against TNF-α, IL-6, and NO. From the results, we propose that the anti-inflammatory activities of the **1** are significantly influenced by the presence of the sulfur groups at the exocyclic double bond in the lactone ring.

Materials and methods

High resolution mass spectra were obtained on Agilent 6540 (Q-TOF) high resolution mass spectrometer, in the electrosprayionization (ESIMS) mode. ^{1}H NMR spectra were recorded (BruckerAvance) DPX FT-NMR were recorded at 400 and ^{13}C NMR at 100 MHz in $CDCl_3$ and MeOD, chemical shifts values were reported in δ (ppm) units and coupling constants values in hertz. Tetramethylsilane (TMS) was used as internal standard. Column chromatography was performed using silica gel (100−200 mesh; Merck). Lipopolysaccharide (LPS), *E. coli* serotype 0111:B4, (3-(4,5,dime-thylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Calbiochem. DMEM and fetal bovine serum was obtained from GIBCO Invitrogen Corporation. Uncoated TNF-α and IL-6 Kits were obtained from e-Bioscience Inc., San Diego, CA. Griess reagent kit from Thermo Fisher Scientific.

General procedure for preparation of Michael addition derivatives of alantolactone (2–8)

In a typical procedure, to a solution of compound **1** (1.2 mmol) in MeOH (10 mL) stirred over a period of 10 min, maintaining the temperature between 0–5 °C, was added triethylamine, followed by the methanolic solution of respective thiols (**2**–**18**) (1.1 mmol). The reaction mixture was stirred at the same temperature for 15 min followed by stirring at room temperature for 3 h. The solvent was evaporated in vacuo, and the crude residue was subjected to column chromatography on silica gel (60–120 mesh) to afford the pure product, characterization was performed using ^{1}H NMR, ^{13}C NMR, and HR-ESI-MS data analysis.

3-((butylthio)methyl)-5,8a-dimethyl-3a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan- 2(3H)-one (2)

^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.32 (d, $J = 3.1$ Hz, 1H), 4.78–4.73 (m, 1H), 3.19 (ddd, $J = 8.5$, 5.6, 3.1 Hz, 1H), 3.02 (dd, $J = 11.7$, 3.3 Hz, 1H), 3.00–2.94 (m, 1H), 2.62–2.55 (m, 3H), 2.55–2.50 (m, 1H), 2.11 (dd, $J = 14.8$, 3.3 Hz, 1H), 1.86–1.78 (m, 1H), 1.61 (s, 1H), 1.59 (d, $J = 1.5$ Hz, 1H), 1.58 (d, $J = 3.4$ Hz, 1H), 1.56–1.55 (m, 1H), 1.54–1.50 (m, 1H), 1.43 (td, $J = 7.1$, 4.0 Hz, 3H), 1.24 (d, $J = 8.5$ Hz, 4H), 1.12 (t, $J = 8.3$ Hz, 4H), 0.93 (t, $J = 7.3$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 176.7, 151.7, 114.5, 77.2, 45.9, 42.8, 42.3, 38.5, 37.4, 33.1, 32.9, 32.4, 31.6, 28.6, 28.4, 23.0, 21.9, 16.8, 13.6; (+) HRESIMS *m/z* 345.1860 $[M+Na]^+$ (calcd for $C_{19}H_{30}NaO_2S^+$, *m/z* 345.1859); HPLC purity 89.22%.

5,8a-dimethyl-3-((pentylthio)methyl)-3a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-2(3H)-one (3)

