

A New Family of Synthetic Diterpenes that Regulates Cytokine Synthesis by Inhibiting I κ B α Phosphorylation

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To the memory of Professor Murray Goodman

The synthesis and the biological evaluation of a new family diterpenes are presented. The synthetic studies were inspired by the structural framework of acanthoic acid (**1**) and yielded a family of compounds that were evaluated as anti-inflammatory agents. Among them, compounds **2**, **10**, **12**, and **16** exhibited a very low nonspecific cytotoxicity and inhibited the synthesis of TNF- α with greater than 65% efficacy at low micromolar concentrations. Cytokine-specificity studies revealed that these compounds also inhibited the synthesis of the proinflammatory cytokines IL-1 β and

IL-6, while inhibition of IL-1ra and IL-8 synthesis was marginal and only occurred at high concentrations. Further studies, through EMSA and Western blot analyses, indicated that these compounds decreased the extent of phosphorylation of I κ B α ; this suggests that they exert their anti-inflammatory profile by inhibiting NF- κ B-mediated cytokine synthesis. These findings imply that these diterpenes represent promising leads for the development of novel anti-inflammatory agents.

Introduction

Inflammation is a protective response of the host immune system against microbial invasion and injury. Its duration and magnitude are crucial to survival of the host, since an inadequate response results in immunodeficiency and can lead to infection and cancer.^[1] At the opposite end of the spectrum, persistent or exaggerated immune activity results in the pathogenesis of several diseases, such as rheumatoid arthritis, Crohn's disease, atherosclerosis, diabetes, and myocardial infarction.^[2] In fact, if inflammation becomes systemic, as occurs in sepsis, severe trauma, and major burn injury, it often results in multisystem organ failure and death.

The initiation and resolution of the inflammatory response is mediated by a network of proinflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-8 and IL-12) and anti-inflammatory cytokines (IL-10 and IL-1ra).^[3] Given the significant role of cytokines in both innate and adaptive immunity, their biosynthesis and action are tightly regulated. Despite such elaborate regulation, inappropriate production of cytokines is often seen and has been associated with significant pathology. These observations have led to the identification of cytokines and related signaling pathways as very important therapeutic targets.

At the intracellular level, the production of most cytokines is regulated by a family of heteromeric transcription factors, collectively referred to as nuclear factor-kappa B (NF- κ B).^[4] In its resting state, NF- κ B is sequestered in the cytoplasm, where it remains bound to inhibitory proteins, termed I κ B. Various inflammatory stimuli induce phosphorylation of I κ B leading to dissociation of NF- κ B from its inhibitors. Once separated from

I κ B, NF- κ B translocates into the nucleus, binds to the target DNA, and initiates gene expression of the inflammatory cytokines.^[5]

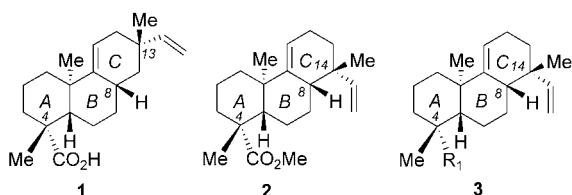
Recent insights into this complex biological event have led to the development of therapeutic strategies aimed at controlling the extent of inflammation by regulating cytokine synthesis.^[6] From a chemistry perspective, these strategies often depart from information on natural products from medicinal herbs, whose extracts have been used in traditional medicines.^[7] One such example is acanthoic acid (**1**; Scheme 1), a novel pimarane diterpene that was isolated from the root bark of *Acanthopanax koreanum* Nakai (Araliaceae).^[8] Crude extracts of this plant have been used in traditional Korean medicine as a tonic and sedative, as well as a remedy for the treatment of

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Scheme 1. Structures of acanthoic acid (**1**) and related analogues.

rheumatism. More recently, studies revealed that **1** suppresses the production of IL-1 β and TNF- α at 10 $\mu\text{g mL}^{-1}$, is orally active, and has no significant toxicity in a rodent model of chronic inflammation.^[9]

Inspired by the medicinal potential of acanthoic acid, we sought to develop a synthetic route to this and related structures with ultimate goal of improving upon the biological effects of the parent molecule.^[10] Our synthetic strategy towards **1** allowed us to access new series of analogues and evaluate the overall structure of the natural product as a function of its activity. These studies led to the identification of a structural motif, represented by **2** (Scheme 1), that differs from the parent molecule only in the composition of the C ring.^[11] Analogues of this family exhibited a low nonspecific cytotoxicity and inhibited TNF- α synthesis at low micromolar concentrations. Among them, the best results were obtained with methyl ester **2**. To further improve upon its activity, we sought to evaluate the role of the ester functionality by studying analogues of generic structure **3**. Herein, we report the synthesis and evaluation of these compounds as modulators of cytokine synthesis.

Results and Discussion

Synthesis of C4 analogues of compound **2**

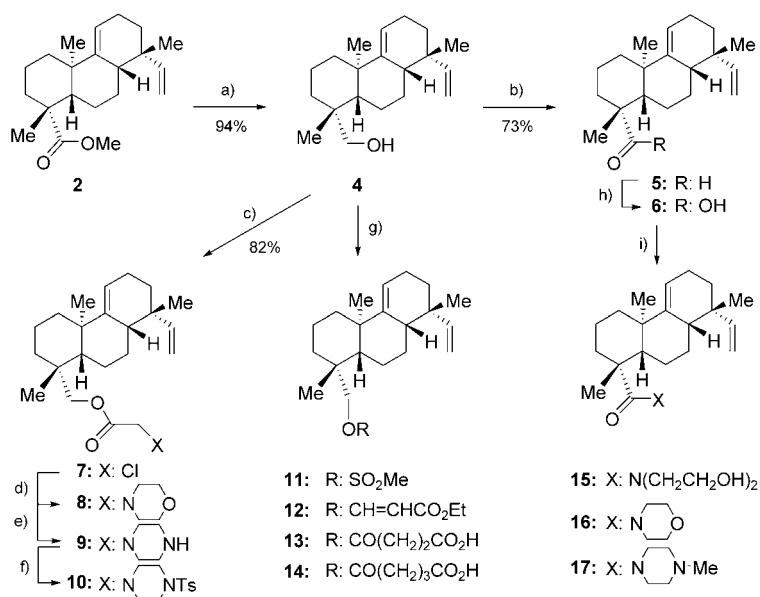
The synthesis of all analogues is highlighted in Schemes 2 and 3, below. Methyl ester **2**, featuring the tricyclic scaffold of acanthoic acid, was synthesized as reported previously^[11] and its structure was further confirmed via X-ray analysis.^[12] Reduction of **2** with diisobutylaluminum hydride (DIBAL-H) produced primary alcohol **4** (94% yield) that was converted to compound **7** upon esterification with chloroacetyl chloride (82% yield, Scheme 2). Treatment of **7** with morpholine and piperazine gave rise to adducts **8** and **9** in 71% and 86% yield, respectively. The latter compound was treated with *p*-toluenesulfonyl chloride to afford the tosylamide **10** in 82% yield. Analogues **11** and **12** were synthesized by treating alcohol **4** with methanesulfonyl chloride and ethyl propiolate, respectively. In a similar manner, treatment of **4** with excess succinic and glutaric anhydride produced the corresponding acids in 78 and 71% yields. Amides **15**–**17** were synthesized by a sequence of reactions that included oxidation of alcohol **4** to acid **6**, conversion of **6** to its acyl chloride and in situ treatment of the latter with excess amine. This sequence

produced the desired amides in three steps and 61–67% combined yield (Scheme 2).

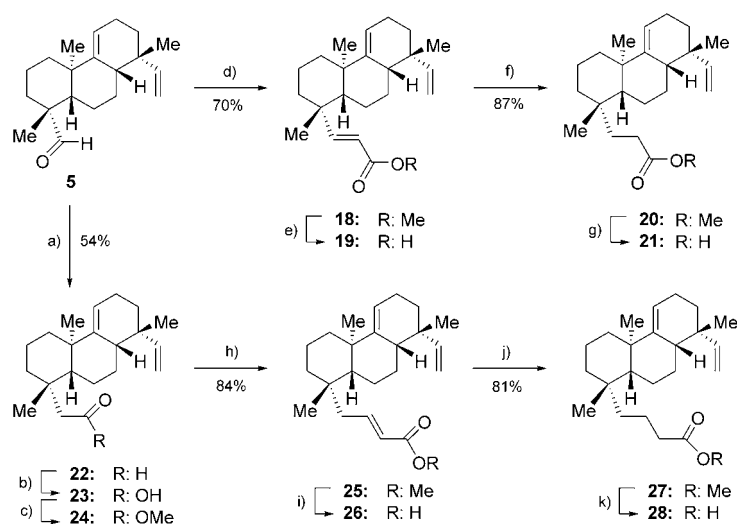
The synthesis of homologated analogues of **2** is shown in Scheme 3. Treatment of aldehyde **5** with MeOCH₂PPh₃Cl and *t*BuOK, followed by acid-induced deprotection of the resulting vinyl ether afforded the one-carbon-extended aldehyde **22** (54% yield). Oxidation of **22** produced carboxylic acid **23** (96% yield) that upon exposure to TMSCHN₂ produced quantitatively methyl ester **24**. Derivative **20**, extended by two carbons, was formed by treating aldehyde **5** with triethyl phosphonoacetate and sodium hydride and reducing the resulting conjugate ester **18** with Mg in methanol (2 steps, 61% yield). Hydrolysis of esters **18** and **20** with lithium hydroxide produced acids **19** and **21**, respectively. Implementation of this strategy to aldehyde **22** allowed the formation of the three-carbon-extended analogues (Scheme 3).

Cytotoxicity and TNF- α inhibition studies

The cytotoxicity of the synthesized compounds was evaluated by using human peripheral blood mononuclear cells (HPBMC). This assay consists of pretreating the cells with various concentrations of the analogues and subsequently evaluating their metabolism and viability by using resazurin-based fluorescence measurements.^[13] Resazurin (Alamar blue) is reduced in mitochondria to a product whose fluorescence intensity is used as an indicator of the cell's energetic capacity and viability.



Scheme 2. Reagents and conditions: a) 4 equiv DIBAL-H, CH₂Cl₂, -78°C, 1 h, 94%; b) 1.3 equiv Dess Martin [O], CH₂Cl₂, 0°C, 3 h, 73%; c) 1.5 equiv chloroacetyl chloride, 2.0 equiv pyridine, DMAP (cat), CH₂Cl₂, 0°C, 2 h, 82%; d) 3.0 equiv morpholine, CH₂Cl₂, reflux, 12 h, 71%; e) 3.0 equiv piperazine, CH₂Cl₂, reflux, 12 h, 86%; f) 1.4 equiv TsCl, 2.0 equiv Et₃N, CH₂Cl₂, 15 h, 25°C, 82%; g) For **11**: 2.0 equiv MsCl, 3.0 equiv pyridine, CH₂Cl₂, 25°C, 3 h, 89%; for **12**: 2.5 equiv 4-methyl morpholine, 1.44 equiv ethyl propiolate, CH₂Cl₂, 0 to 25°C, 15 h, 64%; for **13**: 1.16 equiv succinic anhydride, DMAP (cat), CH₂Cl₂, 25°C, 8 h, 78%; for **14**: 1.2 equiv glutaric anhydride, DMAP (cat), CH₂Cl₂, 25°C, 10 h, 71%; h) 3.0 equiv NaH₂PO₄·H₂O, 3.0 equiv CH₂=C(CH₃)₂, 3.0 equiv NaClO₂, *t*BuOH/H₂O 2/1, 25°C, 1 h, 96%; i) 3.0 equiv (COCl)₂, DMF (cat), 4.0 equiv amine, CH₂Cl₂, 3 h, 25°C, 87% for **15**, 91% for **16**, 95% for **17**.



