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Inhibitory effect of novel 5-*O*-acyl juglones on mammalian DNA polymerase activity, cancer cell growth and inflammatory response

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ABSTRACT

We previously found that vitamin K_3 (menadione, 2-methyl-1,4-naphthoquinone) inhibits the activity of human mitochondrial DNA polymerase γ (pol γ). In this study, we focused on juglone (5-hydroxy-1,4-naphthoquinone), which is a 1,4-naphthoquinone derivative, and chemically synthesized novel juglones conjugated with C2:0 to C22:6 fatty acid (5-*O*-acyl juglones). The chemically modified juglones enhanced mammalian pol inhibition and their cytotoxic and anti-inflammatory activities. The juglone conjugated with oleic acid (C18:1-acyl juglone) showed the strongest inhibition of DNA replicative pol α activity and human colon carcinoma (HCT116) cell growth in 10 synthesized 5-*O*-acyl juglones. C12:0-Acyl juglone was the strongest inhibitor of DNA repair-related pol λ, as well as the strongest suppression of the production of tumor necrosis factor (TNF)-α production induced by lipopolysaccharide (LPS) in the compounds tested. Moreover, this compound caused the greatest reduction in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced acute inflammation in mouse ears. C12:0- and C18:1-Acyl juglones selectively inhibited the activities of mammalian pol species, but did not influence the activities of other pols and DNA metabolic enzymes tested. These data indicate that the novel 5-*O*-acyl juglones target anti-cancer and/or anti-inflammatory agents based on mammalian pol inhibition. Moreover, the results suggest that acylation of juglone is an effective chemical modification to improve the anti-cancer and anti-inflammation of vitamin K_3 derivatives, such as juglone.

1. Introduction

The human genome encodes at least 15 DNA polymerases (pols, i.e., DNA-dependent DNA polymerase, E.C. 2.7.7.7) that participate in cellular DNA synthesis.[1,2] Eukaryotic cells contain 3 replicative pols (α, δ, and ε), 1 mitochondrial pol (γ), and at least 11 non-replicative pols (β, ζ, η, θ, ι, κ, λ, μ, ν, terminal deoxynucleotidyl transferase (TdT), and REV1).[3,4] Pols have a highly conserved structure, which means that their catalytic subunits show little variance among species. Enzymes with conserved structures usually perform important cellular functions, the maintenance of which has evolutionary advantages. On the basis of their sequence homology, eukaryotic pols can be divided into 4 main families, termed A, B, X, and Y.[4] Family A includes mitochondrial pol γ, as well as pols θ and ν. Family B includes 3 replicative pols (α, δ, and ε) and pol ζ. Family X comprises pols β, λ, and μ, as well as TdT, and family Y includes pols η, ι, and κ, in addition to REV1. We have been studying selective inhibitors of each pol derived from natural products including food materials and nutrients for more than 16 years.[5,6] We have found that vitamin K_3, but not K_1 or K_2, is a potent inhibitor of human pol γ.[7–11]

Vitamin K_3 (menadione, 2-methyl-1,4-naphthoquinone) is a fat-soluble compound that contains quinone as its principle chemical feature. Quinones are a class of organic compounds that are derived from aromatic compounds via the exchange of an even number of –CH= groups for –C(=O)– groups, and any necessary rearrangement of double bonds, resulting in a fully conjugated cyclic dione structure. The toxicological properties of quinones, which act as alkylating agents, have also been examined. For example, quinones are known to interact with flavoproteins to generate reactive oxygen species (ROS) that can induce biological injury.[12–15] In this study, we focused on 5-hydroxy-1,4-naphthoquinone (juglone, **1** of Fig. 1), which has the common naphthoquinone skeleton and a hydroxyl group at the C-5 position. Juglone occurs naturally in the leaves, roots, husks, and bark of plants in the Juglandaceae family, particularly the black walnut (*Juglans nigra*), and is toxic or growth stunting in many types of plants. It is

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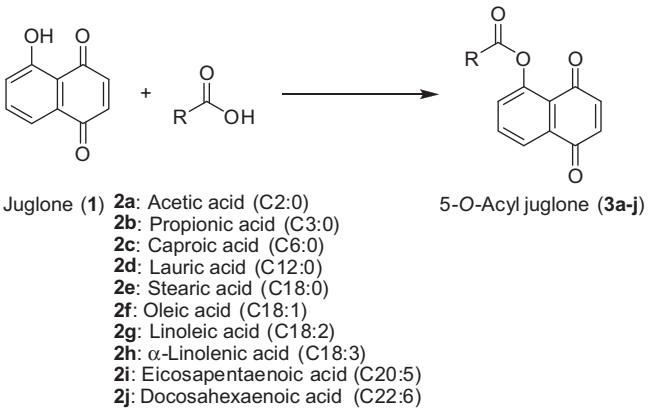


Figure 1. Structure of juglone (**1**), fatty acid (**2**) and 5-*O*-acyl juglone (**3**).

sometimes used as a herbicide, as a dye for cloth and inks, and as a coloring agent for foods and cosmetics. Juglone is known for its wide range of biological activities, such as induction of oxidative stress in many animal cell systems,[16] inhibition of germination of various plant species,[17] and inhibition of the peptidyl-prolyl isomerase Pin1.[18]

We previously found that the catechin and epicatechin derivatives conjugated with fatty acids, such as 3-*O*-acylcatechins and 3-*O*-acylepicatechins, respectively, were stronger pol inhibitors than catechin and epicatechin;[19–21] therefore, the 5-*O*-acylated derivatives of juglone (**3a–j** of Fig. 1) were chemically synthesized from juglone (**1** of Fig. 1) and fatty acids (**2a–j** of Fig. 1). In this study, we investigated the inhibitory effects of 10 5-*O*-acyl juglones on mammalian pol activity, human cancer cell growth and the inflammatory response comparing juglone and fatty acids. The relationship between pol inhibitory and cytotoxic or anti-inflammatory effects of vitamin K_3-based acylated derivatives is discussed.

2. Results and discussion

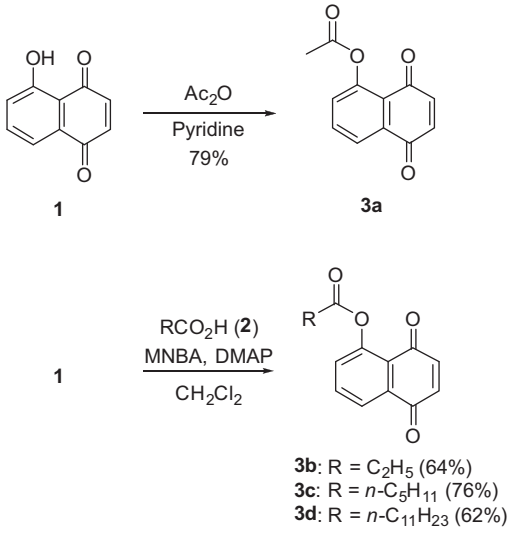
2.1. Synthesis of 5-*O*-acyl juglones

5-*O*-Acetoxy-1,4-naphthoquinone (**3a**)[22] was prepared by treating juglone (**1**) with acetic anhydride in pyridine in 79% yield (Scheme 1). In this reaction, we found that **1** readily decomposes under basic conditions. The instability of **1** prompted us to examine the condensation of **1** with carboxylic acids. After a systematic survey of condensing reagents, catalysts and solvents, we obtained 5-*O*-acyl juglones (**3b–d**) by condensation of **1** with carboxylic acids (**2b–d**) using 2-methyl-6-nitrobenzoic anhydride (MNBA) with DMAP (0.1 equiv) in CH_2Cl_2.[23]