^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.30 (d, $J = 3.1$ Hz, 1H), 4.73 (dd, $J = 5.5$, 2.7 Hz, 1H), 3.17 (ddd, $J = 8.4$, 5.6, 3.1 Hz, 1H), 3.03–2.98 (m, 1H), 2.98–2.92 (m, 1H), 2.61–2.57 (m, 1H), 2.56 (s, 1H), 2.56–2.53 (m, 1H), 2.50 (dd, $J = 9.8$, 5.1 Hz, 1H), 2.09 (dd, $J = 14.8$, 3.2 Hz, 1H), 1.85–1.75 (m, 1H), 1.63 (t, $J = 5.8$ Hz, 1H), 1.61 (d, $J = 5.1$ Hz, 1H), 1.58 (d, $J = 9.2$ Hz, 1H), 1.56 (d, $J = 3.7$ Hz, 1H), 1.54 (s, 1H), 1.50 (q, $J = 5.3$ Hz, 1H), 1.45–1.40 (m, 1H), 1.38 (dd, $J = 4.8$, 3.3 Hz, 1H), 1.35 (d, $J = 4.4$ Hz, 1H), 1.34–1.31 (m, 1H), 1.31–1.23 (m, 1H), 1.22 (s, 3H), 1.15–1.08 (m, 4H), 0.89 (t, $J = 7.0$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 176.6, 151.7, 114.5, 77.2, 45.9, 42.8, 42.3, 38.5, 37.5, 33.1, 32.8, 30.9, 29.2, 28.5, 23.0, 22.2, 16.8, 13.9; (+) HRESIMS *m/z* 359.2014 $[M+Na]^+$ (calcd for $C_{20}H_{32}NaO_2S^+$, *m/z* 359.2021); HPLC purity 83.94%.

5,8a-dimethyl-3-((octylthio)methyl)-3a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-2(3H)-one (4)

^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.29 (t, $J = 7.9$ Hz, 1H), 4.79–4.70 (m, 1H), 3.20–3.13 (m, 1H), 3.03–2.98 (m, 1H), 2.98–2.92 (m, 1H), 2.60–2.56 (m, 1H), 2.55 (s, 1H), 2.55–2.52 (m, 1H), 2.52–2.46 (m, 1H), 2.09 (dd, $J = 14.8$, 3.3

Hz, 1H), 1.79 (ddd, $J = 13.7, 6.7, 3.9$ Hz, 1H), 1.61 (d, $J =$ 6.8 Hz, 1H), 1.57 (dd, $J = 9.1, 2.0$ Hz, 2H), 1.55–1.50 (m, 2H), 1.44–1.38 (m, 2H), 1.25 (dd, $J = 14.1, 4.6$ Hz, 10H), 1.21 (s, 3H), 1.11 (t, $J = 6.6$ Hz, 4H), 0.87 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 176.7, 151.6, 114.5, 77.2, 45.8, 42.8, 42.28, 38.5, 37.4, 33.1, 32.8, 31.8, 29.5, 29.2, 28.7, 28.4, 23.0, 22.65, 16.86, 14.12; (+) HRESIMS *m/z* 379.2660 $[M+H]^+$ (calcd for $C_{23}H_{39}O_2S^+$, *m/z* 379.2665; HPLC purity 88.44%.

3-((cyclohexylthio)methyl)-5,8a-dimethyl-3a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-2(3H)-one (5)

$[\alpha]_D^{25} + 65$ (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.32 (d, $J = 3.1$ Hz, 1H), 4.74 (dt, $J = 5.5, 2.6$ Hz, 1H), 3.18 (ddd, $J = 8.6, 5.6, 3.2$ Hz, 1H), 3.06 (dd, $J = 12.6, 3.8$ Hz, 1H), 2.95 (ddd, $J = 12.1, 8.3, 3.8$ Hz, 1H), 2.75–2.66 (m, 1H), 2.57 (t, $J = 12.3$ Hz, 1H), 2.51 (dd, $J = 7.5, 2.9$ Hz, 1H), 2.11 (dd, $J = 14.8, 3.3$ Hz, 1H), 2.06–1.92 (m, 2H), 1.80 (ddd, $J =$ 13.3, 5.4, 3.1 Hz, 2H), 1.63 (d, $J = 9.5$ Hz, 1H), 1.57 (d, $J =$ 7.2 Hz, 4H), 1.55 (d, $J = 2.5$ Hz, 1H), 1.54–1.48 (m, 1H), 1.46–1.41 (m, 1H), 1.39–1.36 (m, 1H), 1.34 (s, 1H), 1.30 (dd, $J = 5.9, 2.9$ Hz, 1H), 1.28–1.24 (m, 1H), 1.23 (s, 3H), 1.16–1.09 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 176.73, 151.67, 114.64, 77.28, 46.31, 44.35, 42.89, 42.34, 38.58, 37.50, 33.58 (d, $J = 7.6$ Hz), 33.18, 32.92, 28.68, 26.44, 26.24–25.69, 23.06, 16.89; (+) HRESIMS *m/z* 371.2015 $[M+H]^+$ (calcd for $C_{21}H_{32}NaO_2S^+$, *m/z* 371.2021); HPLC purity 95.97%.