Scheme 3. Reagents and conditions: a) 5 equiv $\text{MeOCH}_2\text{PPh}_3\text{Cl}$, 4.8 equiv tBuOK , THF, 0.5 h, 25°C; 1.0 equiv p -toluene sulfonic acid, acetone, 0°C, 54%; b) 3.0 equiv $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 3.0 equiv $\text{CH}_3\text{CH}=\text{C}(\text{CH}_3)_2$, 3.0 equiv NaClO_2 , 1 h, 25°C, 96%; c) 5.0 equiv TMSCHN_2 , 5.0 equiv HCl , MeOH , 0°C, 0.5 h, 100%; d) 1.6 equiv NaH , 2.3 equiv triethyl phosphonoacetate, THF, reflux, 16 h, 70%; e) excess LiOH , THF/ H_2O , 60°C, 12 h, 76%; f) 37 equiv Mg , MeOH , 25°C, 12 h, 87%; g) excess LiOH , THF/ H_2O , 60°C, 12 h, 71%; h) 3 equiv $\text{Ph}_3\text{P}=\text{CHCO}_2\text{Me}$, CH_2Cl_2 , 25°C, 15 h, 84%; i) 5 equiv LiOH , THF/ H_2O , 60°C, 12 h, 95%; j) 10 equiv Mg , MeOH , HCl , 25°C, 15 h, 54%; k) 5 equiv LiOH , THF/ H_2O , 65°C, 12 h, 81%.

The cytotoxicity studies were performed at eight different concentrations of analogues based on a half-log dilution titration (final concentrations: 10, 3.17, and 1 $\mu\text{g mL}^{-1}$, 317, 100, 31.7, 10, and 3.17 ng mL^{-1}). In these experiments, staurosporin at 10 μM was used as a positive control. The data were collected after 28 hours of incubation, and the results are presented as EC_{50} and cytotoxicity efficacy in Table 1.

In a similar manner, we assayed the ability of the synthetic diterpenes to decrease lipopolysaccharide (LPS)-induced $\text{TNF-}\alpha$ production in HPBMC cells (Table 1). It is known that LPS treatment increases the synthesis of $\text{TNF-}\alpha$ in HPBMC cells.^[14] With this in mind, cells were pretreated with our synthetic diterpenes and subsequently incubated with LPS for 4 h. The $\text{TNF-}\alpha$ production was assayed in cell supernatants with a human $\text{TNF-}\alpha$ ELISA kit. These results are presented as IC_{50} and total inhibition efficacy.

The synthesized analogues were evaluated by using the following criteria: a) The IC_{50} value for $\text{TNF-}\alpha$ inhibition should be low and the total inhibition should be greater than 50% and b) inhibition of $\text{TNF-}\alpha$

Table 1. Cytotoxicity and $\text{TNF-}\alpha$ inhibition data of analogues.^[a]

compound (structure)	cytotoxicity (after 28 h)		$\text{TNF-}\alpha$ inhibition (after 4 h)		
	EC_{50} [μM]	Efficacy [%]	IC_{50} [μM]	Efficacy [%]	
1	4: R = H	NT	NA	7.9	38
2	7: R = COCH_2Cl	10.7	92	7.4	94
3	8: R: $\text{COCH}_2\text{-N}$	11.7	99	7.5	98
4	9: R: $\text{COCH}_2\text{-N}$	3.0	99	3.3	99
5	10: R: $\text{COCH}_2\text{-N}$	NT	NA	0.2	65
6	11: R = COCH_2C	7.6	70	4.9	96
7	12: R = COCH_2C	NT	NA	11.4	72
8	13: R = COCH_2C	NT	NA	inactive	NA
9	14: R = COCH_2C	NT	NA	inactive	NA
10	2: X = OMe	5.7	98	2.2	99
11	6: X = OH	25.8	66	18.8	90
12	15: X = $\text{N}(\text{CH}_2\text{CH}_2\text{OH})_2$	8.7	99	3.1	99
13	16: X: N	5.1	99	3.2	99
14	17: X: N	NT	NA	inactive	NA
15	19: R = $\text{CH}=\text{CHCO}_2\text{H}$	NT	NA	inactive	NA
16	21: R = $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$	NT	NA	inactive	NA
17	23: R = $\text{CH}_2\text{CO}_2\text{H}$	NT	NA	inactive	NA
18	24: R = $\text{CH}_2\text{CO}_2\text{OMe}$	6.0	99	3.3	90
19	25: R = $\text{CH}_2\text{CH}=\text{CHCO}_2\text{Me}$	8.1	73	5.1	98
20	26: R = $\text{CH}_2\text{CH}=\text{CHCO}_2\text{H}$	NT	NA	inactive	NA
21	28: R = $\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$	NA	38	6.7	92

[a] Nontoxic (NT) is used when at least 80% of the cells survived after 28 h of incubation with the compound tested at the highest concentration (10 $\mu\text{g mL}^{-1}$). In these cases, the efficacy could not be measured and is not available (NA). "Inactive" is used to indicate that a compound tested at the highest dose (10 $\mu\text{g mL}^{-1}$) did not inhibit $\text{TNF-}\alpha$ synthesis. The efficacy is defined as: $[1 - (\text{fluorescence of reduced resazurin or measured TNF-}\alpha \text{ level} / \text{corresponding value of DMSO control})] \times 100\%$.

α synthesis should occur at concentrations at which the compound does not display a nonspecific cytotoxicity, even after an extended period of incubation (28 h). Using these guidelines we can draw the following conclusions from the data shown in Table 1:

- Comparison of entries 11, 15, 16, 17, 20, and 21 suggests that homologation of the C4 carboxylic acid results in compounds that are inactive. This trend is also observed for the extended carboxylic acids **13** and **14** (entries 8 and 9).
- Comparison of the different esters suggests that homologation decreases the ability of these compounds to suppress TNF- α synthesis (higher IC₅₀ values). Among the esters, the most promising compound is the C4 methyl ester **2** (entry 10).
- Comparison of the C4 carboxylic acid derivatives (entries 10–14) indicates that amides **15** and **16** display comparable biological activities with ester **2**. Compounds **2**, **15**, and **16** are superior to carboxylic acid **6** in terms of TNF- α synthesis inhibition and overall efficacy.
- Comparison of the C17 hydroxyl group derivatives (entries 1–9) indicates that compounds **10** and **12** are the most potent, since they are not toxic and inhibit TNF- α synthesis with greater than 50% overall efficacy.

The above results suggest that across all series compounds **2**, **10**, **12**, and **16** show a promising activity. They were therefore selected for further evaluation in suppressing the production of other cytokines.

Cytokine inhibition studies

The recent availability of pure recombinant cytokines and molecular probes for their genes has led to a better understanding of their role in regulating cell activities.^[3] It is now clear that cytokines have a broad range of properties including pleiotropism (a given cytokine exerts different effects in different cells), redundancy (two or more cytokines mediate similar functions), synergism (the combined effect of two cytokines is greater than the additive effect of each individual protein), and antagonism (the effect of one cytokine inhibits the effect of another). The complexity of such concurrent messages that any one cell can receive may result in a multiplicity of checks and balances that limit the duration and extent of the inflammatory response. Along these lines, it has been suggested that a simultaneous inhibition of multiple proinflammatory cytokines is more beneficial for the control of chronic inflammation. For example, simultaneous inhibition of the proinflammatory cytokines TNF- α and IL-1 β was shown to reduce dramatically inflammation in patients with rheumatoid arthritis.^[15]

To study the profile of our analogues with respect to cytokine inhibition, we compared their ability to modulate the production of the proinflammatory cytokines TNF- α , IL-1 β , IL-8, and IL-6 and that of the anti-inflammatory cytokine IL-1ra. The experiments were performed by using the Cytosets assay kits. The levels of different cytokines were measured from HPBMC cells treated with the above analogues and subsequently stimulated with LPS. These results are expressed in percentage of cytokine inhibition (Figure 1).

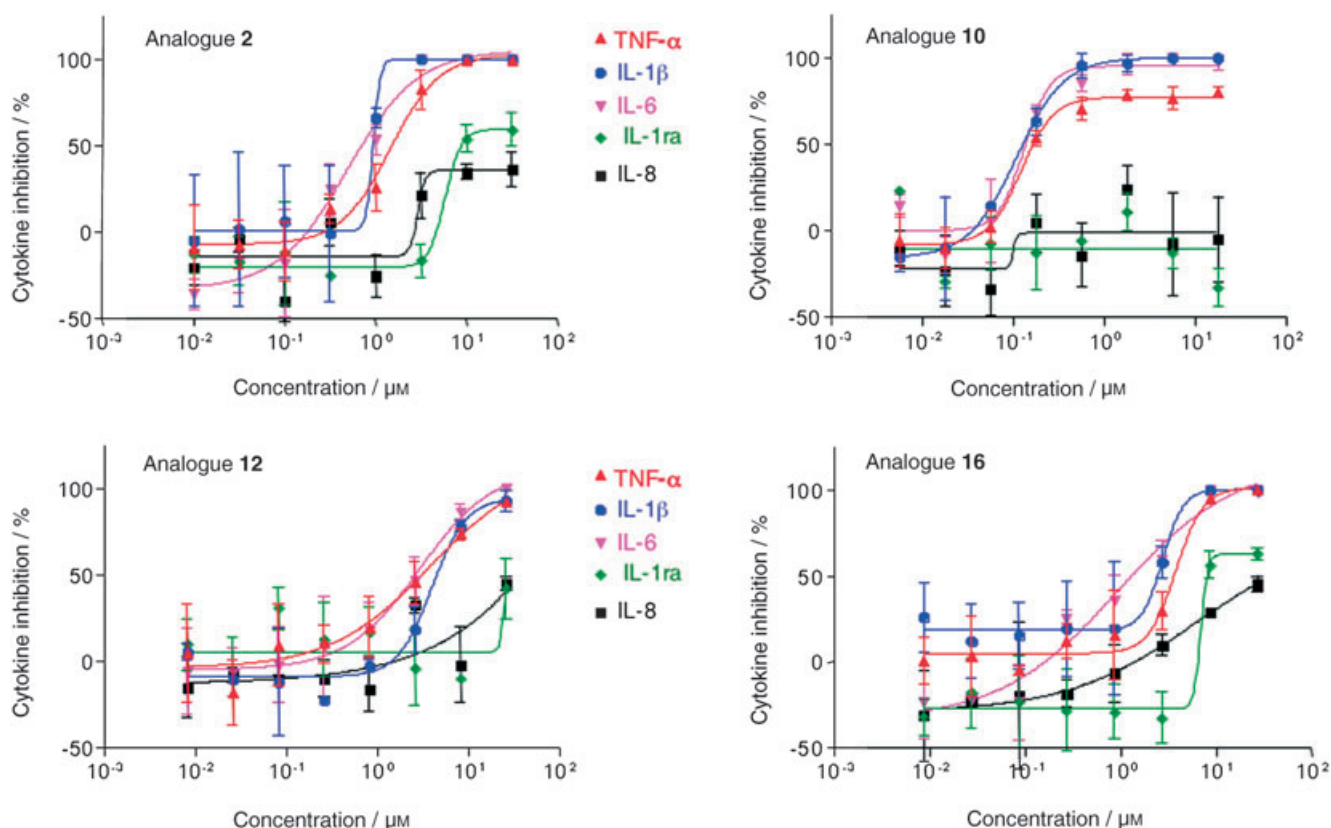


Figure 1. Cytokine selectivity for compounds **2**, **10**, **12**, and **16**.

All compounds showed a dose-dependent inhibition of TNF- α , IL-1 β , and IL-6 synthesis, while in most cases the synthesis of IL-1ra and IL-8 was inhibited only at high concentrations. Administration of 1 μ M of analogue **2** resulted in about 30% inhibition of TNF- α and 60% inhibition of IL-1 β and IL-6. At this concentration, the production levels of IL-1ra and IL-8 were not affected. At higher concentrations of **2** (10 μ M), we observed inhibition of IL-8 and IL-1ra synthesis in the range of 40–50%, while the inhibition of TNF- α , IL-1 β , and IL-6 synthesis reached a plateau between 90–99%.

Analogue **10** showed even more impressive cytokine inhibition data, since it potently inhibited the synthesis of TNF- α , IL-1 β , and IL-6 but did not affect the synthesis of IL-1ra and IL-8 at any concentration up to \sim 18 μ M, which represents the maximum tested concentration. At a concentration of 1 μ M of compound **10**, the inhibition of TNF- α synthesis reached a plateau at about 65%, while IL-1 β and IL-6 were both inhibited by about 95%.

Compound **12** was found to be less active than **2** and **10** since at 1 μ M it inhibited only about 20% of TNF- α and IL-6 synthesis. At this concentration no effect was observed for IL-1 β , IL-1ra, and IL-8 synthesis. At 10 μ M, the inhibition of both IL-1ra and IL-8 was negligible, while the synthesis of TNF- α , IL-1 β , and IL-6 was inhibited by about 80%.

