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Cynaropicrin and inhibition of NF-κB activation: A structure activity relationship study

Jing Li [a], Yusuke Yoshida [b], Manami Kurita [a], Toyonobu Usuki [a,*]

In memory of Professor Koji Nakanishi

[a] *Department of Materials and Life Sciences, Faculty of Science and Technology, Sophia University, 7-1 Kioicho, Chiyoda-ku, Tokyo 102-8554, Japan*

[b] *Adjusted Cell Experiment Laboratory Inc,*

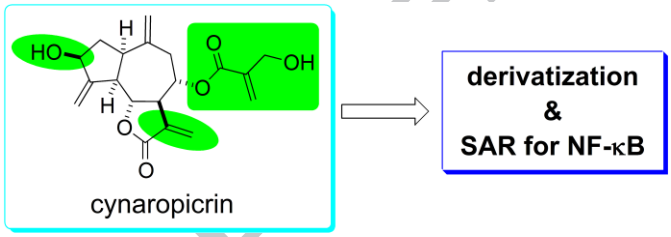
5-4-30 Nishi-Hashimoto, Midori-ku, Sagamihara 252-0131, Japan

* Corresponding author. Tel.: +81 3 3238 3446; fax: +81 3 3238 3361; E-mail address: t-usuki@sophia.ac.jp (T. Usuki)

Abstract:

Cynaropicrin is a guaianolide sesquiterpene lactone with a 5-7-5 tricyclic skeleton, four exo-olefins, and two hydroxyl groups. This natural product has various biological activities including anti-inflammatory properties and antitrypanosomal activity. It was also found to suppress photoaging of the skin by inhibiting the transcription activity of nuclear factor-kappa B (NF-κB). In this paper, nine chemical derivatives of cynaropicrin were prepared, and the structure activity relationship (SAR) study for NF-κB inhibition by measuring relative light units (RLU) was conducted.

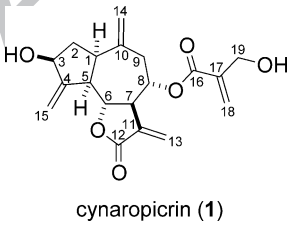
Graphical abstract:



Keywords: cynaropicrin; NF-κB; SAR; derivatization

Main text:

Cynaropicrin (**1**, Figure 1) is one of the sesquiterpene lactone which was first isolated by Šorm and co-workers in 1960 from artichokes (*Cynara scolymus* L).[1] The compound **1** was later also reported to be found in other various Asteraceae, such as *Centaurea solstitialis* L.,[2] *Hemisteptia lyrata* B.,[3] and *Saussurea calcicola*.[4] Cynaropicrin **1**, a guaianolide sesquiterpene lactone, has a 5-7-5 fused tricyclic skeleton with six carbon stereocenters, four exo-olefins, and two hydroxyl groups. **1** has been shown to exert various biological activities, such as anti-inflammatory properties,[5] distinct bitterness mediated through activation of bitter sensory receptors,[6] and potent *in vitro* and *in vivo* inihibitory activity against *Trypanosoma brucei*, the protozoan parasite that causes human African trypanosomiasis (HAT or sleeping sickness).[7] In our previous effort, an *in vitro* structure activity relationship (SAR) study of **1** derivatives activities against *T. brucei* was conducted, the results of which suggested that derivatization of the two hydroxyl groups (OH-3 and OH-19) in **1** does not significantly affect the antitrypanosomal activity.[8] In addition to the antitrypanosomal activity, **1** was also found to suppress photoaging of skin by inhibiting the transcription activity of nuclear factor-kappa B (NF-κB).[9]



