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TITLE: New Triterpenoids for Prevention of Breast Cancer

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REPORT DOCUMENTATION PAGE

We have recently shown that the synthetic triterpenoid, 2-cyano-3,12-dioxoolean-1,9-,dien-28-ic acid (CDDO) is a highly potent inhibitor of the proliferation of several ER-positive and ER-negative human breast cancer cell lines. Furthermore, CDDO at nanomolar levels will block de novo synthesis of two inflammatory enzymes that have recently been implicated in the carcinogenic process, namely inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2). Current efforts are now underway to study whether chronic administration of CDDO can prevent the development of breast cancer in an animal model for this disease. Since we have shown in many other studies that combinations of chemopreventive agents are often more effective than single agents, we have also begun cell culture studies in both ER-positive and ER-negative breast cancer cells to explore the combined use of CDDO together with retinoids or ligands for the nuclear receptor, PPAR-gamma. If we find useful synergisms in cell culture, we will translate these results into suitable animal experiments for prevention of breast cancer in vivo.

 newfound product, 2-cyano-3,12-dioxoolean-1,9-,dien-28-ic acid (CDDO) is a highly potent inhibitor of the proliferation of several ER-positive and ER-negative human breast cancer cell lines. Furthermore, CDDO at nanomolar levels will block de novo synthesis of two inflammatory enzymes that have recently been implicated in the carcinogenic process, namely inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2). Current efforts are now underway to study whether chronic administration of CDDO can prevent the development of breast cancer in an animal model for this disease. Since we have shown in many other studies that combinations of chemopreventive agents are often more effective than single agents, we have also begun cell culture studies in both ER-positive and ER-negative breast cancer cells to explore the combined use of CDDO together with retinoids or ligands for the nuclear receptor, PPAR-gamma. If we find useful synergisms in cell culture, we will translate these results into suitable animal experiments for prevention of breast cancer in vivo.
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For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Michael B. Spur, M.D. 6-29-80
PI - Signature Date
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INTRODUCTION

The principal objective of this project is to show, for the first time, that a synthetic triterpenoid can be used for the prevention of breast cancer in a valid animal model of the human disease. The eventual goal is to extend the use of a synthetic triterpenoid to prevent breast cancer in women at high risk. There is a major need for innovative drug discovery in the field of breast cancer, and a particular need for development of new agents which will inhibit progression of premalignant or early malignant lesions to more invasive and metastatic stages, since genetic and other screening techniques are now identifying large numbers of women who are at risk for eventual development of invasive breast cancer. We have had a long-standing history of professional commitment and involvement in developing and testing new agents for chemoprevention of breast cancer and other malignancies (Moon et al., 1979; Sporn, 1991; Anzano et al., 1994, 1996; Hong and Sporn, 1997). We have been developing a set of synthetic triterpenoids as a new class of chemopreventive agents. Previous studies (Nishino et al., 1988; Huang et al., 1994) have shown that the naturally occurring triterpenoids, oleanolic and ursolic acids, are effective agents for inhibition of experimental skin carcinogenesis. However, they are relatively weak agents, and it is therefore necessary to develop more potent compounds if triterpenoids are to be used effectively in a clinical setting for prevention of breast cancer in women at high risk. We started a new collaborative program at Dartmouth (between the laboratories of Professor Gordon Gribble in the Department of Chemistry and Professor Michael Sporn in the Department of Pharmacology), to synthesize and test new triterpenoids that would be more active than oleanolic acid and ursolic acids. We have made excellent progress resulting in the synthesis of the highly potent new molecule, CDDO. The goal of the project is to determine if CDDO will prevent breast cancer in experimental animals, and if it can be used synergistically with ligands of the nuclear receptor superfamily, such as rexinoids and PPAR-γ agonists, for this purpose.
(6) BODY

a) Experimental Methods

1. Studies on Human Breast Cancer Cells

Cell Maintenance:

MCF-7, T47D, or SK-Br-3 cells were maintained in DMEM/F12 with phenol red, 10% fetal bovine serum (Hyclone), Pen/Strep, in a 37°C, 5% CO₂ humidified incubator.

Treatment for Experiment:

Cells were harvested by trypsinization, resuspended in experimental media (RPMI without phenol red, 10% charcoal/dextran-stripped FBS (Hyclone), Pen/Strep), sedimented and washed once with the same media. Cells were then seeded in experimental media at 1200 cells per well in 96-well plates for MTT assay, 6000 cells per well in 24-well plates for ³H-thymidine incorporation, or 10⁶ cells per 9-cm dish for RNA extraction.

Addition of reagents:

Equal volume of experimental media containing 17 β-estradiol (final concentration = 10 pM), desired triterpenoid compound dissolved in DMSO, or vehicle alone at final concentration = 0.1% was added to the cells. Unstimulated control wells received vehicle in experimental media without 17 β-estradiol. Cells were incubated in compounds for three days (³H-thymidine incorporation and RNA extraction) or five days (MTT assay).

Assay of Thymidine Incorporation into DNA:

5 µCi ³H-thymidine was added to each well. After two hours incorporation time, the media was aspirated, the wells were washed, and the monolayer was fixed with 10% TCA. Nucleic acids were then solubilized with 0.2 N NaOH, 40 µg/ml salmon sperm DNA, and incorporated ³H was measured.

2. Studies on Prevention of Breast Cancer in Rats

We have performed 2 large breast cancer studies in the standard rat model that uses NMU as carcinogen, to evaluate the ability of CDDO, either alone, or in combination with the rexinoid, LG100268, to prevent cancer. The methods for these experiments are attached as Protocols DMS-TP-4 and DMS-TP-5.
Studies of Cancer Prevention by CDDO in Rats
Protocol DMS-TP-4

Synergism of CDDO and LG268 in Ovary-Intact Rats

<table>
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<th>Treatment</th>
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</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>18</td>
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<tr>
<td>B</td>
<td>CDDO, 60 mg/kg diet</td>
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<tr>
<td>C</td>
<td>CDDO, 30 mg/kg diet</td>
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<td>D</td>
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<tr>
<td>E</td>
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<tr>
<td>F</td>
<td>CDDO, 1 mg/kg diet</td>
<td>9</td>
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<tr>
<td>G</td>
<td>LG268 Hi, 50 mg/kg diet</td>
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<tr>
<td>H</td>
<td>CDDO 10 + LG268 Hi</td>
<td>9</td>
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<tr>
<td>I</td>
<td>CDDO 3 + LG268 Hi</td>
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<tr>
<td>J</td>
<td>CDDO 1 + LG268 Hi</td>
<td>9</td>
</tr>
<tr>
<td>K</td>
<td>LG268 Lo, 25 mg/kg diet</td>
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<tr>
<td>L</td>
<td>CDDO 10 + LG268 Lo</td>
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<td>M</td>
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<td>9</td>
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<tr>
<td>N</td>
<td>CDDO 1 + LG268 Lo</td>
<td>9</td>
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<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>135</strong></td>
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Rats and Carcinogen Treatment:
Sprague-Dawley Rats are obtained as a single cohort for the entire experiment. It is essential to know the ages of these animals accurately. When they are 21 day old, they will be injected intraperitoneally with nitroso methyl urea (NMU), 50 milligrams per kilogram body weight. NMU solution = 5 mg/ml in isotonic saline at pH 4 using acetic acid (this should NOT be phosphate buffered saline, PBS). Rats injected on 8/25/99.

Special Diets
These will be started one week after injection of animals with NMU. Chemopreventive agents will be added to the powdered diet in an oily vehicle containing 12.5 ml ethanol, 37.5 ml Neobee oil, and 1.0 ml Tenox 5 for each kilogram of powdered diet.

Duration of Experiment and Autopsy of Rats:
Experiment will be terminated 8 weeks after initial injection of NMU, when tumor incidence in controls approximates 100%. Rats will be palpated weekly to assess tumor incidence. At autopsy all tumors will be counted and weighed.
Studies of Cancer Prevention by CDDO in Rats

Protocol DMS-TP-5

Synergism of CDDO and LG268 in Ovary-Intact Rats

<table>
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<td>Control</td>
<td>24</td>
</tr>
<tr>
<td>B</td>
<td>CDDO, 30 mg/kg diet</td>
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<tr>
<td>C</td>
<td>CDDO, 10 mg/kg diet</td>
<td>12</td>
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<tr>
<td>D</td>
<td>LG268, 60 mg/kg diet</td>
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<tr>
<td>E</td>
<td>9-cis RA, 60 mg/kg diet</td>
<td>12</td>
</tr>
<tr>
<td>F</td>
<td>all-trans-RA, 60 mg/kg diet</td>
<td>12</td>
</tr>
<tr>
<td>G</td>
<td>CDDO 30 + LG268</td>
<td>12</td>
</tr>
<tr>
<td>H</td>
<td>CDDO 30 + 9-cis RA</td>
<td>12</td>
</tr>
<tr>
<td>I</td>
<td>CDDO 30 + all-trans-RA</td>
<td>12</td>
</tr>
<tr>
<td>J</td>
<td>CDDO 10 + LG268</td>
<td>12</td>
</tr>
<tr>
<td>K</td>
<td>CDDO 10 + 9-cis RA</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Total</td>
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Rats and Carcinogen Treatment:
Sprague-Dawley Rats are obtained as a single cohort for the entire experiment. It is essential to know the ages of these animals accurately. When they are 21 day old, they will be injected intraperitoneally with nitroso methyl urea (NMU), 50 milligrams per kilogram body weight. NMU solution = 5 mg/ml in isotonic saline at pH 4 using acetic acid (this should NOT be phosphate buffered saline, PBS). Rats injected on 12/1/99.

Special Diets
These will be started one week after injection of animals with NMU. Chemopreventive agents will be added to the powdered diet in an oily vehicle containing 12.5 ml ethanol, 37.5 ml Neobee oil, and 1.0 ml Tenox 5 for each kilogram of powdered diet.

Duration of Experiment and Autopsy of Rats:
Experiment will be terminated 8 weeks after initial injection of NMU, when tumor incidence in controls approximates 100%. Rats will be palpated weekly to assess tumor incidence. At autopsy all tumors will be counted and weighed.
b) Results and Discussion

1. Synthesis of CDDO and Other New Triterpenoids

During the past year, we have been able to synthesize enough CDDO to allow us to perform studies of prevention of breast cancer in an experimental model in the rat (results described below). Furthermore, we have accomplished the synthesis of more than 50 new triterpenoids, derived from either oleanolic or ursolic acids. These organic syntheses are described in 2 published papers by Tadashi Honda et al.; “Novel Synthetic Oleanane Triterpenoids: A Series of Highly Active Inhibitors of Nitric Oxide Production in Mouse Macrophages” (Bioorganic & Medicinal Chemistry Letters 9, 3429-3434, 1999), and “Novel Synthetic Oleanane and Ursane Triterpenoids with Various Enone Functionalities in Ring A as Inhibitors of Nitric Oxide Production in Mouse Macrophages” (J. Medicinal Chem. 43, 1866-1877, 2000). Reprints of both of these published articles are attached; these include acknowledgement of support from this grant. Activities and structures of some of these new triterpenoids in suppression of growth of human breast cancer cells in culture is described below.