Compound **16** showed similar data with **12**. Incubation with 1 μ M of **16** led to inhibition of IL-1 β and IL-6 synthesis by about 20–50%. At higher concentrations of **16** (10 μ M), TNF- α , IL-1 β , and IL-6 were inhibited by about 90%, while IL-1ra and IL-8 were affected by about 30–50%.

While these data suggest that our compounds inhibit the synthesis of multiple cytokines, it is possible that a potent inhibition of TNF- α and/or IL-1 β might result in inhibition of downstream cytokines like IL-6, IL-8, and IL-1ra. Administration of TNF- α inhibitors has been shown to reduce the levels of IL-1 β and IL-6.^[16,17] In addition, IL-8 release has been shown in vivo to be partially dependent upon TNF- α following LPS administration.^[18] IL-8 levels were also shown to be induced by IL-1 β infusion in primates.^[19] Due to the ability of TNF- α and IL-1 β to induce multiple cytokines, further studies are required to determine the selectivity of these compounds for cytokine inhibition.

Inhibition of NF- κ B activation and I κ B α phosphorylation studies

The induction of most cytokine genes requires activation of a small group of closely related transcription factors, collectively known to as NF- κ B, who play a pivotal role in controlling innate and adaptive immunity.^[4] The NF- κ B pathway is activat-

ed through a series of events that are also mediated by a cascade of kinases, many of which are believed to be unique to that pathway.^[5] In its nonactivated state, NF- κ B is retained in the cytoplasm by interaction with inhibitory proteins, referred to as I κ B.^[20] NF- κ B-activating stimuli lead to phosphorylation of I κ B; the phosphorylated I κ B then undergoes polyubiquitination and is subsequently degraded by the proteasome. This event allows the liberated NF- κ B to translocate to the nucleus, where it binds to its cognate DNA and activates the transcription of the cytokine genes.

In order to investigate the effects of these newly synthesized diterpenes on the NF- κ B pathway, we performed electrophoretic mobility shift assay (EMSA) using specific oligonucleotide probes for the NF- κ B binding regions. As shown in Figure 2,

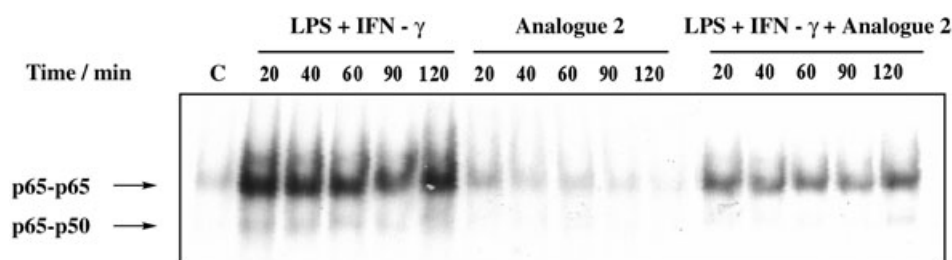


Figure 2. Inhibitory effect on NF- κ B activation in LPS/IFN- γ -stimulated RAW264.7 cells treated with analogue **2**. RAW264.7 cells were pretreated for 15 min with 10 μ M of analogue **2** and then activated for the indicated periods of time with 200 ng mL⁻¹ of LPS and 20 units mL⁻¹ of recombinant murine IFN- γ . C = DMSO control. Nuclear extracts were used to determine the NF- κ B binding activity by EMSA.

the LPS/IFN- γ -dependent activation of NF- κ B, evaluated by EMSAs, was significantly impaired when cells were incubated with analogue **2**. The binding activity was evaluated over a 2 hour period, and binding was decreased by 67% on average, at all time-points analyzed. Because NF- κ B activation is very rapid in these cells (20–30 min after LPS challenge), the lack in activation at later times in cells pretreated with analogue **2** precludes that the effect of this molecule could be attributed to a delayed activation in the pathway. In agreement to this attenuated NF- κ B activity, the degradation of I κ B α and I κ B β was less effective in activated cells treated with analogue **2** (data not shown).

The effects of the selected analogues on the NF- κ B pathway were further evaluated by examining the extent of I κ B α phosphorylation in LPS-stimulated HPBMC cells that were pretreated with compounds **2**, **10**, **12**, and **16** at 10 μ M concentration (Figure 3).^[21] The I κ B α phosphorylation assay was first validated by performing a time-course experiment of LPS stimulation. The optimal condition for LPS-induced I κ B α phosphorylation was then used to evaluate the effect of selected analogues in inhibiting I κ B α phosphorylation. During the course of this study, we also evaluated the cells and did not observe any cell lysis or other morphological changes that might be due to cytotoxicity (data not shown). The Western blot analysis of the phosphorylated I κ B α (p-I κ B α) and total I κ B α is shown in Figure 3. The results demonstrate that all tested analogues inhibited the phosphorylation of I κ B α as compared to the con-

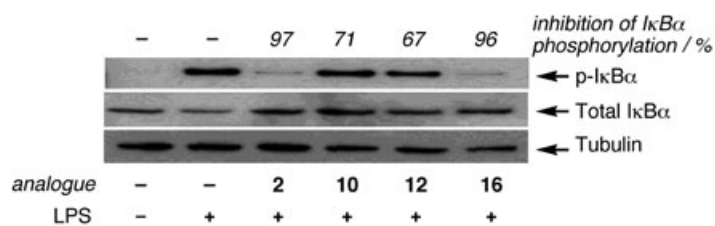


Figure 3. Phosphorylation of $I\kappa B\alpha$ in LPS-stimulated HPBMC treated with analogues **2**, **10**, **12**, and **16**. Cells of HPBMC were pretreated for 1 h with $10\ \mu\text{M}$ of each analogue and then stimulated with $20\ \text{ng mL}^{-1}$ LPS for 1 h. Equal amounts of protein were loaded for Western blot analysis from each cell lysate. DMSO was used as a control (lanes 1 and 2). Lanes 3, 4, 5 and 6 represent analogues **2**, **10**, **12**, and **16**, respectively. The percentage of inhibition of $I\kappa B\alpha$ phosphorylation was comparable to the DMSO/LPS treated cells (lane 2). Anti-tubulin antibody was used to confirm the equal loading of proteins.

trol experiment (DMSO, column 2). The most significant inhibition was observed with compounds **2** and **16** (columns 3 and 6, respectively), followed by **10** and **12**. Specifically, at a concentration of $10\ \mu\text{M}$, compounds **2** and **16** were found to inhibit $I\kappa B\alpha$ phosphorylation by about 96%, while analogues **10** and **12** inhibited this phosphorylation only by 67–71%. These findings suggest a possible mechanism of action involving inhibition of $I\kappa B\alpha$ phosphorylation that is in line with our previous observations on inhibition of cytokine synthesis and EMSA data.

Conclusion

Encouraged by the medicinal potential of acanthoic acid (**1**), we developed a program directed toward the synthesis of related structural motifs (such as **2** and **3**) and their study as novel anti-inflammatory agents. Our synthetic analogues were designed based on the diterpene backbone of the parent structure and aimed to explore the biological significance of the carboxylic acid residue, which is located at the C4 carbon center. Homologation of the carboxylic acid by one, two, or three carbons resulted in considerable loss of the anti-inflammatory properties as evidenced by their decreased ability to inhibit TNF- α synthesis. From the nonhomologated analogues, compounds **2**, **10**, **12**, and **16** at low micromolar concentrations exhibited a very low nonspecific cytotoxicity and inhibited the production of TNF- α with greater than 65% overall efficacy. Cytokine-inhibition studies revealed that the selected compounds also inhibited the synthesis of the proinflammatory cytokines IL-1 β and IL-6, while in most cases IL-8 and the anti-inflammatory cytokine IL-1ra were inhibited marginally and only at high concentrations. Further studies, including EMSA analysis of NF- κ B activation and Western blotting of $I\kappa B\alpha$ phosphorylation status, suggested that these compounds may exert their anti-inflammatory profile by inhibiting NF- κ B-mediated cytokine synthesis. The above findings imply that these compounds and related family members represent promising leads for the development of novel anti-inflammatory agents.

Experimental Section

General techniques: All reagents were commercially obtained (Aldrich, Acros) at highest commercial quality and used without further purification except where noted. Air- and moisture-sensitive liquids and solutions were transferred with a syringe or stainless steel cannula. Organic solutions were concentrated by rotary evaporation below $45\ ^\circ\text{C}$ at approximately 20 mmHg. All nonaqueous reactions were carried out under anhydrous conditions with flame-dried glassware within an argon atmosphere in dry, freshly distilled solvents, unless otherwise noted. Tetrahydrofuran, diethyl ether, dichloromethane, toluene, and benzene were purified by passage through a bed of activated alumina. *N,N*-diisopropylethylamine (DIPEA), diisopropylamine, pyridine, triethylamine (TEA), and boron trifluoride etherate were distilled from calcium hydride prior to use. Dimethyl sulfoxide and dimethylformamide were distilled from calcium hydride under reduced pressure (20 mmHg) and stored over 4 Å molecular sieves until needed. Yields refer to chromatographically and spectroscopically (^1H , ^{13}C NMR) homogeneous materials, unless otherwise stated. Reactions were monitored by thin-layer chromatography carried out on 0.25 mm Merck silica gel plates (60F-254) with UV light as the visualizing agent and 10% ethanolic phosphomolybdic acid (PMA) or *p*-anisaldehyde solution and heat as developing agents. Merck silica gel (60, particle size 0.040–0.063 mm) was used for flash chromatography. Preparative thin-layer chromatography separations were carried out on 0.25 or 0.50 mm Merck silica gel plates (60F-254). NMR spectra were recorded on Varian Mercury 400 and/or Unity 500 MHz instruments and calibrated by using the residual non-deuterated solvent as an internal reference. IR spectra were recorded on a Nicolet 320 Avatar FTIR spectrometer. Optical rotations were recorded on a Jasco P-1010 polarimeter, and values are reported as follows: $[\alpha]_D^{25}$ (c: g per 100 mL, solvent). High-resolution mass spectra (HRMS) were recorded on a VG 7070HS mass spectrometer under chemical ionization (CI) conditions or on a VG ZAB-ZSE mass spectrometer under fast atom bombardment (FAB) conditions. X-ray data were recorded on a Bruker SMART APEX 3 kW sealed tube X-ray diffraction system.

Alcohol 4: A solution of ester **2** (301 mg, 0.95 mmol) in CH_2Cl_2 (5 mL) was cooled at $-78\ ^\circ\text{C}$ and treated with 4.0 equiv DIBAL-H (3.8 mL, 3.8 mmol, of 1.0 M in CH_2Cl_2). After the solution had been stirred for 30 min at $-78\ ^\circ\text{C}$, the reaction was quenched with methanol (0.5 mL). The solution was diluted with ethyl acetate (10 mL), allowed to warm to $25\ ^\circ\text{C}$, and stirred for 1 h with a saturated solution of Rochelle salt (10 mL). The mixture was extracted with ethyl ether ($3 \times 10\ \text{mL}$), and the organic layers were collected, dried (MgSO_4), and concentrated under vacuum. The residue was purified by column chromatography (silica, 2 to 10% diethyl ether in hexanes) to give alcohol **4** (256 mg, 0.89 mmol, 94%) as a white solid. $R_f = 0.55$ (30% Et_2O /hexane); $[\alpha]_D^{25} = +70$ (c = 1.3, benzene); ^1H NMR (400 MHz, CDCl_3): $\delta = 5.95$ (dd, $J = 11.2\ \text{Hz}$, $J = 12\ \text{Hz}$, 1H), 5.46 (m, 1H), 4.98 (t, $J = 8\ \text{Hz}$, 2H), 3.82 (d, $J = 12\ \text{Hz}$, 1H), 3.52 (d, $J = 12\ \text{Hz}$, 1H), 2.07–1.57 (m, 6H), 1.51–1.42 (m, 5H), 1.27–1.23 (m, 6H), 1.05 (s, 3H), 1.02 (s, 3H), 0.95 (s, 3H); ^{13}C NMR (CDCl_3 , 100 MHz): $\delta = 150.4$, 142.5, 117.1, 112.3, 64.9, 45.7, 42.1, 41.1, 38.4, 37.5, 35.4, 29.8, 26.3, 26.0, 25.0, 23.4, 19.1, 18.5; IR (film) $\tilde{\nu}_{\text{max}} = 3495$, 2982, 1597, 1436, 1285, 1128 cm^{-1} ; HRMS calcd for $\text{C}_{20}\text{H}_{32}\text{O}$: 311.2351 [$M + \text{Na}^+$]; found 311.2362.