3-((benzylthio)methyl)-5,8a-dimethyl-3a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-2(3H)-one (6)

$[\alpha]_D^{25} + 115$ (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.33–7.30 (m, 4H), 7.24 (ddd, $J = 10.0, 5.0, 2.5$ Hz, 1H), 5.06 (d, $J = 3.1$ Hz, 1H), 4.66 (dt, $J = 5.4, 2.6$ Hz, 1H), 3.76 (d, J $= 4.3$ Hz, 2H), 3.09–3.05 (m, 1H), 2.98 (dd, $J = 12.5, 3.8$ Hz, 1H), 2.84 (td, $J = 8.0, 3.9$ Hz, 1H), 2.42 (t, $J = 12.2$ Hz, 1H), 2.33 (dd, $J = 9.8, 4.8$ Hz, 1H), 2.08–2.03 (m, 1H), 1.77 (ddd, $J = 10.3, 8.3, 5.3$ Hz, 1H), 1.55 (s, 1H), 1.52 (d, $J = 4.3$ Hz, 1H), 1.49 (d, $J = 2.4$ Hz, 1H), 1.44–1.44 (m, 2H), 1.43–1.38 (m, 1H), 1.26 (s, 1H), 1.17 (s, 3H), 1.07 (dd, $J = 12.6, 2.8$ Hz, 3H), 1.00 (d, $J = 7.6$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 176.4, 151.6, 138.0, 128.8, 128.6, 127.2, 114.3, 77.1, 45.4, 42.8, 42.3, 38.3, 37.4, 37.1, 33.1, 32.9, 28.6, 27.8, 23.0, 16.8; (+) HRESIMS *m/z* 357.17 $[M+H]^+$ (calcd for $C_{22}H_{29}O_2S^+$, *m/z* 357.1883; HPLC purity 92.04%.

5,8a-dimethyl-3-((phenylthio)methyl)-3a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-2(3H)-one (7)

$[\alpha]_D^{25} + 126$ (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.44–7.39 (m, 2H), 7.36–7.30 (m, 2H), 7.24 (ddd, $J = 7.3, 3.9, 1.2$ Hz, 1H), 5.32 (d, $J = 3.1$ Hz, 1H), 4.74–4.70 (m, 1H), 3.53 (dd, $J = 13.1, 3.3$ Hz, 1H), 3.22 (ddd, $J = 8.5, 5.6, 3.2$ Hz, 1H), 2.99 (ddd, $J = 11.6, 8.1, 3.3$ Hz, 1H), 2.91–2.84 (m, 1H), 2.51 (dd, $J = 9.8, 5.0$ Hz, 1H), 2.12 (dd, $J = 14.8, 3.3$ Hz, 1H), 1.88–1.78 (m, 1H), 1.61 (d, $J = 12.3$ Hz, 1H), 1.58 (d, $J = 3.9$ Hz, 1H), 1.55 (s, 1H), 1.55–1.50 (m, 1H), 1.47–1.41 (m, 1H), 1.24 (s, 3H), 1.17–1.10 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 176.67, 152.12, 129.84, 129.24, 126.77, 114.25, 45.17, 42.79, 42.31, 38.57, 37.24, 33.19, 32.89, 30.14, 28.71, 23.12, 16.86; (+) HRESIMS *m/z* 365.1537 $[M+Na]^+$ (calcd for $C_{21}H_{26}NaO_2S^+$, *m/z* 365.1546); HPLC purity 100%.