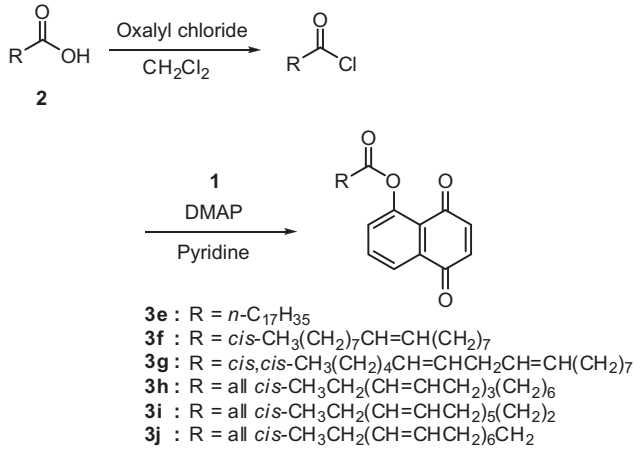
However, this protocol is not effective for condensation of **1** with long fatty acids (**2e–j**). For example, coupling of **1** with eicosapentaenoic acid (**2i**) gave only mixed anhydride of **2i** and 2-methyl-6-nitrobenzoic acid (date not shown). The combination of long acyl moieties with **1** was achieved only when acyl chlorides were used as acylation reagents (Scheme 2). Carboxylic acids (**2e–j**) were converted to the corresponding acyl chlorides, which were treated with **1** (3 equiv) and DMAP (0.1 equiv) in pyridine to give **3e–j**.

2.2. Effect of the synthesized 5-*O*-acyl juglones on mammalian pol activity

Initially, we investigated the in vitro biochemical action of juglone (**1**), 10 fatty acids (**2a–j**) and their 10 chemically synthesized derivatives, 5-*O*-acyl juglones (**3a–j**). The inhibition of four mammalian pols, namely calf pol α, human pol γ, human pol κ and human pol λ, by 10 μM of each compound was investigated. Pols α, γ, κ and λ were used as representatives of the B, A, Y and X families of pols, respectively.[1–3] As shown in Figure 2, juglone, a derivative of vitamin K_3, inhibited the activity of mammalian pols α and γ, with IC_{50} values of less than 10 μM. Although vitamin K_3 selectively inhibited pol γ among the mammalian pols tested,[7–11] juglone was an inhibitor of all families of pols. No fatty acids tested (C2:0 to C22:6) at 10 μM had any effect on pol activity. On the other hand, the synthesized 5-*O*-acyl juglones were more potent pol inhibitors than the individual compounds, such as juglones and fatty acids. In the 5-*O*-acyl juglones, the juglone conjugated with lauric acid (C12:0-acyl juglone) (**3d**) was the strongest inhibitor of pols κ and λ, and C18:1-acyl juglone (**3f**) was the most potent pol inhibitor of pols α and γ. The inhibitory effect of juglone conjugated with saturated fatty acids (**3a–e**) on pols κ and λ ranked as follows: C12:0-acyl juglone (**3d**) > C18:0-acyl juglone (**3e**) > C6:0-acyl juglone (**3c**) > C3:0-acyl juglone (**3b**) > C2:0-acyl juglone (**3a**); and the inhibitory effect of juglone conjugated with unsaturated fatty acids (**3f–j**) on pols α and γ ranked as follows: C18:1-acyl juglone (**3f**) > C20:5-acyl juglone (**3i**) > C22:6-acyl juglone (**3j**) > C18:2-acyl juglone (**3g**) > C18:3-acyl juglone (**3h**). When activated DNA (i.e., bovine deoxyribonuclease I-treated DNA) and dNTP were used as the DNA template–primer and



Scheme 1. Preparation of 5-*O*-acyl juglones (**3a–d**).



Scheme 2. Preparation of 5-*O*-acyl juglones (**3e–j**).

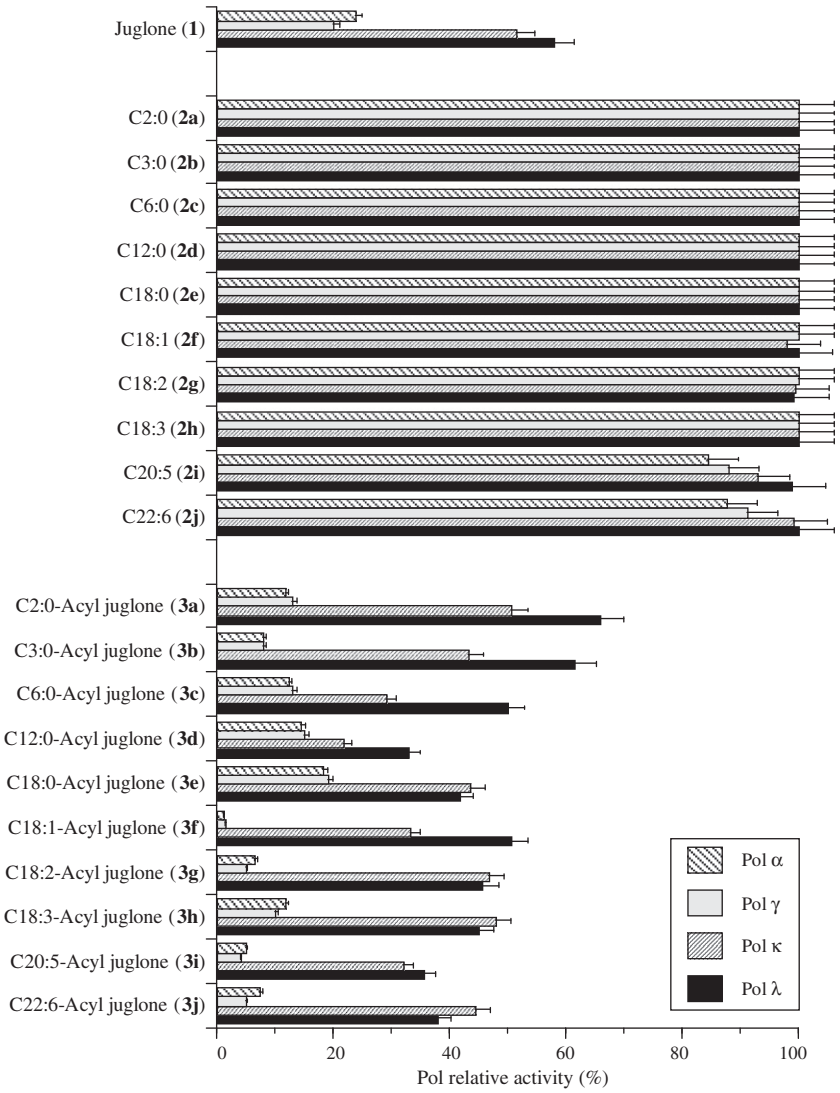


Figure 2. Inhibitory effects of juglone (**1**), fatty acids (**2a**–**j**) and 5-*O*-acyl juglones (**3a**–**j**) on the activity of mammalian pols. Each compound (10 μM) was incubated with calf pol α (B-family pol), human pol γ (A-family pol), human pol κ (Y-family pol) and human pol λ (X-family pol) (0.05 units each). Pol activity in the absence of the compound was taken as 100%, and the relative activity is shown. Data are shown as the mean ± SE (n = 3).

nucleotide substrate instead of synthesized DNA [poly(dA)/oligo(dT)$_{18}$ (A/T = 2/1)] and dTTP, respectively, the inhibitory effects of these compounds did not change (data not shown).