Aldehyde 5: A solution of alcohol **2** (245 mg, 0.85 mmol) in CH_2Cl_2 (5 mL) at $0\ ^\circ\text{C}$ was treated with Dess–Martin periodinane (433 mg, 1.02 mmol) added in three portions over a period of 3 h. After complete consumption of the starting material (TLC chromatography), the reaction was quenched with aqueous saturated sodium

thiosulfate (20 mL). The mixture was extracted with CH_2Cl_2 (3 \times 10 mL) and purified through column chromatography with pure hexane to afford aldehyde **5** (173.8 mg, 0.62 mmol, 73%) as a white solid. $R_f=0.3$ (20% Et_2O /hexane); $[\alpha]_D^{25}=-142$ ($c=1.1$, benzene); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=9.94$ (s, 1H), 5.94 (dd, $J=16.8$, 12 Hz, 1H), 5.53 (d, $J=4.2$ Hz, 1H), 5.01–4.957 (m, 2H), 2.10–2.03 (m, 4H), 1.95–1.86 (m, 3H), 1.71–1.42 (m, 7H), 1.28–1.23 (m, 2H), 1.07 (s, 3H), 1.02 (s, 3H), 0.96 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=206.4$, 148.1, 142.4, 118.1, 112.39, 48.2, 46.6, 41.8, 40.7, 38.2, 37.7, 36.7, 35.1, 25.0, 24.5, 23.9, 23.3, 20.5, 19.6, 18.5; IR (film) $\tilde{\nu}_{\text{max}}=2980$, 1695, 1452, 1361, 1175, 921 cm^{-1} ; HRMS, calcd for $\text{C}_{20}\text{H}_{30}\text{O}$: 287.2375 [$M+\text{H}^+$]; found 287.2392.

Carboxylic acid 6: $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$ (298.6 mg, 2.164 mmol) was added to a solution of aldehyde **3** (207.4 mg, 0.722 mmol) in $t\text{BuOH}/\text{H}_2\text{O}$ (2:1, 6 mL). The reaction mixture was stirred to dissolve completely the salt and then treated at 25 °C with 2-methylbut-2-ene (1.082 mL, 2.164 mmol, 2.0 M in THF) and, after 30 min, with NaClO_2 (195.7 mg, 2.164 mmol). The reaction was completed within 1 h, and at that time the yellow color of the reaction mixture was faded. The reaction mixture was diluted with H_2O (10 mL), and the organic residue was extracted with ethyl acetate (3 \times 10 mL). The organic layers were collected, dried (MgSO_4), and concentrated under vacuum, and the residue was separated by chromatography (silica, 4–10% diethyl ether in hexane) to afford acid **6** (210 mg, 0.69 mmol, 96% yield) as a white solid. $R_f=0.5$ (40% Et_2O); $[\alpha]_D^{25}=-134.2$ ($c=1.1$, benzene); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=5.60$ (dd, $J=17.2$, 10.4 Hz, 1H), 4.97 (dd, $J=10.4$, 2.0 Hz, 1H), 4.76 (dd, $J=17.2$, 2.0 Hz, 2H), 2.2 (d, $J=12.6$ Hz, 1H), 2.0–1.3 (m, 16H), 1.258 (s, 3H), 1.046 (s, 3H), 0.929 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=184.3$, 146.5, 139.0, 129.9, 112.6, 43.8, 41.3, 39.2, 37.6, 37.4, 36.4, 29.8, 28.7, 26.2, 25.0, 20.8, 19.6, 19.0, 18.1, 15.4; IR (film) $\tilde{\nu}_{\text{max}}=3305$, 2987, 1732, 1655, 1070 cm^{-1} ; HRMS: calcd for $\text{C}_{20}\text{H}_{30}\text{O}_2$: 303.2324 [$M+\text{H}^+$]; found 303.2319.

Chloroacetyl ester 7: Chloroacetyl chloride (23 mg, 0.20 mmol) was added to a stirred solution of alcohol **4** (40.1 mg, 0.139 mmol) and DMAP (10 mg) in CH_2Cl_2 (10 mL) at 0 °C. After the mixture had been stirred for 2 h at 25 °C, the reaction was quenched with water (10 mL) and extracted with ethyl ether (3 \times 10 mL). The organic layer was dried (MgSO_4) and concentrated, and the residue was purified through column chromatography (silica, 2–10% diethyl ether in hexane) to produce ester **7** (39 mg, 0.114 mmol, 82%) as a colorless liquid. $R_f=0.35$ (40% Et_2O); $[\alpha]_D^{25}=-95.6$ ($c=1.2$, benzene); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=5.93$ (dd, $J=11.2$, 13 Hz, 1H), 5.48 (brd, 1H), 5.01–4.96 (m, 2H), 4.43 (d, $J=10.4$ Hz, 1H), 4.13 (d, $J=10.8$ Hz, 1H), 4.06 (s, 2H), 2.09–1.96 (m, 3H), 1.92–1.71 (m, 2H), 1.65–1.42 (m, 14H), 1.22 (s, 3H), 1.14 (s, 3H) 0.97 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=167.3$, 150.0, 142.3, 117.4, 112.5, 68.6, 45.9, 42.2, 41.1, 40.9, 38.0, 37.7, 37.4, 36.0, 29.8, 26.7, 26.0, 25.0, 23.4, 19.9, 19.1, 18.8; IR (film) $\tilde{\nu}_{\text{max}}=2985$, 1735, 1708, 1215, 950 cm^{-1} ; HRMS: calcd for $\text{C}_{22}\text{H}_{33}\text{ClO}_2$: 365.2247 [$M+\text{H}^+$]; found 365.2256.

Morpholine 8: Morpholine (15 mg, 0.17 mmol) was added to a solution of chloride **7** (24 mg, 0.066 mmol) in CH_2Cl_2 (5 mL), and the reaction mixture was stirred under reflux for 12 h. The reaction was then quenched with water and extracted with CH_2Cl_2 (3 \times 30 mL). The organic layer was dried (MgSO_4) and concentrated, and the residue was purified through column chromatography (silica, 20% diethyl ether in hexane) to afford morpholine **8** (20 mg, 0.05 mmol, 71%) as an oil. $R_f=0.3$ (80% Et_2O in hexanes); $[\alpha]_D^{25}=-89.5$ ($c=1.2$, benzene); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=5.94$ (dd, $J=17.6$, 11.2 Hz, 1H), 5.47 (m, 1H), 5.00–4.95 (m, 2H), 4.36 (d, $J=10.8$ Hz, 1H), 4.05 (d, $J=10.8$ Hz, 1H), 3.75–3.73 (m, 4H), 3.20 (s, 2H), 2.58–2.56 (m, 4H), 2.08–1.42 (m, 16H), 1.24 (s, 3H), 1.05 (s, 3H), 0.92 (s,

3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=170.2$, 150.0, 142.3, 117.3, 112.4, 81.5, 67.0, 66.8, 59.6, 53.3, 52.0, 45.9, 42.2, 41.0, 38.0, 37.7, 37.2, 29.8, 26.9, 26.0, 25.0, 19.9, 19.1, 18.8; IR (film) $\tilde{\nu}_{\text{max}}=2928$, 2854, 1730, 1640, 1462, 1260, 1095 cm^{-1} ; HRMS, calcd for $\text{C}_{26}\text{H}_{41}\text{NO}_3$: 416.3165 [$M+\text{H}^+$]; found 416.3172.

Piperazine 9: Piperazine (14.2 mg, 0.165 mmol) was added to a solution of chloride **7** (24 mg, 0.066 mmol) in CH_2Cl_2 (5 mL), and the reaction mixture was stirred under reflux for 12 h. The reaction was then quenched with water, and the mixture was extracted with CH_2Cl_2 (3 \times 30 mL) and purified through column chromatography (silica, 0–10% diethyl ether in CH_2Cl_2) to afford piperazine **9** (20 mg, 0.05 mmol, 86%) as a yellow oil. $R_f=0.1$ (2% $\text{MeOH}/\text{CH}_2\text{Cl}_2$); $[\alpha]_D^{25}=-92.5$ ($c=1.0$, benzene); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=5.93$ (dd, $J=17.6$, 11.2 Hz, 1H), 5.48 (brd, 1H), 5.00–4.95 (m, 2H), 4.37 (d, $J=10.8$ Hz, 1H), 4.05 (d, $J=10.8$ Hz, 1H), 3.20 (s, 2H), 2.63 (brd, 2H), 2.60–2.57 (m, 5H), 2.4–2.2 (brd, 3H), 2.08–1.94 (m, 5H), 1.71–1.42 (m, 10H), 1.20 (s, 3H), 1.05 (s, 3H), 0.87 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=170.9$, 150.3, 117.2, 112.4, 66.7, 59.8, 53.8, 53.5, 51.6, 51.3, 45.8, 42.2, 40.9, 37.9, 37.7, 37.2, 36.7, 29.8, 26.9, 26.0, 25.0, 23.3, 19.9, 19.1, 18.8; IR (film) $\tilde{\nu}_{\text{max}}=2953$, 2854, 1730, 1653, 1260, 1095 cm^{-1} ; HRMS: calcd for $\text{C}_{26}\text{H}_{42}\text{N}_2\text{O}_2$: 437.3144 [$M+\text{Na}^+$]; found 437.3163.

Tosyl piperazide 10: Triethylamine (4.8 mg, 0.048 mmol) and *p*-toluenesulfonyl chloride (6.4 mg, 0.036 mmol) were added to a solution of piperazine **9** (10 mg, 0.024 mmol) in CH_2Cl_2 (5 mL), and the mixture was stirred at 25 °C overnight. The reaction was quenched with water (10 mL), and the mixture was extracted with CH_2Cl_2 (3 \times 30 mL). The organic layer was dried (MgSO_4) and concentrated, and the residue was purified through column chromatography (silica, 10–40% diethyl ether in hexanes) to give tosylate **10** (11.1 mg, 0.020 mmol, 82%) as a white solid. $R_f=0.4$ (80% Et_2O in hexanes); $[\alpha]_D^{25}=-79.3$ ($c=1.1$, benzene); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=7.62$ (d, $J=8$ Hz, 2H), 7.31 (d, $J=8$ Hz, 2H), 5.92 (dd, $J=17.6$, 11.2 Hz, 1H), 5.47 (m, 1H), 5.0–4.95 (m, 2H), 4.36 (d, $J=10.8$ Hz, 1H), 4.03 (d, $J=10.8$ Hz, 1H), 3.24 (brd, 1H), 3.08 (brd, 4H), 2.72 (brd, 3H), 2.44 (s, 3H), 2.42–1.90 (m, 4H), 1.63–1.40 (m, 10H), 1.24–1.05 (m, 4H), 1.05 (s, 3H), 1.03 (s, 3H), 0.89 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=194.3$, 149.9, 142.3, 131.9, 129.6, 127.7, 117.3, 112.5, 55.9, 51.9, 45.5, 42.2, 40.9, 37.9, 37.7, 36.2, 29.8, 26.9, 25.9, 25.0, 23.4, 19.9, 19.1, 18.8; IR (film) $\tilde{\nu}_{\text{max}}=3010$, 2958, 2857, 1725, 1648, 1260, 1095 cm^{-1} ; HRMS, calcd for $\text{C}_{33}\text{H}_{48}\text{N}_2\text{O}_4\text{S}$: 591.3232 [$M+\text{Na}^+$]; found 591.3257.

