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13. ABSTRACT ***(Maximum 200 Words)***

We have recently shown that the synthetic triterpenoid, 2-cyano-3,12-dioxoolean-1,9,-dien-28-oic 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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N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

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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. Spinn, M.D. 6-29-00

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% CO_2 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 ^{3}H-thymidine incorporation, or 10^6 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 (^{3}H-thymidine incorporation and RNA extraction) or five days (MTT assay).

Assay of Thymidine Incorporation into DNA:

5 μCi ^{3}H-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 ^{3}H 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

Group	Treatment	No. of Rats
A	Control	18
B	CDDO, 60 mg/kg diet	9
C	CDDO, 30 mg/kg diet	9
D	CDDO, 10 mg/kg diet	9
E	CDDO, 3 mg/kg diet	9
F	CDDO, 1 mg/kg diet	9
G	LG268 Hi, 50 mg/kg diet	9
H	CDDO 10 + LG268 Hi	9
I	CDDO 3 + LG268 Hi	9
J	CDDO 1 + LG268 Hi	9
K	LG268 Lo, 25 mg/kg diet	9
L	CDDO 10 + LG268 Lo	9
M	CDDO 3 + LG268 Lo	9
N	CDDO 1 + LG268 Lo	9
	Total	135

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

Group	Treatment	No. of Rats
A	Control	24
B	CDDO, 30 mg/kg diet	12
C	CDDO, 10 mg/kg diet	12
D	LG268, 60 mg/kg diet	12
E	9-cis RA, 60 mg/kg diet	12
F	all-trans-RA, 60 mg/kg diet	12
G	CDDO 30 + LG268	12
H	CDDO 30 + 9-cis RA	12
I	CDDO 30 + all-trans-RA	12
J	CDDO 10 + LG268	12
K	CDDO 10 + 9-cis RA	12
	Total	144

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

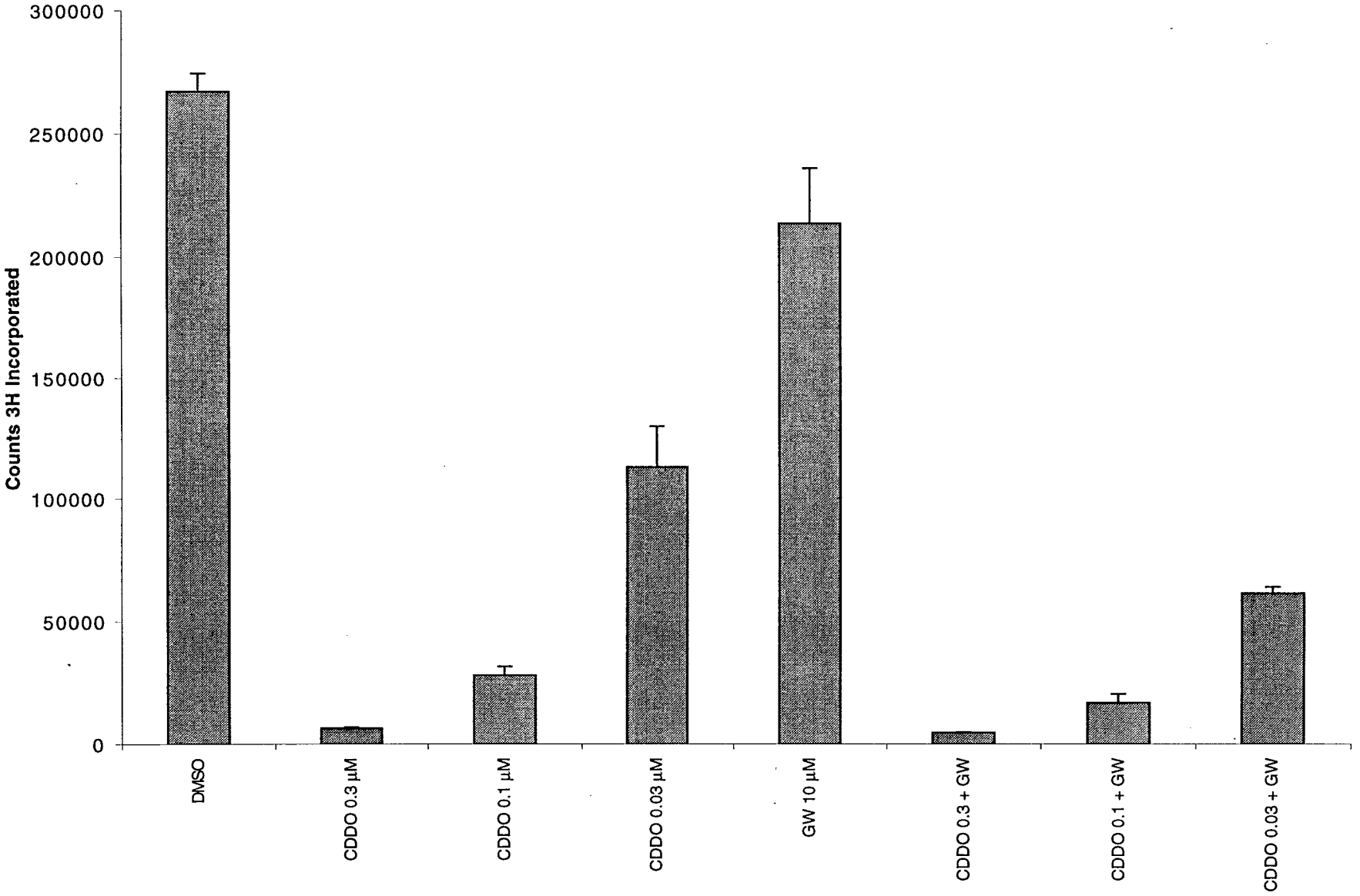
We have tested CDDO in combination with ligands for nuclear receptors (such as PPAR-γ or RXRs) for ability to suppress proliferations of both ER-positive and ER-negative human breast cancer cell lines. Figures 1-4 show that in cell culture, although CDDO itself is a potent inhibitor of breast cancer 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)

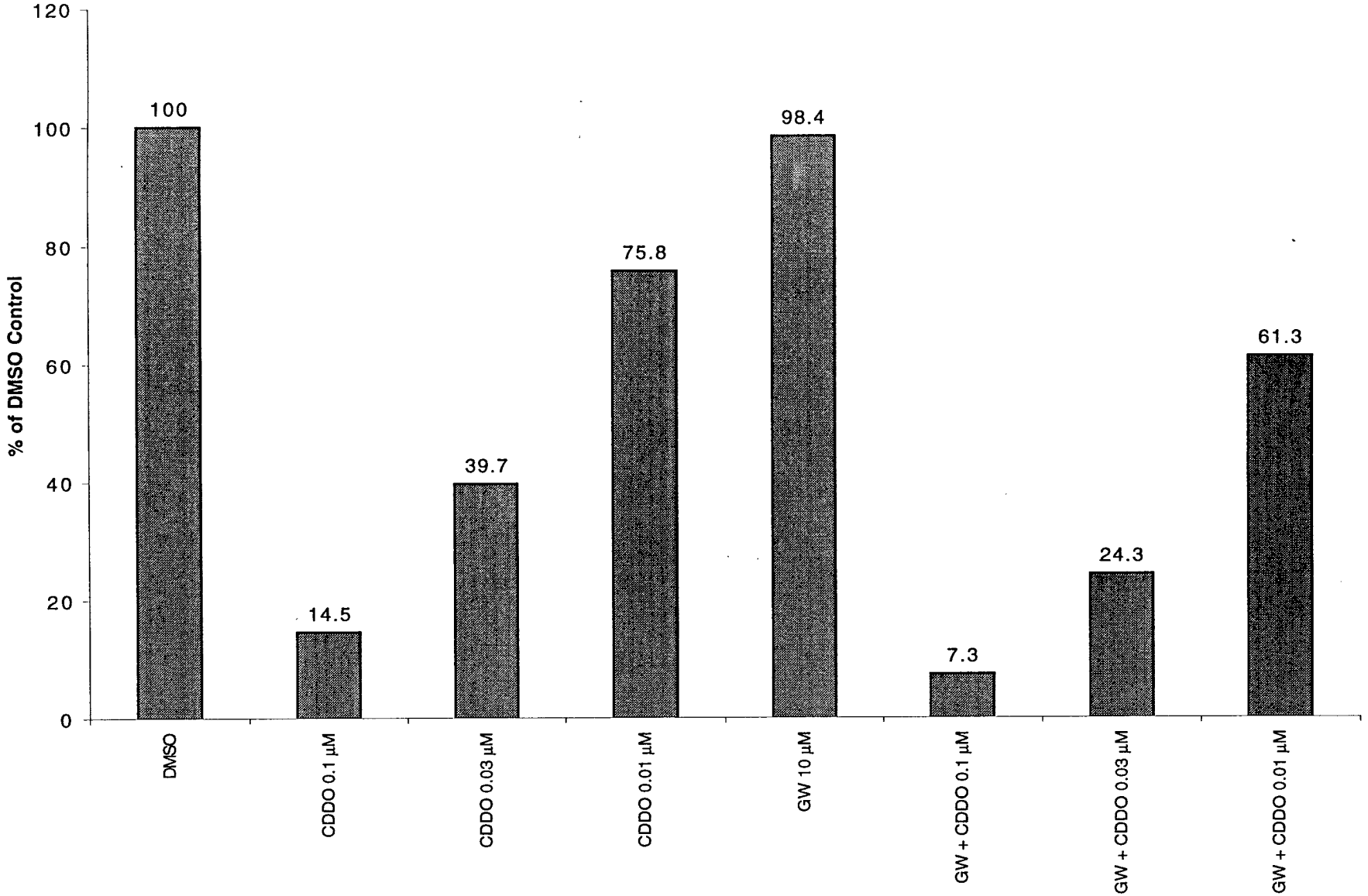


Three days incubation with compounds, 10% stripped FBS, phenol red-free RPMI, 10 pM 17-β estradiol
Two hours thymidine pulse