2. Results with Human Breast Cancer Cells

We have tested CDDO in combination with ligands for nuclear receptors (such as PPAR-γ or RXRs) for ability to suppress proliferations of both ER-positive and ER-negative human breast cancer cell lines. Figures 1-4 show that in cell culture, although CDDO itself is a potent inhibitor of breast cancer cell proliferation, its interactions with other agents, such as the PPAR-γ ligand, GW7845, or the rexinoid, LGD100268, are weak. Furthermore, Figures 5-8 show that although the above 2 agents (GW7845 and LGD100268) interact with each other at a molecular level, they do not have particularly strong synergism in affecting proliferation of various breast cancer cell lines in culture.

In addition to the above results with CDDO itself, we have also assayed a large number of new synthetic triterpenoids, made by Tadashi Honda and Gordon Gribble, for their inhibitory activity on growth of MCF-7 cells in culture. Several new compounds are highly active (TP-190, 192, 155), as shown on the attached Figures 9-18, although none are significantly more active than CDDO itself (TP-151). In this set of figures, we have grouped structures together by resemblances to the chemical structure, rather than by their number which is used to identify them in our laboratory notebooks.
FIGURE 1
CDDO and GW Compound: Combination Effects on Proliferation of MCF-7

(PPAR-γ Ligand)

*Three days incubation with compounds, 10% stripped FBS, phenol red-free RPMI, 10 pM 17-β estradiol
Two hours thymidine pulse
FIGURE 2
Combination Effects of CDDO and GW Compound on Growth of MCF-7 Cells
(PPAR-γ ligand)

Three days incubation with compounds in 10% charcoal-stripped FBS, phenol red-free RPMI, 10 pM 17β-estradiol
Two hours thymidine pulse
FIGURE 3
CDDO and GW Compound: Combination Effects on Proliferation of MDA-231

(PPAR-γ Ligand)

Counts 3H Incorporated

Three days incubation with compounds in 10% FBS growth media
Two hours thymidine pulse
FIGURE 4
Effects of CDDO, GW, and LGD Compounds on Growth of SK-Br-3 Cells

(PAR-Y ligand) (Rexinoid)

Counts 3H Incorporated

Three days incubation with compounds in 10% FBS growth media
Two hours thymidine incorporation
FIGURE 5

Effects of GW and LGD Compounds on Growth of MCF-7 (+ estradiol)

(PPAR-\(\gamma\) (Ligand) (Rexinoid)

Counts 3H Incorporated

- DMSO
- M1.0
- M1.0
- GW 0.1 M
- LGD100288 1 μM
- LGD 0.1 M
- GW 0.1 μM + LGD 0.1 M
- M1.0000

- Three days incubation in compounds, 10% charcoal-stripped FBS, phenol red-free RPMI
- Stimulation with 10 pM 17\(\beta\) estradiol
- Two hours thymidine pulse
FIGURE 6
Effects of GW and LGD Compounds on Growth of T47D

(PPAR-γ Ligand) (Rexinoid)

Counts 3H Incorporated

- DMSO
- GW04804X 10 μM
- GW 1 μM
- GW 0.1 μM
- LGD1069 1 μM
- LGD 0.1 μM
- GW 0.1 μM + LGD 0.1 μM
- CDDO 0.1 μM

Three days incubation with compounds in 10% charcoal stripped FBS, phenol red-free RPMI, + 10 pM 17β estradiol
Two hours thymidine pulse
FIGURE 7
Effects of GW and LGD Compounds on Growth of SK-Br-3

(PPAR-γ ligand)
(Rexinoid)

Counts 3H Incorporated

GW03784X 10 μM
GW 1 μM
GW 0.1 μM
LGD10258 1 μM
LGD 0.1 μM
GW 0.1 μM + LGD 0.1 μM
GW10000

Three days incubation with compounds in 10% FBS growth media
Two hours thymidine pulse
FIGURE 8
Effects of GW and LGD Compounds on Growth of MDA-231

(PPAR-γ ligand) (fexinoid)

Counts 3H Incorporated

DMSO
GW101649 X 10^-6 M
GW 1 μM
GW 0.1 μM
LGD 10699 1 μM
LGD 0.1 μM
GW 0.1 μM + LGD 0.1 μM

Three days incubation with compounds in 10% FBS growth media
Two hours thymidine pulse
FIGURE 9

Effects of structural changes on inhibition levels of TP compounds

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>STRUCTURE</th>
<th>IC50 (μM)</th>
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<tr>
<td>TP-195</td>
<td><img src="image1" alt="Structure of TP-195" /></td>
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<tr>
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<td>TP-155</td>
<td><img src="image5" alt="Structure of TP-155" /></td>
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<td>COMPOUND</td>
<td>STRUCTURE</td>
<td>IC50 (μM)</td>
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<tr>
<td>----------</td>
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<tr>
<td>TP-189</td>
<td><img src="image" alt="Structure of TP-189" /></td>
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<td>TP-192</td>
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<td>TP-194</td>
<td><img src="image" alt="Structure of TP-194" /></td>
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## FIGURE 11

**Effects of structural changes on inhibition levels of TP compounds**

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<th>IC50 (μM)</th>
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<td><img src="image" alt="TP-109 Structure" /></td>
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<tr>
<td>TP-128</td>
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FIGURE 12
Effects of structural changes on inhibition levels of TP compounds

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<td>TP-082</td>
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FIGURE 13
Effects of structural changes on inhibition levels of TP compounds

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<td>TP-175</td>
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FIGURE 14

Effects of structural changes on inhibition levels of TP compounds

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<td>TP-174</td>
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<td>9.0</td>
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FIGURE 15
Effects of structural changes on inhibition levels of TP compounds

<table>
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<tr>
<td>TP-202</td>
<td><img src="image" alt="TP-202 Structure" /></td>
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<tr>
<td>TP-033</td>
<td><img src="image" alt="TP-033 Structure" /></td>
<td>&gt;10</td>
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</table>
FIGURE 16
Effects of structural changes on inhibition levels of TP compounds

<table>
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<tr>
<th>COMPOUND</th>
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<th>IC50 (μM)</th>
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<tr>
<td>TP-216</td>
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<td>TP-163</td>
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<td>11.8</td>
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<tr>
<td>TP-151</td>
<td><img src="image" alt="Structure TP-151" /></td>
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</table>
# FIGURE 17

**Effects of structural changes on inhibition levels of TP compounds**

<table>
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<th>COMPOUND</th>
<th>STRUCTURE</th>
<th>IC$_{50}$ (µM)</th>
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<td>10.1</td>
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<td>11.8</td>
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<tr>
<td>TP-155</td>
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FIGURE 18

Effects of structural changes on inhibition levels of TP compounds

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<th>STRUCTURE</th>
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<td>TP-069</td>
<td><img src="image" alt="TP-069 Structure" /></td>
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3. Results from Studies on Prevention of Experimental Breast Cancer in Rats

We have now performed 2 major long-term studies in vivo with CDDO, to determine if it can prevent experimental breast cancer induced in rats with nitrosomethylurea (NMU). These studies have involved several hundred rats, and have evaluated not only the effects of CDDO when used as a single agent, but also its potential synergy in vivo with the rexinoid, LGD268. The protocols for these two studies (DMS-TP-4 and DMS-TP-5) have been described above, under "Methods." The attached Tables 1 and 2 show the following results: 1) CDDO itself, over a very wide dose range, has little ability to prevent breast cancer induced in rats by NMU; 2) in contrast, CDDO can synergize with LGD268 in vivo to prevent breast cancer in the rat. These synergistic effects can be demonstrated at several different doses of CDDO, and several different doses of LGD268, as seen in Tables 1 and 2. The mechanism of this synergy between the two agents is unknown at the present time. However, these studies are important because they demonstrate for the first time that a synthetic triterpenoid can affect the process of mammary carcinogenesis in an experimental animal.
## Synergism of CDDO and LGD 100268 in Ovary-Intact Rats

**CDDO = 60, 30, 10, 3, 1 mg/kg diet**

**LGD268 = 50, 25 mg/kg diet**

**Data as of 10-28-99**

M.B. Sporn, N. Suh, C. Williams,  
R. Risingsong, Y. Wang, DMS

<table>
<thead>
<tr>
<th>Group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
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<td>(%)</td>
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<td>(89%)</td>
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<td>4.6</td>
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<td>(33%)</td>
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<td>(11%)</td>
<td>(0%)</td>
<td>(25%)</td>
<td>(44%)</td>
<td>(22%)</td>
<td></td>
</tr>
<tr>
<td><strong>Rats with Ulcerated Tumors (%)</strong></td>
<td>1/17</td>
<td>0/9</td>
<td>1/9</td>
<td>1/9</td>
<td>0/9</td>
<td>1/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
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<td>(11%)</td>
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<td>(0%)</td>
<td>(11%)</td>
<td>(0%)</td>
<td>(13%)</td>
<td>(22%)</td>
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### Table 1

**FINAL REPORT: Protocol DMS-TP-4**
# Synergism of CDDO and LGD 100268 in Ovary-Intact Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>CDDO Hi 30 mg/kg</th>
<th>CDDO Lo 10 mg/kg</th>
<th>LGD628 60 mg/kg</th>
<th>9-cis-RA 60 mg/kg</th>
<th>All-transRA 60 mg/kg</th>
<th>CDDO Hi + LGD628</th>
<th>CDDO Hi + 9-cis-RA</th>
<th>CDDO Hi + All-transRA</th>
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<th>CDDO Lo + 9-cis-RA</th>
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<td>Tumor Incidence (%)</td>
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<td>9/12</td>
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<td>10/11</td>
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<td>(75%)</td>
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<td>(83%)</td>
<td>(92%)</td>
<td>(75%)</td>
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<td>(92%)</td>
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<td>0/12</td>
<td>3/12</td>
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<td>1/12</td>
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<td>(25%)</td>
<td>(25%)</td>
<td>(9%)</td>
<td>(17%)</td>
<td>(8%)</td>
<td>(25%)</td>
<td>(50%)</td>
<td>(8%)</td>
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<tr>
<td>No. of Tumors/Rat (average)</td>
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<td>3.4</td>
<td>2.8</td>
<td>1.8</td>
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<td>1.1</td>
<td>2.2</td>
<td>2.5</td>
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<td>3.7</td>
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<tr>
<td>Tumor Burden/Rat (grams, average)</td>
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<tr>
<td>(71%)</td>
<td>(75%)</td>
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<td>(33%)</td>
<td>(50%)</td>
<td>(55%)</td>
<td>(8%)</td>
<td>(25%)</td>
<td>(50%)</td>
<td>(25%)</td>
<td>(67%)</td>
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<tr>
<td>Rats with Tumor Burden &gt; 5 g</td>
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<td>6/12</td>
<td>1/12</td>
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<td>(33%)</td>
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<td>(8%)</td>
<td>(67%)</td>
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</tr>
<tr>
<td>Rats with Ulcerated Tumors</td>
<td>2/24</td>
<td>3/12</td>
<td>1/12</td>
<td>0/12</td>
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<td>(0%)</td>
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<td>(0%)</td>
<td>(0%)</td>
<td>(0%)</td>
<td>(17%)</td>
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</tbody>
</table>
(7) KEY RESEARCH ACCOMPLISHMENTS

- First report of the use of a ligand for peroxisome proliferator-activated receptor-γ (PPAR-γ) to prevent experimental breast cancer.

- Demonstration of synergistic action of a new synthetic triterpenoid, CDDO, together with a new rexinoid, for prevention of experimental breast cancer.