Ethyl ester 12: A solution of alcohol **4** (20 mg, 0.069 mmol) and 4-methylmorpholine (20 mg, 0.173 mmol) in CH_2Cl_2 (10 mL) was cooled to 0 °C and treated with ethyl propiolate (10 mg, 0.10 mmol). The reaction mixture was stirred overnight. The reaction was quenched with water (25 mL), and the mixture was extracted with CH_2Cl_2 (3 \times 10 mL). The combined organic extracts were concentrated and purified through column chromatography (silica, 2% diethyl ether in hexane) to afford ethyl ester **12** (42.8 mg, 0.11 mmol, 64%) as a clear oil. $R_f=0.3$ (20% Et_2O in hexanes); $[\alpha]_D^{25}=-93.1$ ($c=1.0$, benzene); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=7.61$ (d, $J=12.8$ Hz, 1H), 5.93 (dd, $J=17.6$, 11.2 Hz, 1H), 5.48 (brd, 1H), 5.16 (d, $J=12.8$ Hz, 1H), 5.02–4.96 (m, 2H), 4.13 (q, $J=14.4$, 7.2 Hz, 2H), 3.99 (d, $J=10$ Hz, 1H), 3.69 (d, $J=9.6$ Hz, 1H), 2.09–1.78 (m, 6H), 1.62–1.40 (m, 13H), 1.06 (s, 3H), 1.04 (s, 3H), 0.97 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=167.8$, 163.0, 145.0, 142.3, 117.4, 112.5, 95.7, 73.7, 59.7, 45.8, 42.2, 40.9, 37.9, 37.7, 37.6, 37.2, 35.9, 26.7, 26.0, 25.0, 23.4, 19.9, 19.1, 18.8, 14.5; IR (film) $\tilde{\nu}_{\text{max}}=3010$, 2941, 2865, 1750, 1648, 1171, 950 cm^{-1} ; HRMS: calcd for $\text{C}_{25}\text{H}_{38}\text{O}_3$: 387.2899 [$M+\text{H}^+$]; found 387.2902.

Carboxylic acid 13: DMAP (5 mg, cat.) and succinic anhydride (30 mg, 0.28 mmol) were added to a well-stirred solution of alcohol **2** (70 mg, 0.24 mmol) in CH_2Cl_2 (8 mL). The reaction mixture was stirred for 8 h at 25 °C, then washed with water (20 mL) and extracted with CH_2Cl_2 (2 × 40 mL). The desired product was purified through column chromatography (silica, 12–16% diethyl ether in hexane) to afford acid **13** (73 mg, 0.19 mmol, 78% yield) as a white solid. $R_f=0.4$ (70% Et_2O in hexanes); $[\alpha]_D^{25}=-101.3$ ($c=1.0$, CDCl_3); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=5.95$ (dd, $J=17.6$, 11.2 Hz, 1H), 5.48 (m, 1H), 5.0–4.96 (m, 2H), 4.34 (d, $J=10.8$ Hz, 1H), 4.03 (d, $J=11.2$ Hz, 1H), 2.70–2.61 (m, 4H), 2.09–1.91 (m, 4H), 1.80–1.39 (m, 10H), 1.25–1.08 (m, 6H), 1.05 (s, 3H), 0.91 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=177.3$, 172.0, 150.1, 142.3, 117.2, 112.4, 67.1, 45.9, 42.2, 41.0, 38.0, 37.7, 37.2, 36.1, 29.8, 29.0, 26.8, 26.0, 25.0, 23.4, 20.0, 19.9, 19.1, 18.8; IR (film) $\tilde{\nu}_{\text{max}}=3312$, 1732, 1708 cm^{-1} ; HRMS: calcd for $\text{C}_{24}\text{H}_{36}\text{O}_4$: 411.2511 $[\text{M}+\text{Na}^+]$; found 411.2539.

Carboxylic acid 14: This compound was prepared by using the procedure described above for acid **13**. In this case, glutaric anhydride (27.1 mg, 0.24 mmol) was used instead of succinic anhydride. Column chromatography (20% Et_2O in hexanes) gave pure acid **14** (68 mg, 0.17 mmol, 71%) as a white solid. $R_f=0.40$ (80% Et_2O in hexanes); $[\alpha]_D^{25}=-103.5$ ($c=1.1$, CDCl_3); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=6.01$ (dd, $J=17.6$, 11.2 Hz, 1H), 5.46 (brd, 1H), 5.09–4.95 (m, 2H), 4.36 (d, $J=10.8$ Hz, 1H), 4.02 (d, $J=11.2$ Hz, 1H), 2.48–2.36 (m, 3H), 2.16–1.92 (m, 5H), 1.86–1.38 (m, 9H), 1.35–1.11 (m, 10H), 0.98–0.81 (m, 5H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=174.0$, 169.1, 146.3, 138.5, 113.3, 108.4, 93.2, 88.2, 62.6, 41.7, 38.0, 36.8, 33.7, 33.4, 32.9, 29.1, 28.7, 25.5, 25.5, 25.4, 22.5, 20.7, 19.1, 15.6, 14.5; IR (film) $\tilde{\nu}_{\text{max}}=3320$, 1735, 1705 cm^{-1} ; HRMS: calcd for $\text{C}_{25}\text{H}_{38}\text{O}_4$: 425.2668 $[\text{M}+\text{Na}^+]$; found 425.2653.

General procedure for the preparation of amides 15, 16 and 17: A solution of acid **6** (1 equivalent) in anhydrous CH_2Cl_2 was treated under argon with 3.0 equivalent of $(\text{COCl})_2$ and one drop of DMF, and the mixture was stirred at 25 °C for 30 min. The solvent and excess $(\text{COCl})_2$ were evaporated under a gentle heating and by applying vacuum. The dried acyl chloride residue was dissolved in CH_2Cl_2 (5 mL) and treated with 4.0 equiv of freshly distilled amine. After being stirred for 2 h, the reaction was quenched with water and extracted with diethyl ether. The organic layers were dried (MgSO_4) and concentrated, and the residue was purified through silica gel chromatography (20–40% diethyl ether in hexanes) to produce the corresponding amides.

Amide 15: 87% yield, white solid; $R_f=0.2$ (100% Et_2O); $[\alpha]_D^{25}=-41.2$ ($c=0.17$, CDCl_3); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=5.95$ (dd, $J=17.2$, 11.6 Hz, 1H), 5.46 (s, 1H), 4.96 (m, 2H), 2.99 (s, 8H), 2.41–2.32 (m, 1H), 2.18–1.95 (m, 5H), 1.9–1.4 (m, 12), 1.29 (s, 3H), 1.09 (s, 3H), 1.02 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=150.0$, 144.1, 117.0, 111.5, 111.2, 51.7, 46.2, 41.6, 41.4, 39.9, 39.8, 39.2, 37.7, 34.2, 29.8, 27.4, 25.0, 24.8, 23.2, 23.1, 21.4, 21.2; IR (film) $\tilde{\nu}_{\text{max}}=3446$, 2924, 2854, 1730, 1640, 1462, 1360, 1260, 1095 cm^{-1} ; HRMS: calcd for $\text{C}_{24}\text{H}_{39}\text{NO}_3$: 390.3008 $[\text{M}+\text{H}^+]$; found 390.30031.

Amide 16: 91% yield, white solid; $R_f=0.55$ (60% Et_2O in hexanes); $[\alpha]_D^{25}=-47.1$ ($c=0.17$, benzene); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=5.95$ (q, $J=17.2$, 11.6 Hz, 1H), 5.47 (s, 1H), 4.99–4.95 (m, 2H), 3.64–3.60 (m, 8H), 2.34 (d, $J=13.2$ Hz, 1H), 2.1–2.0 (m, 4H), 1.8–1.4 (m, 11H), 1.29 (s, 3H), 1.106 (s, 3H), 1.03 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=175.5$, 149.8, 143.9, 117.1, 111.7, 111.2, 67.0, 51.3, 46.7, 46.5, 41.6, 41.4, 40.4, 39.7, 37.6, 34.4, 27.3, 25.2, 24.9, 23.2, 22.8, 21.2; IR (film) $\tilde{\nu}_{\text{max}}=2926$, 2852, 1733, 1642, 1461, 1358, 1267, 1093 cm^{-1} ; HRMS: calcd for $\text{C}_{24}\text{H}_{37}\text{NO}_2$: 372.2903 $[\text{M}+\text{H}^+]$; found 372.2886.

Amide 17: 95% yield, white solid; $R_f=0.7$ (100% Et_2O); $[\alpha]_D^{25}=+8.6$ ($c=0.1$, benzene); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=5.95$ (dd, $J=17.6$, 11.6 Hz, 1H), 5.46 (d, $J=2.4$ Hz, 1H), 5.00–4.94 (m, 2H), 3.72–3.68 (m, 4H), 2.58–2.30 (m, 6H), 2.25–2.22 (m, 1H), 2.02–1.96 (m, 4H), 1.9–1.4 (m, 11H), 1.29 (s, 3H), 1.25 (s, 3H), 1.09 (s, 3H), 1.03 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=175.4$, 149.8, 143.9, 125.4, 117.1, 111.7, 54.9, 51.3, 46.4, 45.4, 41.6, 40.4, 39.7, 37.6, 34.4, 30.4, 29.8, 27.4, 25.2, 24.9, 23.2, 22.8, 21.2; IR (film) $\tilde{\nu}_{\text{max}}=2925$, 2853, 1731, 1645, 1462, 1360, 1267, 1096 cm^{-1} ; HRMS: calcd for $\text{C}_{25}\text{H}_{40}\text{N}_2\text{O}$: 385.3219 $[\text{M}+\text{H}^+]$; found 385.3103.

Ethyl ester 18: Triethyl phosphonoacetate (500 mg, 0.60 mmol) was added to a well-stirred suspension of NaH (67 mg, 0.43 mmol) in THF (20 mL) at 0 °C. The reaction mixture was allowed to warm slowly to 25 °C, at which point it was treated with a solution of aldehyde **5** (76 mg, 0.27 mmol) in THF (10 mL). The mixture was heated under reflux for 16 h and then quenched with water (30 mL) and extracted with ether (3 × 60 mL). The ether layers were concentrated, and the residue was purified over silica gel (5% diethyl ether in hexane) to afford ester **18** (68 mg, 0.19 mmol, 70%) as an oil. $R_f=0.4$ (10% diethyl ether in hexanes); $[\alpha]_D^{25}=-45.3$ ($c=0.17$, benzene); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=7.34$ (d, $J=16$ Hz, 1H), 5.90 (dd, $J=17.2$, 11.6 Hz, 1H), 5.76 (d, $J=16$ Hz, 1H), 5.48 (m, 1H), 4.97 (m, 2H), 4.18 (q, $J=14.4$, 7.6 Hz, 2H), 2.09–1.74 (m, 6H), 1.66–1.42 (m, 8H), 1.30–1.17 (m, 5H), 1.06 (s, 3H), 0.98 (s, 3H), 0.95 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=167.1$, 154.5, 149.2, 142.4, 118.1, 117.4, 112.3, 60.2, 46.8, 42.4, 41.1, 39.9, 38.7, 38.2, 37.7, 37.0, 29.7, 25.0, 24.8, 23.4, 20.2, 19.2, 19.1, 14.5; IR (film) $\tilde{\nu}_{\text{max}}=2928$, 2850, 1735, 1650, 1465, 1264, 1082 cm^{-1} ; HRMS: calcd for $\text{C}_{24}\text{H}_{36}\text{O}_2$: 357.2794 $[\text{M}+\text{H}^+]$; found 357.2762.

Carboxylic acid 19: LiOH (40 mg, excess) was added to a solution of methyl ester **18** (60 mg, 0.17 mmol) in THF/ H_2O (1:1, 8 mL). After being stirred for 30 min, the reaction mixture was heated under reflux for 12 h. The reaction mixture was diluted with water, acidified with HCl solution (3 N, 40 mL), and extracted with diethyl ether (2 × 50 mL). After column chromatography (silica, 50% diethyl ether in hexane), carboxylic acid **19** was obtained (42 mg, 0.13 mmol, 76%) as a white solid. $R_f=0.52$ (100% diethyl ether); $[\alpha]_D^{25}=-67.5$ ($c=0.17$, benzene); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=7.45$ (d, $J=16$ Hz, 1H), 5.94 (dd, $J=17.2$, 11.6 Hz, 1H), 5.76 (d, $J=4$ Hz, 1H), 5.48 (m, 1H), 5.00–4.95 (m, 2H), 2.15–1.74 (m, 5H), 1.64–1.50 (m, 7H), 1.47–1.42 (m, 5H), 1.06 (s, 3H), 0.99 (s, 3H), 0.95 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): $\delta=171.0$, 157.4, 149.0, 142.4, 117.6, 117.2, 112.4, 46.8, 42.4, 41.0, 40.2, 38.5, 38.2, 37.8, 37.0, 29.8, 29.5, 25.0, 24.8, 23.4, 20.2, 19.1; IR (film) $\tilde{\nu}_{\text{max}}=3332$, 2935, 2867, 1730, 1648, 1463, 1154, 1080 cm^{-1} ; HRMS: calcd for $\text{C}_{22}\text{H}_{32}\text{O}_2$: 351.2300 $[\text{M}+\text{Na}^+]$; found 351.2329.

