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Synthesis of a Novel Family of Diterpenes and Their Evaluation as Anti-Inflammatory Agents

Thanh Lam,[a] Taotao Ling,[a] Chinmay Chowdhury,[a] Ta-Hsiang Chao,[b] F. R. Bahjat,[b] G. K. Lloyd,[b] Lyle L. Moldawer,[c] Michael A. Palladino[b] and Emmanuel A. Theodorakis[a,*]

[a]*Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0358, USA*
[b]*Nereus Pharmaceuticals, Inc, 10480 Wateridge Circle, San Diego, CA 92121, USA*
[c]*Department of Surgery, University of Florida College of Medicine, Gainesville, FL 32610, USA*

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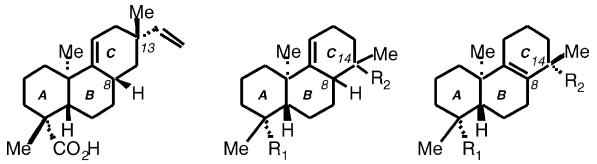
Abstract—The synthesis and biological evaluation of a new family of diterpenes, represented by structures **2** and **3**, is presented. These compounds constitute isomeric analogues of acanthoic acid (**1**) and were examined as potent anti-inflammatory agents. Among them, methyl ester **12** exhibited a low non-specific cytotoxicity, inhibited TNF-α synthesis and displayed good specificity in suppressing cytokine expression.

Inflammation is a central immune response that protects the host against tissue injury and microbial invasion and as such it should exhibit a rapid onset and short duration (acute inflammation). However, its persistent or uncontrolled activity (chronic inflammation) has detrimental effects to the body and results in the pathogenesis of several immune diseases, including septic shock, rheumatoid arthritis, inflammatory bowel diseases and congestive heart failure.[1] The unfolding of an appropriate inflammatory response is mediated and carefully controlled by different families of cytokines that have either pro-inflammatory (such as TNF-α, IL-β, IL-8 and IL-12), or anti-inflammatory (such as IL-10 and IL-1ra) effect.[2]

Our increased understanding of the involvement of cytokines in the inflammatory response has led to the development of therapeutic strategies based on their selective inhibition.[3] For example, several products have been approved or are currently under development for controlling the adverse effects of TNF-α overproduction, including soluble receptors (Enbrel®),[4] monoclonal antibodies to TNF-α (Remicade®, Humira®),[5] immunoconjugates[6] and small molecules inhibitors.[7]

Our studies are focused on the development of novel anti-inflammatory agents based on the privileged structures of natural products. The starting point for our research was the structure of acanthoic acid (**1**) (Fig. 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 rheumatism. More recently, studies revealed that **1** suppresses the production of IL-1 and TNF-α at 10 μg/mL, 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 goals to improve upon the biological effects of the parent molecule. Our synthetic strategy[10] allows us to access new families of acanthoic



1: acanthoic acid **2** **3**

Figure 1. Chemical structures of acanthoic acid and related analogues.

*Corresponding author. Tel.: +1-858-822-0456; fax: +1-858-822-0386; e-mail: etheodor@chem.ucsd.edu