- Scale up of laboratory synthesis of CDDO to produce gram quantities for in vivo studies.
(8) REPORTABLE OUTCOMES

We are attaching copies of the following 3 publications, all of which have resulted for support provided by this grant:


(9) CONCLUSIONS

We have now established that a new synthetic triterpenoid, CDDO, not only has potent anti-proliferative activity on human breast cancer cells in culture, but also that this same agent can be used to potentiate the chemopreventive action of a member of another class of agents, namely the rexinoid, LGD10028. We have developed a practical synthesis that can be used to make enough CDDO so that studies of its pharmacology can now be pursued in vivo. Finally, since further new synthetic triterpenoids are still being made in our collaboration with Professor Gribble, there is the hope that even more potent and useful compounds will be made in the future. We believe our studies are important, in that they are establishing the triterpenoids as a class of new agents that may eventually have practical clinical use for prevention of breast cancer in women.


(11) APPENDICIES

Attached are reprints of 3 references we put in "Reportable Outcomes".
A New Ligand for the Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ), GW7845, Inhibits Rat Mammary Carcinogenesis

Nanjo Suh, Yongping Wang, Charlotte R. Williams, Renee Risingsong, Tona Gilmer, Timothy M. Willson, and Michael B. Sporn

Abstract

We have tested a new ligand for peroxisome proliferator-activated receptor-γ, GW7845, as an inhibitor of experimental mammary carcinogenesis, using the classic rat model with nitrosomethyurea as carcinogen. Rats were first treated with a single dose of nitrosomethyurea (50 mg/kg body weight, i.p.). Starting 1 week later, they were fed GW7845, at either 60 or 30 mg/kg of diet, for 2 months. This agent significantly reduced tumor incidence, tumor number, and tumor weight at both doses. This is the first report of the use of a ligand for peroxisome proliferator-activated receptor-γ to prevent experimental breast cancer.

Introduction

The continuing magnitude of the breast cancer problem with respect to incidence, morbidity, and mortality requires further drug discovery to prevent this disease (1). The use of tamoxifen, raloxifene, and fenretinide as clinically proven, effective agents to suppress breast carcinogenesis (2–4) indicates that chemoprevention is a viable strategy for the prevention of breast cancer in women. Current research in this area is driven by the need to discover new agents that will be more effective and have fewer side effects. In this brief communication, we report the first use of a new and highly potent ligand for the nuclear receptor, PPAR-γ, GW7845 to inhibit experimental mammary carcinogenesis in vivo.

PPAR-γ is a transcription factor belonging to the nuclear receptor superfamily (5–7) and forms functional heterodimers with the retinoid X receptor (8). PPAR-γ is of great current interest because it mediates the antidiabetic effects of several TZDs that are now in widespread clinical use for treatment of type 2 diabetes (9, 10). The PPARs bind a variety of naturally occurring fatty acids and eicosanoids with low micromolar affinity (6). Interestingly, PPAR-γ has a preference for polyunsaturated fatty acids (11), dietary components that have been shown to lower the incidence of cancer in experimental animals (12, 13), although the clinical relevance of these observations remains unclear (12, 14).

Synthetic PPAR-γ ligands have been shown to inhibit growth of several human tumor cell lines in culture (15–17) and, most notably, to induce growth arrest and differentiation in primary cultures of human liposarcoma cells, both in vitro and in vivo (18, 19). In contrast, there have been conflicting reports on the effects of the TZD class of PPAR-γ ligands in experimental colon carcinogenesis (20–22). The mechanism of inhibition of growth of tumor cells by ligands for PPAR-γ is not well understood (23). For the present study, reported here, the availability of a potent member of a new class of ligands for PPAR-γ, GW7845 (24), has enabled us to test this agent for inhibition of mammary carcinogenesis in the classic rat model that uses NMU as carcinogen.

Materials and Methods

Cell Culture and Differentiation Assays. GW7845 was dissolved in DMSO (0.01%), and aliquots were frozen at −20°C. Serial dilutions were made in DMSO before addition to cell culture media. The 3T3-L1 preadipocyte cells were obtained from American Type Culture Collection, grown to confluency in DMEM/5% calf serum, and then treated once with compounds in DMEM/10% fetal bovine serum. Every 2 days thereafter, medium was changed to DMEM/10% fetal bovine serum without added compounds. Cells were harvested on day 6, and as a marker of differentiation, glyceraldehyde 3-phosphate dehydrogenase was measured in lysates, using a standard assay for consumption of NADH at 340 nm (25).

Mammary Carcinogenesis Studies. A total of 159 female Sprague-Dawley rats (Taconic Farms, Germantown, NY) received i.p. injections of NMU (50 mg/kg body weight) when 21 days old, as described by Thompson et al. (26). One week later, the rats were randomly assigned to one of six experimental groups (Table 1). GW7845 and tamoxifen were blended into the diets as described previously (27) and were fed to the rats continuously, either alone or in combination, for the duration of the experiment. Rats were killed after 2 months (CO₂ inhalation), and breast cancers were enumerated and weighed at autopsy.

Other. The Fisher exact test and the Mann-Whitney rank test were used to evaluate the statistical differences between the treatment groups; all P values shown are two-sided. Institutional guidelines for proper and humane use of rats were observed.

Results and Discussion

GW7845 is a tyrosine analogue (Fig. 1), rather than a TZD such as troglitazone, rosiglitazone, and pioglitazone (the ligands for PPAR-γ in current clinical use). Unlike the TZDs, GW7845 has been optimized for potency on PPAR-γ (24) and is significantly more potent than either rosiglitazone or troglitazone when assayed for induction of adipogenic differentiation in the fibroblastic cell line, 3T3-L1 (25), as shown in Fig. 2.

We have performed two separate but identical long-term experiments to demonstrate the chemopreventive efficacy of GW7845. Given the widespread use of tamoxifen as an agent to prevent breast cancer, we have also looked at potential synergism between GW7845 and tamoxifen. The results in both experiments were essentially identical; therefore, we have pooled the data in Table 1.

GW7845 was well tolerated at the doses fed (Table 1), and rats treated with this agent weighed the same as controls. Table 1 shows that GW7845 had significant inhibitory effects on mammary carcinogenesis regardless of whether tumor incidence, numbers of tumors per rat, or ATB (the average weight of a rat's tumor at autopsy) was
Table 1 Prevention of breast cancer by GW7845 and tamoxifen

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of tumor-free rats (total no. of rats (P1; P2)(^a))</th>
<th>Average no. of tumors (P1; P2)(^b)</th>
<th>ATB (P1; P2)(^b)</th>
<th>Rats with 3 or more tumors (P1; P2)(^b)</th>
<th>Rats with tumor burden (&gt;5) g (P1; P2)(^b)</th>
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</thead>
<tbody>
<tr>
<td>Control (vehicle)</td>
<td>5/42</td>
<td>2.4</td>
<td>5.6</td>
<td>22/42</td>
<td>18/42</td>
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<tr>
<td>GW7845 Hi</td>
<td>8/21 (0.02)</td>
<td>1.1 (0.002)</td>
<td>1.7 (0.002)</td>
<td>2/21 (0.0009)</td>
<td>1/21 (0.0002)</td>
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<tr>
<td>GW7845 Lo</td>
<td>7/21 (0.08)</td>
<td>0.8 (&lt;0.0001)</td>
<td>1.5 (&lt;0.0004)</td>
<td>2/21 (&lt;0.0001)</td>
<td>2/21 (0.0009)</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>5/33</td>
<td>1.6 (0.02)</td>
<td>2.4 (0.02)</td>
<td>7/33 (0.008)</td>
<td>6/33 (0.03)</td>
</tr>
<tr>
<td>Tamoxifen + GW7845 Hi</td>
<td>9/21 (0.009; 0.03)</td>
<td>0.9 (0.0002; 0.03)</td>
<td>0.9 (0.0002; 0.03)</td>
<td>2/21 (&lt;0.0001; 0.03)</td>
<td>2/21 (0.0002)</td>
</tr>
<tr>
<td>Tamoxifen + GW7845 Lo</td>
<td>12/21 (0.003; 0.002)</td>
<td>0.6 (&lt;0.0001; 0.001)</td>
<td>1.3 (0.001; 0.01)</td>
<td>21/20 (0.0002)</td>
<td>32/21 (0.03)</td>
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</tbody>
</table>

\(^a\) Doses used were as follows: 60 mg GW7845/kg diet (GW7845 Hi); 30 mg GW7845/kg diet (GW7845 Lo); and 0.5 mg tamoxifen/kg diet. All animals (21 days old) received an i.p. injection of 50 mg NMU/kg body weight 1 week before starting the feeding of chemopreventive agents.

\(^b\) P2 is the value for the comparison of rats treated with chemopreventive agents with control rats treated with vehicle alone; P2 is the value for the comparison of rats treated with tamoxifen + GW7845 with rats treated with tamoxifen alone.

Fig. 1. Structure of GW7845.

measured. The effects on ATB are particularly interesting: GW7845 effected a 70% reduction in this index. Striking effects of GW7845 on tumor multiplicity and weight were seen (Table 1) when the number of rats with three or more tumors or the number of rats with a tumor burden \(>5\) g were scored. Both doses of GW7845 appeared equally effective in all parameters measured. To evaluate possible synergy with tamoxifen, we deliberately chose a very low dose of this agent, which is only marginally effective (27, 28). As seen in Table 1, although some statistically significant additive effects were seen with the combination of GW7845 and tamoxifen, there was little evidence in these experiments for a strong synergy between the two.

These initial experiments in vivo establish GW7845 as an agent worthy of further consideration for chemoprevention of cancer. Further studies in other organ systems in which PPAR-\(\gamma\) plays an important role, as well as potential synergy with other agents for which there is a mechanistic basis (e.g., selective ligands for the retinoid X receptor), should now be pursued, as well as further evaluation of the mechanism of suppression of carcinogenesis by PPAR-\(\gamma\).

Acknowledgments

We thank Tammy Frazer for expert assistance in preparation of the manuscript. Marilyn Brown and her staff, especially Jennifer Marocco and Catherine LaBarre, have provided excellent animal care.

References

**GW7845, A NEW PPARγ LIGAND**


NOVEL SYNTHETIC OLEANANE TRITERPENOIDS:
A SERIES OF HIGHLY ACTIVE INHIBITORS OF
NITRIC OXIDE PRODUCTION IN MOUSE MACROPHAGES

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nanjo suh,* yongping wang,* and michael b. sporn*

*Department of Chemistry, Dartmouth College, Hanover, NH 03755, U.S.A. and
†Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03755, U.S.A.

Received 16 August 1999; accepted 2 November 1999

Abstract: Novel oleanane triterpenoids with modified rings A and C were designed and synthesized. Among them, methyl 2-carboxy-3,12-dioxygenolana-1,9-dien-28-oate showed similar high inhibitory activity (IC50 = 0.8 nM) to 2-cyano-3,12-dioxygenolana-1,9-dien-28-oic acid (CDDO), which we have synthesized previously, against production of nitric oxide induced by interferon-γ in mouse macrophages. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

In a previous communication1 we reported that 2-cyano-3,12-dioxygenolana-1,9-dien-28-oic acid (CDDO) (1) has high inhibitory activity against production of nitric oxide (NO) induced by interferon-γ (IFN-γ) in mouse macrophages (IC50 = 0.1 nM level). We also showed that CDDO is a potent, multifunctional agent.2 For example, CDDO induces monocytic differentiation of human myeloid leukemia cells and adipogenic differentiation of mouse 3T3-L1 fibroblasts. CDDO inhibits proliferation of many human tumor cell lines. CDDO blocks de novo synthesis of inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. CDDO will protect rat brain hippocampal neurons from cell death induced by β-amyloid. The above activities have been found at concentrations ranging from 10^-6 to 10^-9 M in cell culture.