Methyl ester 20: Magnesium powder (50 mg, 6.25 mmol) was added to a well-stirred solution of ester **18** (60 mg, 0.168 mmol) in methanol (10 mL), and the reaction mixture was stirred at 25 °C for 12 h. HCl (10 mL, 2 M) was added to the reaction mixture to dissolve the remaining magnesium. The reaction mixture was concentrated under reduced pressure and extracted with diethyl ether (2 × 50 mL). Column chromatography of the residue (10% diethyl ether in hexane) afforded methyl ester **20** (50 mg, 0.15 mmol, 87%) as an oil. $R_f=0.4$ (10% diethyl ether in hexanes); $[\alpha]_D^{25}=-70.4$ ($c=0.18$, benzene); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): $\delta=5.95$ (dd, $J=17.6$, 11.2 Hz, 1H), 5.45 (m, 1H), 4.98 (m, 2H), 3.66 (s, 3H), 3.26–1.87 (m, 7H), 1.69–1.51 (m, 11H), 1.24–1.19 (m, 2H), 1.09 (s, 3H), 1.04 (s, 3H), 0.93 (s, 3H); IR (film) $\tilde{\nu}_{\text{max}}=2930$, 2852, 1734, 1647, 1265, 982 cm^{-1} ; HRMS: calcd for $\text{C}_{23}\text{H}_{36}\text{O}_2$: 345.2794 $[\text{M}+\text{H}^+]$; found 345.2763.

Carboxylic acid 21: Ester **20** (50 mg, 0.14 mmol) was dissolved in THF/H₂O (1:1, 4 mL), and the solution was treated with LiOH (30 mg). After being stirred for 30 min, the reaction mixture was heated under reflux for 12 h. The reaction mixture was diluted with water, acidified with HCl solution (3 N), and extracted with diethyl ether (2×50 mL). The organic residue was purified through column chromatography (silica, 50% diethyl ether in hexane) to give carboxylic acid **21** (34 mg, 0.10 mmol, 71%) as a white solid. $R_f=0.55$ (100% diethyl ether); $[\alpha]_D^{25}=-60.5$ ($c=1$, benzene); ¹H NMR (CDCl₃, 400 MHz): $\delta=5.95$ (dd, $J=17.2, 11.2$ Hz, 1H), 5.46 (d, $J=4.4$ Hz, 1H), 5.00–4.95 (m, 2H), 2.27–2.17 (m, 3H), 2.09–1.88 (m, 10H), 1.69–1.37 (m, 7H), 1.24–1.18 (m, 1H), 1.09 (s, 3H), 1.04 (s, 3H), 0.83 (s, 3H); ¹³C NMR (CDCl₃, 100 MHz): $\delta=178.6, 152.1, 143.7, 118.1, 113.5, 67.1, 48.2, 43.7, 42.5, 39.4, 38.4, 37.0, 31.0, 30.5, 29.5, 28.4, 27.3, 26.3, 24.6, 21.2, 20.4, 19.9$; IR (film) $\tilde{\nu}_{\text{max}}=3360, 2928, 2850, 1735, 1708, 1452, 1134, 1080$ cm⁻¹; HRMS: calcd for C₂₂H₃₄O₂: 331.2637 [M+H]⁺; found 331.2650.

Aldehyde 22: A suspension of ethoxy methyl triphenyl phosphonium chloride (476.1 mg, 1.39 mmol) in THF (10 mL) was treated with 4.8 equiv of potassium *tert*-butoxide (152.2 mg, 1.34 mmol), followed by addition of aldehyde **5** (80 mg, 0.28 mmol). The reaction was completed within 30 min at 25 °C. After the reaction mixture had been concentrated, the residue was redissolved in ethyl ether (20 mL) and extracted with brine (20 mL). The organic layer was concentrated and dried, and the residue was purified by flash chromatography on silica gel to produce the corresponding vinyl ether (87 mg, 0.28 mmol, 99% yield). *p*-Toluenesulfonic acid (50 mg, 0.26 mmol) was added to a well-stirred solution of the vinyl ether (50 mg, 0.159 mmol) in acetone (10 mL), and the reaction was stirred for 2 h at 0 °C. TLC indicated the complete formation of the desired product. After the solvent had been removed, the reaction mixture was extracted with diethyl ether (3×40 mL) and washed first with aqueous saturated NaHCO₃ and then with brine. The crude product was purified through column chromatography (silica, 4% diethyl ether in hexane) to give aldehyde **22** (42 mg, 0.14 mmol, 88%) as an oil. $R_f=0.50$ (20% diethyl ether in hexanes); $[\alpha]_D^{25}=+4.4$ ($c=1.0$, benzene); ¹H NMR (CDCl₃, 400 MHz): $\delta=5.95$ (dd, $J=17.2, 11.2$ Hz, 1H), 5.48 (m, 1H), 5.01–4.96 (m, 2H), 2.55 (dd, $J=14, 3.2$ Hz, 1H), 2.37 (dd, $J=3.6, 2$ Hz, 1H), 2.09–1.93 (m, 3H), 1.76–1.71 (m, 2H), 1.63–1.13 (m, 12H), 1.09 (s, 3H), 1.07 (s, 3H), 1.06 (s, 3H); ¹³C NMR (CDCl₃, 100 MHz): $\delta=204.3, 150.2, 142.2, 117.3, 112.5, 47.2, 47.1, 42.4, 40.8, 39.0, 38.1, 37.7, 37.2, 37.0, 29.1, 25.6, 25.0, 23.4, 19.9, 19.2, 19.1$; IR (film) $\tilde{\nu}_{\text{max}}=2927, 2863, 1730, 1651, 1450, 1135, 1082$ cm⁻¹; HRMS: calcd for C₂₁H₃₂O: 301.2531 [M+H]⁺; found 301.2563.

Carboxylic acid 23: NaH₂PO₄·H₂O (23.3 mg, 0.17 mmol) was added to a solution of aldehyde **22** (17 mg, 0.056 mmol) in *t*BuOH/H₂O (3:1, 3 mL). The reaction mixture was stirred to dissolve completely the salt and then treated at 25 °C with 2-methylbut-2-ene in THF (84.5 μL, 0.17 mmol, 2.0 M in THF). The reaction mixture was stirred for 30 min and then treated with NaClO₂ (15.3 mg, 0.17 mmol). The reaction was completed within 1 h, at which time the yellow color of the reaction mixture had faded. The product was collected by extraction with CH₂Cl₂, the organic layers were dried, and the residue was purified through column chromatography (silica, 12% diethyl ether in hexane) to give acid **23** (17.0 mg, 0.054 mmol, 96%) as a white solid. $R_f=0.60$ (40% diethyl ether in hexanes); $[\alpha]_D^{25}=-4.75$ ($c=0.64$, CDCl₃); ¹H NMR (CDCl₃, 400 MHz): $\delta=6.00-5.90$ (m, 1H), 5.48 (d, $J=3$ Hz, 1H), 5.02–4.95 (m, 2H), 2.58 (d, $J=12.6$ Hz, 1H), 2.30 (d, $J=12.6$ Hz, 1H), 2.07–1.83 (m, 5H), 1.8–1.4 (m, 12H), 1.25 (s, 3H), 1.07 (s, 3H), 1.02 (s, 3H); ¹³C NMR (CDCl₃, 100 MHz): $\delta=181.2, 145.4, 143.3, 117.5, 111.7, 50.1, 45.1, 40.4, 38.7, 38.5, 38.1,$

37.4, 37.0, 36.2, 28.8, 25.7, 25.1, 23.5, 20.9, 19.3, 18.9; IR (film) $\tilde{\nu}_{\text{max}}=3350, 2925, 2861, 1745, 1643, 1350, 1130, 1085$ cm⁻¹; HRMS: calcd for C₂₁H₃₂O₂: 317.2481 [M+H]⁺; found 317.2453.

Methyl ester 24: HCl (78.6 μL, 1 M in water) and trimethylsilyl diazomethane (40 μL, 0.8 mmol, 2 M in hexane) were added to a solution of carboxylic acid **23** (5 mg, 0.16 mmol) in methanol (2 mL) at 0 °C. The reaction mixture was stirred at 25 °C for 30 min, then neutralized with aqueous saturated NH₄Cl and extracted with ethyl ether (3×10 mL). The organic layers were dried (MgSO₄) and concentrated, and the residue was purified by column chromatography (5% diethyl ether in hexane) to afford methyl ester **24** (5.5 mg, 0.016 mmol, 100%) as a clear oil. $R_f=0.36$ (40% diethyl ether in hexanes); $[\alpha]_D^{25}=-7.0$ ($c=0.18$, CDCl₃); ¹H NMR (CDCl₃, 400 MHz): $\delta=5.95$ (dd, $J=17.2, 11.6$ Hz, 1H), 5.48 (d, $J=4.4$ Hz, 1H), 5.00–4.96 (m, 2H), 3.63 (s, 3H), 2.55 (d, $J=12.8$ Hz, 1H), 2.30 (d, $J=12.8, 1$ Hz), 2.08–1.92 (m, 4H), 1.83–1.41 (m, 12H), 1.06 (s, 3H), 1.05 (s, 3H), 0.96 (s, 3H); ¹³C NMR (CDCl₃, 100 MHz): $\delta=173.5, 150.5, 142.4, 117.2, 112.4, 51.2, 46.8, 42.5, 41.1, 38.2, 38.1, 38.0, 37.7, 37.2, 36.5, 28.8, 25.6, 25.0, 23.4, 20.0, 19.2, 19.1$; IR (film) $\tilde{\nu}_{\text{max}}=2950, 2863, 1730, 1645, 1450, 1233, 1162$ cm⁻¹; HRMS: calcd for C₂₂H₃₄O₂: 331.2638 [M+H]⁺; found: 331.2651.

Methyl ester 25: Methyl(triphenylphosphoronylidene) acetate (90.7 mg, 0.27 mmol) and aldehyde **22** (27.3 mg, 0.09 mmol) were dissolved in CH₂Cl₂ (5 mL), and the solution was stirred at 25 °C. The reaction was completed within one day, then the mixture was neutralized with aqueous saturated NH₄Cl and extracted with CH₂Cl₂ (3×10 mL). The organic layers were combined, dried (MgSO₄) and concentrated under reduced pressure. The residue was purified through column chromatography (silica, 2–4% diethyl ether in hexane) to afford **25** as a mixture of *trans* and *cis* isomers (24.4 mg of *trans* and 2.5 mg of *cis*, 0.23 mmol, 84% total yield).

trans-**25**: white solid. $R_f=0.47$ (20% diethyl ether in hexanes); $[\alpha]_D^{25}=-12.1$ ($c=0.21$, CDCl₃); ¹H NMR (CDCl₃, 400 MHz): $\delta=7.00-6.92$ (m, 1H), 5.96 (dd, $J=17.2, 11.6$ Hz, 1H), 5.80 (d, $J=15.2$ Hz, 1H), 4.47 (d, $J=4.4$ Hz, 1H), 5.00–4.96 (m, 2H), 3.72 (s, 3H), 2.45 (dd, $J=14, 8$ Hz, 1H), 2.19 (dd, $J=14, 8$ Hz, 1H), 2.12–1.9 (m, 4H), 1.76–1.35 (m, 12H), 1.08 (s, 3H), 1.06 (s, 3H), 0.89 (s, 3H); ¹³C NMR (CDCl₃, 100 MHz): $\delta=166.7, 150.7, 148.0, 142.4, 122.4, 117.0, 112.4, 51.4, 46.6, 42.4, 41.0, 38.1, 37.8, 37.7, 37.2, 35.9, 29.8, 28.8, 25.9, 25.0, 23.4, 19.9, 19.0, 18.8$; IR (film) $\tilde{\nu}_{\text{max}}=2955, 2861, 1732, 1646, 1454, 1202, 1165$ cm⁻¹; HRMS: calcd for C₂₄H₃₆O₂: 379.2613 [M+Na]⁺; found 379.2655.

cis: ¹H NMR (CDCl₃, 400 MHz): $\delta=6.32-6.25$ (m, 1H), 5.97 (dd, $J=17.2, 11.6$ Hz, 1H), 5.83 (d, $J=11.6$ Hz, 1H), 5.47 (d, $J=8$ Hz, 1H), 5.00–4.96 (m, 2H), 3.71 (s, 3H), 3.15 (dd, $J=14.8, 9.2$ Hz, 1H), 2.58 (dd, $J=14.8, 9.2$ Hz, 1H), 2.12–1.9 (m, 4H), 1.98–1.46 (m, 12H), 1.15 (s, 3H), 1.07 (s, 3H), 0.90 (s, 3H).