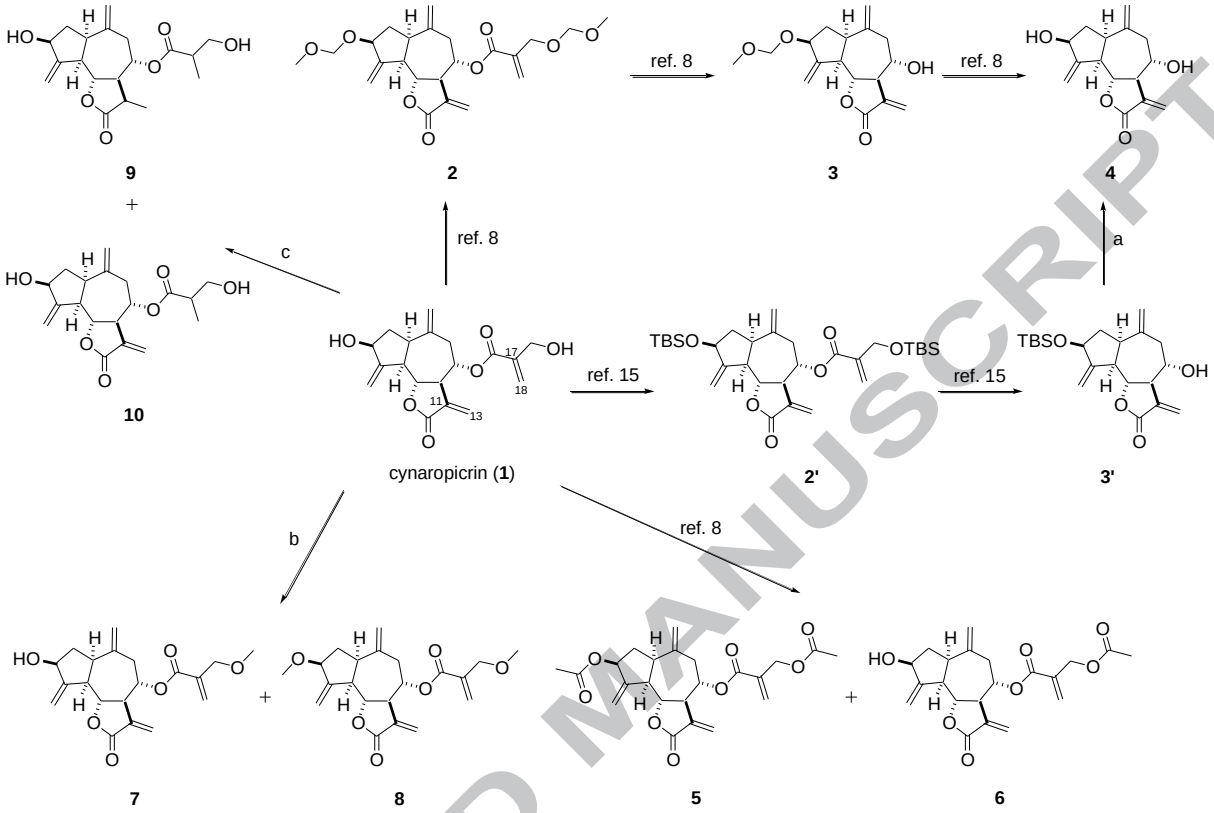
cynaropicrin (**1**)

Figure 1. Structure of cynaropicrin (**1**). The carbon numbering of **1** was adapted from ref. 2.

NF-κB was first identified by Barnes as a transcription factor regulating the expression of K light chains in mouse B lymphocytes in 1986.[10] NF-κB plays a central role in regulating of diverse biological processes including immune response, cell development, growth, and survival.[11] NF-κB is activated in keratinocytes by stimulation such as by ultraviolet (UV) rays, which causes photoaging of the skin, characterized by skin wrinkling, laxity, and pigmentation. In addition, it is activated by inflammatory cytokines such as interleukin-2 (IL-2), IL-6, IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF),[12] and induces the expression of various genes such as those of basic fibroblast growth factor (bFGF) and matrix metalloprotease-1 (MMP-1). Moreover, UV irradiation induces skin cells to secrete bFGF, which is responsible for the proliferation of melanocytes and keratinocytes[9] and promotes the proliferation of skin keratinocytes and melanocytes.[13] It was found that UVB, IL-1, and tumor necrosis factor (TNF) induced NF-κB activation, and produced MMP-1 and bFGF in HaCaT keratinocytes and skin fibroblasts. Thus, inhibition of the NF-κB activation pathway would block the vicious cycle elicited by UV irradiation and effectively prevent UVB-mediated cutaneous alterations.[13] Herein, we screened the effects of nine derivatizations of cynaropicrin **1** on TNFα-mediated NF-κB dependent gene expression by measuring relative light units (RLU).[9]

As the starting material for preparation of the derivatives, a gram-scale isolation of cynaropicrin **1** was carried out from *Cynara scolymus* L. (Asteraceae) according to the literature procedure.[8,14] Derivatization of the obtained natural product **1** was then carried out as shown in Scheme 1. We envisaged that the side chain of cynaropicrin would be essential for inhibition of NF-κB activation, and aimed to synthesize deacylcynaropicrin **4** at first. For the synthesis of **4**, protection of the two hydroxyl groups (OH-3 and OH-19) was conducted.