In the communication,1 we also reported that the combination of a 1-en-3-one functionality with a nitrile group at C-2 in ring A and a 9-en-12-one functionality in ring C enhances activity very strongly in comparison with the enhancement by each functionality alone. We therefore designed and synthesized a series of novel oleanane triterpenoids to survey what combination of ring A with ring C provides highly active compounds. We have found that methyl 2-carboxy-3,12-dioxygenolana-1,9-dien-28-oate (2) has similar high inhibitory activity to CDDO and methyl 2-cyano-3,12-dioxygenolana-1,9-dien-28-oate (CDDO methyl ester) (3).13 The new compound 2 is expected to be an alternative agent to CDDO. In this communication, the synthesis, inhibitory activity, and structure–activity relationships (SAR) are reported for these analogs.

Chemistry

Modification of Ring A (Schemes 1 and 2)

Initially, we designed and synthesized new olean-12-ene derivatives with a 1-en-3-one functionality having a substituent at C-2 in ring A, 6–9 and 12–18, to discover which substituents enhance activity in comparison with the lead compound 4, which was reported previously.4 Chloride 6 was synthesized in 81% yield from
Scheme 1.

5 11 12 19 20

\[ a \rightarrow b \]

6 \( X = \text{Cl} \)
8 \( X = \text{Br} \)

7 \( X = \text{Cl} \)
9 \( X = \text{Br} \)

Scheme 2.

\[ c \rightarrow d, e, f \rightarrow g \rightarrow h \]

10

11

13

12

14

16

17

15

18

Epoxide 5 with hydrogen chloride in acetic acid and CHCl₃.\(^5\) Halogenolysis of 6 with LiI in DMF\(^6\) gave chloride 7 in 77% yield. Similarly, bromides 8 and 9 were prepared from 5 and 8 (yield, 96% and 76%), respectively. Compound 11\(^7\) was prepared in 95% yield by formylation of C-3 ketone 10\(^8\) with ethyl formate in the presence of sodium methoxide in benzene.\(^8\) Nitrile 12 was synthesized in three steps (yield, 30%) from 11 according to the same synthetic route as for 30, which was prepared previously.\(^1\) Enal 13 was prepared from 11 by phenylselenenyl chloride-pyridine in CH₂Cl₂ and sequential addition of 30% H₂O₂\(^9\) (yield, 71%; 79% based on recovered 11). Jones oxidation of 13 gave acid 14 in 30% yield. Methylation of 14 with MeOH under acidic conditions gave ester 15 in 80% yield. Halogenolysis of 14 gave dicarboxylic acid 16 in 58% yield. Methylation of 16 with MeOH under acidic conditions gave ester 17 selectively in 70% yield because the carboxylic acid at C-17 of 16 is very sterically hindered. Amide 18 was prepared selectively in 72% yield from 15 with saturated ammonia-MeOH. Compounds 12 and 14–17 were found to be more active than the lead compound 4 (see Table 1).
Scheme 3.

\[
\begin{align*}
19 & \xrightarrow{c, d} 20 & \xrightarrow{e} 21 & \xrightarrow{f} 22 \\
& + \xrightarrow{b} 23 & & 24 \\
\end{align*}
\]

Scheme 4.

\[
\begin{align*}
25 & \xrightarrow{k, l} 26 & \xrightarrow{g} 27 & \xrightarrow{m} 29 \\
& \xrightarrow{b} 28 & & \\
\end{align*}
\]

a: HX/ACOH/CHCl₃, b: Li/l/DMF, c: HCO₂Et/NaOMe/PhH, d: NH₂OH.HCl/aq EtoH, e: NaOMe/ Et₂O/MeOH, f: PhSeCl/AcOEt; 30%H₂O₂/THF, g: PhSeCl/pyr./CH₂Cl₂; 30%H₂O₂/CH₂Cl₂, h: Jones, i: H₂SO₄/MeOH, j: NH₂/MeOH, k: Stiles' reagent/DMF, l: CH₂N₂/Et₂O/THF, m: KOH/aq MeOH

Modification of Ring C

We already reported the synthesis and inhibitory activity of 3-oxoolean-1-ene derivatives with various structures of ring C, and among them enones 31–33 are more active than the lead compound 4 (see Table 2).⁴

Combination of Modified Ring A with Ring C (Schemes 3 and 4)

On the basis of the above results, new oleanane derivatives with modified rings A and C, 22–24, and 27–29, were designed and synthesized. Isoxazole 20 was prepared from C-3 ketone 19 by formylation (yield, 98%), followed by condensation with hydroxylamine (yield, 74%).¹⁰ Cleavage of the isoxazole moiety of 20 with sodium methoxide gave nitrile 21 in 92% yield.¹⁰ Nitrile 22 was prepared from 21 by phenylselenenyl
Table 1. IC₅₀ (µM)² Values of Olean-12-ene Derivatives with Modified Ring A

<table>
<thead>
<tr>
<th>compd</th>
<th>R₁ at C-2</th>
<th>R₂ at C-17</th>
<th>Taft's α⁺ value of R₂</th>
<th>activity IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>OH</td>
<td>CO₂H</td>
<td>1.34</td>
<td>27</td>
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<tr>
<td>18</td>
<td>CONH₂</td>
<td>CO₂Me</td>
<td>1.68</td>
<td>14</td>
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<td>35</td>
<td>OMe</td>
<td>CO₂H</td>
<td>1.81</td>
<td>30</td>
</tr>
<tr>
<td>15</td>
<td>CO₂Me</td>
<td>CO₂Me</td>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>17</td>
<td>CO₂Me</td>
<td>CO₂H</td>
<td>2.2</td>
<td>0.9</td>
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<tr>
<td>14</td>
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<tr>
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<td>CO₂H</td>
<td>CO₂H</td>
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<td>0.07</td>
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<tr>
<td>13</td>
<td>CHO</td>
<td>CO₂Me</td>
<td>2.15</td>
<td>toxic⁺</td>
</tr>
<tr>
<td>36 ¹</td>
<td>CHO</td>
<td>CO₂H</td>
<td>2.15</td>
<td>toxic⁺</td>
</tr>
<tr>
<td>8</td>
<td>Br</td>
<td>CO₂Me</td>
<td>2.84</td>
<td>&gt; 40</td>
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<tr>
<td>9</td>
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<td>&gt; 40</td>
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<tr>
<td>6</td>
<td>Cl</td>
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<tr>
<td>7</td>
<td>Cl</td>
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<td>2.96</td>
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<td>12</td>
<td>CN</td>
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<tr>
<td>30 ¹</td>
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<td>CO₂H</td>
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<td>0.6</td>
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<td>4 ²</td>
<td>H</td>
<td>CO₂H</td>
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<td>&gt; 40</td>
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<td>oleanolic acid</td>
<td>-</td>
<td></td>
<td>-</td>
<td>&gt; 40</td>
</tr>
<tr>
<td>hydrocortisone</td>
<td>-</td>
<td></td>
<td>-</td>
<td>0.01</td>
</tr>
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</table>

chloride in ethyl acetate and sequential addition of 30% H₂O₂,¹¹ (yield, 33%; 57% based on recovered 21). Halogenolysis of 22 gave acids 23 and 24 in 37% and 16% yield, respectively. Compounds 2 and 27–29 could not be synthesized according to the similar synthetic route as for 14–17 because Jones oxidation of the precursor of 2 (aldehyde at C-2) gives an unknown compound instead of 2. They were synthesized according to the alternative route illustrated in Scheme 4. Ester 26 was prepared in 78% yield from C-3 ketone 25¹ by Stiles’ reagent (methoxymagnesium methyl carbonate) in DMF,¹⁷ followed by methylation with diazomethane. Enone 27 was prepared from 26 according to the same method as for 13 (yield, 71%; 88% based on recovered 26). Hydrolysis of 27 with potassium hydroxide in aqueous MeOH gave acid 2 selectively in 78% yield again because of the steric hindrance of the methoxycarbonyl group at C-17 of 27. Halogenolysis of 2 gave dicarboxylic acid 28 and monocarboxylic acid 31 in 47% and 24% yield, respectively. Methylation of 28 with MeOH under acidic conditions gave ester 29 selectively in 82% yield.

Biological Results and Discussion

Inhibitory Activity of Olean-12-ene Derivatives with Modified Ring A

The inhibitory activities [IC₅₀ (µM) value] of olean-12-ene derivatives with a 1-en-3-one functionality with a substituent at C-2 in ring A,¹³ oleanolic acid, and hydrocortisone (a positive control) on production of NO induced by IFN-γ in mouse macrophages¹⁴ are shown in Table 1. These derivatives are arranged according to
Table 2. IC$_{50}$ (µM)$^a$ Values of Oleanane Derivatives with Modified Rings A and C

<table>
<thead>
<tr>
<th>compd</th>
<th>structure of ring C</th>
<th>R$_1$ at C-2</th>
<th>R$_2$ at C-17</th>
<th>activity IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3$^b$</td>
<td>CN</td>
<td>CN</td>
<td>CO$_2$Me</td>
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<tr>
<td>1$^b$</td>
<td>CN</td>
<td>CO$_2$H</td>
<td>0.0002</td>
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</tr>
<tr>
<td>27</td>
<td>CO$_2$Me</td>
<td>CO$_2$Me</td>
<td>toxic$^a$</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>CO$_2$Me</td>
<td>CO$_2$H</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>CO$_2$Me</td>
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<td></td>
</tr>
<tr>
<td>28</td>
<td>CO$_2$H</td>
<td>CO$_2$H</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>31$^a$</td>
<td>H</td>
<td>H</td>
<td>0.2</td>
<td></td>
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<tr>
<td>22</td>
<td>CN</td>
<td>CO$_2$Me</td>
<td>0.02</td>
<td></td>
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<td>CN</td>
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</tr>
<tr>
<td>32$^a$</td>
<td>H</td>
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<td>1.4</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>CN</td>
<td>CO$_2$H</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>33$^a$</td>
<td>H</td>
<td>CO$_2$H</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dexamethasone</td>
<td></td>
<td></td>
<td>0.0001</td>
</tr>
</tbody>
</table>

$^a$IC$_{50}$ (µM) values of compounds 1-3, 16, 22-24, hydrocortisone, and dexamethasone were determined in the range of 0.1 µM–1 µM (tenfold dilutions). The other compounds were assayed in the range of 0.01–40 µM (fourfold dilutions). Values are an average of two separate experiments.

The strength of Taft’s σ$^*$ values$^1$ of substituents at C-2. These results provide the following interesting SAR:

(1) The relationship between Taft’s σ$^*$ value and activity is not observed.
(2) Methoxycarbonyl, carboxyl, and nitrile groups at C-2 enhance activity. Compounds 12, 14-16, and 30 are about 10–100 times more active than the lead compound 4.
(3) Hydroxyl, aminocarbonyl, methoxy, chloride, and bromide groups decrease activity.
(4) Formyl group does not show activity, but only toxicity.
(5) Methoxycarbonyl and carboxyl groups at C-17 show similar activity.

Inhibitory Activity of Oleanane Derivatives with Modified Rings A and C

The inhibitory activities [IC$_{50}$ (µM) value] of oleanane derivatives with modified rings A and C,$^{13}$ and dexamethasone (a positive control) on production of NO induced by IFN-γ in mouse macrophages are shown in Table 2. These results provide the following interesting SAR:

(1) A 9-en-12-one functionality is the strongest enhancer of activity among structures of ring C. Compound 31 is about 10 times more active than 4.
(2) 12-En-11-one and 13-en-11-one functionalites also enhance activity. Compounds 32 and 33 are about 2–4 times more active than 4.