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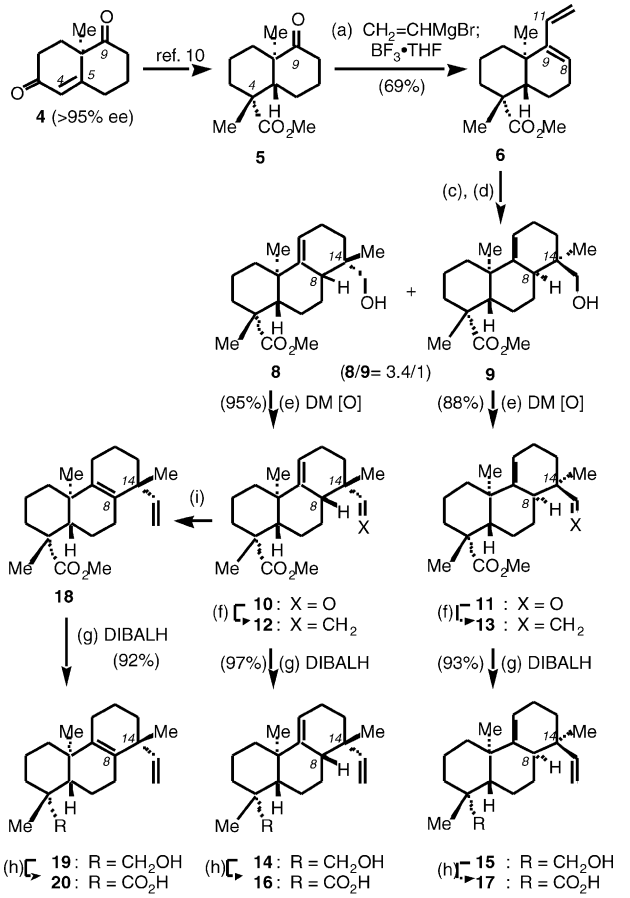
acid analogues, best represented by the generic structures **2** and **3** (Fig. 1). These analogues differ from **1** in the conformation or composition of the rigid tricyclic core, allowing us to evaluate the overall shape of the natural product as a function of its activity. Herein, we report the synthesis and evaluation of these compounds as TNF-α modulators.

The synthesis of all compounds is shown in Scheme 1. Compound **5**, representing the bicyclic scaffold of acanthoic acid and related compounds, was synthesized as one enantiomer in 6 steps from enone **4**. Treatment of **5** with vinyl Grignard followed by dehydration ($BF_3{\cdot}Et_2O$) produced diene **6** in 69% yield. The Diels-Alder cycloaddition between **6** and methacrolein proceeded under neat conditions and afforded a mixture of diastereomeric aldehydes which after reduction afforded alcohols **8** and **9** in 94% combined yield (**8**:**9** = 3.4:1).[10] The major isomer **8** was subsequently functionalized at the C14 stereocenter by a two step sequence involving oxidation of the primary alcohol (Dess–Martin periodinane) and Wittig olefination of the resulting aldehyde, to produce compound **12** in 86% yield. Conversion of the methyl ester functionality of **12** to carboxylic acid **16** was best achieved by a three steps process involving initial reduction to the primary alcohol **14** (DIBAL-H) and subsequent oxidation (Dess–Martin periodinane, sodium hypochlorite). This process produced acid **16** in 80% overall yield. The same sequence of reactions was applied for the conversion of the minor alcohol **9** to carboxylic acid **17**. Base-induced epimerization of the double bond of **12** produced compound **18** in which the C9–C11 double bond had migrated in the more substituted C8–C9 position. This compound was then converted to acid **20** using the same strategy as previously described.



Scheme 1. Reagents and conditions: (a) 1.5 equiv CH_2=CHMgBr (1.0 M in THF), THF, 0 °C; 4.4 equiv $BF_3{\cdot}Et_2O$, benzene/THF: 4/1, 80 °C, 5 h, 69%; (c) 13 equiv **7**, neat, 8 h, 25 °C, **8**/**9**: 3.4/1, 100%; (d) 1.4 equiv $NaBH_4$, THF/MeOH: 10/1, 30 min, 25 °C, 94%; (e) 1.6 equiv Dess–Martin [O], CH_2Cl_2, 0 °C, 3 h, 95% for **10**, 88% for **11**; (f) 2.7 equiv Ph_3PCH_3Br, 2.2 equiv NaHMDS (1.0 M in THF), THF, 25 °C, 18 h, 86% for **12**, 89% for **13**; (g) 4.0 equiv DIBALH, CH_2Cl_2, −78 °C, 1 h, 95% for **14**, 97% for **15**, 92% for **19**; (h) 2.5 equiv Dess–Martin [O], CH_2Cl_2, 0 °C, 3 h; 2.0 equiv $NaClO_2$, 2.0 equiv NaH_2PO_4, 2.0 equiv CH_3CH=$C(CH_3)_2$, *t*BuOH/H_2O 2/1 25 °C, 1 h, 83% for **16**, 76% for **17**, 78% for **20**; (i) LiOH, DMF, 100 °C, 6 h, 83%.