We have previously reported the synthesis of **4** via introduction of a methoxymethyl (MOM) group on the two hydroxyl groups.[8] However, optical rotation ($[\alpha]_D^{20}$ = +14.5) of **4** via di-MOM cynaropicrin (**2**) and deacylcynaropicrin with MOM (**3**) was not in good agreement with that of the natural product ($[\alpha]_D^{20}$ = +40.6)[3] probably due to the epimerization and/or racemization of chiral carbon caused by intense conditions during MOM group removal (pyridinium *p*-toluenesulfonate (PPTS), *tert*-buthanol, reflux, 24 h). As an alternative route, the synthesis of **4** by introduction of a *tert*-butyl dimethylsilyl (TBS) group on the two hydroxyl groups of **1**[15] was carried out via compound **3'** followed by removal of the TBS using tetrabutyl ammonium fluoride (TBAF) in tethrahydrofuran (THF), and thus the optical rotation ($[\alpha]_D^{20}$ = +37.5) was in agreement with that of the natural one.[3]



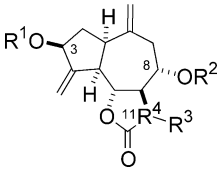
Scheme 1. Reagents and conditions: (a) TBAF, THF, rt, 6 h, 75%; (b) $(MeO)_2SO_2$, K_2CO_3, acetone, reflux, 6 h, 6% (**7**), 30% (**8**); (c) $NaBH_4$, MeOH, rt, 24 h, 45% (**9**), 6% (**10**).

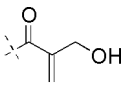
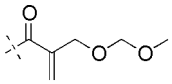
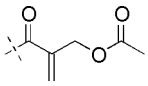
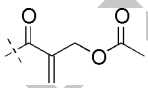
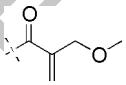
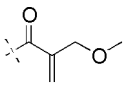
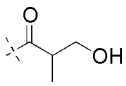
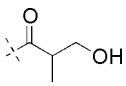
We next turned our attention to the derivatization of the two hydroxyl groups (OH-3 and OH-19). 3,19-Di-Ac cynaropicrin **5** and 19-Ac cynaropicrin **6** were prepared as reported in our previous study.[8] In addition to these derivatives, methylation of **1** was conducted with $(MeO)_2SO_2$ in acetone at reflux for 31 h[16] to give 3,19-di-Me cynaropicrin **7** and 19-Me cyaropicrin **8** in 6% and 30% yield, respectively. Attempt of reduction of exo-olefines of **1** with $NaBH_4$ in MeOH at room temperature for 24 h afforded **9** and **10** in 45% and 6% yield, respectively. Exo-olefines of both C17-C18 and C11-C13 were much more reactive than other exo-olefines, probably due to the chelation between borane and α-carbonyl group.

Moreover, tetrapropylammonium perruthenate (TPAP) oxidation,[17] oxidation by Dess-Martin periodinane,[18] Trost-Masuyama oxidation,[19] and palladium-catalyzed hydrogenation of **1** were carried out to acquire more derivatives for SAR study. However, no reaction occurred at all or complex mixtures were obtained. Preparation of the nine derivatives from natural **1** was achieved through high performance liquid chromatography (HPLC) purification.[20]