10

FIGURE 2

Combination Effects of CDDO and GW Compound on Growth of MCF-7 Cells (PPAR-γ ligand)

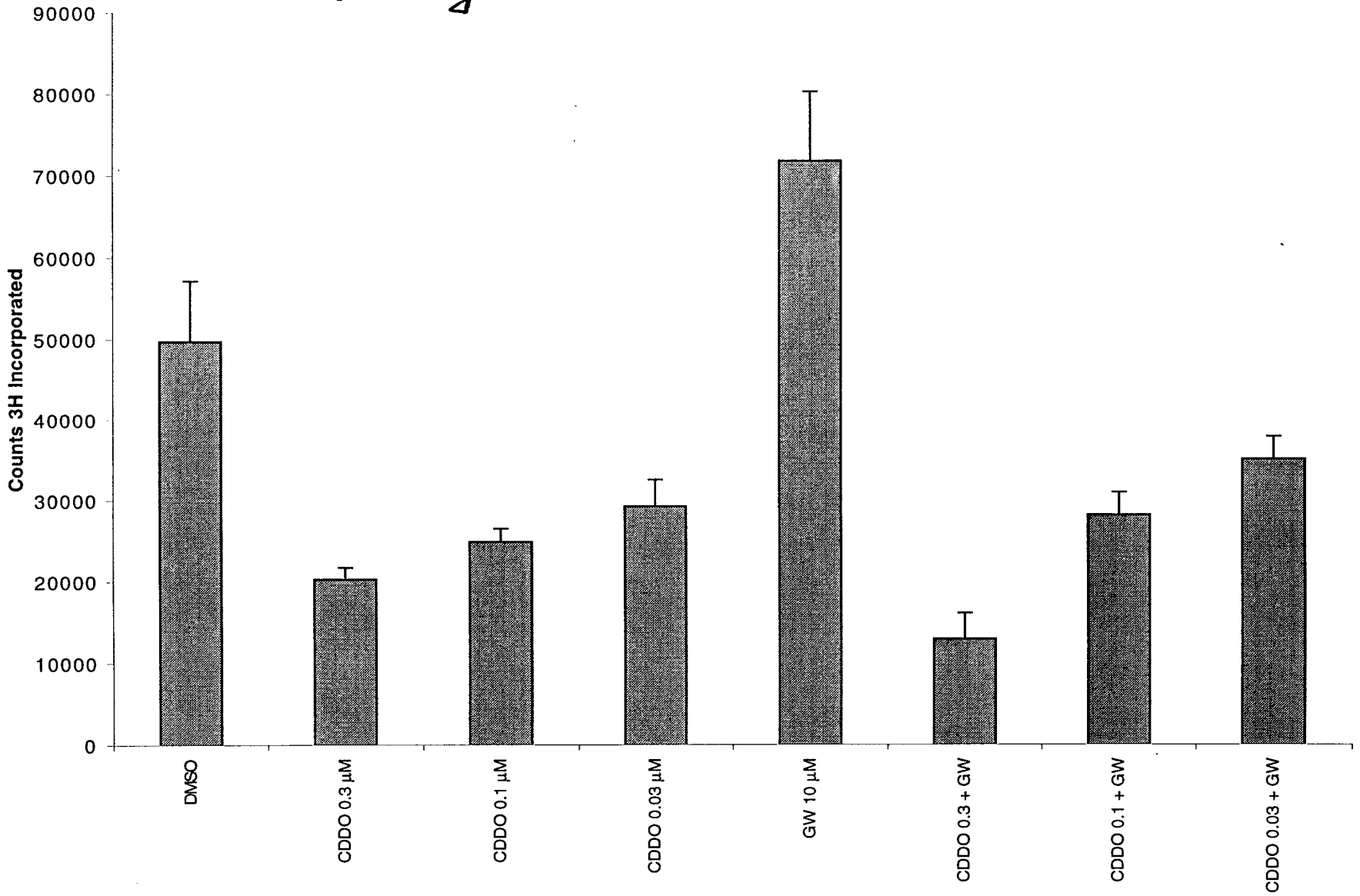


Three days incubation with compounds in 10% charcoal-stripped FBS, phenol red-free RPMI, 10 pM 17β-estradiol
Two hours thymidine pulse

FIGURE 3

CDDO and GW Compound: Combination Effects on Proliferation of MDA-231

(PPAR-γ Ligand)

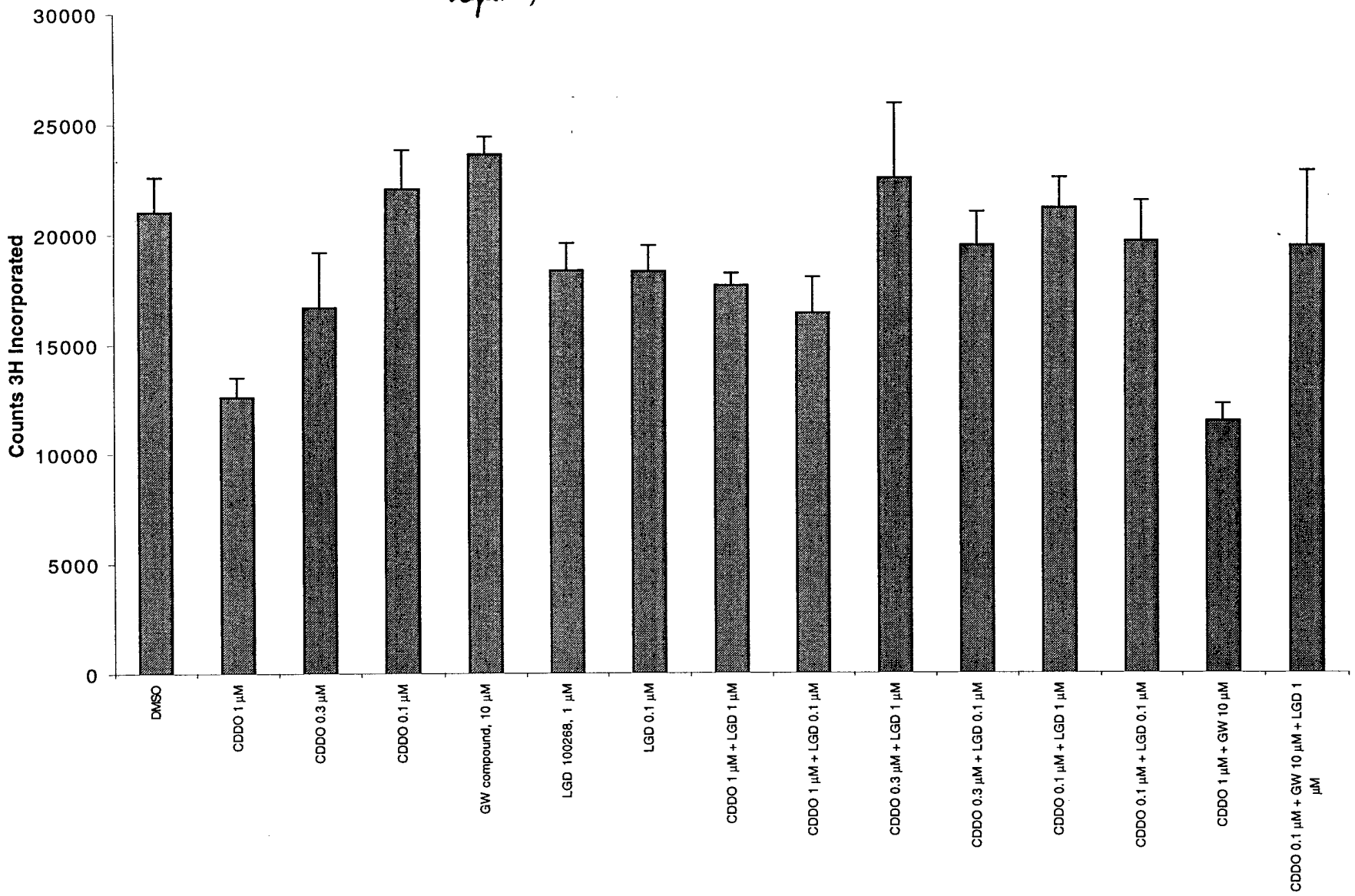


Three days incubation with compounds in 10% FBS growth media
Two hours thymidine pulse