5,8a-dimethyl-3-((m-tolylthio)methyl)-3a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-2(3H)-one (8)

$[\alpha]_D^{25} + 106$ (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.20 (dd, $J = 4.9, 4.3$ Hz, 3H), 7.04 (dd, $J = 5.4, 2.5$ Hz, 1H), 5.32 (d, $J = 3.1$ Hz, 1H), 4.71 (dd, $J = 5.5, 2.7$ Hz, 1H), 3.51 (dd, $J = 13.1, 3.4$ Hz, 1H), 3.20 (ddd, $J =$ 8.5, 5.6, 3.2 Hz, 1H), 2.97 (td, $J = 8.3, 4.1$ Hz, 1H), 2.89–2.82 (m, 1H), 2.54–2.48 (m, 1H), 2.34 (s, 3H), 2.10 (dd, $J = 14.8, 3.3$ Hz, 1H), 1.86–1.78 (m, 1H), 1.61 (d, $J =$ 12.1 Hz, 1H), 1.58 (d, $J = 3.9$ Hz, 1H), 1.56–1.54 (m, 1H), 1.54–1.49 (m, 1H), 1.46–1.41 (m, 1H), 1.24 (s, 3H), 1.12 (t, $J = 8.0$ Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 176.5, 152.0, 139.0, 134.5, 130.4, 129.0, 127.6, 126.8, 114.3, 77.26, 45.2, 42.8, 42.3, 38.5, 37.3, 33.2, 32.9, 30.2, 28.6, 23.0, 21.3, 16.8; (+) HRESIMS *m/z* 379.1695 $[M+Na]^+$ (calcd for $C_{22}H_{28}NaO_2S^+$, *m/z* 379.5132; HPLC purity 95.23%.

3-(((4-ethylphenyl)thio)methyl)-5,8a-dimethyl-3a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-2(3H)-one (9)

$[\alpha]_D^{25} + 90$ (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.34 (t, $J = 8.5$ Hz, 2H), 7.14 (d, $J = 8.1$ Hz, 2H), 5.30 (d, J $= 3.0$ Hz, 1H), 4.69 (dd, $J = 5.4, 2.6$ Hz, 1H), 3.46 (dd, $J =$ 13.1, 3.3 Hz, 1H), 3.20 (ddd, $J = 8.5, 5.6, 3.2$ Hz, 1H), 2.94 (ddd, $J = 11.7, 8.2, 3.4$ Hz, 1H), 2.86–2.78 (m, 1H), 2.62 (q, $J = 7.6$ Hz, 2H), 2.52–2.45 (m, 1H), 2.09 (dd, $J = 14.8$, 3.3 Hz, 1H), 1.86–1.77 (m, 1H), 1.59 (d, $J = 10.5$ Hz, 1H), 1.56 (d, $J = 3.8$ Hz, 1H), 1.56–1.54 (m, 1H), 1.53–1.49 (m, 1H), 1.45–1.39 (m, 1H), 1.22 (t, $J = 3.8$ Hz, 5H), 1.20 (s, 1H), 1.15–1.08 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 176.2, 152.3, 132.5, 117.7, 117.5, 115.0, 114.8, 114.10, 77.3, 45.2, 42.7, 42.2, 38.5, 37.3, 33.1, 32.8, 30.6, 30.5, 30.0, 28.6, 24.0, 22.8, 16.84; (+) HRESIMS *m/z* 371.2039 $[M+H]^+$ (calcd for $C_{23}H_{31}O_2S^+$, *m/z* 371.2045; HPLC purity 95.71%.

3-(((4-isopropylphenyl)thio)methyl)-5,8a-dimethyl-3a,5,6,7,8,8a,9,9a-octahydronaphtho [2,3-b]furan-2(3H)-one (10)