2.3. Effect of the synthesized 5-*O*-acyl juglones on pols and other DNA metabolic enzymes

Among the 10 5-*O*-acyl juglones investigated, C12:0-acyl juglone (**3d**) and C18:1-acyl juglone (**3f**) displayed the strongest inhibitory effect on mammalian pols (Fig. 2) and was therefore the focus of this section. As described briefly in the introduction, we succeeded in obtaining eleven mammalian pol species, including pols α, β, γ, δ, ε, η, ι, κ, λ and μ, and TdT; however, pols ζ, θ and ν, and REV1 are not yet available. Currently, eukaryotes are thought to express at least 15 species of pols,[1,2] and we are still in an era when most pols are very difficult to obtain in their purified form in a laboratory. Table 1 shows the inhibitory effect (IC$_{50}$ value) of these 5-*O*-acyl juglones against various pol species including the eleven mammalian pols that could be obtained. C12:0-Acyl juglone (**3d**) inhibited the activity of all of the pols from mammals with IC$_{50}$ values of 6.3–8.4 μM. C18:1-Acyl juglone (**3f**) also inhibited all mammalian pols tested, and 50% inhibition of the A, B, X and Y families of pols was observed at a dose of 0.72, 0.68–0.74, 10.2–11.9 and 8.1–8.7 μM, respectively; therefore, the inhibitory effect of this compound on the A- and B-families of pols was more than 10-fold stronger than that on X- and Y-families of pols.

By contrast, these compounds had no effect on plant pols, such as cauliflower pol α or rice pol λ, or prokaryotic pols, such as *Escherichia coli* pol I, *Taq* pol or T4 pol (Table 1). The three-dimensional structures of eukaryotic pols are likely to differ greatly from those of prokaryotic pols. These compounds did not inhibit the activity of other DNA metabolic enzymes, such as calf primase pol α, HIV-1 reverse transcriptase, T7 RNA polymerase, mouse IMP dehydrogenase (type II), T4 polynucleotide kinase, or bovine deoxyribonuclease I. These results suggest that 5-*O*-acyl juglones may be selective inhibitors of mammalian pols; in particular, juglone conjugated with unsaturated fatty acids, such as C18:1-acyl juglone (**3f**), potently inhibited the activities of A- and B-families of pols.

To test whether these 5-*O*-acyl juglones are intercalating agents that distort DNA and subsequently inhibits enzyme activity, we measured the thermal transition of DNA in the presence or absence of these compounds. The thermal transition profile of DNA was the same with or without the compound (data not shown). Therefore,

Table 1
IC_{50} values of C12:0-acyl juglone (**3d**) and C18:1-acyl juglone (**3f**) for mammalian pols, various pols and other DNA metabolic enzymes

Enzyme	IC_{50} values (μM)	
	C12:0-Acyl juglone (**3d**)	C18:1-Acyl juglone (**3f**)
Mammalian pols		
[A-Family of pol]		
Human pol γ	6.5 ± 0.40	0.72 ± 0.04
[B-Family of pols]		
Calf pol α	6.3 ± 0.38	0.68 ± 0.04
Human pol δ	6.9 ± 0.41	0.74 ± 0.05
Human pol ε	6.6 ± 0.40	0.70 ± 0.04
[X-Family of pols]		
Rat pol β	7.6 ± 0.46	11.5 ± 0.69
Human pol λ	7.0 ± 0.43	10.2 ± 0.61
Human pol μ	7.9 ± 0.48	10.8 ± 0.65
Calf TdT	8.4 ± 0.50	11.9 ± 0.72
[Y-Family of pols]		
Human pol η	6.9 ± 0.42	8.4 ± 0.51
Mouse pol ι	7.1 ± 0.43	8.7 ± 0.53
Human pol κ	6.8 ± 0.41	8.1 ± 0.49
Plant pol		
Cauliflower pol α	>100	>200
Rice pol λ	>100	>200
Prokaryotic pols		
E. coli pol I	>100	>100
Taq pol	>100	>100
T4 pol	>100	>100
Other DNA metabolic enzymes		
Calf primase of pol α	>100	>100
HIV-1 reverse transcriptase	>100	>100
T7 RNA polymerase	>100	>100
Mouse IMP dehydrogenase (type II)	>100	>100
T4 polynucleotide kinase	>100	>100
Bovine deoxyribonuclease I	>100	>100

Each compound was incubated with each enzyme (0.05 units). Enzyme activity in the absence of the compounds was taken as 100 %. Data are shown as the means ± SE of three independent experiments.

the inhibition of pols by 5-*O*-acyl juglones is not due to DNA distortion, but seems to be due to the direct effect of this compound on the enzymes themselves.

2.4. Effect of the synthesized 5-*O*-acyl juglones on cultured human cancer cells

Pols have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents.[5] The chemically synthesized 5-*O*-acyl juglones could therefore be useful in chemotherapy, and we investigated the cytotoxic effect of juglone (**1**), fatty acids (**2a–j**) and 5-*O*-acyl juglones (**3a–j**) against HCT116 human colon carcinoma cultured cell line. As shown in Figure 3, 100 μM juglone suppressed cell growth with an LD_{50} value of less than 100 μM, whereas the 10 fatty acids had no effect on cell viability. In the synthesized 5-*O*-acyl juglones, C18:1-acyl juglone (**3f**) had the strongest growth inhibitory effect on HCT116 cells. The influence of 5-*O*-acyl juglones on HCT116 cell growth showed the same tendency as that on the inhibition of pols α and γ (Fig. 2), suggesting that cancer cell growth prevention by these compounds may be related to inhibition of the activities of DNA replicative pols, such as pol α; in particular, the inhibition of both A- and B-families of pols by these compounds must be important for HCT116 cell proliferation. C18:1-Acyl juglone (**3f**) more strongly suppressed the growth of HCT116 cells than the same concentrations of aphidicolin, camptothecin and etoposide, which are inhibitors of replicative pols, DNA topoisomerase I and DNA topoisomerase II, respectively (data not shown).

2.5. Effect of the synthesized 5-*O*-acyl juglones on the LPS-induced inflammatory response in cultured macrophage cells

Next, we investigated whether chemically synthesized 5-*O*-acyl juglones can inhibit TNF-α production induced by lipopolysaccharide (LPS) stimulation in cultured mouse macrophage RAW264.7 cells. The inflammatory cytokine TNF-α activates the major inflammation signaling pathway by binding to the TNF-α receptor (TNFR), resulting in various inflammatory diseases.[24] In RAW264.7 cells, no compound showed cytotoxicity at 5 μM, because the LD_{50} values of these compounds were >10 μM. As shown in Figure 4, juglone (**1**) at 5 μM moderately suppressed the LPS-stimulated production of TNF-α, although 10 fatty acids (**2a-j**) displayed no inhibitory effect on TNF-α production. The inhibitory effects of C12:0-acyl juglone (**3d**) were the strongest among these 10 5-*O*-acyl juglones. The effect of the compounds on the suppression of LPS-evoked TNF-α production showed almost the same tendency as their inhibitory effect on mammalian pol λ. These results suggest that 5-*O*-acyl juglones, such as C12:0-acyl juglone (**3d**), inhibit the activities of mammalian pols, and then prevent TNF-α production in LPS-induced macrophages, but do not affect cell growth.

2.6. Effect of the synthesized 5-*O*-acyl juglones on TPA-induced anti-inflammatory activity

In a previous pol inhibitor study, we found a relationship between pol λ inhibitors and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced acute anti-inflammatory activity.[6,25,26] Thus, using the mouse ear inflammatory test, we examined the anti-inflammatory activity of juglone (**1**), fatty acids (**2a–j**) and 5-*O*-acyl juglones (**3a–j**) at 500 μg/ear. Application of TPA (0.5 μg) to the mouse ear induced edema, resulting in a 241% increase in the weight of the ear disk 7 h after application. As shown in Figure 5, pretreatment with juglone moderately suppressed inflammation, but 10 fatty acids had a weak effect on the level of inflammation. The anti-inflammatory effect of the chemically synthesized 5-*O*-acyl juglones was stronger than that of juglone and fatty acid, and C12:0-acyl juglone (**3d**) was the strongest among these 10 5-*O*-acyl juglones. Therefore, the mouse ear anti-inflammatory effect of these compounds correlated with their inhibitory effects on both mammalian pol λ (Fig. 2) and TNF-α production in macrophages (Fig. 4), and C12:0-acyl juglone (**3d**) had the strongest effect among the synthesized 5-*O*-acyl juglones. These results suggest that inhibition of pol λ activity has a positive correlation with the anti-inflammatory activity observed.