(3) The combination of a 9-en-12-one functionality with nitrile and carboxyl groups at C-2 provides extremely highly active compounds. Compounds 2, 3, and CDDO (1) are about 10,000 times more active than 4.

(4) The combination of 12-en-11-one and 13-en-11-one functionalities with a nitrile group at C-2 also provides highly active compounds. Compounds 22–24 are about 100 times more active than 4.

(5) Although compounds 27–29 were also expected to show similar high activity to CDDO from the perspective of SAR, they did not show high activity.

Currently, further evaluation in vivo for both antiinflammation and chemoprevention of CDDO, 2, and 3 are in progress. Studies on the mode of action of these compounds also are in progress.

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References and Notes
3. CDDO methyl ester (3) was found to show the same high activity as CDDO after our previous communication was published.
7. 'H and 13C NMR of compound 11 in CDCl3 showed that it is the single isomer as depicted in Scheme 2.
13. All new compounds, 2, 6–9, 12–18, 22–24, and 27–29 exhibited satisfactory spectral data including high-resolution mass spectra and elemental analyses.
14. Briefly, the procedure for this assay is as follows: Macrophages were harvested from female mice injected intraperitoneally four days earlier with 4% thioglycolate. These cells were seeded in 96-well tissue culture plates and incubated with 20 μg/mL IFN-γ in the presence or absence of inhibitory test compounds. After 48 hours NO production (measured as nitrite by the Griess reaction) was determined. Full details of the assay are given in reference 16.
Novel Synthetic Oleanane and Ursane Triterpenoids with Various Enone Functionalities in Ring A as Inhibitors of Nitric Oxide Production in Mouse Macrophages

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Novel Synthetic Oleanane and Ursane Triterpenoids with Various Enone Functionalities in Ring A as Inhibitors of Nitric Oxide Production in Mouse Macrophages

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Received January 7, 2000

We initially randomly synthesized about 60 oleanane and ursane triterpenoids as potential anti-inflammatory and cancer chemopreventive agents. Preliminary screening of these derivatives for inhibitory production of nitric oxide induced by interferon-γ in mouse macrophages revealed that 3-oxoolean-1,12-dien-28-oic acid (B-15) showed significant activity (IC50 = 5.6 μM). On the basis of the structure of B-15, 19 novel olean- and urs-12-ene triterpenoids with a 1-en-3-one functionality having a substituent at C-2 in ring A have been designed and synthesized. Among them, 3-oxoolean-1,12-diene derivatives with carboxyl, methoxycarbonyl, and nitrite groups at C-2 showed higher activity than the lead compound B-15. In particular, 2-carboxy-3-oxoolean-1,12-dien-28-oic acid (3) had the highest activity (IC50 = 0.07 μM) in this group of triterpenoids. The potency of 3 was similar to that of hydrocortisone (IC50 = 0.01 μM), although 3 does not act through the glucocorticoid receptor. Interesting structure-activity relationships of these novel synthetic triterpenoids are also discussed.

Introduction

Oleanane and ursane triterpenoids are pentacyclic compounds with 30 carbon atoms, which are derived biosynthetically from the cyclization of squalene.¹ The group includes a very large number of naturally occurring members that cover an impressive variety of functional groups.² Many compounds of this group are reported to have interesting biological, pharmacological, or medicinal activities similar to those of retinoids and steroids, such as anti-inflammatory activity, suppression of tumor promotion, suppression of immunoglobulin synthesis, protection of the liver against toxic injury, induction of collagen synthesis, and induction of differentiation in leukemia or teratocarcinoma cells.³ However, the potency of these triterpenoids is relatively weak. There are no systematic studies of structure-activity relationships based on chemical modification of oleanane and ursane triterpenoids.⁴ We have therefore considered that bioassay-directed systematic drug design and synthesis of derivatives of oleanolic acid (1) and ursolic acid (2), which are commercially available, could be of great value in discovering novel structures with high biological potency.


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The high output of nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS), which is expressed in activated macrophages, plays an important role in host defense. However, excessive production of NO also can destroy functional normal tissues during acute and chronic inflammation.⁵ This phenomenon is also closely related mechanistically to carcinogenesis.⁶ Thus, inhibitors of NO production in macrophages are potential anti-inflammatory and cancer chemopreventive drugs. Because oleanolic and ursolic acids are already known to have weak anti-inflammatory and antitumorigenic activity,³a,³b,³e,³f we focused our attention on therapeutic agents of these diseases. For this purpose, we have adopted an assay system that measures inhibition of NO production induced by interferon-γ (IFN-γ) in mouse macrophages⁷ as a preliminary screening assay system. We synthesized various oleanolic and ursolic acid derivatives and tested them as inhibitors of NO production. As a result, we have identified a series of novel olean-12-ene triterpenoids with a 1-en-3-one functionality having carboxyl, methoxycarbonyl, and nitrite groups at C-2 in ring A that show significant inhibitory activity (IC50 = 0.01–0.1 μM level) against production of NO induced by IFN-γ in mouse macrophages. In particular, 2-carboxy-3-oxoolean-1,12-dien-28-oic acid (3) had the highest activity (IC50 = 0.07 μM) in this group of compounds. The potency of 3 was similar to that of hydrocortisone (IC50 = 0.01 μM), although 3 does not act through the glucocorticoid receptor. We report here the synthesis, inhibitory activity, and structure-activity relationships of these novel triterpenoids in detail.

Chemistry

Discovery of Lead Compound. When we started this project, we had no information about a lead
### Table 1. Preliminary Screening Results of Synthetic Oleanane and Ursane Triterpenoids

<table>
<thead>
<tr>
<th>compd</th>
<th>skeleton</th>
<th>C-3</th>
<th>C-12</th>
<th>C-13</th>
<th>C-17</th>
<th>inhibition (%) at 10 μM²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>olean-12-ene</td>
<td>β-OH</td>
<td>H</td>
<td>C-12</td>
<td>C-13</td>
<td>CO₂H</td>
</tr>
<tr>
<td>2</td>
<td>urs-12-ene</td>
<td>β-OH</td>
<td>H</td>
<td>C-12</td>
<td>C-13</td>
<td>CO₂H</td>
</tr>
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<td>H</td>
<td>C-12</td>
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compound. Therefore, about 60 oleanolic and ursolic acid derivatives were initially randomly synthesized. They are divided into seven categories: 3-hydroxy derivatives, A; 3-oxo derivatives, B; chloro derivatives, C; dehydroxyoleanane derivatives, D; A-ring cleaved derivatives, E; C-ring cleaved oleanane derivatives, F; and lactams, G (see Table 1). In the preliminary screen of these derivatives for inhibition of production of NO induced by IFN-γ in mouse macrophages, 3-oxooleana-1,12-dien-28-oic acid (B-15) was found to show significant activity (inhibition: 85% at 10 μM, IC₅₀ = 5.6 μM). (See Tables 1 and 2.)

Design and Synthesis of New Derivatives. When B-15 is compared with the other derivatives, it has the following features: first, it is an oleanane; second, it has a 1-en-3-one functionality in ring A; third, it has a carboxyl group at C-17. We focused our attention on the 1-en-3-one functionality in ring A among these features. We therefore designed novel olean- and urs-12-ene triterpenoids with a 1-en-3-one functionality having a substituent at C-2 in ring A, 3-19, and novel triterpenoid—steroid hybrid compounds, 20 and 21 (see Table 2). The syntheses of these newly designed derivatives and compounds B-13–B-16 are illustrated in Schemes 1–6.

Ester B-13⁹ was synthesized in 62% yield by introduction of a double bond at C-1 of methyl oleanonate (B-3)¹⁰ with phenylethlenenyl chloride (PhSeCl) in ethyl acetate and sequential addition of pyridine and m-chloroperbenzoic acid.¹¹,¹² Acid B-15 was synthesized in 85% yield by halogenolysis of B-13 with lithium iodide in N,N-dimethylformamide (DMF).¹³ Similarly, acid B-16 was synthesized in 58% yield via ester B-14 from methyl ursonate (B-4).¹⁵ Epoxide 22 was prepared in 99% yield by epoxidation of B-13 with alkaline hydrogen peroxide. Treatment of 22 with sodium methoxide gave enone 23 (yield, 87%; 98% based on recovered 22). Diosphenol 24 was synthesized by demethylation of the methyl enol ether at C-2 of 23 with hydrochloric acid in acetic acid (yield, 81%). Halogenolysis of 24 gave acid 4 (yield, 18%). Halogenolysis of 23 gave a desired partial demethylated product 5 in 26% (41% based on recovered 26).
Scheme 2

\[ \text{Reagents: (a) } 30\% \text{ H}_2\text{O}_2, \text{ NaOH(aq), THF; (b) } \text{NaOMe, MeOH; (c) } \text{HCl, AcOH; (d) LiI, DMF.} \]

Scheme 3

\[ \text{Reagents: (a) } \text{HX, AcOH, CHCl}_3; \text{ (b) } \text{LiI, DMF.} \]

23) yield. Chloride 6 was synthesized in 81% yield from 22 with hydrogen chloride in acetic acid and chloroform. Halogenolysis of 6 gave chloride 7 in 77% yield. Similarly, bromides 8 and 9 were prepared from 22 and 8 (yield, 96% and 76%), respectively. Hydroxy-methylene 25,19,20 was prepared in 95% yield by formylation of B-3 with ethyl formate in the presence of sodium methoxide in benzene. Isoxazole 26 was prepared in 86% yield by condensation of 25 with hydroxylamine. Cleavage of the isoxazole moiety of 26 with sodium methoxide gave nitrile 27 in 99% yield. 1H NMR showed that 27 is a mixture of three tautomers [27a, 27b (2a-cyano), and 27c (2a-cyano)] and that 27a is the major one in CDCl3. Enone 10 was prepared in 88% yield by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) oxidation of 27 in benzene, although the same method as for B-13 gave 10 in only 35% yield. Halogenolysis of 10 gave acid 11 in 71% (91% based on recovered 10) yield. Similarly, ursane derivative 12 was synthesized in 52% yield via 28,20,23 28, and 30 from B-4. Acid 13 was prepared in 74% yield by halogenolysis of 12. Enal 14 was prepared from 25 by PhSeCl1-pyridine in methylene chloride and sequential addition of 30% hydrogen peroxide24 (yield, 71%; 79% based on recovered 25). Halogenolysis of 14 did not give acid 15 but a complex mixture. Therefore, the synthesis of acid 15 from olelanic acid (B-1)10 was attempted. Formylation of B-1 with ethyl formate in the presence of sodium methoxide in tetrahydrofuran gave 3220 (yield, 45%; 66% based on recovered B-1). Acid 15 was prepared from 32 according to the same method as for 14 (yield, 71%; 84% based on recovered 32). Jones oxidation of 14 gave acid 16 in 30% (39% based on recovered 14) yield. Because this yield was not enough to synthesize derivatives 3 and 17–19 from 16, an alternative route was adopted. Ester 31 was prepared in 74% (89% based on recovered B-3) yield from B-3 by Stiles’ reagent (methoxymagnesium methyl carbonate) in DMF,25 followed by methylation with diazomethane. 1H NMR showed that 31 is the single tautomer in CDCl3 as depicted in Scheme 5. Enone 17 was prepared from 31 according to the same method as for 14 (yield, 83%; 90% based on recovered 31). Hydrolysis of 17 with potassium hydroxide in aqueous methanol gave acid 16 selectively in 97% yield because the methoxycarbonyl group at C-17 of 17 is sterically hindered. Halogenolysis of 16 gave dicarboxylic acid 3 in 58% yield. Methylation of 3 with methanol under acidic conditions gave ester 18 selectively in 78% yield because of the steric hindrance of the carboxylic acid at C-17 of 3. Amide 19 was prepared selectively in 96% yield from 17 with saturated ammonia–methanol.