Nine compounds prepared in the synthetic study and natural **1** were tested with luciferase reporter gene (*luc2*) for *in vitro* antiphotoaging activity via inhibition of NF-κB activation (Table 1).[21] The DNA element specifically binds to NF-κB upstream and introduce the expression vector having a luciferase report gene downstream into HEK293 cell (transfection). NF-κB activated by TNF-α stimulation then binds to upstream of an expression to guide onset of gene. The expression of the luciferase gene produces luciferase protein, which will emit light in the presence of luciferin. Herein the emission of light was detected for NF-κB activity. We examined three other concentration of samples (0.75 μM, 1.5 μM, and 3.0 μM), however, no compound showed effective or acceptable activity for 0.75 μM. Parent compound **1** had strong antiphotoaging activity of 72.85 ± 4.02 and 5.67 ± 1.16 (mean ± standard error of the mean) for 1.5 μM and 3.0 μM, respectively. Di-MOM compound **2** showed moderate activity of 98.35 ± 3.52 for 1.5 μM and 8.97 ± 1.85 for 3.0 μM. Meanwhile, deacyl derivative **3** showed weak inhibition of NF-κB activation (125.06 ± 1.91 for 1.5 μM and 100.42 ± 4.42 for 3.0 μM, respectively), suggesting that the side chain at OH-8 of **1** was necessary for potent inhibition of NF-κB activation.[8] Accordingly, deacylcynaropicrin **4** did not show inhibitory of NF-κB activation (118.54 ± 1.80 for 1.5 μM and 96.38 ± 2.06 for 3.0 μM, respectively).

Table 1. Relative light units (RLU) of cynaropicrin (**1**) and derivatives **1-10**.



Compd	R^1	R^2	R^3-R^4	0.75 μM	1.5 μM	3.0 μM
1	H		$C=CH_2$	134.36 ± 8.06	72.85 ± 4.02	5.67 ± 1.16
2			$C=CH_2$	154.19 ± 6.19	98.35 ± 3.52	8.97 ± 1.85
3		H	$C=CH_2$	126.80 ± 3.59	125.06 ± 1.91	100.42 ± 4.42
4	H	H	$C=CH_2$	126.83 ± 1.90	118.54 ± 1.80	96.38 ± 2.06
5			$C=CH_2$	170.79 ± 2.49	141.92 ± 6.60	93.49 ± 1.68
6	H		$C=CH_2$	130.03 ± 5.33	109.99 ± 2.31	33.84 ± 2.47
7	CH_3		$C=CH_2$	160.54 ± 7.34	151.32 ± 8.69	137.26 ± 4.02
8	H		$C=CH_2$	173.60 ± 3.39	158.28 ± 4.89	133.55 ± 6.38
9	H		CH-CH_3	122.95± 4.74	138.86 ± 3.88	136.65 ± 4.02
10	H		$C=CH_2$	122.95 ± 4.74	135.03 ± 2.50	136.88 ±7.26

[a] Each value corresponds to the mean ± standard derivation and error of the mean from at

least three independent bioassays.

Surprisingly, the activity of di-Ac compound **5** was weaker (RLU 141.92 ± 6.60 for 1.5 μM and 93.49 ± 1.68 for 3.0 μM, respectively) than that of compound **2** which has di-MOM groups at the same positions. As well as compound **5**, compound **7** having di-Me groups at the same positions showed weaker activity (RLU 151.32 ± 8.69 for 1.5 μM and 137.26 ± 4.02 for 3.0 μM, respectively) than compound **2**, whereas compound **6** which has a Me group at OH-19 showed slightly weaker activity (RLU 109.99 ± 2.31 for 1.5 μM and 33.84 ± 2.47 for 3.0 μM, respectively) than **2**. It was thus found that protecting groups at OH-3 and OH-19 positions affect NF-κB activity when compared to compound **1**.

We also screened derivatives **9** and **10**, and found that neither of them inhibited NF-κB activation (**9**: RLU 138.86 ± 3.88 for 1.5 μM and 136.65 ± 4.02 for 3.0 μM; **10**: RLU 135.03 ± 2.50 for 1.5 μM and 136.88 ±7.26 for 3.0 μM, respectively). This was thus suggested that exo-olefin at C-17 affect NF-κB activity while exo-olefin at C-11 did not.

Based on these results of the SAR study, the following conclusions could be drawn: 1) an acyl side chain at OH-8 is essential for inhibition of NF-κB activation; 2) di-MOM cynaropicrin showed similar activity with natural cynaropicrin **1**; 3) an exo-olefine at C-17 affects inhibition of NF-κB activation; 4) an exo-olefine at C-11 does not affect NF-κB activity.

In summary, synthesis of derivatives of cynaropicrin **1** and determination of their antiphotoaging activities via inhibitory NF-κB activity were carried out as part of a SAR study. The results suggest that side chain of **1**, the hydrophilicity of the OH-3 and OH-19 substituent, and the exo-olefin (C17-C18) significantly affect inhibitory activity against NF-κB. These results will be useful for the design of labeled derivatives, such as photo-affinity, spin-probe labels, etc, for further biological studies of sesquiterpene lactones.