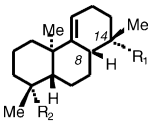
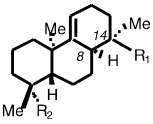
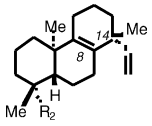
The cytotoxicity of the synthesized compounds was evaluated using human peripheral blood mononuclear cells (HPBMC). This assay consists of pretreating the cells with various concentrations of the analogues and subsequently evaluating their metabolism and viability using resazurin-based fluorescence measurements.[11] Resazurin is metabolized in mitochondria to a fluorescent dye whose fluorescence intensity is used as an indicator of the cell's energy metabolism. The study was performed at eight different concentrations of analogues based on a half-log dilution titration (final concentrations: 10, 3.17, 1 μg/mL, 317, 100, 31.7, 10 and 3.17 ng/mL). In these experiments staurosporin at 10 μM was used as a positive control. The data were collected after 4 h and 28 h of incubation. No cytotoxicity was observed after 4 h of incubation. The results obtained after 28 hr of incubation are presented as EC_{50} and cytotoxicity efficacy in Table 1.[12]

In a similar manner we assayed the ability of the synthetic diterpenes to decrease lipopolysaccharide (LPS)-induced TNF-α production in HPBMC cells. It is known that LPS treatment increases the synthesis of TNF-α in HPBMC cells.[13] With this in mind, cells were pretreated with our synthetic diterpenes and subsequently incubated with LPS for 4 h. The TNF-α production was assayed in cell supernatants using Human TNF ELISA cytosets kit. These results are presented as IC_{50} and total inhibition efficacy in Table 1.[14]

The efficacy of TNF-α inhibition was determined 4 h post-LPS stimulation. During that time no cytotoxicity was observed for any of the analogues tested. The cytotoxicity observed for some analogues after longer incubation period (28 h) may be due to inhibition of signaling pathways, such as NF-kB, an effect known to induce apoptosis.

An ideal TNF-α inhibitor should inhibit TNF-α production at concentrations in which it does not display non-specific cytotoxicity.[15] To this end, the ratio between EC_{50} and IC_{50} should be high (arbitrarily chosen as greater than 2) and the inhibition efficacy should be greater than 90%. Based on these guidelines, correlation of the data presented in Table 1 allowed us to draw the following conclusions: (a) Most of the synthetic analogues displayed better activities than these of

Table 1. Cytotoxicity[12] and TNF-α inhibition[14] data of analogues

Entry	Compd	(Structure)		Cytotoxicity		TNF-α inhibition	
				EC_{50}	% Efficacy	IC_{50}	% Efficacy
1	Abietic acid			NT	NA	3.5	34
2	Podocarpic acid			NT	NA	Inactive	NA
3	Forskolin			NT	NA	Inactive	NA
4		**8**	R_1: CH_2OH R_2: CO_2Me	NA	NA	Inactive	NA
5		**12**	R_1: $CH{=}CH_2$ R_2: CO_2Me	1.8	98	0.7	99
6		**14**	R_1: $CH{=}CH_2$ R_2: CH_2OH	NT	NA	2.3	38
7		**16**	R_1: $CH{=}CH_2$ R_2: CO_2H	7.8	66	5.7	90
8		**13**	R_1: $CH{=}CH_2$ R_2: CO_2Me	3.1	71	1.4	99
9		**15**	R_1: $CH{=}CH_2$ R_2: CH_2OH	2.3	93	2.2	99
10		**17**	R_1: $CH{=}CH_2$ R_2: CO_2H	NT	35	3.7	86
11		**18**	R_2: CO_2Me	NT	NA	0.6	47
12		**19**	R_2: CH_2OH	3.4	99	3.1	99
13		**20**	R_2: CO_2H	2.2	99	2.8	98

related diterpene natural products, such as abietic acid, podocarpic acid and forskolin; (b) From the series of analogues arising from manipulation of the major diastereomer of the Diels–Alder reaction between diene **6** and methacrolein, only compounds **12** and **14**, exhibited a good EC_{50}/IC_{50} ratio. Among them, the most efficacious is methyl ester **12** which inhibits up to 99% of TNF-α production at non-cytotoxic concentrations; (c) From the series of analogues arising from manipulation of the minor diastereomer of the Diels–Alder reaction between diene **6** and methacrolein, the most promising analogue is again methyl ester **13**. Alcohol **15** displays TNF-α inhibition at concentrations in which it is cytotoxic ($EC_{50}/IC_{50} \approx 1$), while carboxylic acid **17**, although non-toxic, it only inhibits TNF-α synthesis up to 86%; (d) From the series of compounds arising from isomerization of the double bond of the major diastereomer of the Diels-Alder reaction only methyl ester **18** exhibits an acceptable EC_{50}/IC_{50} ratio, while compounds **19** and **20** are cytotoxic. Nonetheless, compound **18** has reduced efficacy since it only inhibits TNF-α synthesis up to 47%.