2.7. Three-dimensional structures of juglone, fatty acids and 5-*O*-acyl juglones

To obtain information about the molecular basis of the inhibitory properties of 5-*O*-acyl juglones, computational analyses were performed using molecular simulation. We focused on the calculated log*P* (*C* log *P*) value (partition coefficients for octanol/water) of the synthesized 10 5-*O*-acyl juglones (**3a–j**) and their part compounds, such as juglone (**1**) and fatty acids (**2a–j**), as chemical properties (Table 2). The values of *C*log*P*, which indicate hydrophobicity, in 5-*O*-acyl juglones had almost the same tendency and numerical values as those of fatty acids, which are consisted of 5-*O*-acyl juglones; therefore, the *C*log*P* value of 5-*O*-acyl juglones depends on the acyl-chain length, and does not affect bioactivity, such as pol inhibition, human cancer cell growth suppression and anti-inflammatory response.

The length and width of the three-dimensional structures, from which the energy-minimized compounds were calculated, are shown in Table 2. The molecular length of 5-*O*-acyl juglones was approximately 5–6 Å longer than that of fatty acids consisting of

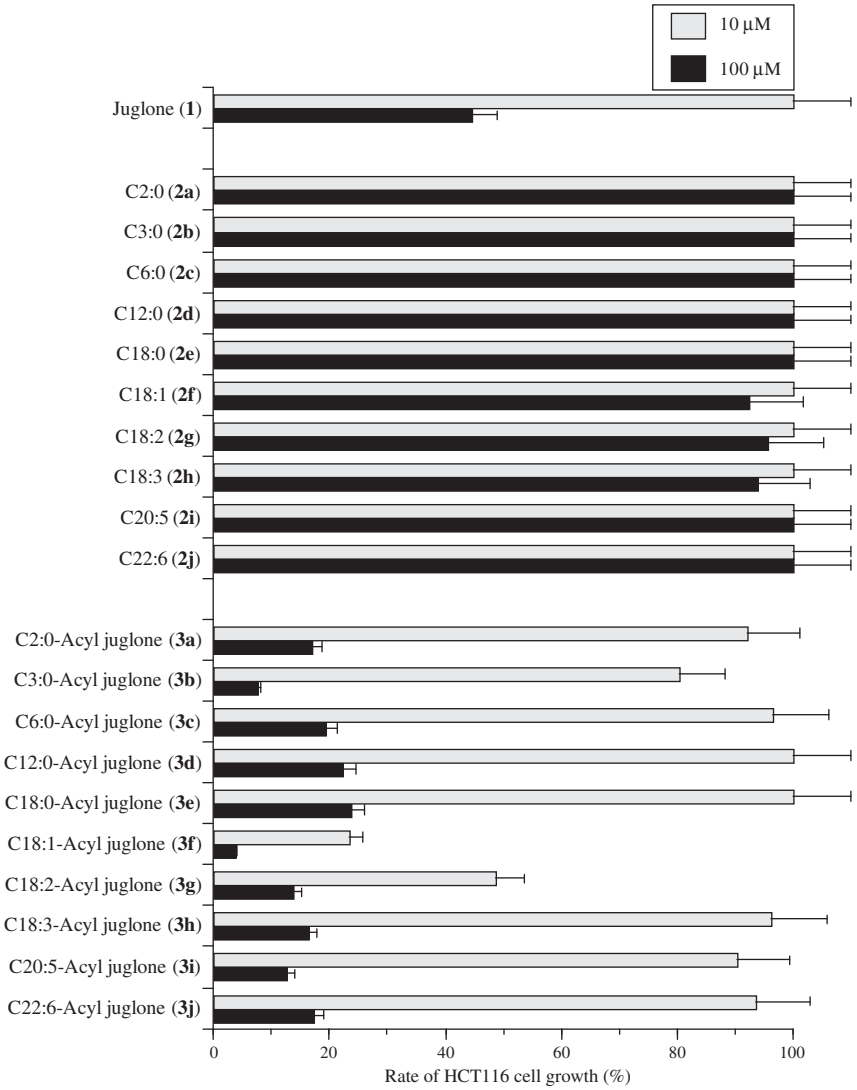


Figure 3. Effect of juglone (**1**), fatty acids (**2a**–**j**) and 5-*O*-acyl juglones (**3a**–**j**) on the proliferation of HCT116 human colon carcinoma cultured cell growth. Each compound (10 and 100 μM) was added to the culture of HCT116 cells. The cells were incubated for 24 h, and the rate of cultured cell growth inhibition was determined by WST-1 assay.44 Cell growth inhibition of the cancer cells in the absence of the compound was taken as 100%. Data are shown as the mean ± SE ($n = 5$).

the 5-*O*-acyl juglones. The 5-*O*-acyl juglones conjugated with saturated fatty acid (i.e., **3a–e**) were almost the same molecular width (5.07–5.13 Å). The length and width difference between 5-*O*-acyl juglones and fatty acids is caused by the juglone moiety (5.15 × 5.23 Å) of 5-*O*-acyl juglones, and the molecule of both length and width of >8.28 and >5.07 Å, respectively, might be necessary to have the bioactivity. Moreover, the molecular length and width of C12:0-acyl juglone (**3d**) and C18:1-acyl juglone (**3f**), which are the strongest inhibitors of mammalian pols κ, λ and pols α, γ, respectively (Fig. 2), are 20.54 × 5.13 and 25.49 × 7.04 Å, respectively; therefore, the three-dimensional length and width of these compounds might be important for the inhibition of mammalian pol species.

3. Conclusion

Recently, we found that vitamin K_3, but not K_1 or K_2, suppressed inflammation in in vitro cell culture experiments and in vivo animal experiments.[10] Since vitamin K_3 (2-methyl-1,4-naphthoquinone) has a 1,4-naphthoquinone backbone, this structure must be important for the abovementioned activities. Although the vitamin K group of vitamins shares a methylated 1,4-naphthoquinone ring structure, the aliphatic side chains attached to the 3-positions of vitamins K_1 and K_2 may reduce their pol inhibitory and anti-inflammatory activities.[10] 5-Hydroxy-1,4-naphthoquinone (juglone) conjugated with fatty acid (i.e., 5-*O*-acyl juglone) was found to be a stronger inhibitor of mammalian pols, human cancer cell growth and inflammation than other 1,4-naphthoquinone based compounds, such as vitamin K_3 and juglone; therefore, 5-*O*-acyl juglones could be chemotherapy agents for anti-cancer and/or anti-inflammation based on mammalian pol inhibition.

4. Experimental procedure

4.1. Materials

A chemically synthesized DNA template, such as poly(dA), was purchased from GE Healthcare Bio-Sciences (Little Chalfont, UK). The oligo$(dT)_{18}$ DNA primer was customized by Sigma–Aldrich Japan K.K. (Hokkaido, Japan). The synthesized radioactive nucleotides, such as [^{3}H]-deoxythymidine 5′-triphosphate (dTTP) (43 Ci/mmol), were obtained from MP Biomedicals LLC (Solon, OH, USA). All other reagents were of analytical grade and purchased from Nacalai Tesque Inc. (Kyoto, Japan).