$[\alpha]_D^{25}$ + 75 (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.34 (d, J = 8.2 Hz, 2H), 7.18 (d, J = 8.3 Hz, 2H), 5.30 (d, J = 3.1 Hz, 1H), 4.71 (dt, J = 5.5, 2.6 Hz, 1H), 3.48 (dd, J = 13.2, 3.4 Hz, 1H), 3.22 (ddd, J = 8.5, 5.6, 3.2 Hz, 1H), 2.95 (ddd, J = 11.4, 7.5, 4.0 Hz, 1H), 2.86 (dt, J = 25.4, 9.5 Hz, 2H), 2.53–2.47 (m, 1H), 2.13–2.07 (m, 1H), 1.86–1.77 (m, 1H), 1.60 (d, J = 12.7 Hz, 1H), 1.57 (s, 1H), 1.54–1.49 (m, 1H), 1.46–1.40 (m, 1H), 1.23 (t, J = 6.7 Hz, 10H), 1.11 (t, J = 8.4 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 176.7, 151.9, 148.0, 131.7. 130.5, 127.4, 114.3, 77.2, 45.2, 42.8, 42.3, 38.56, 37.2, 33.7, 33.1, 32.9, 30.7, 28.7, 23.9, 23.1, 16.8; (+) HRESIMS *m/z* 385.2187$[M+H]^+$ (calcd for $C_{24}H_{33}O_2S^+$, *m/z* 385.2196; HPLC purity 95.86%.

3-(((4-(tert-butyl)phenyl)thio)methyl)-5,8a-dimethyl-3a,5,6,7,8,8a,9,9a-octahydronaphtho [2,3-b]furan-2(3H)-one (11)

$[\alpha]_D^{25}$ + 40 (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.34 (s, 4H), 5.30 (d, J = 3.1 Hz, 1H), 4.71 (dd, J = 5.5, 2.7 Hz, 1H), 3.48 (dd, J = 13.2, 3.4 Hz, 1H), 3.22 (ddd, J = 8.5, 5.6, 3.2 Hz, 1H), 2.96 (ddd, J = 11.7, 7.8, 4.2 Hz, 1H), 2.90–2.81 (m, 1H), 2.53–2.48 (m, 1H), 2.13–2.08 (m, 1H), 1.82 (ddd, J = 13.3, 8.3, 3.0 Hz, 1H), 1.58 (d, J = 3.7 Hz, 1H), 1.55 (d, J = 3.0 Hz, 1H), 1.46–1.41 (m, 1H), 1.31 (s, 9H), 1.22 (d, J = 11.9 Hz, 3H), 1.12 (d, J = 7.5 Hz, 3H), 1.08 (d, J = 7.6 Hz, 1H); ^{13}C NMR (100 MHz, CDC_{l3}) δ: 176.6, 151.9, 150.2, 130.9, 130.2, 126.2, 114.4, 77.2, 45.3, 42.8, 42.3, 38.5, 37.2, 33.1, 32.9, 31.2, 30.6, 28.6, 23.0, 16.8; (+) HRESIMS *m/z* 399.2329 $[M+H]^+$ (calcd for $C_{24}H_{34}O_2S^+$, *m/z* 399.2352; HPLC purity 94.64%.

3-(((4-hydroxyphenyl)thio)methyl)-5,8a-dimethyl-3a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-2(3H)-one (12)

$[\alpha]_D^{25}$ + 105 (c 0.1, $CHCl_3$);^{1}H NMR (400 MHz, CDCl3) δ: 7.37–7.33 (m, 2H), 6.83–6.78 (m, 2H), 5.40 (s, 1H), 5.32 (d, J = 3.1 Hz, 1H), 4.72 (dt, J = 5.5, 2.6 Hz, 1H), 3.37 (dd, J = 13.2, 3.3 Hz, 1H), 3.24–3.19 (m, 1H), 2.91 (ddd, J = 11.6, 8.1, 3.4 Hz, 1H), 2.83–2.76 (m, 1H), 2.57–2.50 (m, 1H), 2.11 (dd, J = 14.8, 3.2 Hz, 1H), 1.87–1.78 (m, 1H), 1.59 (d, J = 3.8 Hz, 1H), 1.57–1.55 (m, 1H), 1.52 (d, J = 2.4 Hz, 1H), 1.47–1.41 (m, 1H), 1.31–1.24 (m, 1H), 1.23 (s, 3H), 1.12 (t, J = 7.0 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 177.0, 155.7, 151.9, 134.0, 116.3, 114.3, 77.3, 45.3, 42.8, 42.3, 38.5, 37.1, 33.1, 32.9, 32.1, 28.6, 23.0, 16.8; (+) HRESIMS *m/z* 359.1679 $[M+H]^+$ (calcd for $C_{21}H_{27}O_2S^+$, 359.1675; HPLC purity 98.91%.