The above results suggest that across the three series the methyl ester derivatives **12**, **13** and **18** show a more promising activity. The eight-point dose response titration curves for these analogues illustrate clearly their differences in terms of cytotoxicity and TNF-α inhibition (Fig. 2). Since the overall functionalization of these molecules is identical, the different efficacy in TNF-α inhibition is likely due to conformational changes of the tricyclic scaffold. Moreover, among these three compounds, methyl ester **12** had better efficacy and led to almost complete inhibition of TNF-α production.

Compound **12**, displaying the most promising profile, underwent further evaluation in terms of regulating cytokine synthesis. This was accomplished by examining its ability to suppress the production of various pro- and anti-inflammatory cytokines using the related Cytosets assay kits.[16] Similarly to the TNF-α study, the levels of different cytokines were measured from HPBMC cells treated with **12** and subsequently stimulated with LPS. These results are expressed in percentage of cytokine inhibition (Fig. 3).[16] Administration of 316 ng/mL of **12** resulted in 30–40% inhibition of TNF-α and 60–65% inhibition of IL-1β and IL-6. This inhibition is cytokine selective since at this concentration the production levels IL-1ra and IL-8 were not affected.

Recent data indicate that simultaneous inhibition of TNF-α and IL-1β reduces dramatically inflammation in patients suffering from rheumatoid arthritis and related diseases.[17,18] With this in mind, the selectivity manifested by **12** against production of both TNF-α and IL-1β could be beneficial to the treatment of inflammatory diseases.

In conclusion, inspired by the chemical structure and medicinal potential of acanthoic acid, we developed a new family of non-natural analogues of **1**. Being isomeric to the parent molecule at the C13 and C14 centers, these compounds cannot be synthetically available from manipulation of the natural product and cannot be biosynthetically accessible. Among them, the C4 methyl ester analogues were found to display promising TNF-α inhibitory profile. Of particular interest is methyl ester **12**, which maintains the overall shape and conformation of the tricyclic core of the natural