FIGURE 4

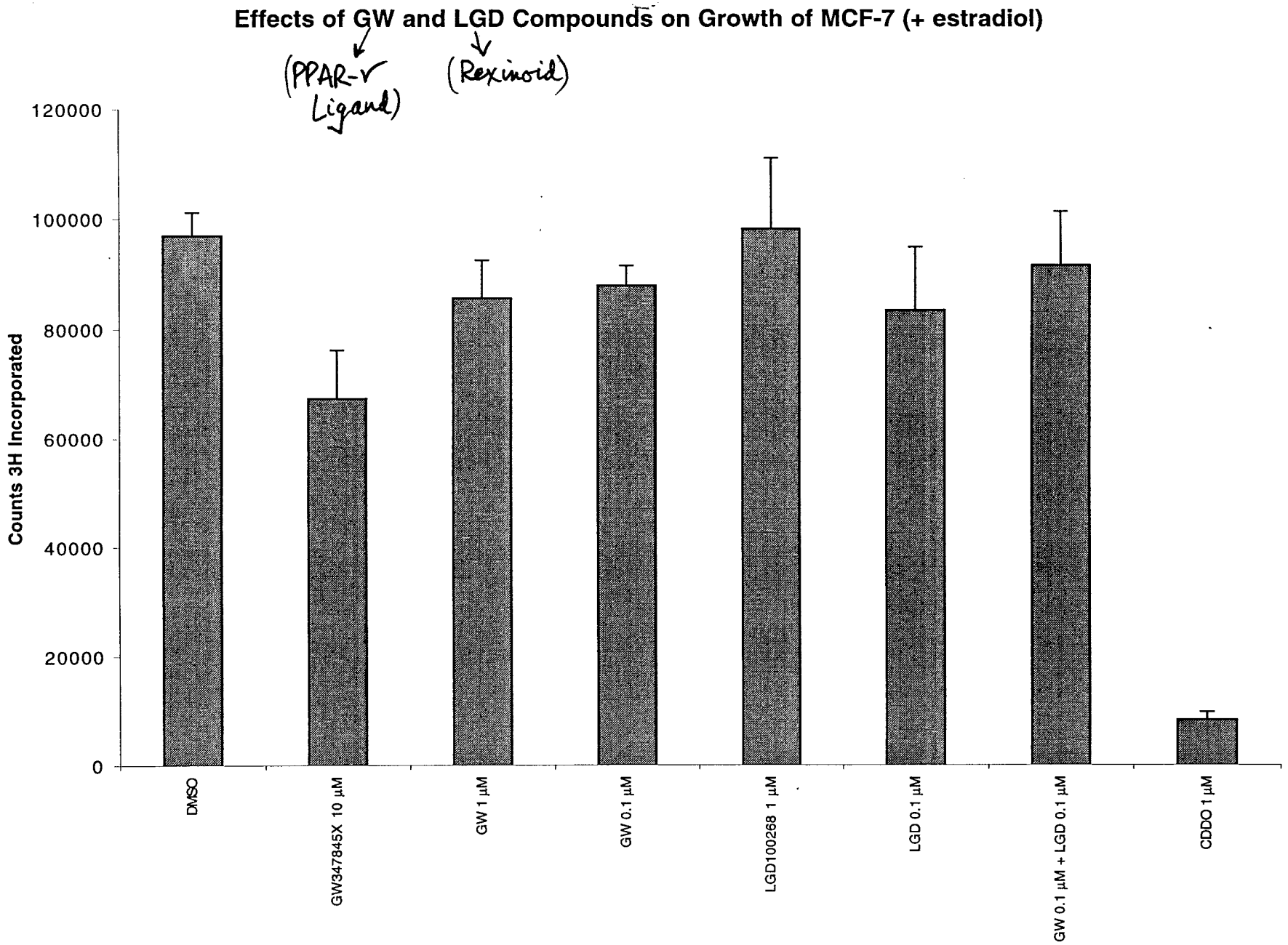
Effects of CDDO, GW, and LGD Compounds on Growth of SK-Br-3 Cells

(PPAR-γ ligand) (Rexinoid)



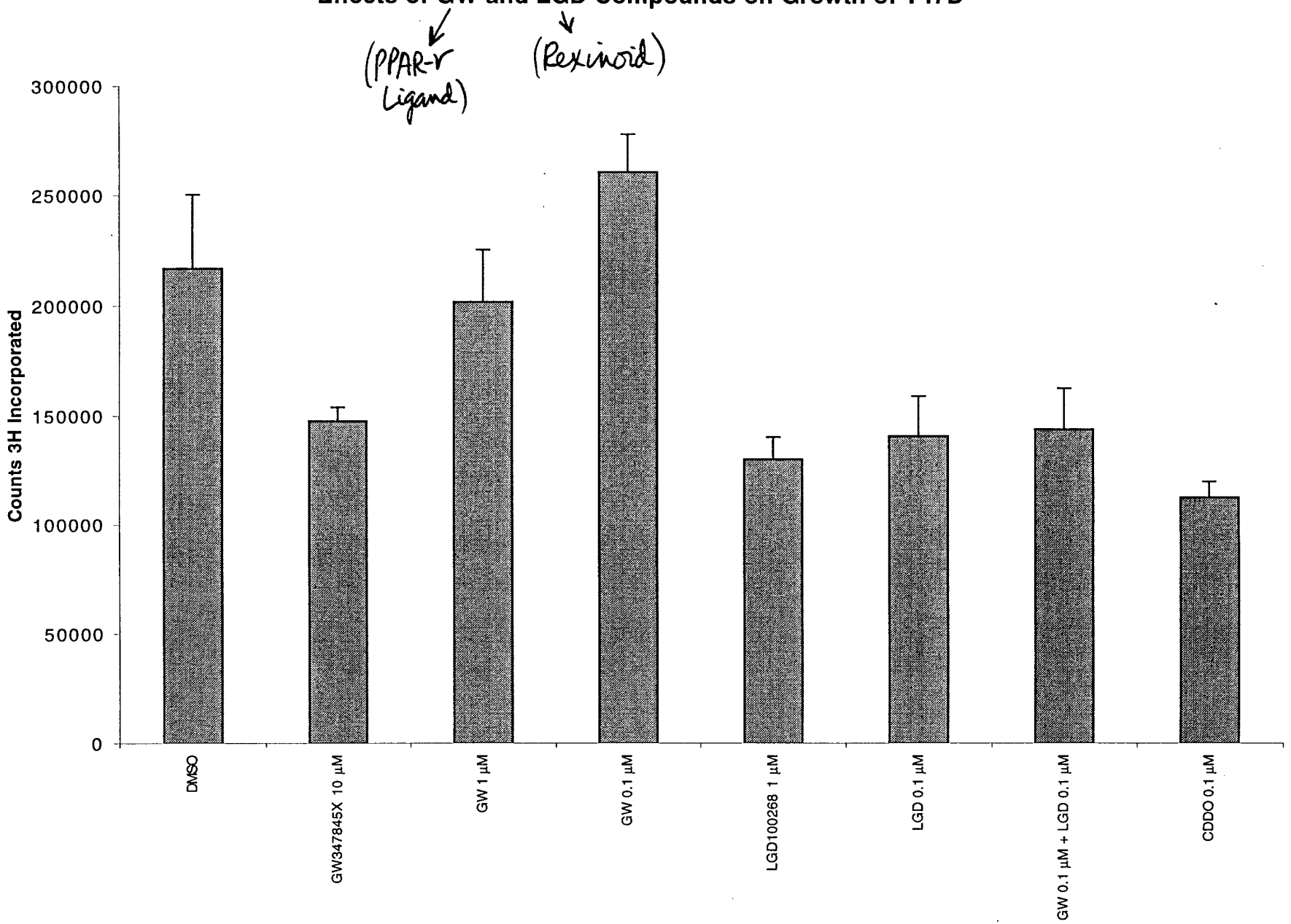
Three days incubation with compounds in 10% FBS growth media
Two hours thymidine incorporation

FIGURE 5



- Three days incubation in compounds, 10% charcoal-stripped FBS, phenol red-free RPMI
  Stimulation with 10 pM 17β estradiol
  Two hours thymidine pulse

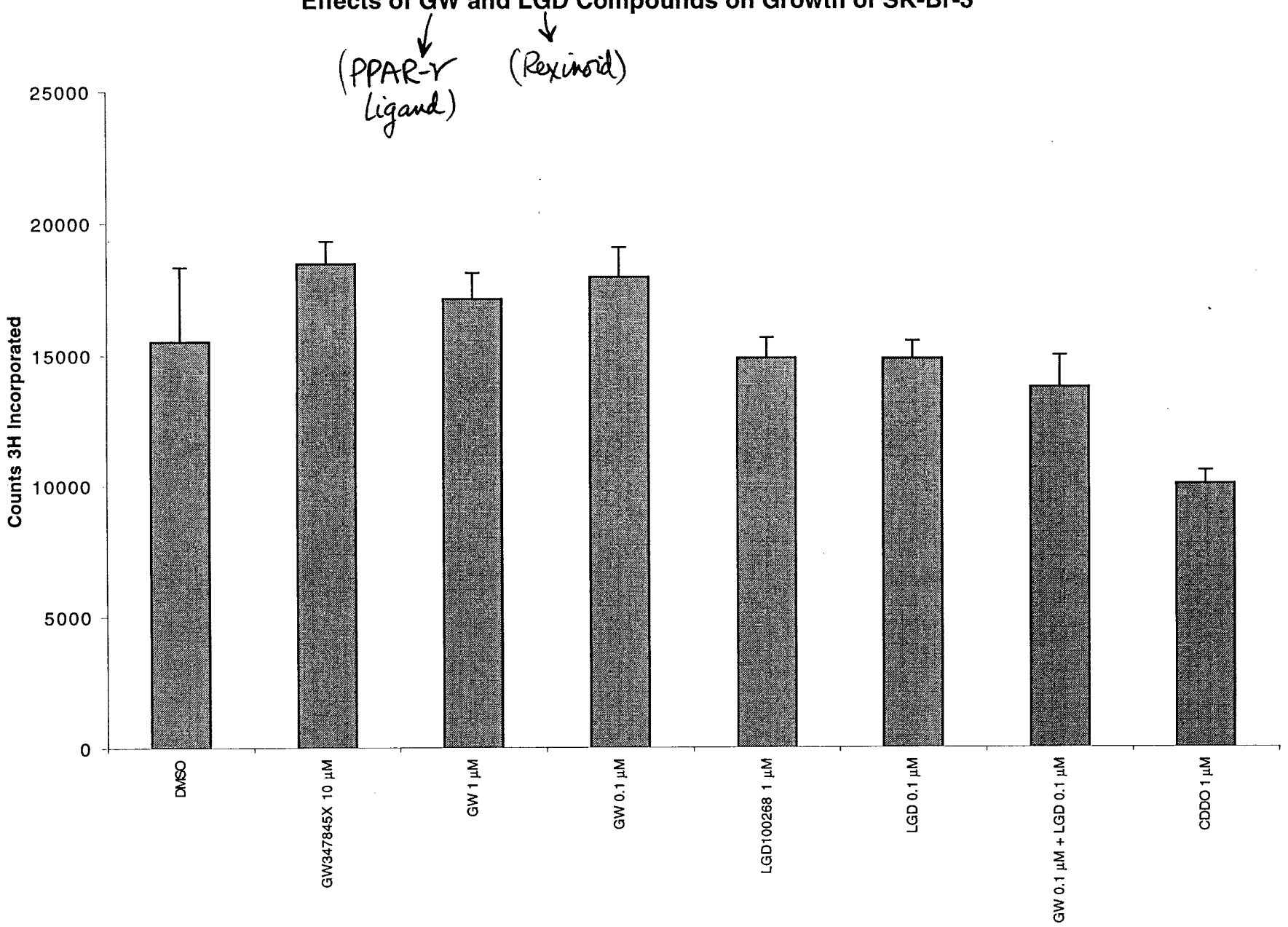
FIGURE 6



Three days incubation with compounds in 10% charcoal stripped FBS, phenol red-free RPMI, + 10 pM 17β estradiol
Two hours thymidine pulse