Biological Results and Discussion

The inhibitory activities [IC50 (μM) value] of compounds B-1, B-13, B-15, B-16, 1–21, and hydrocortisone (a positive control) on NO production induced by IFN-γ in mouse macrophages are shown in Table 2. These derivatives are arranged according to the strength of Taft’s σ* values46 of substituents at C-2. These results provide the following interesting structure–activity relationships:

(1) In the A ring, a 1-en-3-one functionality is important for significant activity. The lead compound B-15 is much more potent than the C-3 ketone B-1 and the
Scheme 4

Reagents: (a) HCO₂Et, NaOMe, PhH; (b) NH₂OH·HCl, aq EtOH; (c) NaOMe, Et₂O, MeOH; (d) DDQ, PhH; (e) LiI, DMF.

Scheme 5

Reagents: (a) PhSeCl, pyr, CH₂Cl₂; 30% H₂O₂, CH₂Cl₂; (b) Jones; (c) Stiles’ reagent, DMF; (d) CH₃N₂, Et₂O, THF; (e) KOH, aq MeOH; (f) LiI, DMF; (g) H₂SO₄, MeOH; (h) NH₃, MeOH.

Scheme 6

Reagents: (a) HCO₂Et, NaOMe, THF; (b) PhSeCl, pyr, CH₂Cl₂; 30% H₂O₂, CH₂Cl₂.

C-3 alcohol 1 (oleanolic acid). Also, the ursene derivative B-16 is more potent than the C-3 alcohol 2 (ursolic acid).

2 A correlation between Taft’s σₚ values of substituents at C-2 and biological activity is not observed. This result shows that the activity does not depend on the strength of electron-withdrawing effect of a substituent at C-2.

3 Carboxyl, methoxycarbonyl, and nitrile groups at C-2 enhance activity. Compounds 3, 10, 11, 16, and 17 are about 10–100 times more potent than B-15. In particular, 3 showed the highest activity (IC₅₀ = 0.07 µM) in this series of compounds. The potency of 3 was similar to that of hydrocortisone (IC₅₀ = 0.01 µM).

4 Hydroxyl, amino carbonyl, methoxy, chloride, and bromide groups decrease activity. Compounds 4–9 and 19 are much less potent than B-15.

5 A formyl group does not confer activity but only toxicity.

6 23,24-Dimethyl groups are important for signifi-
Table 2. Activity of Olean- and Urs-12-ene Triterpenoids with Various 1-En-3-one Functionalities

<table>
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<th>R at C-17</th>
<th>Taft’s value of R3</th>
<th>Formula</th>
<th>Analyses</th>
<th>Activityb</th>
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a O, 3-oxoolean-1,12-diene; D, 23,24-dinor-3-oxoolean-1,4,12-triene; U, 3-oxoursa-1,12-diene. b C, H, and N analyses were within ±0.4% of the theoretical values. c Details of the evaluation method are described in the Experimental Section. IC50 values of 3 and hydrocortisone were determined in the range of 0.1–1 μM (10-fold dilutions). The other compounds were assayed in the range of 0.01–40 μM (4-fold dilutions). Values are an average of two separate experiments. d Compounds 14 and 15 were toxic to cells above 1 μM and were not active below 1 μM. e Ursolic acid (2) was toxic to cells above 10 μM and was not active below 10 μM.

Experimental Section

General Experimental Procedures. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were measured with a Jasco DIP-181 digital polarimeter. UV and IR spectra were recorded on a Hewlett-Packard 8451A UV/VIS spectrophotometer and a Perkin-Elmer 600 series FTIR spectrophotometer, respectively. 1H (300 MHz) and 13C (75 MHz) NMR spectra were recorded on a Varian XL-300 Fourier transform spectrometer. The chemical shifts are reported in δ (ppm) using the δ 7.27 signal of CDCl3 (1H NMR) and the δ 77.33 signal of CDC13 (13C NMR) as internal standards. Low-resolution mass spectra and high-resolution MS data were obtained on a Micromass 70-VSE unless otherwise stated. Elemental microanalysis was performed by Atlantic Microlab Inc. TLC and preparative TLC (prep-TLC) were performed with Merck precoated TLC plates silica gel 60 F254. Flash column chromatography was done with Select Scientific silica gel (230–400 mesh). The standard work up method was as follows: an organic extract was washed with saturated aqueous NaHCO3 solution (three times) followed by saturated aqueous NaCl solution (three times), then dried over anhydrous MgSO4 and filtered. The filtrate was evaporated in vacuo.

Methyl 3-Oxoolean-1,12-dien-28-oate (B-1). A solution of methyl oleanonate (B-3)n (2.00 g, 4.27 mmol) and phenyliselenenyl chloride (98%) (1.00 g, 5.12 mmol) in EtOAc (85 mL) was stirred at room temperature for 3 h. To the stirred
23.8, 23.5, 21.8, 19.1, 18.8, 17.5. EIMS (70 eV) m/z: 437 [M]+ (9), 436 (11), 435 (27), 262 (57), 203 (100). HREIMS: Caled for C23H30O2: 466.3447. Found: 466.3446.

**Methyl 3-Oxoura-1,12-dien-28-oate (B-14).** B-14 was prepared from methyl ursonate (B-4) according to the same method as for B-13 to give an amorphous solid (66%): d18 O +98 (c 0.77, CHCl3). UV (EtOH) λmax (log ε): 222 (3.95) nm. IR (KBr): 2974, 2935, 2871, 1725, 1669 cm−1. 1H NMR (CDCl3): δ 7.06 (1H, d, J = 10.1 Hz), 6.81 (1H, d, J = 10.1 Hz), 5.63 (1H, t, J = 11.5 Hz), 1.17, 1.15 (each 3H, s), 1.10 (6H, s), 0.95 (3H, d, J = 5.4 Hz). 13C NMR (CDCl3): δ 205.5, 178.4, 159.3, 144.5, 123.6, 115.9, 114.5, 115.3, 121.5, 52.6, 51.8, 47.0, 45.9, 44.7, 42.2, 42.0, 41.7, 40.3, 39.7, 34.1, 33.3, 32.7, 32.5, 30.8, 28.2, 28.1, 24.4, 23.7, 23.5, 21.8, 21.4, 19.1, 19.0, 17.7. EIMS (70 eV) m/z: 496 [M]+ (14), 406 (12), 262 (74), 203 (100). HREIMS: Caled for C27H36O4: 466.3447. Found: 466.3446.

**3-Oxyoleana-1,12-dien-28-oic Acid (B-15).** A mixture of B-13 (100 mg, 0.21 mmol) and LIL (500 mg) in dry DMP (2 mL) was heated under reflux for 6 h. The mixture was acidified with 5% aqueous HCl solution and then extracted with a mixture of CH2Cl2 and EtOAc (1:2) three times. The extract was worked up according to the standard method to give a solid (110 mg). The solid was subjected to flash column chromatography (hexanes–EtOAc (5:1)) followed by hexanes–EtOAc (2:1) to give B-15 as an amorphous solid (82 mg, 85%): d18 O+ +98 (c 0.45, CHCl3). UV (EtOH) λmax (log ε): 230 (3.75) nm. IR (KBr): 2941, 2868, 1732, 1685, 1671 cm−1. 1H NMR (CDCl3): δ 7.04 (1H, d, J = 10.2 Hz), 5.81 (1H, d, J = 11.5 Hz), 2.86 (1H, dd, J = 4.2, 13.4 Hz), 1.16, 1.152, 1.147, 1.07, 0.94, 0.91, 0.84 (each 3H, s). 13C NMR (CDCl3): δ 205.5, 184.5, 159.2, 144.2, 125.3, 122.1, 53.5, 46.8, 45.8, 44.7, 42.1, 41.9, 41.3, 40.2, 39.7, 34.0, 33.3, 32.6, 32.5, 30.9, 28.0, 27.8, 26.0, 23.7, 23.5, 23.0, 21.8, 19.0, 18.9, 17.7. EIMS (70 eV) m/z: 462 [M]+ (8.5), 437 (3.8), 406 (6.6), 248 (60), 233 (14), 205 (100). HREIMS: Caled for C27H38O4: 462.2390. Found: 462.2389 (Table 2).

**3-Oxyoleana-1,12-dien-28-oic Acid (B-16).** B-16 was prepared from B-14 according to the same method as for B-15 to give an amorphous solid (88%): d18 O +91 (c 0.84, CHCl3). UV (EtOH) λmax (log ε): 230 (3.99) nm. IR (KBr): 3306, 2973, 2930, 2870, 1729, 1695, 1669 cm−1. 1H NMR (CDCl3): δ 7.07 (1H, d, J = 10.1 Hz), 6.52 (1H, d, J = 10.1 Hz), 5.33 (1H, t, J = 3.7 Hz), 2.24 (1H, d, J = 11.2 Hz), 1.18, 1.16, 1.11, 1.09 (each 3H, s), 0.96 (3H, d, J = 6.1 Hz), 0.88 (3H, s). 13C NMR (CDCl3): δ 249.5, 184.3, 159.3, 125.3, 53.6, 52.9, 48.5, 44.7, 42.5, 41.9, 41.5, 39.6, 39.2, 39.0, 36.8, 32.9, 30.8, 28.2, 28.1, 24.2, 23.7, 23.4, 21.8, 21.3, 19.0, 17.8, 17.2. FABMS (NBA) m/z: 453 [M+H]+ (100) (by a Micromass ZAB-SE). HRFABMS: Caled for C27H38O4: 453.3369. Found: 453.3355 (by a Micromass 70-SE-4F).

**2-Carboxy-3-oxyoleana-1,12-dien-28-oic Acid (C).** A mixture of 16 (109 mg, 0.21 mmol) and LIL (520 mg) in dry DMP (1.5 mL) was heated under reflux for 1 h. After 5% aqueous HCl solution was added, the acidic mixture was extracted with EtOAc three times. The extract was washed with water (three times) and saturated aqueous NaCl solution (three times), dried over anhydrous MgSO4, and filtered. The filtrate was evaporated in vacuo to give a residue (108 mg). The residue was subjected to flash column chromatography (CH2Cl2–MeOH (15:1)) followed by CH2Cl2–MeOH (10:1) to afford 3 as a crystalline solid (61 mg, 56%): mp 260–265°C dec; d18 O +91 (c 0.53, CHCl3). UV (EtOH) λmax (log ε): 234 (4.06). IR (KBr): 3359, 2943, 2872, 1752 cm−1. 1H NMR (CDCl3): δ 8.43 (1H, s), 5.37 (1H, t, J = 3.5 Hz), 2.87 (1H, dd, J = 5.8, 13.9 Hz), 1.25, 1.22, 1.18, 1.15, 0.95, 0.93, 0.88 (each 3H, s). 13C NMR (CDCl3): δ 209.0, 183.9, 173.2, 163.2, 144.2, 124.8, 52.4, 46.8, 45.7, 45.5, 42.3, 41.4, 41.0, 40.6, 40.4, 34.0, 33.2, 32.5, 32.3, 30.9, 28.4, 27.8, 26.0, 23.7, 23.5, 23.0, 22.0, 19.0, 18.4, 17.8. EIMS (70 eV) m/z: 496 [M]+ (3.0), 478 (3.4), 452 (7.6), 248 (56), 231 (50), 203 (100). HREIMS: Caled for C27H36O4: 496.3198. Found: 496.3198 (Table 2).