3-(((3-fluorophenyl)thio)methyl)-5,8a-dimethyl-3a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-2(3H)-one (13)

$[\alpha]_D^{25}$ + 95 (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.35–7.29 (m, 1H), 7.20–7.16 (m, 1H), 7.14–7.09 (m, 1H), 6.95 (td, J = 8.4, 2.4 Hz, 1H), 5.32 (d, J = 3.1 Hz, 1H), 4.77 (dt, J = 5.5, 2.6 Hz, 1H), 3.55 (dd, J = 13.0, 3.4 Hz, 1H), 3.24 (ddd, J = 8.5, 5.6, 3.2 Hz, 1H), 3.04 (ddd, J = 11.6, 8.1, 3.4 Hz, 1H), 2.92 (dd, J = 15.2, 9.7 Hz, 1H), 2.57–2.51 (m, 1H), 2.15 (dd, J = 14.8, 3.2 Hz, 1H), 1.84 (ddd, J = 10.0, 8.1, 5.2 Hz, 1H), 1.66–1.63 (m, 1H), 1.60 (d, J = 4.7 Hz, 1H), 1.58–1.54 (m, 1H), 1.49–1.45 (m, 1H), 1.27 (s, 3H), 1.22 (dd, J = 15.5, 3.7 Hz, 1H), 1.16 (d, J = 7.6 Hz, 3H), 1.15–1.10 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 176.3, 152.4, 130.5, 124.6, 116.1, 115.9, 114.0, 113.6, 113.4, 77.3, 45.1, 42.7, 42.3, 38.6, 37.3, 33.2, 32.9, 29.8, 28.6, 23.1, 16.8; (+) HRESIMS *m/z* 383.1452 $[M+Na]^+$ (calcd for $C_{21}H_{25}FNaO_2S^+$, *m/z* 383.1452; HPLC purity 91.91%.

3-(((4-chlorophenyl)thio)methyl)-5,8a-dimethyl-3a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-2(3H)-one (14)

$[\alpha]_D^{25}$ + 105 (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.35–7.31 (m, 2H), 7.31–7.26 (m, 2H), 5.28 (d, J = 3.1 Hz, 1H), 4.74–4.70 (m, 1H), 3.48 (dd, J = 12.8, 3.2 Hz, 1H), 3.22–3.17 (m, 1H), 2.99–2.92 (m, 1H), 2.90–2.83 (m, 1H), 2.54–2.47 (m, 1H), 2.11 (dd, J = 14.8, 3.2 Hz, 1H), 1.86–1.77 (m, 1H), 1.61 (d, J = 12.8 Hz, 1H), 1.54–1.51 (m, 1H), 1.46–1.41 (m, 1H), 1.34–1.25 (m, 2H), 1.24 (s, 3H), 1.16–1.10 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 176.4, 152.2, 133.1, 132.7, 131.0, 129.3, 114.0, 77.0, 45.1, 42.7, 42.2, 38.6, 37.2, 33.2, 32.8, 30.4, 28.6, 23.1, 16.8; (+) HRESIMS *m/z* 377.1337 $[M+H]^+$ (calcd for $C_{21}H_{26}ClO_2S^+$ *m/z* 377.1342; HPLC purity 96.85%.

3-(((4-bromophenyl)thio)methyl)-5,8a-dimethyl-3a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-2(3H)-one (15)

$[\alpha]_D^{25}$ + 94 (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.47–7.39 (m, 2H), 7.26–7.22 (m, 2H), 5.27 (d, J = 3.0 Hz, 1H), 4.74–4.68 (m, 1H), 3.47 (dd, J = 12.9, 3.2 Hz, 1H), 3.22–3.15 (m, 1H), 2.95 (ddd, J = 11.4, 8.1, 3.3 Hz, 1H), 2.89–2.82 (m, 1H), 2.49 (dd, J = 9.8, 4.9 Hz, 1H), 2.10 (dd, J = 14.8, 3.2 Hz, 1H), 1.80 (ddd, J = 10.4, 8.3, 5.3 Hz, 1H), 1.58 (d, J = 3.0 Hz, 1H), 1.56 (s, 1H), 1.54 (s, 1H), 1.54–1.50 (m, 1H), 1.46–1.40 (m, 1H), 1.22 (s, 3H), 1.15–1.08 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 176.2, 152.3, 134.2, 132.2, 131.2, 120.6, 114.0, 77.2, 45.1, 42.8, 42.3, 38.6, 37.2, 33.2, 32.9, 30.3, 28.6, 23.0, 16.8; (+) HRESIMS

m/z 423.0806 [M+H$^+$ (calcd for $C_{21}H_{26}BrO_2S^+$, *m/z* 423.0816; HPLC purity 98.45%.