FIGURE 7

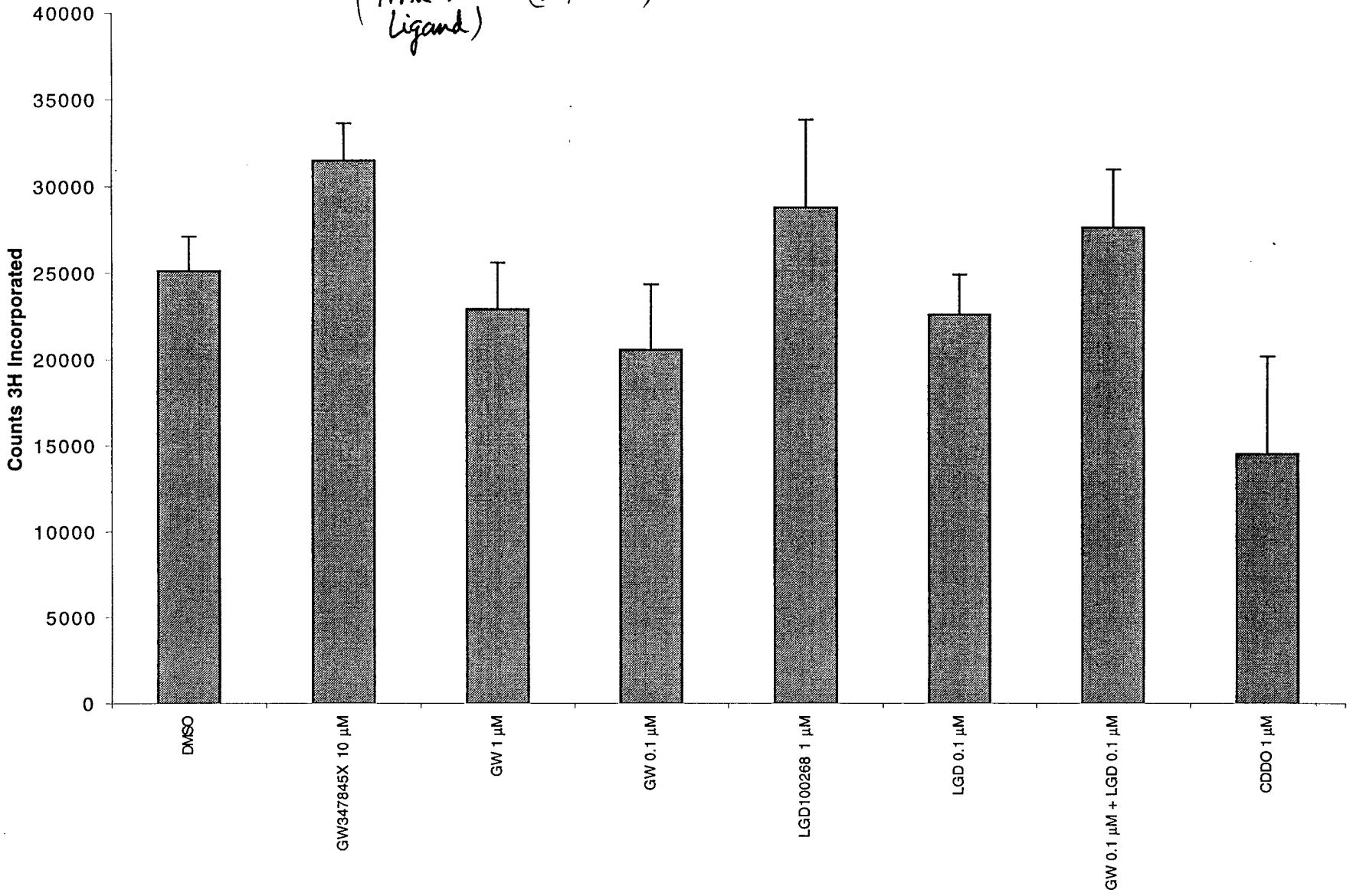
Effects of GW and LGD Compounds on Growth of SK-Br-3



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



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

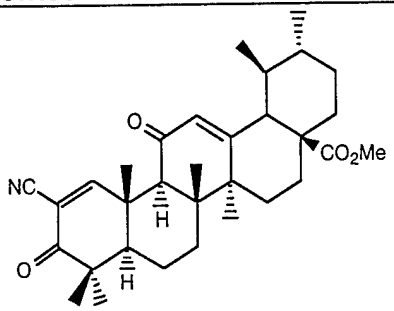
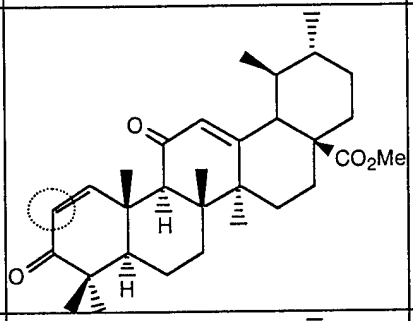
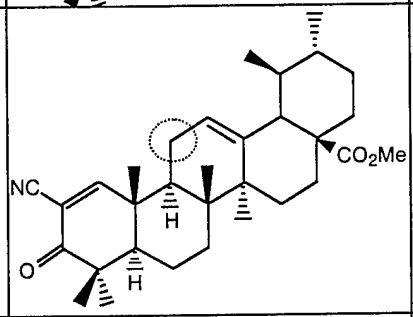
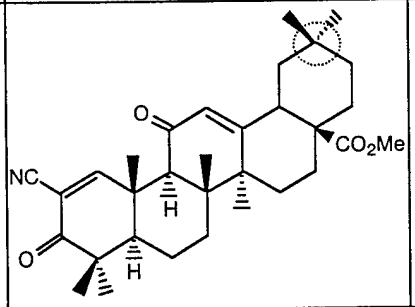
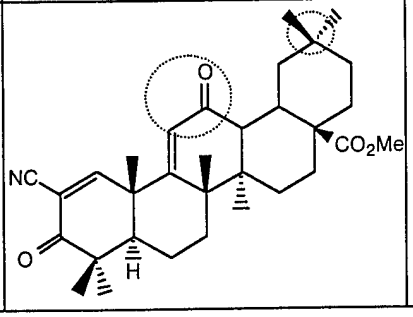
COMPOUND	STRUCTURE	IC50 (μM)
TP-195		0.4
TP-217		5.5
TP-167		2.1
TP-162		0.6
TP-155		0.1

FIGURE 10

Effects of structural changes on inhibition levels of TP compounds

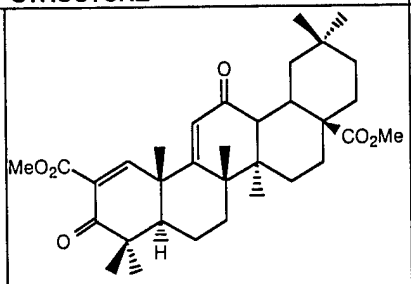
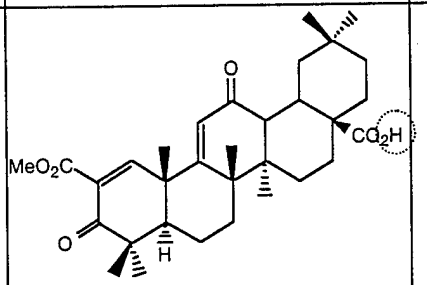
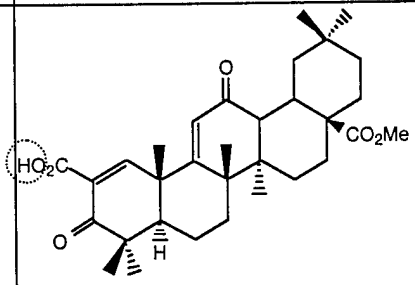
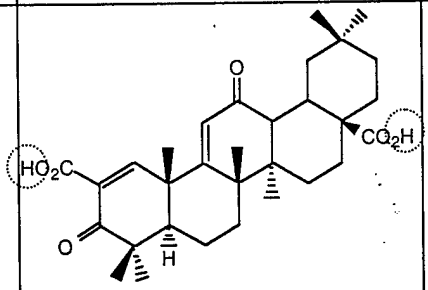
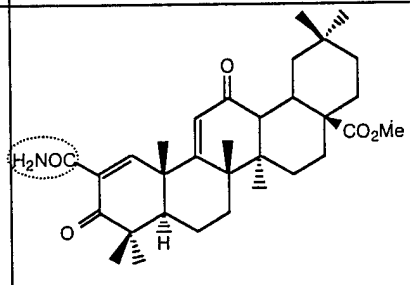
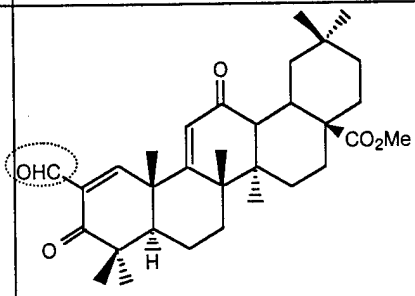
COMPOUND	STRUCTURE	IC50 (μM)
TP-189		0.46
TP-192		0.04
TP-190		0.44
TP-191		3.85
TP-193		0.86
TP-194		0.32