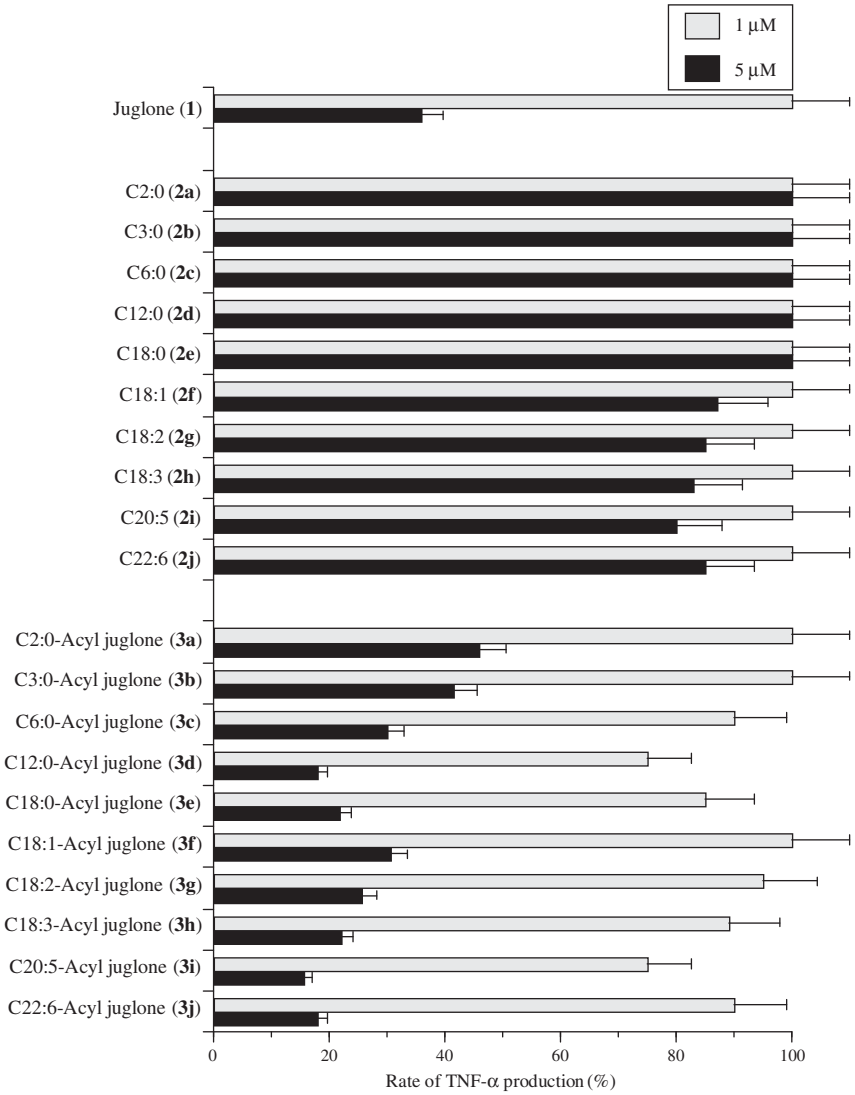


Figure 4. Inhibitory effects of juglone (**1**), fatty acids (**2a**–**j**) and 5-*O*-acyl juglones (**3a**–**j**) on LPS-induced production of TNF-α in the mouse macrophage cell line RAW264.7. These cells were pretreated with 1 and 5 μM of each compound as a vehicle control (TNF-α level, 43 pg/mL) for 30 min and then treated with 100 ng/mL LPS for 24 h (LPS-evoked TNF-α level, 538 pg/mL). The TNF-α concentration in the cell medium was measured by ELISA. The relative effect in the absence of the compound was taken as 100%. Data are shown as the mean ± SE (n = 4).

4.2. Preparation of enzymes

Pol α was purified from the calf thymus by immuno-affinity column chromatography, as described by Tamai et al.[27] Recombinant rat pol β was purified from *E. coli* JMpβ5, as described by Date et al.[28] The human pol γ catalytic gene was cloned into pFastBac (Invitrogen Japan K.K., Tokyo Japan). Histidine-tagged enzyme was expressed using the BAC-TO-BAC HT Baculovirus Expression System according to the supplier's manual (Life Technologies, MD, USA) and was purified with ProBoundresin (Invitrogen Japan K.K.).[29] Human pols δ and ε were purified by nuclear fractionation of human peripheral blood cancer cells (Molt-4) using the second subunit of pols δ and ε-conjugated affinity column chromatography, respectively.[30] A truncated form of human pol η (residues 1–511), tagged with His_6 at its C-terminus, was expressed in *E. coli* cells and was purified as described by Kusumoto et al.[31] A recombinant mouse pol ι, tagged with His_6 at its C-terminus, was expressed and purified by Ni-NTA column chromatography as described elsewhere (Masutani et al. in preparation). A truncated form of pol κ (residues 1–560) tagged with His_6 at its C-terminus was overproduced in *E. coli* and purified as described by Ohashi et al.[32] Recombinant human His-pol λ was overexpressed and purified according to a method described by Shimazaki et al.[33] Recombinant human His-pol μ was overexpressed in *E. coli* BL21 and purified by Glutathione SepharoseTM 4B (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA) column chromatography (Maezawa et al. in preparation). Pol α from a higher plant, cauliflower inflorescence, was purified according to the method outlined by Sakaguchi et al.[34] Recombinant rice (*Oryza sativa* L. cv. Nipponbare) pol λ tagged with His_6 at the C-terminus was expressed in *E. coli* and purified as described by Uchiyama et al.[35] Calf thymus TdT and bovine pancreas deoxyribonuclease I were obtained from Stratagene Cloning Systems (La Jolla, CA, USA). The Klenow fragment of pol I from *E. coli* and human immunodeficiency virus type-1 (HIV-1) reverse transcriptase were purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). *Taq* pol, T4 pol, T7 RNA polymerase, and T4 polynucleotide kinase were purchased from Takara Bio (Tokyo, Japan).

4.3. DNA polymerase assays

The reaction mixtures for pol α, pol β, plant pols and prokaryotic pols have been described previously.[36,37] Those for pol γ, and pols δ and ε were as described by Umeda et al.[29] and Ogawa et