3-(((3,4-dichlorophenyl)thio)methyl)-5,8a-dimethyl-3a,5,6,7,8,8a,9,9a-octahydronaphtho [2,3-b]furan-2(3H)-one (16)

$[\alpha]_D^{25}$ + 95 (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 7.45 (d, *J* = 2.1 Hz, 1H), 7.38 (d, *J* = 8.4 Hz, 1H), 7.20 (dd, *J* = 8.4, 2.2 Hz, 1H), 5.26 (d, *J* = 3.0 Hz, 1H), 4.74 (dd, *J* = 5.4, 2.7 Hz, 1H), 3.48 (dd, *J* = 12.7, 3.2 Hz, 1H), 3.21–3.16 (m, 1H), 2.98 (ddd, *J* = 11.4, 8.0, 3.3 Hz, 1H), 2.92–2.85 (m, 1H), 2.50 (dd, *J* = 9.9, 4.7 Hz, 1H), 2.12 (dd, *J* = 14.8, 3.3 Hz, 1H), 1.81 (ddd, *J* = 10.3, 8.3, 2.5 Hz, 1H), 1.61 (d, *J* = 13.1 Hz, 1H), 1.58 (d, *J* = 3.6 Hz, 1H), 1.56 (s, 1H), 1.55–1.51 (m, 1H), 1.46–1.41 (m, 1H), 1.24 (s, 3H), 1.12 (t, *J* = 7.6 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 176.2, 152.6, 135.3, 133.2, 130.9, 130.5, 128.4, 113.8, 77.3, 45.07, 42.7, 42.2, 38.6, 37.2, 33.2, 32.8, 30.2, 28.6, 23.1, 16.8; (+) HRESIMS *m/z* 411.0971 [M+H]$^+$ (calcd for $C_{21}H_{25}Cl_2O_2S^+$, *m/z* 411.0947; HPLC purity 95.94%.

3-(((3,5-dichlorophenyl)thio)methyl)-5,8a-dimethyl-3a,5,6,7,8,8a,9,9a-octahydronaphtho [2,3-b]furan-2(3H)-one (17)

$[\alpha]_D^{25}$ + 70 (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 7.24–7.16 (m, 3H), 5.24 (d, *J* = 2.9 Hz, 1H), 4.77–4.71 (m, 1H), 3.49 (dd, *J* = 12.6, 3.4 Hz, 1H), 3.20–3.14 (m, 1H), 2.99 (ddd, *J* = 11.5, 8.1, 3.4 Hz, 1H), 2.94–2.86 (m, 1H), 2.53–2.46 (m, 1H), 2.12 (dd, *J* = 14.8, 3.2 Hz, 1H), 1.87–1.77 (m, 1H), 1.60 (d, *J* = 13.2 Hz, 1H), 1.56 (d, *J* = 5.3 Hz, 2H), 1.53–1.49 (m, 1H), 1.46–1.40 (m, 1H), 1.23 (s, 3H), 1.11 (t, *J* = 9.1 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 176.0, 152.7, 138.9, 135.5, 126.5, 113.7, 77.3, 45.0, 42.7, 42.3, 38.6, 37.3, 33.2, 32.8, 29.7, 28.6, 23.1, 16.8; (+) HRESIMS *m/z*411.0947 [M+H]$^+$ (calcd for $C_{21}H_{25}Cl_2O_2S^+$, *m/z* 411.0952; HPLC purity 82.24%.