FIGURE 11

Effects of structural changes on inhibition levels of TP compounds

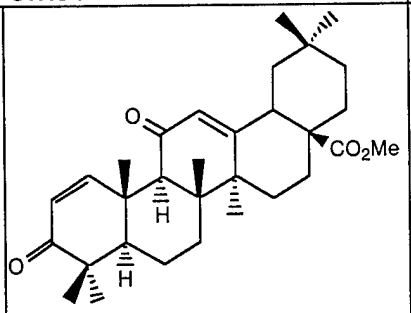
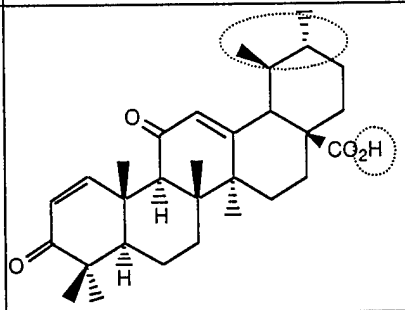
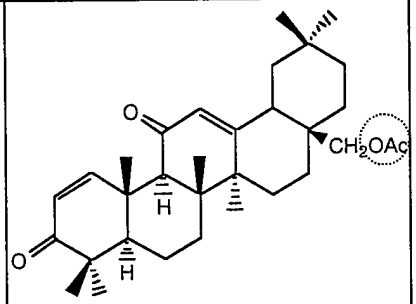
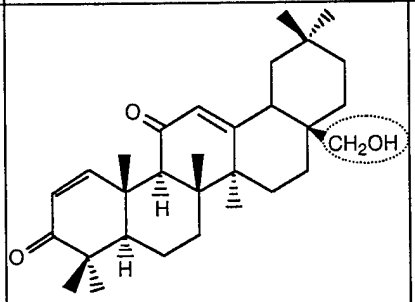
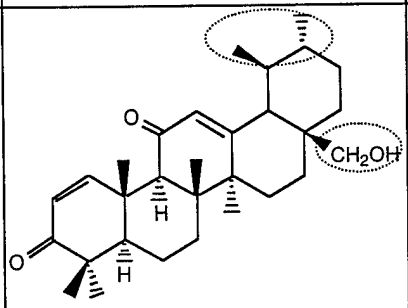
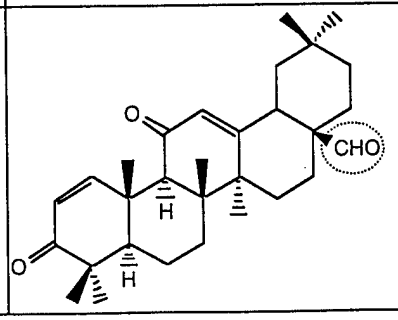
COMPOUND	STRUCTURE	IC50 (μM)
TP-196		4.9
TP-81		11.8
TP-107		4.2
TP-108		3.3
TP-109		10.1
TP-128		2.9

FIGURE 12

Effects of structural changes on inhibition levels of TP compounds

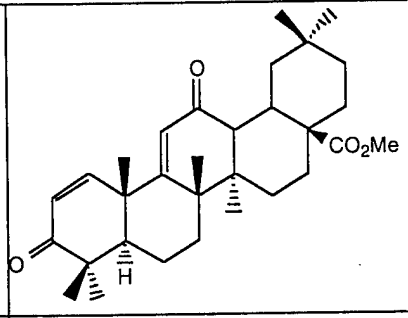
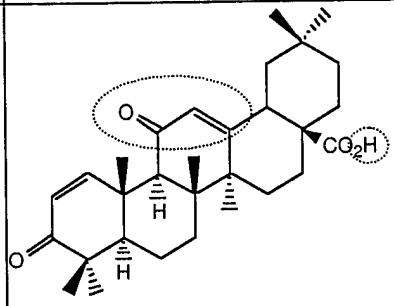
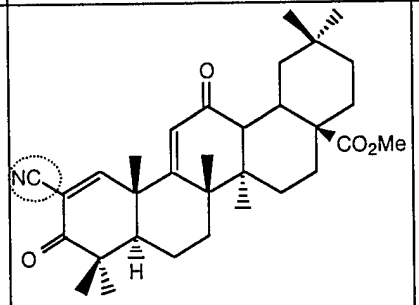
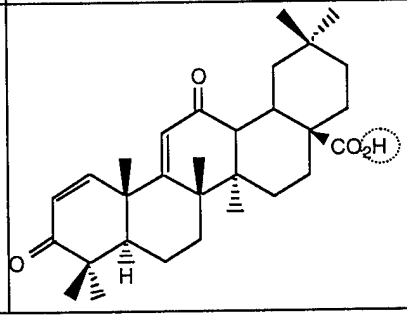
COMPOUND	STRUCTURE	IC50 (μM)
TP-198		7.5
TP-072		10.7
TP-155		0.1
TP-082		3.2

FIGURE 13

Effects of structural changes on inhibition levels of TP compounds

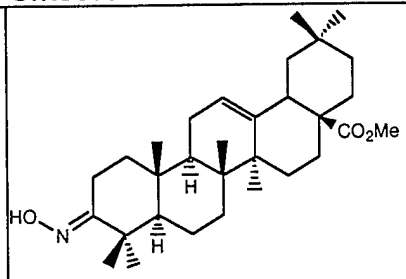
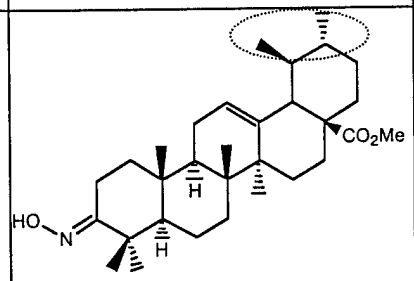
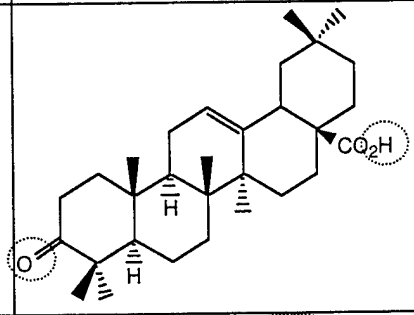
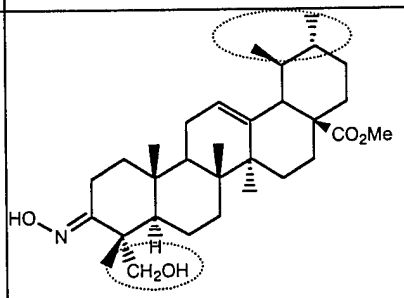
COMPOUND	STRUCTURE	IC50 (μM)
TP-199		8.3
TP-174		9.0
TP-018		>10
TP-175		5.9

FIGURE 14

Effects of structural changes on inhibition levels of TP compounds

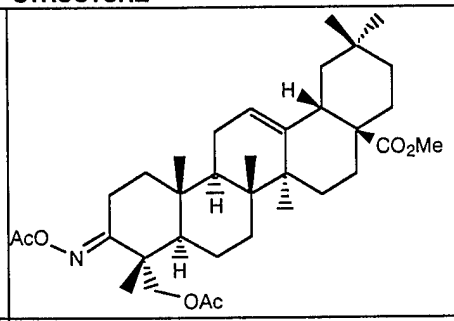
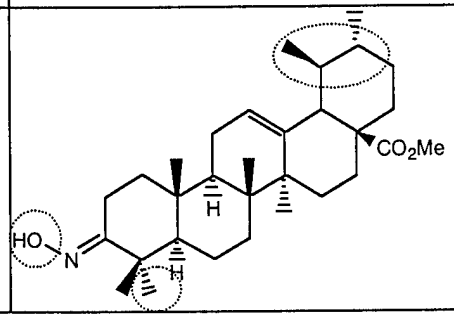
COMPOUND	STRUCTURE	IC50 (μM)
TP-200		4.7
TP-174		9.0

FIGURE 15

Effects of structural changes on inhibition levels of TP compounds

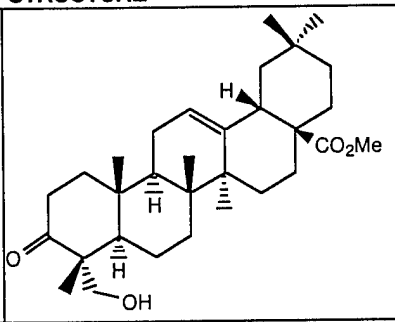
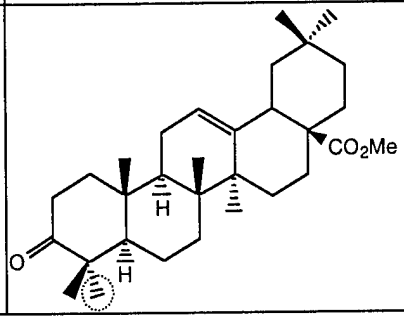
COMPOUND	STRUCTURE	IC50 (μM)
TP-202		6.6
TP-033		>10

FIGURE 16

Effects of structural changes on inhibition levels of TP compounds

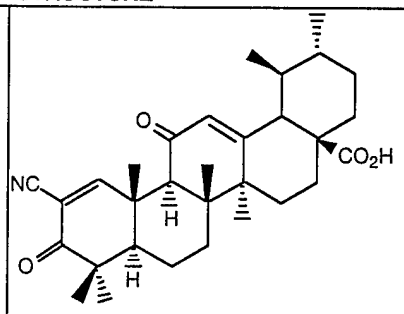
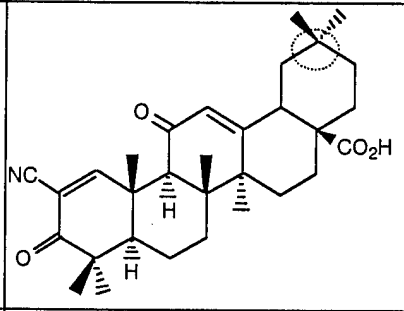
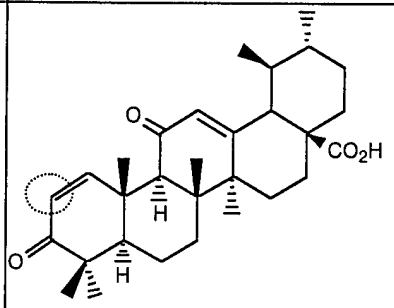
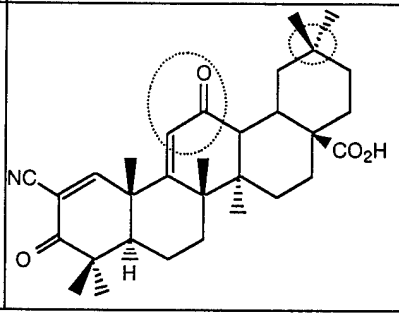
COMPOUND	STRUCTURE	IC50 (μM)
TP-216		0.4
TP-163		2.6
TP-081		11.8
TP-151		0.1