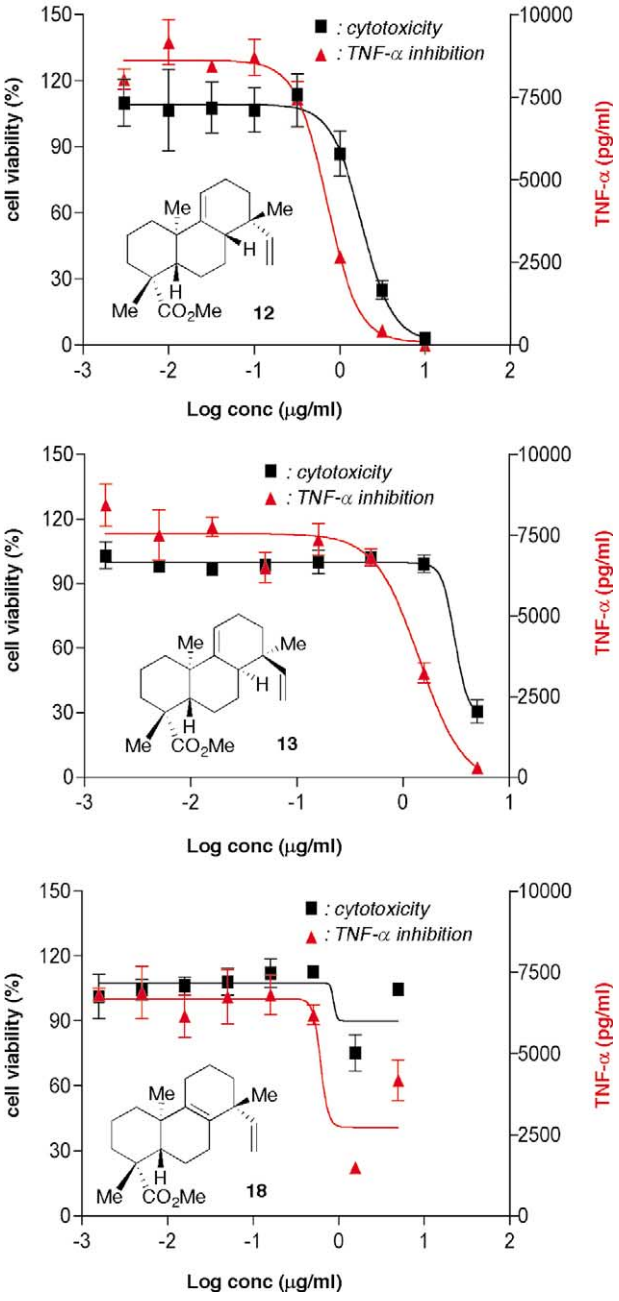


Figure 2. Titration curves for cytotoxicity and TNF-α inhibition of analogues **12**, **13** and **18**. Note that the cytotoxicity was measured 28 h after incubation with analogues, while the level of TNF-α was measured 4 h post-LPS stimulation. See refs 12 and 14 for more detail.

product. This analogue inhibited up to 99% of TNF-α production at concentrations in which it was not cytotoxic and manifested a promising cytokine selectivity. Moreover, being a small molecule, compound **12** could overcome the long-term immunosuppressive and immunogenic effects of protein-based therapeutics and may show improved tissue penetration and pharmacology.[4–7] The above studies suggest that compound **12** or related family members can be used as starting points for the development of novel anti-inflammatory drugs.

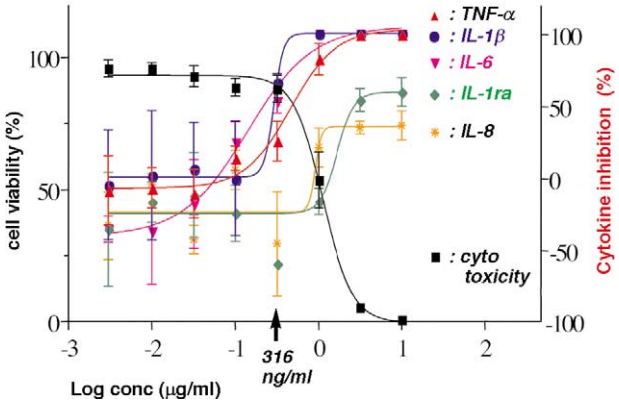


Figure 3. Cytokine selectivity data for methyl ester analogue **12**.

Acknowledgements

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12. Cytotoxicity assay: HPBMC (Human Peripheral Blood Mononuclear Cells) purchased from Cambrex (Walkersville, MD, USA) were seeded in 96-well Costar 3904 tissue culture plates at 25-K cell/well in 80-μL volume of LGM-3 medium and then recovered in the tissue culture incubator for 12 h. At the end of recovery, 10 μL of eight-point dose response titration curves of analogues (starting from10 μg/mL with half-log dilution) were added to corresponding wells. The 96-well tissue culture plates were then returned to the incubator for 24-h incubation. 10 μL of resauzurin dye (Sigma, St. Louis, MO, USA) was then added to each well at the end of incubation and the plate was again returned to the incubator for another 4 h incubation before fluorescence measurement. The fluorescence of resauzurin was measured by using 530-mm excitation and 590-mm emission filters. Staurosporin (Sigma, St. Louis, MO, USA) 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 using the Sigmoidal dose response model with variable slope.
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15. Cytokine selectivity measurements: Cytokines such as IL-1β, IL-6, IL-1ra, and IL-8 were analyzed using the Cytosets kits purchased from Biosource International (Camarillo, CA). Like TNF-α, the levels of IL-1β 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.
16. Non-toxic (NT) is used when at least 80% of the cells survived after 24 h of incubation with the compound tested at the highest concentration (10 μg/mL). 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 μg/mL) did not inhibit TNF-α synthesis.
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