Carboxylic acid 26: *trans*-ester **25** (8 mg, 0.023 mmol) and LiOH (2.70 mg, 0.113 mmol) were dissolved in THF/H₂O (1:1, 1 mL). The reaction mixture was refluxed at 60 °C overnight. Upon completion of the reaction, the mixture was neutralized with HCl (1 M) and extracted with CH₂Cl₂ (3×5 mL). The organic layers were dried (MgSO₄) and concentrated, and the residue was purified by column chromatography (30% diethyl ether in hexane) to afford acid **26** (7.3 mg, 0.021 mmol, 95%) as a white solid. $R_f=0.76$ (60% diethyl ether); $[\alpha]_D^{25}=-15.2$ ($c=0.29$, CDCl₃); ¹H NMR (CDCl₃, 400 MHz): $\delta=7.12-7.04$ (m, 1H), 5.96 (dd, $J=17.2, 11.6$ Hz, 1H), 5.82 (d, $J=15.6$ Hz, 1H), 5.48 (d, $J=4.4$ Hz, 1H), 5.01–4.97 (m, 2H), 2.53–2.46 (m, 1H), 2.23–2.19 (m, 1H), 2.10–1.93 (m, 4H), 1.76–1.36 (m, 12H), 1.09 (s, 3H), 1.07 (s, 3H), 0.88 (s, 3H); ¹³C NMR (CDCl₃, 100 MHz): $\delta=169, 150.7, 142.5, 122.0, 117.2, 114.5, 46.5, 42.4, 40.9,$

38.1, 37.9, 37.7, 37.2, 36.0, 29.7, 28.8, 25.8, 25.7, 24.9, 24.9, 23.3, 19.8, 18.9; IR (film) $\tilde{\nu}_{\max}$ = 3310, 2967, 2860, 1732, 1646, 1454 cm^{-1} ; HRMS: calcd for $\text{C}_{23}\text{H}_{34}\text{O}_2$: $[M+H]^+$ 343.2637; found 343.2601.

Methyl ester 27: In a moisture-free flask, ester **25** (5 mg, 0.14 mmol) was dissolved in dry methanol (2 mL) and treated with Mg (34 mg, 1.4 mmol), which was freshly activated by heat. The solution was stirred overnight at 25 °C. After completion of the reaction, the mixture was acidified with HCl (2 M in water), to dissolve the remaining Mg, and then extracted with ether (3 × 5 mL). The organic layers were dried (MgSO_4) and concentrated, and the residue was purified by column chromatography (1–10% diethyl ether in hexane) to afford ester **27** (2.9 mg, 0.076 mmol, 54%) as a yellow solid. R_f = 0.4 (10% diethyl ether in hexanes); $[\alpha]_D^{25}$ = –22.1 (c = 0.12, CDCl_3); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): δ = 5.96 (dd, J = 17.2, 11.6 Hz, 1H), 5.45 (d, J = 4 Hz, 1H), 5.00–4.95 (m, 2H), 3.67 (s, 3H), 2.27 (m, 2H), 2.15–1.88 (m, 4H), 1.70–1.38 (m, 16H), 1.25 (s, 3H), 1.05 (s, 3H), 0.83 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 400 MHz): δ = 200.0, 151.1, 142.6, 116.7, 112.3, 51.5, 46.9, 42.5, 41.4, 38.3, 37.7, 37.4, 36.1, 35.2, 32.0, 29.8, 28.6, 26.2, 25.0, 23.4, 20.3, 20.0, 19.3, 18.6; IR (film) $\tilde{\nu}_{\max}$ = 2985, 2847, 1730, 1651, 1450 cm^{-1} ; HRMS: calcd for $\text{C}_{24}\text{H}_{38}\text{O}_2$: 359.2950 $[M+H]^+$; found 359.2977.

Carboxylic acid 28: Ester **27** (5 mg, 0.014 mmol) and LiOH (1.70 mg, 0.07 mmol) were dissolved in THF/ H_2O (1 mL of 1:1 ratio). The reaction mixture was refluxed at 65 °C overnight. Upon completion of the reaction, the mixture was neutralized with HCl (1 M) and extracted with CH_2Cl_2 (3 × 5 mL). The organic layers were dried (MgSO_4) and concentrated, and the residue was purified by column chromatography (50% diethyl ether in hexane) to give acid **28** (3.9 mg, 0.011 mmol, 81%) as a white solid. R_f = 0.55 (80% diethyl ether in hexanes); $[\alpha]_D^{25}$ = +3.4 (c = 0.19, CDCl_3); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): δ = 5.96 (dd, J = 17.2, 11.6 Hz, 1H), 5.45 (d, J = 4 Hz, 1H), 5.00–4.95 (m, 2H), 2.37 (m, 2H), 2.15–1.90 (m, 6H), 1.70–1.40 (m, 15H), 1.25 (s, 3H), 1.05 (s, 3H), 0.84 (s, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): δ = 178.3, 151.1, 142.6, 125.4, 116.8, 112.3, 47.0, 42.5, 41.4, 38.3, 37.7, 36.1, 34.9, 32.0, 30.4, 29.8, 28.6, 26.1, 25.0, 23.8, 20.0, 19.3, 18.6; IR (film) $\tilde{\nu}_{\max}$ = 3350, 2987, 1720, 1643 cm^{-1} ; HRMS: calcd for $\text{C}_{23}\text{H}_{36}\text{O}_2$: 367.2613 $[M+\text{Na}^+]$; found 367.1629.

Cytotoxicity assay: HPBMC (Human Peripheral Blood Mononuclear Cells, lot # 2F1391 from pre-selected donors), purchased from Cambrex (Walkersville, MD), were seeded in 96-well Costar 3904 tissue-culture plates at 2.5×10^4 cells per well in 80 μL volume of LGM-3 medium (Cambrex) and then allowed to recover in the tissue-culture incubator. After 12 h of recovery, 10 μL of eight-point half-log serial dilutions of analogues were added to corresponding wells. The 96-well tissue-culture plates were then returned to the incubator for 24 h of incubation. Resazurin dye (10 μL , Sigma, St. Louis, MO) was then added to each well at the end of incubation, and the plate was again returned to the incubator for another 4 h of incubation before fluorescence measurement. The fluorescence of resazurin was measured by using 530 nm excitation and 590 nm emission filters. Staurosporin (Sigma, St. Louis, MO) at a final concentration of 10 μM was used as a positive control. Data analysis: Microsoft Excel and Graphpad Prism 3.0 were used to analyze the data generated from the cytokine measurements and cytotoxicity assays. The EC_{50} s for both cytotoxicity and TNF- α synthesis inhibition were calculated by using the Sigmoidal dose-response model with variable slope.

TNF- α measurement assay: HPBMC cells were seeded in 96-well Costar 3904 tissue-culture plates at 2.5×10^4 cells per well in 80 μL volume of LGM-3 medium (Cambrex) and then allowed to recover in the tissue-culture incubator. After 12 h of recovery, 10 μL of

eight-point half-log serial dilutions of analogues were added to corresponding wells for another hour's incubation, and LPS (from *E. coli*, strain 0111:B4, Sigma-Aldrich, St. Louis, MO) at 50 ng mL^{-1} final concentration was added for 4 h to stimulate TNF- α synthesis. At the end of incubation, supernatants were collected and stored at –80 °C until use. A p38 kinase inhibitor, SB203580 (Sigma, St. Louis, MO), was used as a positive control at a final concentration of 10 μM . The TNF- α measurement was performed by using the human TNF- α cytosets kit purchased from Biosource International (Camarillo, CA) according to manufacturer's suggestions.

Cytokine selectivity measurements: Cytokines such as IL-1 β , IL-6, IL-1ra, and IL-8 were analyzed by using the Cytosets kits purchased from Biosource International (Camarillo, CA). The levels of IL-1 β and IL-8 were measured from the same samples that were stimulated with LPS for 4 h. IL-1ra and IL-6 levels were analyzed from cells treated with LPS for 12 and 24 h, respectively.

Electrophoretic mobility shift assay: Compound-treated RAW 264.7 cells (ATCC #TIB-71) were stimulated with LPS (from *Salmonella typhimurium*, Sigma-Aldrich, St. Louis) and recombinant murine IFN- γ at different time points. To detect NF- κB , nuclear extracts were prepared by first washing cells (1.5×10^6) with phosphate buffered solution (PBS) and collected by centrifugation. Cell pellets were homogenized with buffer A (100 μL ; 10 mM Hepes; pH 7.9, 1 mM EDTA, 1 mM ethyleneglycol bis(2-aminoethylether)-tetraacetic acid, 100 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 $\mu\text{g mL}^{-1}$ aprotinin, 10 $\mu\text{g mL}^{-1}$ leupeptin, 2 $\mu\text{g mL}^{-1}$ Na-*p*-tosyl-L-lysine chloromethyl ketone, 5 mM NaF, 1 mM Na_2VO_4 , 10 mM Na_2MoO_4). After 10 min at 4 °C, Nonidet P-40 was added to reach a 0.5% concentration. The tubes were gently vortexed for 15 s, and nuclei were collected by centrifugation at 8000 g for 15 min. The pellets were resuspended in buffer A (50 μL) supplemented with 20% glycerol and KCl (0.4 M) and gently shaken for 30 min at 4 °C. Nuclear protein extracts were obtained by centrifugation at 13000 g for 15 min. Protein content was assayed by using the Bio-Rad protein reagent. All cell fractionation steps were carried out at 4 °C.

The sequence 5'-TGCTAGGGGATTTCCTCTCTGT-3', corresponding to the consensus NF- κB binding site (nucleotides –978 to –952) of the murine NOS-2 promoter was used. Oligonucleotides were annealed with their complementary sequence by incubation for 5 min at 85 °C in Tris-HCl (10 mM, pH 8.0), NaCl (50 mM), MgCl_2 (10 mM), 1,4-dithiothreitol (DTT; 1 mM). Aliquots of 50 ng of these annealed oligonucleotides were end-labeled with Klenow enzyme fragment in the presence of $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ (50 μCi) and the other unlabeled dNTPs in a final volume of 50 μL . A total of 5×10^4 dpm of the DNA probe was used for each binding assay of nuclear extracts as follows: nuclear protein (3 μg) was incubated for 15 min at 4 °C with the DNA and of poly(dI-dC) (2 μg), 5% glycerol, EDTA (1 mM), KCl (100 mM), MgCl_2 (5 mM), DTT (1 mM), Tris-HCl (10 mM; pH 7.8) in a final volume of 20 μL . The DNA-protein complexes were separated on native 6% polyacrylamide gels in 0.5% Tris-borate-EDTA buffer. Supershift assays were carried out after incubation of the nuclear extracts with 2 μg of antibodies (anti-p50, anti-c-Rel, anti-p65) for 1 h at 4 °C.

IkB α phosphorylation assay LPS stimulation: 3×10^6 human peripheral blood cells resuspended in LGM-3 medium (1 mL, Cambrex) were seeded in 6-well tissue-culture plates for 12 h in a 95% humidified atmosphere incubator at 37 °C and with 5% CO_2 . After 1 h of pretreatment of selected analogues at a final concentration of 10 μM , cells were stimulated with LPS (20 ng mL^{-1} , from *E. coli*, strain 0111:B4, Sigma-Aldrich, St. Louis) for another hour. The cells

were then harvested, washed once in ice-cold 1X PBS, and lysed in RIPA buffer (0.9% NaCl, Tris-HCl, pH 7.4, 1% Triton X-100, 1 mL EDTA, 0.25% Na deoxycholic acid, 2 mM Na₃VO₄) supplemented with 1X protease inhibitor cocktail (Calbiochem, San Diego, CA).