Acknowledgements

This paper is dedicated to Professor Koji Nakanishi (Columbia University). We thank Dr. Michael Adams (University of Basel, currently Bacoba AG, Switzerland) for his supports and suggestions.

Supplementary data

Supplementary data (^{1}H and ^{13}C NMR spectra of compounds **7**, **8**, **9** and **10**, and summary of bioassay) associated with this article can be found in the online version, at http://

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20. **General:** All non-aqueous reactions were conducted under an atmosphere of nitrogen with magnetic stirring. CH_2Cl_2 was dried using activated molecular sieves. All reagents were obtained from commercial suppliers and used without further purification unless otherwise stated. Analytical thin layer chromatography (TLC) was performed on silica

gel 60 F_{254} plates produced by Merck KGaA (Darmstadt, Germany). Column chromatography was performed with acidic silica gel 60 (spherical, 40-50 μm; Kanto Chemicals Co. Ltd, Tokyo, Japan). Optical rotations were measured on a JASCO P-2200 digital polarimeter at the sodium lamp (λ = 589 nm) D line and are reported as follows: $[\alpha]_D^T$ (*c* g/100 mL, solvent). Infrared (IR) spectra were recorded on a JASCO FT-IR 4100 spectrometer and are reported in wavenumbers (cm^{-1}). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-EXC 300 spectrometer (300 MHz). ^1H NMR data are reported as follows: chemical shift (δ, ppm), integration, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), coupling constants (*J*) in Hz, assignments. ^{13}C NMR data are reported in terms of chemical shift (δ, ppm). Fast atom bombardment (FAB) MS spectra were recorded on a JEOL JMS-700 instrument. Electrospray ionization (ESI) MS spectra were recorded on a JEOL JMS-T100LC instrument.

Isolation of cynaropicrin (1): Extraction and isolation of cynaropicrin from artichoke leaves were achieved by the following published protocol.[8,14] Dried artichoke leaves (100 g) obtained from Dixa AG (Gallen, Switzerland) through Dr. Michael Adams were exhaustively extracted three times with ethyl acetate (900 mL) yielding 5.4 g of crude extract. The extract was separated on a silica gel column chromatography with ethyl acetate as mobile phase. Fractions containing **1** were concentrated to give 1.7 g of impure cynaropicrin **1**. Purification via silica gel column chromatography (hexane/EtOAc = 1:2) afforded **1** as a yellow solid (1.4 g).

(1*R*,3*S*,5*R*,6*R*,7*R*,8*S*)-3-Hydroxy-13,14,15-trimethylene-12-oxododecahydroazuleno[6,7-β]furan-8-yl methacrylate (4): To a solution of **3'** (24 mg, 0.064 mmol, 1.0 eq) under dry nitrogen atmosphere in THF (1.0 mL) was added TBAF (46.0 μL, 0.92 mmol, 2.5 eq) at 0 °C. After stirring 6 h under room temperature, the mixture was diluted with EtOAc and quenched with saturated NH_4Cl solution. The aqueous layer was then extracted with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. Purification on silica gel column chromatography (hexane/EtOAc = 1:1) afforded **4** as a colorless oil (12.7 mg, 0.048 mmol, 75%); R_f 0.20 (hexane/EtOAc = 1:1); $[\alpha]_D^{20}$ +37.5 (*c* 1.2, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) δ 6.27-6.26 (1H, d, *J* = 3.0 Hz, H13), 6.15-6.14 (1H, d, *J* = 3.0 Hz, H13), 5.48 (1H, s, H15), 5.34 (1H, s, H15), 5.13 (1H, s, H14), 4.98 (1H, s, H14), 4.55-4.54 (1H, m, H3), 4.18-4.08 (1H, m, H6), 3.99-3.93 (1H, m, H8), 3.01 (1H, m, H1), 2.92-2.67 (3H, m, H5/7/9), 2.32-2.18 (2H, m, H2/9), 1.78-1.67 (1H, m, H2); FAB-MS (*m*/*z*) calcd for $C_{15}H_{19}O_4$ $[M+H]^+$ 263.12, found 263.12.