FIGURE 17

Effects of structural changes on inhibition levels of TP compounds

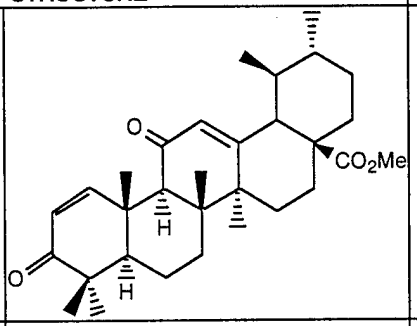
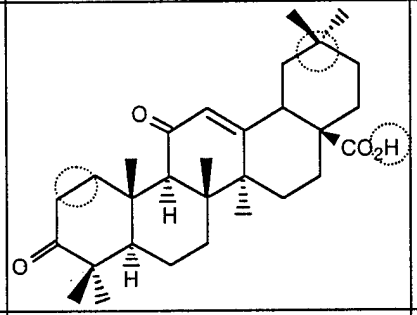
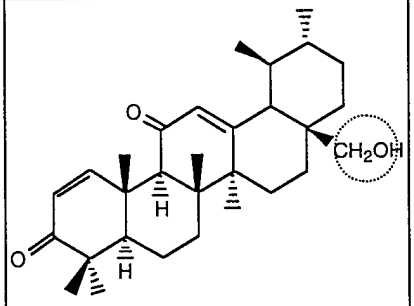
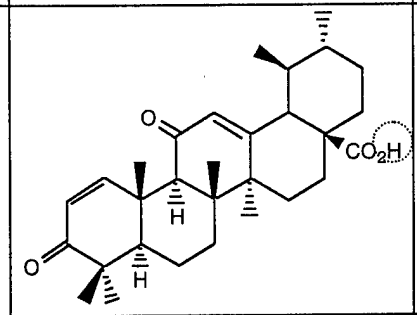
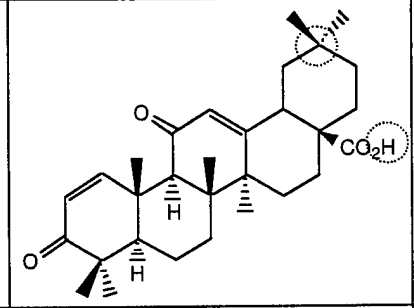
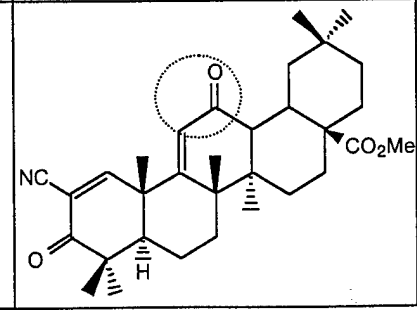
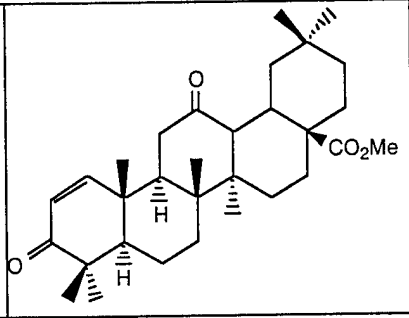
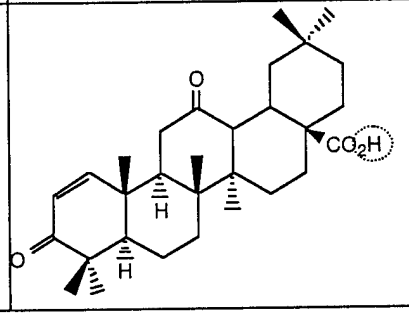
COMPOUND	STRUCTURE	IC50 (μM)
TP-217		5.5
TP-087		>10
TP-109		10.1
TP-081		11.8
TP-072		10.7
TP-155		0.1

FIGURE 18

Effects of structural changes on inhibition levels of TP compounds

COMPOUND	STRUCTURE	IC50 (μM)
TP-197		1.0
TP-069		6.6

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.

FINAL REPORT: Protocol DMS-TP-4 (TABLE 1)

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

	Control	CDDO 60 mg/kg	CDDO 30 mg/kg	CDDO 10 mg/kg	CDDO 3 mg/kg	CDDO 1 mg/kg	LG268 Hi 50 mg/kg	LG(50)+ CDDO(10)	LG(50)+ CDDO(3)	LG(50)+ CDDO(1)	LG268Lo 25mg/kg	LG(25)+ CDDO(10)	LG(25)+ CDDO(3)	LG(25)+ CDDO(1)
Group	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Tumor Incidence (%)	17/17 (100%)	8/9 (89%)	8/9 (89%)	8/9 (89%)	9/9 (100%)	8/9 (89%)	7/9 (78%)	5/9 (56%)	3/9 (33%)	6/9 (67%)	8/9 (89%)	6/8 (75%)	6/9 (67%)	6/9 (67%)
No. Tumor Free (%)	0/17 (0%)	1/9 (11%)	1/9 (11%)	1/9 (11%)	0/9 (0%)	1/9 (11%)	2/9 (22%)	4/9 (44%)	6/9 (67%)	3/9 (33%)	1/9 (11%)	2/8 (25%)	3/9 (33%)	3/9 (33%)
No. of Tumors/Rat (average)	3.2	2.8	2.1	2.7	3.0	2.7	1.4	0.8	0.6	1.3	2.1	1.5	1.4	1.0
Tumor Burden/Rat (grams, average)	5.5	7.0	4.3	4.6	6.4	6.1	2.0	0.3	1.2	2.0	1.5	1.8	5.5	3.0
Rats with Three or More Tumors	9/17 (53%)	5/9 (56%)	3/9 (33%)	5/9 (56%)	4/9 (44%)	5/9 (56%)	1/9 (11%)	0/9 (0%)	1/9 (11%)	2/9 (22%)	4/9 (44%)	2/8 (25%)	2/9 (22%)	1/9 (11%)
Rats with Tumor Burden > 5 g	7/17 (41%)	5/9 (56%)	4/9 (44%)	3/9 (33%)	3/9 (33%)	4/9 (44%)	1/9 (11%)	0/9 (0%)	1/9 (11%)	1/9 (11%)	0/9 (0%)	2/8 (25%)	4/9 (44%)	2/9 (22%)
Rats with Ulcerated Tumors	1/17 (6%)	0/9 (0%)	1/9 (11%)	1/9 (11%)	0/9 (0%)	1/9 (11%)	0/9 (0%)	0/9 (0%)	1/9 (11%)	0/9 (0%)	0/9 (0%)	1/8 (13%)	2/9 (22%)	0/9 (0%)

Synergism of CDDO and LGD 100268 in Ovary-Intact Rats

CDDO =30 and 10 mg/kg diet
LGD268 = 60 mg/kg diet
9-cis-RA = 60 mg/kg diet
All-trans-RA = 60 mg/kg diet

Data as of 2-3-00
M.B. Sporn, N. Suh, C. Williams,
R. Risingsong, Y. Wang, DMS

	Control	CDDO Hi 30 mg/kg	CDDO Lo 10 mg/kg	LG268 60 mg/kg	9-cis-RA 60 mg/kg	All-transRA 60 mg/kg	CDDO Hi + LG268	CDDO Hi + 9cis-RA	CDDO Hi + All-transRA	CDDO Lo + LG268	CDDO Lo + 9cis-RA
Group	A	B	C	D	E	F	G	H	I	J	K
Tumor Incidence (%)	24/24 (100%)	12/12 (100%)	12/12 (100%)	9/12 (75%)	9/12 (75%)	10/11 (91%)	10/12 (83%)	11/12 (92%)	9/12 (75%)	6/12 (50%)	11/12 (92%)
No. Tumor Free (%)	0/24 (0%)	0/12 (0%)	0/12 (0%)	3/12 (25%)	3/12 (25%)	1/11 (9%)	2/12 (17%)	1/12 (8%)	3/12 (25%)	6/12 (50%)	1/12 (8%)
No. of Tumors/Rat (average)	3.4	3.4	2.8	1.8	2.9	2.4	1.1	2.2	2.5	1.5	3.7
Tumor Burden/Rat (grams, average)	8.7	9.1	10.5	2.1	10.5	7.8	0.7	3.5	9.8	1.8	14.1
Rats with Three or More Tumors	17/24 (71%)	9/12 (75%)	6/12 (50%)	4/12 (33%)	6/12 (50%)	6/11 (55%)	1/12 (8%)	3/12 (25%)	6/12 (50%)	3/12 (25%)	8/12 (67%)
Rats with Tumor Burden > 5 g	13/24 (54%)	8/12 (67%)	6/12 (50%)	1/12 (8%)	7/12 (58%)	6/11 (55%)	0/12 (0%)	4/12 (33%)	4/12 (33%)	1/12 (8%)	8/12 (67%)
Rats with Ulcerated Tumors	2/24 (8%)	3/12 (25%)	1/12 (8%)	0/12 (0%)	1/12 (8%)	0/11 (0%)	0/12 (0%)	0/12 (0%)	1/12 (8%)	0/12 (0%)	2/12 (17%)

(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:

1) Suh, N., Wang, Y., Williams, C.R., Risingsong, R., Gilmer, T., Willson, T. M., and Sporn, M. B.: A new ligand for the peroxisome proliferator-activated receptor-γ (PPAR-γ), GW7845, inhibits rat mammary carcinogenesis. Cancer Res. 59: 5671-5673, 1999.