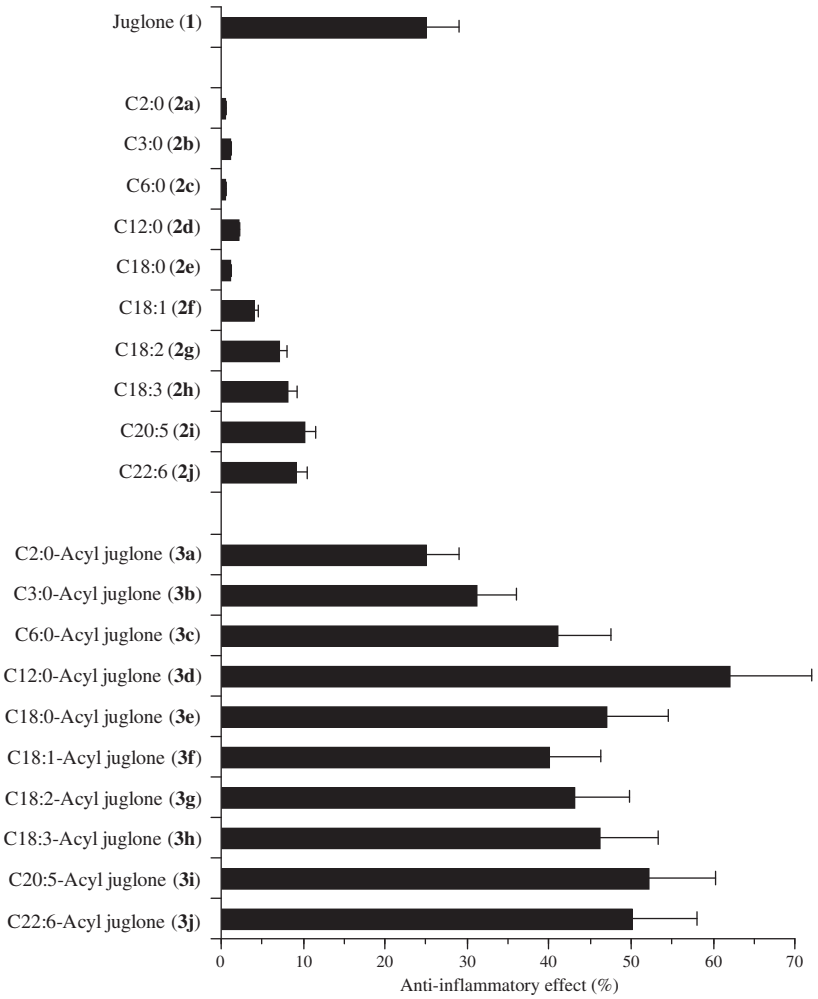


Figure 5. Anti-inflammatory activity of juglone (**1**), fatty acids (**2a**–**j**) and 5-*O*-acyl juglones (**3a**–**j**) toward TPA-induced edema on mouse ear. Each compound (500 μg) was applied individually to one ear of a mouse, and after 30 min TPA (0.5 μg) was applied to both ears. Edema was evaluated after 7 h. The inhibitory effect is expressed as the percentage of edema. Data are shown as the means ± SE (n = 6).

al.[38] respectively. The reaction mixtures for pols η, ι and κ were the same as for pol α, and the reaction mixture for pols λ and μ was the same as for pol β. For pols, poly(dA)/oligo(dT)$_{18}$ (A/T = 2/1) and dTTP were used as the DNA template–primer and nucleotide (i.e., dNTP) substrate, respectively. For TdT, oligo(dT)$_{18}$ (3′-OH) and dTTP were used as the DNA primer and nucleotide substrate, respectively. For HIV-1 reverse transcriptase, poly(rA)/oligo(dT)$_{18}$ (A/T = 2/1) and dTTP were used as the template–primer and nucleotide substrate, respectively.

Each compound was dissolved in distilled dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 s. Aliquots of 4 μL sonicated samples were mixed with 16 μL of each enzyme (final amount 0.05 units) in 50 mM Tris–HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol and 0.1 mM ethylenediamine tetraacetic acid (EDTA), and kept at 0 °C for 10 min. These inhibitor–enzyme mixtures (8 μL) were added to 16 μL of each of the enzyme standard reaction mixtures, and incubation was carried out at 37 °C for 60 min, except for *Taq* pol, which was incubated at 74 °C for 60 min. Activity without the inhibitor was considered 100%, and the remaining activity at each concentration of the inhibitor was determined relative to this value. One unit of pol activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol dNTP (i.e., dTTP) into synthetic DNA template–primers in 60 min at 37 °C under the normal reaction conditions for each enzyme.[33,34]

4.4. Other DNA metabolic enzymes assays

The activities of calf primase of pol α, T7 RNA polymerase, mouse IMP dehydrogenase (type II), T4 polynucleotide kinase and bovine deoxyribonuclease I were measured in standard assays according to the manufacturer's specifications, as described by Tamiya-Koizumi et al.[39] Nakayama and Saneyoshi,[40] Mizushina et al.[41] Soltis et al.[42] and Lu and Sakaguchi,[43] respectively.

4.5. Cell culture and measurement of cell viability

The cultured human cancer cell line, HCT116 (colon carcinoma cells), was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human cancer cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 mg/mL). HCT116 cells were cultured at 37 °C in a humid atmosphere of 5% CO_2/95% air. For the cell growth assay, the cells were plated at 1×10^4 cells into each well of 96-well microplates with various concentrations of the isolated compounds. These compounds were dissolved in DMSO at a concentration of 10 mM as a stock solution. The stock solutions were diluted to the appropriate final concentrations with growth medium as 0.5% DMSO just before use. Cell viability was determined by WST-1 assay.[44]

Table 2
C log *P* values and molecular length and width of the three-dimensional structure of juglone (**1**), fatty acids (**2a–j**) and 5-*O*-acyl juglones (**3a–j**)

Compound	*C* log *P*[a]	Length[b] (Å)	Width[b] (Å)
Juglone (**1**)	0.52	5.15	5.23
*Fatty acid (**2**)*			
2a	−0.07	2.41	1.97
2b	0.39	3.73	2.24
2c	1.76	7.41	1.95
2d	4.49	14.94	2.01
2e	7.23	22.48	2.02
2f	6.52	20.61	4.47
2g	5.80	16.42	7.00
2h	5.65	15.46	8.05
2i	5.85	9.91	8.51
2j	6.36	9.19	8.51
*5-O-Acyl juglone (**3**)*			
3a	0.50	8.25	5.11
3b	1.15	9.50	5.07
3c	2.40	13.17	5.12
3d	4.91	20.54	5.13
3e	7.41	28.11	5.13
3f	7.09	25.49	7.04
3g	6.77	21.58	8.08
3h	6.45	20.78	8.43
3i	6.65	16.51	7.17
3j	7.16	14.53	8.65

[a] The *C* log *P* values were obtained using CS ChemDraw version 5.0 software.
[b] The length and width were calculated using CS Chem3D version 5.0 software.

4.6. Measurement of TNF-α level in the cell culture medium of mouse macrophages

A mouse macrophage cell line, RAW264.7, was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, U.S.). The cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 4.5 g glucose per liter plus 10% fetal calf serum, 5 mM L-glutamine, 50 units/mL penicillin and 50 units/mL streptomycin. The cells were cultured at 37 °C in standard medium in a humidified atmosphere of 5% CO_2–95% air.

RAW264.7 cells were placed in a 12-well plate at 5×10^4 cells/well and incubated for 24 h. The cells were pretreated with the compounds (final concentrations of 1 and 5 μM) for 30 min before the addition of 100 ng/mL of LPS. After stimulation with LPS for 24 h, the cell culture medium was collected to measure the amount of TNF-α secreted. The concentration of TNF-α in the culture medium was quantified by using a commercially available enzyme-linked immunosorbent assay (ELISA) development system (Bay Bioscience Co., Ltd, Kobe, Japan) in accordance with the manufacturer's protocol.

4.7. TPA-induced anti-inflammatory assay in mouse

The mouse inflammatory test was performed according to Gschwendt's method.[45] In brief, an acetone solution containing the compounds (500 μg in 20 μL) or 20 μL acetone as a vehicle control was applied to the inner part of the mouse ear. Thirty minutes after the test compound had been applied, a TPA solution (0.5 μg/20 μL of acetone) was applied to the same part of the ear. Acetone, followed by TPA solution, was applied to the other ear of the same mouse as a control. After 7 h, a disk (6 mm diameter) was obtained from the ear and weighed. The inhibitory effect (IE) is presented as the ratio of the increase in weight of the ear disks:

$$\mathrm{IE} = [(\text{TPA only}) - (\text{tested compound} + \text{TPA})]/[(\text{TPA only}) - (\text{vehicle})] \times 100$$

4.8. Molecular modeling

Structures were minimized by using the molecular dynamics and MM2 force field incorporated in Chem3D version 5.0 software (Cambridge Soft, USA). To obtain theoretical molecular properties, further semiempirical minimization with MOPAC (Chem3D) was carried out. For semiempirical calculations, the PM3 method was used for compounds **2a–h**. The AM1 method was used for compounds **1**, **2i**, **2j**, and **3a–j**.