3-(((2-chloro-4-fluorophenyl)thio)methyl)-5,8a-dimethyl-3a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-2(3H)-one (18)

$[\alpha]_D^{25}$ + 90 (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 7.44 (dd, *J* = 8.7, 5.8 Hz, 1H), 7.21–7.18 (m, 1H), 7.00 (td, *J* = 8.3, 2.7 Hz, 1H), 5.33 (d, *J* = 3.0 Hz, 1H), 4.81–4.72 (m, 1H), 3.44 (dd, *J* = 12.3, 2.8 Hz, 1H), 3.28–3.20 (m, 1H), 3.00–2.94 (m, 1H), 2.89 (t, *J* = 12.2 Hz, 1H), 2.55–2.48 (m, 1H), 2.12 (dd, *J* = 14.8, 3.3 Hz, 1H), 1.86–1.77 (m, 1H), 1.61 (d, *J* = 13.0 Hz, 1H), 1.58 (d, *J* = 3.9 Hz, 1H), 1.56 (d, *J* = 2.3 Hz, 1H), 1.55–1.51 (m, 1H), 1.46–1.41 (m, 1H), 1.24 (s, 3H), 1.16–1.10 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ:* 176.1, 162.5 (d), 152.3, 136.5, 132.5, 129.4, 117.7 (d), 115.0 (d), 114.1, 77.3, 45.3, 42.7, 42.3, 38.5, 37.39, 33.1, 32.8, 30.4, 28.6, 23.0, 16.8; (+) HRESIMS *m/z* 395.1242 [M+H]$^+$ (calcd for $C_{21}H_{25}ClFO_2S^+$, *m/z* 395.1242); HPLC purity 91.53%.

Cell culture

RAW 264.7 macrophage cells (ATCC, Rockwille, MD, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 ng/mL streptomycin and incubated at 37 °C under 5% CO_2.

Effect of compounds on nitric oxide inhibition in LPS-induced RAW 246.7 cell line

The level of nitrite accumulation in cell culture supernatant was analyzed using Griess Reagent Kit (Thermo Fisher Scientific). Briefly, 150 µL of cell culture supernatant was mixed with 20 µL of Griess reagent and 130 µL of distilled water and incubated for 30 min at room temperature. The absorbance was measured at 548 nm. Nitrite concentration was calculated with reference to a standard curve obtained using $NaNO_2$.

The measurement of TNF-α and IL-6 production

The amount of TNF-α and IL6 in cell culture supernatant was measured using commercially available uncoated TNF-α and IL-6 Elisa kits (Bioscience Inc., San Diego, CA) according to manufacturer's instructions.

Cell viability by MTT assay

RAW 264.7 macrophage cells seeded into 96-well plates (20,000 cells/well) were treated with test compounds or vehicle control (0.1% DMSO) for 48 h before being added with 20 µL of MTT solution (final concentration of 0.25 mg/ml). After 4 h incubation the supernatant was removed, the formazan crystals were dissolved in 100 µL of DMSO and absorbance at 570 nm was determined by Synergy Mx microplate reader. Cell viability was determined using an equation shown below. The data were expressed as mean of quadruplicate determinations ± SEM.

$$\%\ \text{cell viability} = (\text{absorbance of treated cells}/\text{absorbance of untreated cells}) * 100$$

In vivo study

Female Balb/C mice, 10–12 weeks old and weight is 20–25 g, were housed under standard laboratory conditions, 23 ± 1 °C, 55 ± 10% relative humidity, 12/12 h light/dark cycles and fed with pellet diet (Lipton India Ltd) and water

ad libitum. Experiments were designed in such a way so as to use minimum number of animals. The experimental protocols were approved by Institutional Animal Ethic Committee (IAEC reg. no. 74/152/2/19). Female Balb/C mice were randomly grouped with six mice containing each group. Mice were fasted overnight and drugged with different doses of **1**, **3**, **4**, **6**, and **18** (5 and 10 mg/kg) and normal saline as vehicle control. After 1 h, LPS (10 mg/kg) was administered by intraperitoneal (i.p.) injection to induce experimental sepsis. Blood for serum samples were collected after 3 h from retro-orbital plexus. The serum was used to study inhibition of TNF-α and IL-6 cytokines using invitrogen (Mouse Kits).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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