2) Honda, T., Rounds, B. V., Bore, L., Favaloro, F.G. Jr., Gribble, G.W., Suh, N., Wang, Y., and Sporn, M. B.: Novel synthetic oleanane triterpenoids, a series of highly active inhibitors of nitric oxide production in mouse macrophages, Bioorg. Med. Chem. Lett. 9: 3429-3434, 1999.

3. Honda, T., Gribble, G. W., Suh, N., Finlay, H. J., Rounds, B. V., Bore, L., Favaloro, F. G., Wang, Y., and Sporn, M. B.: Novel synthetic oleanane and ursane triterpenoids with various enone functionalities in ring A as inhibitors of nitric oxide production in mouse macrophages. J. Med. Chem. 43: 1866-1877, 2000.

(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.

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(11) APPENDICIES

Attached are reprints of 3 references we put in "Reportable Outcomes".

[CANCER RESEARCH 59, 5671–5673, November 15, 1999]

Advances in Brief

A New Ligand for the Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ), GW7845, Inhibits Rat Mammary Carcinogenesis[1]

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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 nitrosomethylurea as carcinogen. Rats were first treated with a single dose of nitrosomethylurea (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,[3] 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 M), 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, glycerol 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 (CO_2 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

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[3] The abbreviations used are: PPAR-γ, peroxisome proliferator-activated receptor-γ; TZD, thiazolidinedione; NMU, nitrosomethylurea; ATB, average tumor burden.

Table 1 *Prevention of breast cancer by GW7845 and tamoxifen*

Treatment[a]	No. of tumor-free rats/total no. of rats (P_1; P_2)[b]	Average no. of tumors (P_1; P_2)[b]	ATB (P_1; P_2)[b]	Rats with 3 or more tumors (P_1; P_2)[b]	Rats with tumor burden >5 g (P_1; P_2)[b]
Control (vehicle)	5/42	2.4	5.6	22/42	18/42
GW7845 Hi	8/21 (0.02)	1.1 (0.002)	1.7 (0.002)	2/21 (0.0009)	1/21 (0.002)
GW7845 Lo	7/21 (0.08)	0.8 (<0.0001)	1.5 (0.0004)	0/21 (<0.0001)	2/21 (0.009)
Tamoxifen	5/33	1.6 (0.02)	2.4 (0.02)	7/33 (0.008)	6/33 (0.03)
Tamoxifen + GW7845 Hi	9/21 (0.009; 0.03)	0.9 (0.0002; 0.03)	0.9 (0.0002; 0.05)	0/21 (<0.0001; 0.03)	0/21 (0.0002)
Tamoxifen + GW7845 Lo	12/21 (0.0003; 0.002)	0.6 (<0.0001; 0.001)	1.3 (0.0001; 0.01)	1/21 (0.0002)	3/21 (0.03)

[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] P_1 is the value for the comparison of rats treated with chemopreventive agents with control rats treated with vehicle alone; P_2 is the value for the comparison of rats treated with tamoxifen + GW7845 with rats treated with tamoxifen alone.

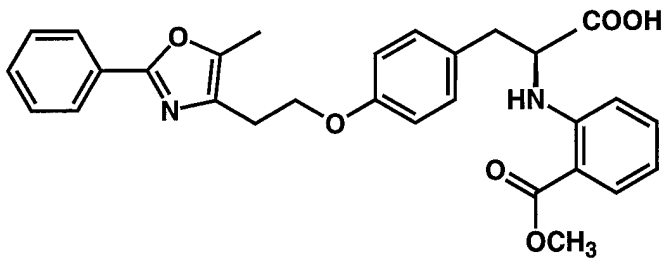

Fig. 1. Structure of GW7845.

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-γ 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-γ.

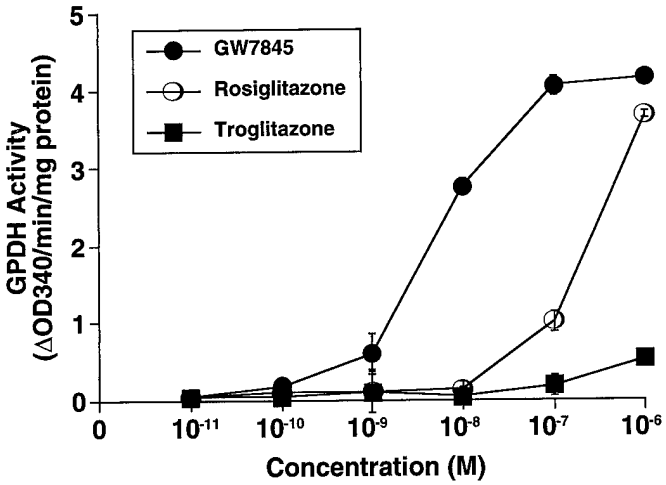

Fig. 2. GW7845 is more potent than either rosiglitazone or troglitazone in induction of adipogenic differentiation in 3T3-L1 fibroblasts. Adipogenesis was measured after 6 days of treatment, as described (25), using a glycerol 3-phosphate dehydrogenase assay as a marker. *OD340*, absorbance at 340 nm; *bars*; SE.

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BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

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

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Abstract: Novel oleanane triterpenoids with modified rings A and C were designed and synthesized. Among them, methyl 2-carboxy-3,12-dioxooleana-1,9-dien-28-oate showed similar high inhibitory activity (IC_{50} = 0.8 nM) to 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), which we have synthesized previously, against production of nitric oxide induced by interferon-γ in mouse macrophages.

Introduction

In a previous communication[1] we reported that 2-cyano-3,12-dioxooleana-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 (IC_{50} = 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 (*i*-NOS) 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-dioxooleana-1,9-dien-28-oate (**2**) has similar high inhibitory activity to CDDO and methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO methyl ester) (**3**).[1,3] 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

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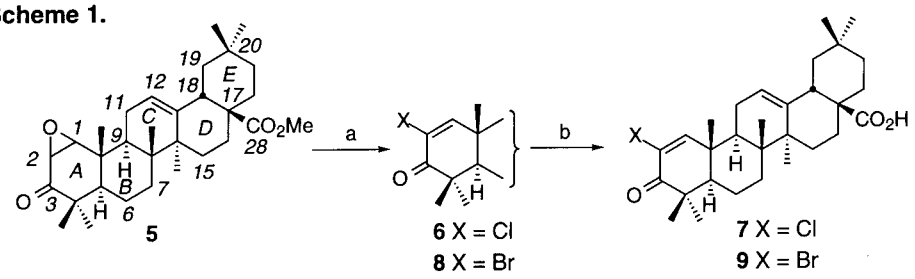
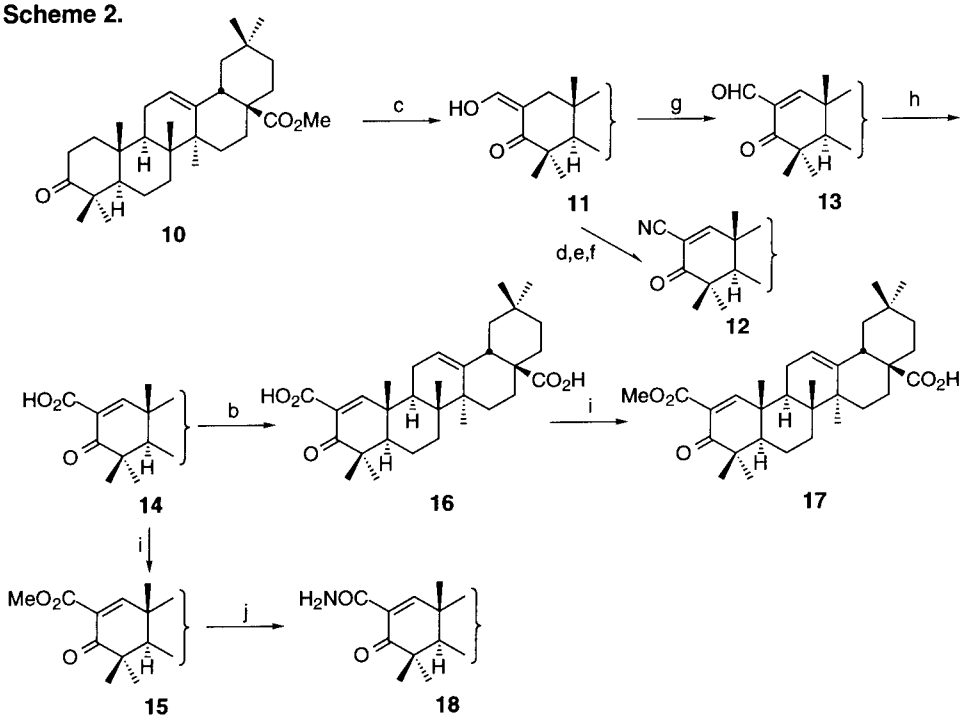
Scheme 1.