4.9. Instrumental analyses

^{1}H and ^{13}C NMR spectra were recorded on a JEOL 270 MHz spectrometer (EX-270 W). Chemical shifts are expressed in δ (ppm) relative to Me_4Si or the residual solvent resonance, and coupling constants (*J*) are expressed in Hz. Melting point (Mp) data were determined with a Yanaco MP-3S instrument and are uncorrected. Infrared (IR) spectra were recorded on a Horiba FT210 spectrometer, using NaCl (neat) or KBr pellet (solid). Mass spectra (MS) for compound **3b–d**, **3h**, and **3i** were obtained on an Applied Biosystems mass spectrometer (API QSTAR pulsar i). Mass spectra (MS) for compound **3e–3g** and **3j** were obtained on JEOL's high-resolution double-focusing mass spectrometer, the JMS-700 ('MStation'). Analytical thin-layer chromatography was performed on Silica Gel 60 F254 plates (Merck, Germany). Flash chromatography was carried out on PSQ 100B (Fuji Silysia Co., Japan).

4.10. General procedure for the preparation of 5-*O*-acyl juglones using MNBA

To a solution of juglone (1 equiv) and carboxylic acid (1.2 equiv) in CH_2Cl_2, Et_3N (2.2 equiv), MNBA (1.2 equiv) and DMAP (0.1 equiv) were added at room temperature. After the mixture had been stirred for 24 h, the reaction was quenched by the addition of H_2O. The resulting mixture was extracted with $CHCl_3$. The combined extracts were washed with brine, dried (Na_2SO_4), and concentrated in vacuo. The residue was purified by silica gel chromatography using hexanes/ethyl acetate as the eluent.

4.10.1. 5-*O*-Propionyloxy-1,4-naphthoquinone (3b)

The title compound was prepared in 64% yield according to the general procedure described above. Yellow solid; Mp = 113–114 °C; IR (KBr) 2972, 1757, 1662, 1601, 1452, 1367, 1329, 1290, 1234, 1142, 1038, 1009, 926, 872, 781 cm^{-1}; ^{1}H NMR (270 MHz, $CDCl_3$) δ 8.05 (dd, *J* = 7.6 Hz, 1.4 Hz, 1H), 7.76 (t, *J* = 7.6 Hz, 1H), 7.39 (dd, *J* = 7.6 Hz, 1.4 Hz, 1H), 6.94 (d, *J* = 10.3 Hz, 1H), 6.84 (d, *J* = 10.3 Hz, 1H), 2.78 (q, *J* = 7.6 Hz, 2H), 1.33 (t, *J* = 7.6 Hz, 3H); ^{13}C NMR (68 MHz, $CDCl_3$) δ 184.0, 183.4, 172.6, 149.4, 139.7, 137.1, 134.7, 133.4, 129.6, 124.8, 123.2, 27.7, 8.8; HRMS, calcd for $C_{13}H_{10}O_4Na$ ($[M+Na]^+$) 253.0471, found 253.0455.

4.10.2. 5-*O*-Hexanoyloxy-1,4-naphthoquinone (3c)

The title compound was prepared in 76% yield according to the general procedure described above. Ocher solid; Mp = 44–46 °C; IR (KBr) 2951, 2866, 1768, 1662, 1597, 1456, 1375, 1331, 1286, 1230, 1120, 1099, 1043, 939, 889, 849, 791 cm^{-1}; ^{1}H NMR (270 MHz, $CDCl_3$) δ 8.04 (dd, *J* = 7.8 Hz, 1.4 Hz, 1H), 7.76 (t, *J* = 7.8 Hz, 1H), 7.38 (dd, *J* = 7.8 Hz, 1.4 Hz, 1H), 6.94 (d, *J* = 10.5 Hz, 1H), 6.84 (d, *J* = 10.5 Hz, 1H), 2.74 (t, *J* = 7.3 Hz, 2H), 1.83 (quin, *J* = 7.3 Hz, 2H), 1.51–1.34 (br m, 4H), 0.95 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (68 MHz, $CDCl_3$) δ 183.9, 183.4, 171.9, 149.4, 139.7, 137.1, 134.6, 133.3, 129.7, 124.7, 123.2, 34.2, 31.3, 24.1, 22.4, 14.0; HRMS, calcd for $C_{16}H_{16}O_4Na$ ($[M+Na]^+$) 295.0940, found 295.0937.

4.10.3. 5-*O*-Dodecanoyloxy-1,4-naphthoquinone (3d)

The title compound was prepared in 62% yield according to the general procedure described above. Yellow solid; Mp = 63–65 °C; IR (KBr) 3076, 2922, 2856, 1766, 1666, 1599, 1375, 1333, 1296, 1232, 1134, 1111, 993, 939, 895, 783 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 8.04 (dd, J = 7.6 Hz, 1.4 Hz, 1H), 7.75 (t, J = 7.6 Hz, 1H), 7.37 (dd, J = 7.6 Hz, 1.4 Hz, 1H), 6.93 (d, J = 10.5, 1H), 6.84 (d, J = 10.5, 1H), 2.74 (t, J = 7.6 Hz, 2H), 1.82 (quin, J = 7.6 Hz, 2H), 1.47–1.28 (br m, 16H), 0.88 (t, J = 6.8 Hz, 3H); ^{13}C NMR (68 MHz, $CDCl_3$) δ 184.0, 183.4, 171.9, 149.4, 139.7, 137.1, 134.6, 133.3, 129.7, 124.7, 123.2, 34.2, 31.9, 29.6 (2C), 29.5, 29.4, 29.3, 29.2, 24.5, 22.7, 14.2; HRMS, calcd for $C_{22}H_{28}O_4Na$ ($[M+Na]^+$) 379.1879, found 379.1914.

4.11. General procedure for the preparation of 5-*O*-acyl juglones via acyl chlorides

To a solution of carboxylic acid (1 equiv) in CH_2Cl_2, oxalyl chloride (3.1 equiv) was added at 0 °C, and the mixture was stirred at room temperature for 6 h. The solvent was removed to yield crude acyl chloride.

A solution of the acyl chloride (1 equiv), juglone (3 equiv) and DMAP (0.1 equiv) in pyridine was stirred at room temperature. After the mixture had been stirred for 3 h, the reaction was quenched by the addition of 1 M aqueous HCl solution. The resulting mixture was extracted with EtOAc. The extract was washed with brine, dried (Na_2SO_4), and concentrated in vacuo. The residue was purified by silica gel chromatography using hexanes/ethyl acetate as the eluent.

4.11.1. 5-*O*-Octadecanoyloxy-1,4-naphthoquinone (3e)

The title compound was prepared in 24% yield according to the general procedure described above. Yellow solid; Mp = 72–75 °C; IR (KBr) 3074, 2924, 2852, 1763, 1666, 1597, 1460, 1371, 1331, 1292, 1144, 1107, 1036, 939, 897 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 8.04 (dd, J = 7.8 Hz, 1.4 Hz, 1H), 7.76 (t, J = 7.8 Hz, 1H), 7.38 (dd, J = 7.8 Hz, 1.4 Hz, 1H), 6.94 (d, J = 10.3 Hz, 1H), 6.84 (d, J = 10.3 Hz, 1H), 2.74 (t, J = 7.8 Hz, 2H), 1.82 (quin, J = 7.8 Hz, 2H), 1.33–1.25 (br m, 28H), 0.88 (t, J = 7.0 Hz, 3H); ^{13}C NMR (68 MHz, $CDCl_3$) δ 184.1, 183,5, 172.0, 149.5, 139.8, 137.2, 134.7, 133.4, 129.7, 124.8, 123.3, 34.3, 32.0, 29.8 (5C), 29.7 (2C), 29.7, 29.6, 29.4, 29.4, 29.2, 24.5, 22.8, 14.2. HRMS, calcd for $C_{28}H_{40}O_4$ (M^+) 440.2927, found 440.2941.