Western blot analysis: Cell lysates prepared as above were cleared by centrifugation at 14000 rpm and 4°C for 10 min. The protein concentration was normalized by determination of the total protein amount in the supernatant by using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein per lane were separated by electrophoresis in 10% NuPage MES pre-cast gels and transferred to nitrocellular membranes (Invitrogen, San Diego, CA). Primary antibodies against p-I κ B α and total I κ B α were purchased from Cell Signaling (Beverly, MA) and anti-tubulin antibody was purchased from NeoMarkers (Fremont, CA). HRP-conjugated goat-anti-mouse secondary antibody was from Pierce Biotechnology (Rockford, IL), and proteins were detected by chemiluminescence with Supersignal West Dura substrate (Pierce Biotechnology, Rockford, IL). The ratio between p-I κ B α /total I κ B α among different treatments was determined by using ImageQuant TL software (Amersham Biosciences) and the percentage inhibition of I κ B α phosphorylation was calculated in comparison to the DMSO/LPS treated cells.

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- [1] For selected references on the role of inflammation in disease pathogenesis see: G. S. Firestein, R. Maki, J. M. Alvaro-Gracia, *J. Immunol.* **1990**, *144*, 3347; G. Camussi, E. Lupin, *Drugs* **1998**, *55*, 613–620; A. M. Armstrong, K. R. Gardiner, S. J. Kirk, M. J. Halliday, B. J. Rowlands, *Br. J. Surg.* **1997**, *84*, 1051–1058; R. Ferrari, *Cardiovasc. Res.* **1998**, *37*, 554–559; K. J. Tracey, *Nature* **2002**, *420*, 853–859.
- [2] For selected recent reviews of the role of inflammation in such diseases see: E. H. S. Choy, G. S. Panayi, *N. Engl. J. Med.* **2001**, *344*, 907–916; A. Belluzzi, S. Boschi, C. Bringola, A. Munarini, G. Cariani, F. Miglio, *Am. J. Clin. Nutr.* **2000**, *71*, 339S–242S; Y. Sherer, Y. Shoenfeld, *Ann. Rheum. Dis.* **2002**, *61*, 97–99; A. E. Becker, O. J. De Boer, A. C. Van der Val, *Annu. Rev. Med.* **2001**, *52*, 289–297; M. F. White, *Science* **2003**, *302*, 1710–1711; N. Ouchi, S. Kihara, T. Funahashi, Y. Matsuzawa, K. Walsh, *Curr. Opin. Lipidol.* **2003**, *14*, 561–566; N. C. Riedemann, P. A. Ward, *Expert Opin. Biol. Ther.* **2003**, *3*, 339–350; H. Yang, H. Wang, C. J. Czura, K. J. Tracey, *J. Endotoxin Res.* **2002**, *8*, 469–472; P. E. Morris, R. D. Hite, C. Ohl, *BioDrugs* **2002**, *16*, 403–417; P. P. Graczyk, *Prog. Med. Chem.* **2002**, *39*, 1–72; R. Nau, H. Eiffert, *Clin. Microbiol. Rev.* **2002**, *15*, 95–110.
- [3] F. R. Balkwill, F. Burke, *Immunol. Today* **1989**, *10*, 299–304; Z. Szekanecz, A. E. Kosh, S. L. Kunkel, R. M. Strieter, *Clin. Pharmacol.* **1998**, *12*, 377–390; R. Kurzrock, M. Talpaz, *Cytokines: Interleukins and their Receptors*, Kluwer, Boston, **1995**; C. A. Dinarello, L. L. Moldawer, *Proinflammatory and Anti-inflammatory Cytokines in Rheumatoid Arthritis*, 3rd ed., Amgen, Thousand Oaks, **2001**.
- [4] P. P. Tak, G. S. Firestein, *J. Clin. Invest.* **2001**, *107*, 7–11; X. Li, G. R. Stark, *Exp. Hematol.* **2002**, *30*, 285–296; Y. Yamamoto, R. B. Gaynor, *J. Clin. Invest.* **2001**, *107*, 135–142; F. Chen, V. Castranova, X. Shi, L. M. Demers, *Clin. Chem.* **1999**, *45*, 7–17; A. S. Baldwin, *J. Clin. Invest.* **2001**, *107*, 135–142; C. Y. Wang, M. W. Mayo, A. S. Baldwin, Jr., *Science* **1996**, *274*, 784–787; D. J. van Antwerp, S. J. Martin, T. Kafri, D. R. Green, I. M. Verma, *Science*, **1996**, *274*, 787–789.
- [5] Q. Li, I. M. Verma, *Nat. Rev. Immunol.* **2002**, *2*, 725–734; A. S. Baldwin, Jr., *Annu. Rev. Immunol.* **1996**, *14*, 649–683; H. C. Liou, *J. Biochem. Mol. Biol.* **2002**, *35*, 537–546; A. A. Beg, D. Baltimore, *Science* **1996**, *274*, 782–784; U. Siebenlist, G. Franzoso, K. Brown, *Annu. Rev. Cell Biol.* **1994**, *10*, 405–455; P. A. Bauerle, T. Henkel, *Annu. Rev. Immunol.* **1994**, *12*, 141–179.
- [6] For selected references on this topic see: M. A. Palladino, F. R. Bahjat, E. A. Theodorakis, L. L. Moldawer, *Nat. Rev. Drug Discovery* **2003**, *2*, 736–746; *Tumor Necrosis Factors. The Molecules and their Emerging Role in Medicine*, (Ed.: B. Beutler), Raven Press, New York, **1992**; B. Aggarwal, R. Puri, *Human Cytokines: Their Role in Disease and Therapy*, Blackwell, Cambridge, UK, **1995**; R. Thorpe, A. Mire-Sluis, *Cytokines*, Academic Press, San Diego, **1998**; D. W. Galvani, J. C. Cawley, *Cytokine Therapy*, Cambridge University Press, Cambridge, UK, **1992**; G. Camussi, E. Lupin, *Drugs* **1998**, *55*, 613–620.
- [7] S. Habtemariam, *Planta Med.* **2000**, *66*, 303–313; P. Bremmer, M. Heinrich, *J. Pharm. Pharmacol.* **2002**, *54*, 453–472; R. L. Mazor, I. Y. Menendez, M. A. Ryan, M. A. Fielder, H. R. Wong, *Cytokine* **2000**, *12*, 239–245; M. Sheehan, H. R. Wong, P. W. Hake, V. Malhotra, M. O'Connor, B. Zingarelli, *Mol. Pharmacol.* **2002**, *61*, 953–963; S. K. Manna, C. Bueso-Ramos, F. Alvarado, B. B. Aggarwal, *Cancer Lett.* **2003**, *190*, 171–182; V. Castro, P. Rungeler, R. Murillo, E. Hernandez, G. Mora, H. L. Pahl, I. Merfort, *Phytochemistry* **2000**, *53*, 257–263; J.-H. Lee, T. H. Koo, B. Y. Hwang, J. J. Lee, *J. Biol. Chem.* **2002**, *277*, 18411–18420; S. P. Hehner, M. Heinrich, P. M. Bork, M. Vogt, F. Ratter, V. Lehmann, K. S. K. Schulze-Osthoff, W. Droge, M. L. Schmitz, *J. Biol. Chem.* **1998**, *273*, 1288–1297; C. Delporte, N. Backhouse, P. Salinas, A. San-Martin, J. Borquez, A. Loyola, *Bioorg. Med. Chem.* **2003**, *11*, 1187–1190; I. Posadas, M. C. Terencio, A. Randazzo, L. Gomez-Paloma, M. Paya, M. J. Alcaraz, *Biochem. Pharmacol.* **2003**, *65*, 887–895; D. Yang, X.-Y. Ye, M. Xu, *J. Org. Chem.* **2000**, *65*, 2208–2217; T. Krakauer, B. Q. Li, H. A. Young, *FEBS Lett.* **2001**, *500*, 52–55; A. Castrillo, B. de las Heras, S. Hortelano, B. Rodriguez, A. Villar, L. Bosca, *J. Biol. Chem.* **2001**, *276*, 15854–15860; M.-C. Liang, S. Bardhan, C. Li, E. A. Pace, J. A. Porco, Jr., T. D. Gilmore, *Mol. Pharmacol.* **2003**, *64*, 123–131.
- [8] Y.-H. Kim, B. S. Chung, U. Sankawa, *J. Nat. Prod.* **1988**, *51*, 1080–1083.
- [9] H.-S. Kang, Y.-H. Kim, C.-S. Lee, J.-J. Lee, I. Choi, K.-H. Pyun, *Cell. Immunol.* **1996**, *170*, 212–221; H.-S. Kang, H. K. Song, J.-J. Lee, K.-H. Pyun, I. Choi, *Mediators Inflammation* **1998**, *7*, 257–259; Y.-G. Suh, Y.-H. Kim, M.-H. Park, Y.-H. Choi, H.-K. Lee, J.-Y. Moon, K.-H. Min, D.-Y. Shin, J.-K. Jung, O.-H. Park, R.-O. Jeon, H.-S. Park, S.-A. Kang, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 559–562.
- [10] T. Ling, B. A. Kramer, M. A. Palladino, E. A. Theodorakis, *Org. Lett.* **2000**, *2*, 2073–2076; T. Ling, C. Chowdhury, B. A. Kramer, B. G. Vong, M. A. Palladino, E. A. Theodorakis, *J. Org. Chem.* **2001**, *66*, 8843–8853.
- [11] T. Lam, T. Ling, C. Chowdhury, T.-H. Chao, F. R. Bahjat, G. K. Lloyd, L. L. Moldawer, M. A. Palladino, E. A. Theodorakis, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3217–3221.
- [12] CCDC-230392 (2) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.
- [13] S. A. Ahmed, R. M. Gogal, Jr., J. E. Walsh, *J. Immunol. Methods* **1994**, *170*, 211–224.
- [14] K. Rutault, C. A. Hazzalin, L. C. Mahadevan, *J. Biol. Chem.* **2001**, *276*, 6666–6674.
- [15] A. C. Allison, E. M. Eugui, *Ann. N.Y. Acad. Sci.* **1995**, *762*, 331–341; W. B. van den Berg, *Arthritis Res.* **2001**, *3*, 18–26; T. Saxne, M. A. Palladino, D. Hernegard, N. Talal, F. A. Wollheim, *Arthritis Rheum.* **1988**, *31*, 1041–1045; C. A. Dinarello, J. A. Gelfand, S. M. Wolff, *J. Am. Med. Assoc.* **1993**, *269*, 1829–1835.
- [16] Y. Fong, K. J. Tracey, L. L. Moldawer, D. G. Hesse, K. B. Manogue, J. S. Kenney, A. T. Lee, G. C. Kuo, A. C. Allison, S. F. Lowry, E. Cerami, *J. Exp. Med.* **1989**, *170*, 1627–1633.

- [17] K. J. Van Zee, L. L. Moldawer, H. S. Oldenburg, W. A. Thompson, S. A. Stackpole, W. J. Montegut, M. A. Rogy, C. Meschter, H. Gallati, C. D. Schiller, W. F. Richter, H. Loetscher, A. Ashkenazi, S. M. Chamow, F. Wurm, S. E. Calvano, S. F. Lowry, W. Lesslauer, *J. Immunol.* **1996**, *156*, 2221–2230.
- [18] H. Redl, G. Schlag, M. Ceska, J. Davies, W. A. Burman, *J. Infect. Dis.* **1993**, *167*, 1464–1466.
- [19] K. J. Van Zee, L. E. DeForge, E. Fischer, M. A. Marano, J. S. Kenney, D. G. Remick, S. F. Lowry, L. L. Moldawer, *J. Immunol.* **1991**, *146*, 3478–3482.
- [20] For selected reviews on the I κ B-NF- κ B complex see: M. Karin, Y. Yamamoto, Q. M. Wang, *Nat. Rev. Drug Discovery* **2004**, *3*, 17–26; J. R. Burke, *Curr. Opin. Drug Discov. Dev.* **2003**, *6*, 720–728.
- [21] For selected recent studies on I κ B inhibition with small molecules see: A. Castrillo, M. Mojena, S. Hortelano, L. Bosca, *J. Biol. Chem.* **2001**, *276*, 34082–34088; J. R. Burke, M. A. Pattoli, K. R. Gregor, P. J. Brassil, J. F. McMaster, K. W. McIntyre, X. Yang, V. S. Iotzova, W. Clarke, J. Strnad, Y. Qiu, F. C. Zusi, *J. Biol. Chem.* **2003**, *278*, 1450–1456; B. H. B. Kwok, B. Koh, M. I. Ndubuisi, M. Elofsson, C. M. Crews, *Chem. Biol.* **2001**, *8*, 759–766.

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