(1*R*,3*S*,5*R*,6*R*,7*R*,8*S*)-3-Methoxy-13,14,15-trimethylene-12-oxododecahydroazuleno[6,7-β]furan-8-yl 17-(methoxy)acrylate (7) and

(1*R*,3*S*,5*R*,6*R*,7*R*,8*S*)-3-hydroxy-13,14,15-trimethylene-12-oxododecahydroazuleno[6,7-β]furan-8-yl 17-(methoxy)acrylate (8): To a solution of cynaropicrin **1** (50.0 mg, 0.144 mmol, 1.0 eq) and K_2CO_3 (80.0 mg, 0.576 mmol) in acetone (2.0 mL) was added $(MeO)_2SO_2$ (55.0 µL, 0.576 mmol). After stirring at reflux for 6 h, the reaction mixture was cooled to room temperature and filtered. After concentration to remove acetone, EtOAc and saturated $NaHCO_3$ solution were added and stirred for 5 min, then extracted with EtOAc. The combined organic layers were washed with 2 M HCl, saturated $NaHCO_3$ solution, brine, dried over Na_2SO_4, and concentrated *in vacuo*. Purification by silica gel column chromatography (hexane/EtOAc = 1:1) afforded **7** as a colorless oil (3.0 mg, 0.008 mmol, 6%); $[\alpha]_D^{20}$ +108.1 (*c* 0.5, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.37 (1H, s, H19), 6.21-6.20 (1H, d, *J* = 6.0 Hz, H13), 5.93 (1H, s, H19), 5.62-5.61 (1H, d, *J* = 3.0 Hz, H13), 5.42 (1H, s, H15), 5.27 (1H, s, H15), 5.16-5.10 (2H, m, H8, H14), 4.90-4.89 (1H, m, H14), 4.32-4.26 (1H, m, H3), 4.17-4.03 (2H, m, H18), 4.01-4.00 (1H, t, *J* = 4.5 Hz, H6), 3.40 (1H, s, Me), 3.31 (1H, s, Me), 3.17-3.10 (1H, m, H7), 3.04-2.98 (1H, m, H1), 2.78-2.66 (1H, m, H5), 2.43-2.24 (1H, m, H9), 1.81-1.72 (1H, m, H2), 1.59 (1H, m, H2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.6, 165.3, 150.3, 148.6, 144.1, 137.6, 126.1, 122.6, 117.6, 115.6, 81.4, 60.1, 56.9, 52.1, 46.9, 44.9, 38.2, 37.3, 34.7, 14.5; ESI-HRMS (*m*/*z*) calcd for $C_{20}H_{24}NaO_6$ $[M+Na]^+$ 383.1471, found 383.1451. Purification on silica gel column chromatography (hexane/EtOAc = 1:2) afforded **8** as a colorless oil (15.4 mg, 0.043 mmol, 30%); R_f 0.21 (hexane/EtOAc = 1:2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.37-6.36 (1H, m, H19), 6.22-6.21 (1H, d, *J* = 3.0 Hz, H13), 5.94 (1H, m, H19), 5.65-5.64 (1H, d, *J* = 3.0 Hz, H13), 5.49 (1H, m, H15), 5.35 (1H, m, H15), 5.14-4.94 (2H, m, H8/H14), 4.58-4.53 (1H, m, H14), 4.27-4.16 (2H, m, H18), 3.39 (3H, s, Me), 3.23-3.15 (1H, m, H7), 3.02-2.93 (1H, m, H1), 2.87-2.81 (1H, m, H5), 2.73-2.62 (1H, m, H9), 2.42-2.36 (1H, m, H9), 2.27-2.18 (1H, m, H2), 1.78-1.67 (1H, m, H2); ESI-HRMS (*m*/*z*) calcd for $C_{21}H_{26}NaO_6$ $[M+Na]^+$ 397.1627, found 397.1630.

(1*R*,3*S*,5*R*,6*R*,7*R*,8*S*)-3-Hydroxy-14,15-trimethylene-13-methyl-12-oxododecahydroazuleno[6,7-β]furan-8-yl 17-methyl-methacrylate (9) and (1*R*,3*S*,5*R*,6*R*,7*R*,8*S*)-3-hydroxy-13,14,15-trimethylene-12-oxododecahydroazuleno[6,7-β]furan-8-yl 17-methyl-methacrylate (10): To a solution of **1** (48.0 mg, 0.139 mmol, 1.0 eq) under dry nitrogen atmosphere, were dissolved in 1.0 ml of MeOH. $NaBH_4$ (10.5 mg, 0.278 mmol, 2.0 eq) was added and the mixture was magnetically stirred for 24 h at room temperature. The reaction was monitored by TLC, elunted with hexane/EtOAc = 1:3 (R_f **9** = 0.40, R_f **10** = 0.30). For work-up, the reacted mixture was diluted with H_2O and acidified with 2 M H_2SO_4. The organic phase was washed with H_2O and saturated brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification by silica gel column chromatography (hexane/EtOAc = 1:3) afforded **9** as a colorless oil (22.0 mg,