**2-Hydroxy-3-oxyoleana-1,12-dien-28-oic Acid (D).** A mixture was prepared from 4 according to the same method as for B-15

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**Figure 1.** Blockade by glucocorticoid antagonist RU486 of hydrocortisone-inhibited NO production but not of triterpenoid (3 and 11) inhibited NO production in primary mouse macrophages. Macrophage cells were incubated with IFN-γ (20 ng/mL) together with hydrocortisone or triterpenoids without RU466 (●); in some cases RU466 (1 μM) was added simultaneously to both hydrocortisone- and triterpenoid-treated cell wells (○). RU486 itself does not interfere with NO production at the concentration tested.
except that the reaction time was 2 h. The reaction mixture was subjected to flash column chromatography [hexanes–EtOAc (5:1)] followed by hexanes–EtOAc (4:1) to give 4 as an amorphous solid (18%): [α]D +99° (c 0.46, CHCl3). UV (EtOH) λmax (log e), 366 (6.1) nm; IR (KBr): 2943, 2870, 1733, 1691, 1601 cm−1. 1H NMR (CDCl3): δ 7.49 (1H, s), 5.35 (1H, t, J = 3.5 Hz), 3.63 (3H, s), 2.90 (1H, dd, J = 4.0, 13.8 Hz), 1.20, 1.15, 0.94, 0.91, 0.83 (each 3H, s). 13C NMR (CDCl3): δ 179.3, 178.3, 169.5, 144.6, 121.8, 121.6, 53.5, 51.6, 46.9, 46.5, 45.8, 43.1, 42.3, 42.1, 41.7, 40.3, 34.0, 33.2, 32.4, 28.7, 27.8, 26.0, 23.6, 23.2, 22.3, 19.1, 18.7, 17.5. EIMS (70 eV) m/z: 546 (5.0) and 544 (4.5) [M]+, 262 (8.5), 203 (24), 118 (100), 116 (100), 110 (100). HREIMS: Caled for C36H30O8Br: 544.5525. Found: 544.5535. Anal. (Table 2).

2-Bromo-3-o xooleana-1,12-dien-28-oic Acid (9). 9 was prepared from 8 according to the same method as for B-15 except that the reaction time was 4 h. The reaction mixture was subjected to flash column chromatography [hexanes–EtOAc (4:1)] followed by hexanes–EtOAc (3:1) to give 9 as an amorphous solid (76%): [α]D +82° (c 0.31, CHCl3). UV (EtOH) λmax (log e), 366 nm (IR): 2943, 2930, 2870, 1727, 1686, 1601 cm−1. 1H NMR (CDCl3): δ 7.49 (1H, s), 5.35 (1H, t, J = 3.4 Hz), 2.86 (1H, dd, J = 4.2, 13.7 Hz), 1.21 (6H, s), 1.16, 1.14, 0.94, 0.83 (each 3H, s). 13C NMR (CDCl3): δ 177.1, 184.4, 143.3, 144.3, 121.8, 121.7, 53.5, 46.9, 46.5, 43.1, 42.2, 42.0, 41.3, 40.3, 34.0, 33.2, 32.4, 28.7, 27.8, 26.0, 23.6, 23.2, 22.3, 19.1, 18.7, 17.5. EIMS (70 eV) m/z: 532 (13) and 530 (14) [M]+, 285 (5.6), 283 (2.6), 248 (100), 235 (10), 233 (11), 203 (84). HREIMS: Caled for C35H29O8Br: 530.5004. Found: 530.5006. Anal. (Table 2).
2-Cyano-3-oxoala-1,12-dien-28-oic Acid (13). 13 was prepared from 12 according to the same method as for B-15 except that the reaction time was 4 h. The reaction mixture was subjected to prep-TLC [hexanes—EtOAc (1:5)] to give 13 as an amorphous solid (74%). [α]D +148° (c 0.50, CHCl3).

UV (EtOH) λmax (log ε): 238 (3.86) nm. IR (KBr): 3417, 2973, 2926, 2870, 2233, 1731, 1689 cm−1. H NMR (CDCl3): δ 7.77 (1H, s), 4.39 (2H, d, J = 5.3 Hz), 1.22, 1.20, 1.12, 1.11 (each 3H, s), 0.95, 0.88 (each 3H, d, J = 5.7 Hz), 0.87 (9H, s). 13C NMR (CDCl3): δ 198.2, 184.2, 170.2, 139.0, 124.4, 115.1, 114.1, 52.8, 52.8, 45.2, 45.0, 42.6, 42.6, 40.68, 40.65, 39.1, 39.1, 36.7, 32.5, 30.7, 28.1, 28.0, 24.5, 21.6, 21.8, 21.5, 18.9, 18.2, 17.7, 17.2, 17.5. EIMS (70 eV) m/z: 477 [M]+ (22), 431 (23), 248 (100), 203 (48). HREIMS: Caled for C39H32O7N: 577.2343. Found: 577.2340. (Anal. Table 2).

2-Methoxy-carbonyl-3-oxoala-1,12-dien-28-oic Acid (17). 17 was prepared from 16 by the similar method as for 14. The reaction mixture was subjected to flash column chromatography [hexanes—EtOAc (4:1)] to give 17 as an amorphous solid (83%, 90% based on recovered 31): [α]D +65° (c 0.78, CHCl3). UV (EtOH) λmax (log ε): 230 (3.97) nm. IR (KBr): 2947, 2866, 1727, 1684, 1624 cm−1. H NMR (CDCl3): δ 7.73 (1H, s), 5.37 (1H, t, J = 3.5 Hz), 3.79, 3.64 (each 3H, s), 2.90 (1H, dd, J = 3.9, 13.7 Hz), 1.16 (6H, s), 1.13, 0.94, 0.91, 0.84 (each 3H, s). 13C NMR (CDCl3): δ 201.2, 176.4, 166.0, 144.8, 145.6, 122.8, 121.7, 52.7, 52.4, 51.8, 49.4, 45.3, 44.5, 42.9, 41.8, 41.5, 40.3, 39.5, 34.1, 33.2, 32.4, 29.2, 30.9, 28.7, 27.8, 25.9, 23.8, 23.2, 23.1, 19.4, 18.0, 17.5. EIMS (70 eV) m/z: 524 [M]+ (24), 492 (23), 465 (13), 262 (35), 203 (100). HREIMS: Caled for C42H40O8: 524.3502. Found: 524.3494. (Anal. Table 2).

2-Methoxy-carbonyl-3-oxoala-1,12-dien-28-oic Acid (18). A solution of 3 (52 mg, 0.10 mmol) in MeOH (5.2 mL) containing concentrated H2SO4 (0.15 mL) was heated under reflux for 30 min. After saturated aqueous NaCl solution was added to the mixture, it was extracted with EtOAc three times. The extract was worked up according to the standard method to give a residue (53 mg). The residue was subjected to flash column chromatography [hexanes—EtOAc (2:1)] to give 18 as an amorphous solid (42 mg, 78%): [α]D +61° (c 0.56, CHCl3). UV (EtOH) λmax (log ε): 230 (3.83) nm. IR (KBr): 3233, 2947, 2866, 1733, 1695, 1622 cm−1. H NMR (CDCl3): δ 7.73 (1H, s), 5.37 (1H, t, J = 3.4 Hz), 3.79 (3H, s), 2.86 (1H, dd, J = 4.1, 13.7 Hz), 1.16, 1.05, 1.14, 0.54, 0.82 (each 3H, s). 13C NMR (CDCl3): δ 200.1, 184.4, 166.2, 144.7, 129.2, 122.0, 52.7, 52.4, 46.9, 45.9, 45.8, 42.2, 41.5, 41.4, 40.3, 39.5, 34.9, 33.3, 32.5, 32.3, 30.9, 28.7, 27.8, 26.0, 23.6, 23.6, 23.2, 21.4, 19.4, 18.0, 17.7. EIMS (70 eV) m/z: 510 [M]+ (2.6), 495 (2.0), 478 (2.5), 432 (3.0), 263 (29), 248 (58), 231 (37), 203 (100). HREIMS: Caled for C42H36O8: 510.3345. Found: 510.3344. (Anal. Table 2).

2-Methyl-3-oxoala-1,12-dien-28-oic Acid (16). (From 14) To a solution of 14 (357 mg, 0.72 mmol) in acetone (71 mL) was added Jones reagent (0.5 mL) dropwise in an ice bath. The mixture was stirred in the ice bath for 20 min. After excess Jones reagent was decomposed with MeOH, the acetone was evaporated in vacuo. Under water was added to the resultant mixture, the aqueous mixture was extracted with EtOAc three times. The extract was washed with water (three times) and saturated aqueous NaCl solution (three times), dried over anhydrous MgSO4, and filtered. The filtrate was evaporated in vacuo to give a residue (294 mg). The residue was subjected to flash column chromatography [hexanes—EtOAc (1:1) followed by EtOAc] to afford 14 (89 mg) and 16 as a crystalline solid (109 mg; 30%, 39% based on recovered 14): mp 230–231 °C; [α]D +85° (c 0.61, CHCl3). UV (EtOH) λmax (log ε): 234 (3.78) nm. IR (KBr): 3436, 2946, 2876, 1756, 1722, 1639 cm−1. H NMR (CDCl3): δ 8.43 (1H, s), 5.36 (1H, t, J = 3.5 Hz), 3.64 (3H, s), 2.90 (1H, dd, J = 5.9, 13.7 Hz), 1.24, 1.21, 1.19, 1.13, 0.94, 0.91, 0.85 (each 3H, s). 13C NMR (CDCl3): δ 209.2, 178.4, 173.4, 165.2, 144.5, 123.3, 121.4, 52.4, 51.8, 47.0, 45.7, 45.5, 42.3, 41.7, 41.1, 40.6, 40.4, 34.0, 33.3, 32.4, 32.3, 30.9, 28.3, 27.8, 26.0, 23.8, 23.5, 23.1, 22.0, 19.0, 18.3, 17.8. EIMS (70 eV) m/z: 510 [M]+ (16), 492 (15), 451 (14), 433 (14), 262 (27), 203 (100). HREIMS: Caled for C39H28O7N: 510.2386. Found: 510.2387. (Anal. Table 2).