4.11.2. 5-*O*-{(*Z*)-Octadecenoyloxy}-1,4-naphthoquinone (3f)

The title compound was prepared in 29% yield according to the general procedure described above. Brown oil; IR (neat) 3005, 2924, 2856, 1766, 1668, 1599, 1456, 1327, 1284, 1113, 1036, 941, 850 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 8.05 (dd, J = 8.1 Hz, 1.4 Hz, 1H), 7.76 (t, J = 8.1 Hz, 1H), 7.38 (dd, J = 8.1 Hz, 1.4 Hz, 1H), 6.94 (d, J = 10.3 Hz, 1H), 6.84 (d, J = 10.3 Hz, 1H), 5.39–5.34 (m, 2H), 2.74 (t, J = 7.8 Hz, 2H), 2.03 (br m, 4H), 1.82 (quin, J = 7.8 Hz, 2H), 1.45–1.26 (br m, 20H), 0.88 (t, J = 7.0 Hz, 3H); ^{13}C NMR (68 MHz, $CDCl_3$) δ 184.1, 183.5, 172.0, 149.5, 139.8, 137.2, 134.7, 133.4, 129.9, 129.7, 129.7, 124.8, 123.2, 34.3, 32.0, 29.8, 29.8, 29.6, 29.4 (2C), 29.3, 29.2 (2C), 27.3, 27.3, 24.5, 22.8, 14.2. HRMS, calcd for $C_{28}H_{38}O_4$ (M^+) 438.2770, found 438.2770.

4.11.3. 5-*O*-Linoleoyloxy-1,4-naphthoquinone (3g)

The title compound was prepared in 31% yield according to the general procedure described above. Brown oil; IR (neat) 3008, 2925, 2858, 1768, 1670, 1599, 1456, 1329, 1286, 1113, 1038, 941, 852 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 8.04 (dd, J = 7.8 Hz, 1.4 Hz, 1H), 7.76 (t, J = 7.8 Hz, 1H), 7.38 (dd, J = 7.8 Hz, 1.4 Hz, 1H), 6.94 (d, J = 10.3 Hz, 1H), 6.84 (d, J = 10.3 Hz, 1H), 5.39 (m, 4H), 2.80–2.71 (m, 2H), 2.74 (t, J = 8.1 Hz, 2H), 2.06–2.04 (m, 4H), 1.82 (quin, J = 7.3 Hz, 2H), 1.48–1.26 (br m, 14H), 0.89 (t, J = 7.0 Hz, 3H); ^{13}C NMR (68 MHz, $CDCl_3$) δ 184.1, 183.5, 171.9, 149.5, 139.8, 137.2, 134.7, 133.4, 130.1, 130.0, 129.7, 127.9, 127.8, 124.8, 123.2, 34.2, 31.6, 29.7, 29.4, 29.3, 29.2 (3C), 27.3, 25.7, 24.5, 22.7, 14.2. HRMS, calcd for $C_{28}H_{36}O_4$ (M^+) 436.2614, found 436.2632.

4.11.4. 5-*O*-Linolenoyloxy-1,4-naphthoquinone (3h)

The title compound was prepared in 34% yield according to the general procedure described above. Brown oil; IR (neat) 3012, 2927, 2858, 1766, 1670, 1601, 1454, 1327, 1284, 1114, 1037, 939, 852 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 8.04 (dd, J = 7.8 Hz, 1.4 Hz, 1H), 7.76 (t, J = 7.8 Hz, 1H), 7.38 (dd, J = 7.8 Hz, 1.4 Hz, 1H), 6.94 (d, J = 10.3 Hz, 1H), 6.84 (d, J = 10.3 Hz, 1H), 5.37 (m, 6H), 2.84–2.71 (br m, 4H), 2.74 (t, J = 7.8 Hz, 2H), 2.08 (br m, 4H), 1.82 (quin, J = 7.8 Hz, 2H), 1.36 (br m, 8H), 0.98 (t, J = 7.6 Hz, 3H); ^{13}C NMR (68 MHz, $CDCl_3$) δ 184.0, 183.4, 171.9, 149.4, 139.8, 137.2, 134.7, 133.4, 131.8, 130.2, 129.7, 128.2, 128.1, 127.6, 127.0, 124.8, 123.2, 34.2, 29.7, 29.3, 29.2 (2C), 27.3, 25.7, 25.6, 24.6, 20.6, 14.4. HRMS, calcd for $C_{28}H_{34}O_4Na$ ($[M+Na]^+$) 457.2349, found 457.2335.

4.11.5. 5-*O*-Eicosapentaenoyloxy-1,4-naphthoquinone (3i)

The title compound was prepared in 54% yield according to the general procedure described above. Brown oil; IR (neat) 3012, 2962, 1768, 1668, 1599, 1450, 1327, 1284, 1203, 1117, 1038, 937, 891, 781, 715 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 8.05 (dd, J = 8.0 Hz, 1.1 Hz, 1H), 7.76 (t, J = 8.0 Hz, 1H), 7.38 (dd, J = 8.0 Hz, 1.1 Hz, 1H), 6.94 (d, J = 10.3 Hz, 1H), 6.84 (d, J = 10.3 Hz, 1H), 5.50–5.26 (br m, 10H), 2.86–2.73 (br m, 10H), 2.26 (q, J = 7.0 Hz, 2H), 2.07 (quin, J = 7.0 Hz, 2H), 1.91 (quin, J = 7.6 Hz, 2H), 0.97 (t, J = 7.6 Hz, 3H); ^{13}C NMR (68 MHz, $CDCl_3$) δ 184.0, 183.4, 171.7, 149.4, 139.8, 137.2, 134.7, 133.4, 131.9, 129.7, 128.9, 128.8, 128.4, 128.1 (2C), 128.0, 127.9, 127.7, 126.9, 124.8, 33.6, 26.6, 25.7 (3C), 25.7, 25.6, 24.4, 20.6, 14.4; HRMS, calcd for $C_{30}H_{34}O_4Na$ ($[M+Na]^+$) 481.2349, found 481.2366.

4.11.6. 5-*O*-Docosahexaenoyloxy-1,4-naphthoquinone (3j)

The title compound was prepared in 62% yield according to the general procedure described above. Brown oil; IR (neat) 3012, 2964, 2924, 1768, 1670, 1601, 1450, 1363, 1327, 1284, 1225, 1119, 1038, 935, 850 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 8.05 (dd, J = 8.0 Hz, 1.4 Hz, 1H), 7.76 (t, J = 8.0 Hz, 1H), 7.38 (dd, J = 8.0 Hz, 1.4 Hz, 1H), 6.94 (d, J = 10.3 Hz, 1H), 6.84 (d, J = 10.3 Hz, 1H), 5.41 (m, 12H), 2.92–2.76 (m, 12H), 2.59 (q, J = 7.0 Hz, 2H), 2.07 (quin, J = 7.6 Hz, 2H), 0.97 (t, J = 7.8 Hz, 3H); ^{13}C NMR (68 MHz, $CDCl_3$) δ 184.0, 183.4, 171.3, 149.4, 139.8, 137.2, 131.2, 134.7, 133.4, 131.9, 129.7, 129.4, 128.4, 128.2, 128.1, 128.1, 128.0, 127.9 (3C), 127.7, 127.7, 126.9, 34.2, 30.4, 25.7, 25.7, 25.7, 25.6, 22.4, 20.6, 14.4. HRMS, calcd for $C_{32}H_{36}O_4$ (M^+) 484.2613, found 484.2632.

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