0.063 mmol, 45%); ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.40 (1H, m, H15), 5.32-5.29 (1H, m, H15), 5.09-4.98 (3H, m, H8/14), 4.56-4.51 (1H, m, H3), 4.05-4.02 (1H, m, H6), 3.77-3.73 (2H, m, H19), 2.93-2.87 (1H, m, H1), 2.83-2.80 (1H, m, H17), 2.74-2.68 (2H, m, H7/11), 2.58/2.52 (1H, m, H5), 2.31-2.18 (3H, m, H2/9), 1.79 (1H, m, H2), 1.42-1.39 (3H, t, J = 9.0 Hz, H13), 1.23 (3H, m, H18); ^{13}C NMR (75 MHz, $CDCl_3$): δ 177.5, 165.1, 152.5, 141.9, 139.1, 126.3, 117.3, 112.3, 78.7, 76.2, 73.4, 62.1, 53.2, 50.4, 44.1, 41.1, 40.3, 38.6, 15.3; ESI-MS (*m*/*z*) calcd for $C_{19}H_{26}O_6$ $[M]^+$ 350.17, found 350.17. Purification by silica gel column chromatography (hexane/EtOAc = 1:3) afforded **10** as a colorless oil (2.8 mg, 0.008 mmol, 6%); ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.14 (1H, m, H13), 5.65-5.64 (1H, m, H13), 5.42 (1H, m, H15), 5.34-5.32 (1H, m, H15), 5.14 (1H, m, H8), 5.04-5.02 (2H, m, H14), 4.55 (1H, m, H3), 4.08 (1H, m, H6), 2.95-2.87 (2H, m, H19), 2.84-2.77 (1H, m, H1), 2.74-2.72 (1H, m, H17), 2.55-2.49 (1H, m, H5), 2.34-2.31 (1H, m, H7), 2.27-2.25 (1H, m, H9), 2.23-2.20 (1H, m, H2), 1.97 (1H, m, H2), 1.23 (3H, m, H18).

21. **NF-kB assay**: HEK 293 cells (RIKEN RBC, Japan) were dispensed into the wells of a multi-well plate (1.5×10^4 cells/well in 96-well plate) in minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA) and incubated at 37 °C for 24 h. Each well of 96-well plate was added with 10μL of transfection reagent containing 0.01 μg pGL4.32 [luc2P/NF-κB-RE/Hygro] (Promega, USA), 10 μL of MEM and 0.1μL of X-tremeGENE HP reagent (Roche, Switzerland) and incubated at 37 °C for 24 h. After the supernatants were exchanged for MEM including 10% FBS, 1% NEAA and compounds, and then incubated at 37 °C for 2 h, 5 μL of 200 ng/mL TNF-α (Sigma-Aldrich, USA) was added to each well of 96-well plate. After 5 h of culture, 30 μL of supernatant was removed from each well, and 70 μL of Bright-Glo Luciferase Assay Reagent (Promega, USA) was added. After 2 min of mixing, the relative light units (RLU) as a NF-κB activity were measured using a microplate reader Varioskan (Thermo Fisher Scientific, USA).

    Cytotoxicity: HEK 293 cells (1.5 X 10^4 cells/well in 96-well plate) were plated in MEM supplemented with 10% FBS, 1% NEAA and incubated at 37 °C for 24 h. The supernatant of each well was changed for MEM including 10% FBS, 1% NEAA and compounds, and then incubated at 37 °C for 7 h. The viability of the cultures was determined colorimetrically using WST-8 reagent (Nacalai Tesque, Japan) in a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) reduction assay. In brief, culture supernatants were replaced by MEM medium including 10% WST-8 reagent and incubated at 37 °C for 1 h. The absorbance was measured at 450 nm using a microplate reader. Cell viabilities are presented as absorbance changes per hour.

Graphical abstract