From 17: A solution of 17 (500 mg, 0.95 mmol) in MeOH (29 mL) and aqueous KOH solution (KOH, 2.9 g, water, 10 mL) was heated under reflux for 15 min. After removal of MeOH in vacuo, the mixture was acidified with 5% aqueous HCl solution. It was extracted with EtOAc (three times). The extract was washed with water and saturated aqueous NaCl solution (each three times), dried over MgSO4, and filtered. The filtrate gave 16 as a crystalline solid (470 mg, 97%). It was used for the next reaction without further purification.
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- **30% H$_2$O$_2$ (1.4 mL) in MeOH (2.8 mL) in an ice bath. The mixture was stirred at room temperature for 4 h. To the mixture were added saturated aqueous NaH$_2$SO$_4$ and 5% aqueous NaOH solutions, successively. After removal of THF and MeOH, the resultant mixture was acidified with 6 M aqueous HCl solution. The acetylated layer was extracted with CH$_2$Cl$_2$ three times. The extract was worked up according to the standard method (90). The resultant solid was washed with hot MeOH (228 mg, 99%). This material was used for the next reaction without further purification. An analytically pure sample was obtained by recrystallization from MeOH as colorless needles: mp 212–213 °C; [α]$_{D}^{20}$ +157° (c 0.80, CHCl$_3$). IR (KBr): 2934, 2866, 1727, 1699 cm$^{-1}$. 1H NMR (CDCl$_3$): δ 5.36 (1H, t, J = 3.3 Hz), 3.64 (3H, s), 3.50 (1H, d, J = 4.5 Hz), 3.37 (1H, d, J = 4.5 Hz), 2.90 (1H, dd, J = 4.2, 13.9 Hz), 1.21, 1.11, 0.97, 0.94, 0.92, 0.80 (each 3H, s). 13C NMR (CDCl$_3$): δ 213.0, 178.4, 144.5, 121.8, 64.1, 57.1, 51.8, 46.7, 43.3, 42.1, 41.7, 40.0, 41.6, 41.7, 39.8, 39.7, 38.8, 34.1, 33.5, 32.9, 30.9, 28.2, 26.0, 24.0, 22.8, 23.3, 21.1, 19.1, 17.4, 15.1. EIMS (70 eV) m/z: 482 [M$^+$] (7.7), 472 (13), 262 (31), 249 (11), 203 (100). HREIMS: Calcd for C$_{24}$H$_{30}$O: 482.3396. Found: 482.3391.

**Methyl 2-Methoxy-3-oxoolean-1,12-dien-28-oate (23).** A mixture of 22 (300 mg, 0.62 mmol) and Na (360 mg in MeOH (36 mL) was heated under reflux for 48 h. After removal of MeOH in vacuo, the resultant mixture was diluted with water and then acidified with 6 M aqueous HCl solution. The aqueous solution was extracted with a mixture of CH$_2$Cl$_2$ and Et$_2$O (1:2) two times. The extract was worked up according to the standard method (90) to give a solid (270 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (6:1) followed by hexanes–EtOAc (5:1)] to give 26 as an amorphous solid (934 mg, 86%): UV (EtOH) $\lambda_{max}$ (log e): 228 (3.65) nm. IR (KBr): 2940, 2864, 1725 cm$^{-1}$. 1H NMR (CDCl$_3$): δ 7.98 (1H, s), 5.34 (1H, t, J = 3.5 Hz), 3.63 (3H, s), 2.89 (1H, dd, J = 4.4, 13.7 Hz), 2.42 (1H, d, J = 15.1 Hz), 1.30, 1.21, 1.15, 0.93, 0.90, 0.87, 0.79 (each 3H, s). 13C NMR (CDCl$_3$): δ 178.4, 173.2, 150.4, 144.0, 122.3, 109.0, 53.7, 51.7, 49.8, 46.3, 46.0, 42.0, 41.6, 39.5, 38.9, 35.5, 34.9, 34.0, 33.3, 32.5, 32.1, 30.9, 29.0, 27.9, 25.9, 23.8, 23.5, 23.2, 21.9, 17.6, 16.8, 14.7. EIMS (70 eV) m/z: 493 [M$^+$] (11), 434 (18), 262 (28), 249 (16), 203 (100). HREIMS: Calcd for C$_{24}$H$_{26}$O$_2$: 493.3556. Found: 493.3556.

**Methyl 2-Cyano-3-oxoolean-1,12-dien-28-oate (27).** A mixture of 26 (887 mg, 1.80 mmol) in Et$_2$O (50 mL) and MeOH (25 mL) was added NaOMe (3.2 g) in an ice bath. The mixture was stirred at room temperature for 1 h. The mixture was diluted with a mixture of CH$_2$Cl$_2$ and Et$_2$O (1:2) (50 mL). After the extract was washed with 5% aqueous HCl solution, it was worked up according to the standard method to afford 27 as an amorphous solid (679 mg, 59%). This material was used for the next reaction without further purification. An analytically pure sample was obtained by flash column chromatography [hexanes–EtOAc (5:1)] as an amorphous solid: UV (EtOH) $\lambda_{max}$ (log e): 238 (3.88) nm. IR (KBr): 2946, 2870, 2202, 1724, 1633 cm$^{-1}$. 1H NMR of major tautomer $\Delta$7a (CDCl$_3$): δ 6.15 (1H, brs), 5.31 (1H, t, J = 3.6 Hz), 3.83 (3H, s), 2.88 (1H, dd, J = 4.0, 13.6 Hz), 2.09 (1H, d, J = 15.0 Hz), 1.16, 1.13, 1.07, 0.95, 0.93, 0.90, 0.76 (each 3H, s), EIMS (70 eV) m/z: 493 [M$^+$] (6.3), 434 (17), 262 (19), 249 (20), 203 (100). HREIMS: Calcd for C$_{24}$H$_{26}$O$_2$: 493.3556. Found: 493.3548.

**Methyl 2-Hydroxy methylenyl-3-oxoolean-12-en-28-oate (28).** 28 was prepared from B-4 according to the same method as for 25 to give an amorphous solid (quantitative). This material was used for the next reaction without further purification. An analytically pure sample was obtained by flash column chromatography [hexanes–EtOAc (7:1)] and subsequent recrystallization from MeOH as colorless needles: mp 170–171 °C. UV (EtOH) $\lambda_{max}$ (log e): 294 (0.96) nm. IR (KBr): 2921, 2857, 1747, 1726, 1705, 1609, 1575, 1454, 1371, 1271, 1235, 1020, 787, 776, 766, 720, 685, 674, 642, 617, 593, 587, 571, 565, 559, 547, 538, 522, 509, 487, 473, 465, 451, 433, 407, 384, 367, 354, 338, 326, 32.5, 30.9, 28.5, 27.8, 26.0, 23.81, 23.76, 23.2, 22.0, 20.4, 19.2, 17.4. EIMS (70 eV) m/z: 493 [M$^+$] (80), 436 (21), 328 (19), 262 (36), 203 (100). HREIMS: Calcd for C$_{24}$H$_{26}$O$_2$: 493.3556. Found: 493.3548.

**Methyl 2-Hydroxymethylene-3-oxoolean-12-en-28-oate (29).** 29 was prepared from 28 according to the same method as for 26 to give an amorphous solid (84%): UV (EtOH) $\lambda_{max}$ (log e): 228 (3.70) nm. IR (KBr): 2969, 2922, 2870, 1725 cm$^{-1}$. 1H NMR (CDCl$_3$): δ 7.98 (1H, s), 5.31 (1H, t, J = 3.4 Hz), 3.62 (3H, s), 2.46 (1H, d, J = 15.0 Hz), 2.27 (1H, d, J = 11.1 Hz), 1.31, 1.22.
Methyl 2-Cyano-3-oxo-12-en-28-oate (31). A mixture of B-3 (2.0 g, 4.27 mmol) and 1.8 M DMF solution of methoxymethylmethyl carbonate (Stiles' reagent) (20 mL, 36 mmol) was heated under reflux for 2 h while a slow stream of N₂ was bubbled through the mixture with a pipet. To the mixture were added 5% aqueous HCl solution and EtOAc. The aqueous layer was extracted with EtOAc (three times). The combined organic layers were washed with water (three times) and saturated aqueous NaCl solution (three times), dried over MgSO₄, and filtered. The filtrate was evaporated in vacuo to give a solid (2.26 g). To a solution of the solid in THF (30 mL) was added excess amount of ethereal diazomethane. The mixture was kept at room temperature for 10 min. The mixture was evaporated in vacuo to give a solid (2.38 g). The solid was subjected to flash column chromatography [hexane-EtOAc (7:1) to give B-3 (330 mg) and 31 as crystals (1.66; 74%; 79% based on recovered B-3). mp 160–162 °C. UV (EtOH) λmax (log e) = 282 (4.01) nm. IR (KBr): 2948, 2858, 1737, 1660, 1615 cm⁻¹. ¹³C NMR (CDCl₃): δ 12.51 (1H, s), 5.33 (1H, J, = 3.7 Hz), 3.74, 3.63 (each 3H, s), 2.89 (1H, dd, J = 4.2, 13.9 Hz), 2.35 (1H, d, J = 15.7 Hz), 1.18, 1.14, 1.10, 0.94 (each 3H, s), 0.91 (6H, s), 0.78 (3H, s). ¹³C NMR (CDCl₃): δ 178.5, 177.9, 174.2, 143.8, 122.6, 94.3, 52.5, 51.8, 51.7, 47.0, 46.13, 46.09, 42.0, 41.7, 39.4, 38.6, 38.4, 35.7, 34.1, 33.3, 32.6, 32.1, 31.0, 28.8, 27.9, 26.0, 23.8, 23.6, 23.5, 20.4, 19.8, 16.8, 15.1. EIMS (70 eV) m/z: 526 [M⁺] (0.8), 494 (5.6), 479 (3.1), 460 (2.0), 455 (5.6), 453 (10.6). (100) HREIMS: Caled for C₃₉H₄₃O₉: 526.3658. Found: 526.3658. 2-Hydroxyethylene-3-oxoole-12-en-28-ionic Acid (32). To a stirred mixture of oleic acid (B-1) (840 mg, 1.59 mmol) and ethyl formate (97%) (357 mg, 4.66 mmol) in THF (12 mL) was added NaOMe (258 mg, 4.78 mmol). The mixture was stirred until room temperature overnight. The mixture was acidified with 10% aqueous HCl solution. The mixture was extracted with EtOAc three times. The extract was worked up according to the standard method. The solid was referred to as a solid (600 mg). The solid was subjected to flash column chromatography [hexane-EtOAc (5:1) followed by hexane-EtOAc (4:1)] to afford B-1 (168 mg) and 32 as a crystalline solid (260 mg; 45%; 66% based on recovered B-1): mp 200–203 °C dec; UV (EtOH) λmax (log e) = 292 (3.95) nm. IR (KBr): 2946, 2654, 1732, 1684, 1587 cm⁻¹. H NMR (CDCl₃): δ 14.91 (1H, brs), 8.59 (1H, s), 5.34 (1H, t, J = 0.5 Hz), 2.86 (1H, dd, J = 4.5, 13.9 Hz), 2.28 (1H, d, J = 14.0 Hz), 1.30 (1H, m), 1.16 (CH₃, s), 1.10 (1H, 0.92, 0.91, 0.92 (each 3H), s). ¹³C NMR (CDCl₃): δ 190.7, 188.7, 184.7, 143.8, 122.6, 105.9, 52.2, 46.8, 46.0, 45.9, 41.9, 41.2, 40.2, 39.34, 39.30, 36.5, 34.0, 33.3, 32.6, 32.0, 30.9, 28.6, 27.8, 25.9, 23.7, 23.5, 23.1, 21.0, 19.6, 17.0, 14.6. EIMS (70 eV) m/z: 482 [M⁺] (1.8), 438 (2.7), 436 (0.6), 248 (77), 203 (100). HREIMS: Caled for C₂₈H₄₃O₉: 482.3939. Found: 482.3932.
Synthetic Oleanane and Ursane Triterpenoids


(12) Deeq oxidation of B-3 in benzene (reflux) did not give B-13 at all.


(16) In addition to 5, these conditions also gave the completely demethylated product 4 (yield, 12%; 18% based on recovered 23) and the other partially demethylated product 24 (yield, 19%; 25% based on recovered 23) from 23.