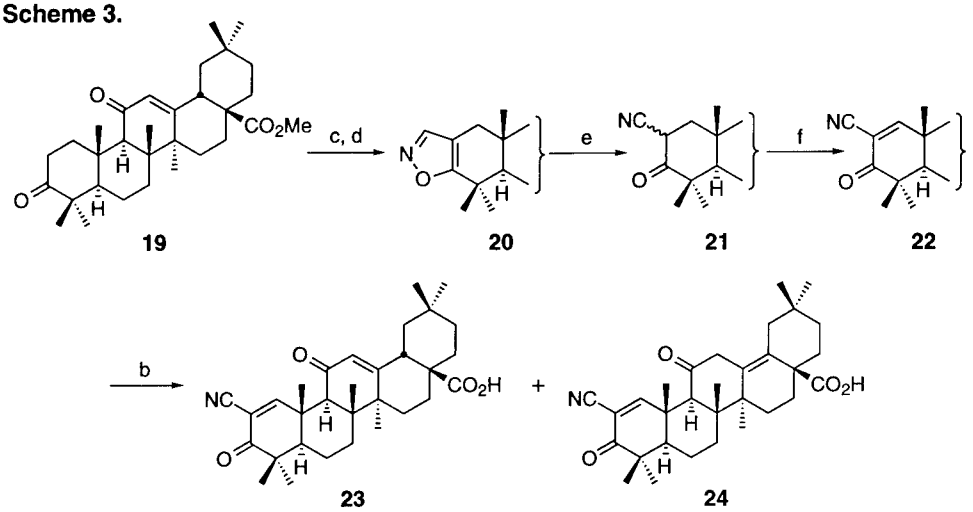


Scheme 2.

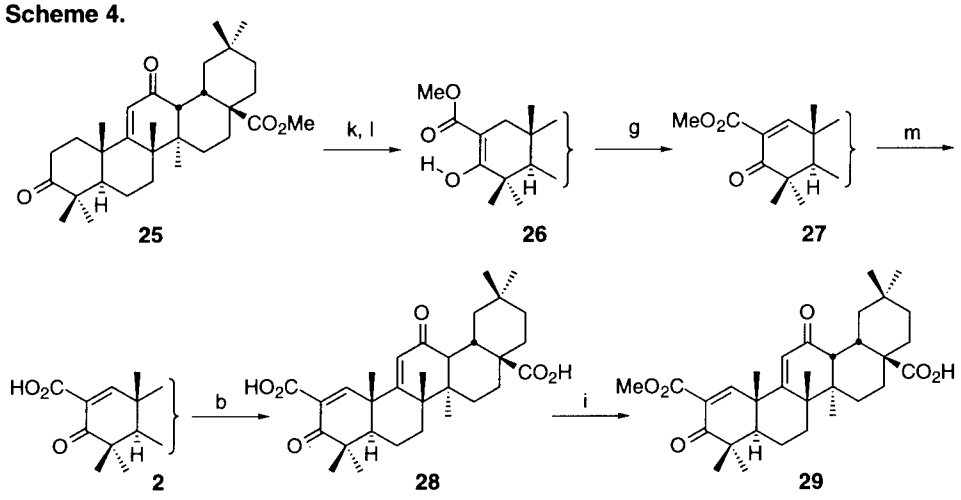


epoxide **5**[4] with hydrogen chloride in acetic acid and $CHCl_3$.[5] Halogenolysis of **6** with LiI in DMF[6] gave chloride **7** in 77% yield. Similarly, bromides **8** and **9** were prepared from **5** and **8** (yield, 96% and 76%), respectively. Compound **11**[7] was prepared in 95% yield by formylation of C-3 ketone **10**[4] 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 CH_2Cl_2 and sequential addition of 30% H_2O_2[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.



Scheme 4.



a: HX/AcOH/$CHCl_3$, b: LiI/DMF, c: HCO_2Et/NaOMe/PhH, d: NH_2OH·HCl/aq EtOH, e: NaOMe/Et_2O/MeOH, f: PhSeCl/AcOEt; 30%H_2O_2/THF, g: PhSeCl/pyr./CH_2Cl_2; 30%H_2O_2/CH_2Cl_2, h: Jones, i: H_2SO_4/MeOH, j: NH_3/MeOH, k: Stiles' reagent/DMF, l: CH_2N_2/Et_2O/THF, m: KOH/aq MeOH

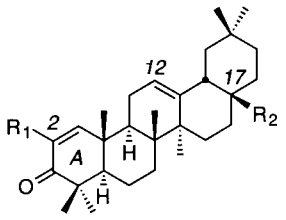
Modification of Ring C

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, **2**, **22–24**, and **27–29**, were designed and synthesized. Isoxazole **20** was prepared from C-3 ketone **19**[4] 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. IC_{50} (μM)[a] Values of Olean-12-ene Derivatives with Modified Ring A



compd	R_1 at C-2	R_2 at C-17	Taft's σ* value of R_1	activity IC_{50} (μM)
34[4]	OH	CO_2H	1.34	27
18	$CONH_2$	CO_2Me	1.68	14
35[4]	OMe	CO_2H	1.81	30
15	CO_2Me	CO_2Me	2	0.9
17	CO_2Me	CO_2H		2.2
14	CO_2H	CO_2Me	2.08	0.8
16	CO_2H	CO_2H		0.07
13	CHO	CO_2Me	2.15	toxic[b]
36[1]	CHO	CO_2H		toxic[b]
8	Br	CO_2Me	2.84	> 40
9	Br	CO_2H		7.3
6	Cl	CO_2Me	2.96	> 40
7	Cl	CO_2H		> 40
12	CN	CO_2Me	3.3	0.7
30[1]	CN	CO_2H		0.6
4[4]	H	CO_2H	-	5.6
oleanolic acid			-	> 40
hydrocortisone			-	0.01

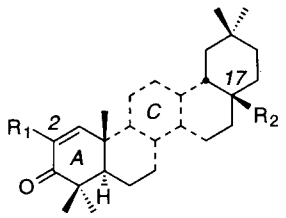
chloride in ethyl acetate and sequential addition of 30% H_2O_2[11] (yield, 33%; 57% based on recovered **21**). Halogenolysis of **22** gave acids **23** and **24** in 37% and 16% yield, respectively. Compounds **2** and **27–29** could not be synthesized according to the similar synthetic route as 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 **25**[4] by Stiles' reagent (methoxymagnesium methyl carbonate) in DMF,[12] 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.

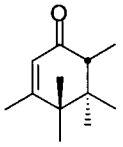
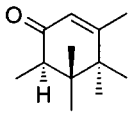
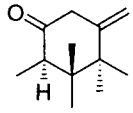
Biological Results and Discussion

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

The inhibitory activities [IC_{50} (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 macrophages[14] 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



compd	structure of ring C	R_1 at C-2	R_2 at C-17	activity IC_{50} (μM)
3 [1]		CN	CO_2Me	0.0001
1 [1]		CN	CO_2H	0.0002
27		CO_2Me	CO_2Me	toxic[b]
29		CO_2Me	CO_2H	0.1
2		CO_2H	CO_2Me	0.0008
28		CO_2H	CO_2H	0.2
31 [4]		H	CO_2H	0.2
22		CN	CO_2Me	0.02
23		CN	CO_2H	0.04
32 [4]		H	CO_2H	1.4
24		CN	CO_2H	0.07
33 [4]		H	CO_2H	2.6
dexamethasone				0.0001

[a]IC_{50} (μM) values of compounds **1–3**, **16**, **22–24**, hydrocortisone and dexamethasone were determined in the range of 0.1 pM–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.
[b]Compounds **13**, **27** and **36** were toxic to cells above 1 μM and were not active below 1 μM.

the strength of Taft's σ* values[15] 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 functionalities 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.

Acknowledgments: We thank Drs. Carl Nathan and Qiao-wen Xie for expert advice on the preparation of macrophages and the nitric oxide assay. We also thank Dr. Steven Mullen (University of Illinois) for the mass spectra. This investigation was supported by funds from the NIH Grant 1 R01-CA78814, the Norris Cotton Cancer Center, U.S. Dept. of Defense Grants # DAMD17-96-1-6163, # DAMD17-98-1-8604, the Oliver and Jennie Donaldson Charitable Trust, the National Foundation for Cancer Research, and a Zenith Award from the Alzheimer's Association. M. B. S. is Oscar M. Cohn Professor, F. G. F., Jr. is Oscar M. Cohn Scholar, and Y. W. is a Howard Hughes Medical Institute Predoctoral Fellow.

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3. CDDO methyl ester (**3**) was found to show the same high activity as CDDO after our previous communication[1] was published.
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7. ^{1}H and ^{13}C NMR of compound **11** in $CDCl_3$ showed that it is the single isomer as depicted in Scheme 2.
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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% thioglycollate. These cells were seeded in 96-well tissue culture plates and incubated with 20 ng/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.
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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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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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We initially randomly synthesized about 60 oleanane and ursane triterpenoids as potential anti-inflammatory and cancer chemopreventive agents. Preliminary screening of these derivatives for inhibition of production of nitric oxide induced by interferon-γ in mouse macrophages revealed that 3-oxooleana-1,12-dien-28-oic acid (**B-15**) showed significant activity (IC_{50} = 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-oxooleana-1,12-diene derivatives with carboxyl, methoxycarbonyl, and nitrile groups at C-2 showed higher activity than the lead compound **B-15**. In particular, 2-carboxy-3-oxooleana-1,12-dien-28-oic acid (**3**) had the highest activity (IC_{50} = 0.07 μM) in this group of triterpenoids. The potency of **3** was similar to that of hydrocortisone (IC_{50} = 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 by 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.

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 anticarcinogenic 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 macrophages[7] 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 nitrile groups at C-2 in ring A that show significant inhibitory activity (IC_{50} = 0.01−0.1 μM level) against production of NO induced by IFN-γ in mouse macrophages. In particular, 2-carboxy-3-oxooleana-1,12-dien-28-oic acid (**3**) had the highest activity (IC_{50} = 0.07 μM) in this group of compounds. The potency of **3** was similar to that of hydrocortisone (IC_{50} = 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

† Part of this work has been reported in preliminary form: (a) Honda, T.; Finlay, H. J.; Gribble, G. W.; Suh, N.; Sporn, M. B. New enone derivatives of oleanolic acid and ursolic acid as inhibitors of nitric oxide production in mouse macrophages. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1623−1628. (b) Honda, T.; Rounds, B. V.; Bore, L.; Favaloro, F. G., Jr.; Gribble, G. W.; Suh, N.; Wang, Y.; Sporn, M. B. Novel synthetic oleanane triterpenoids: a series of highly active inhibitors of nitric oxide production in mouse macrophages. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3429−3434.

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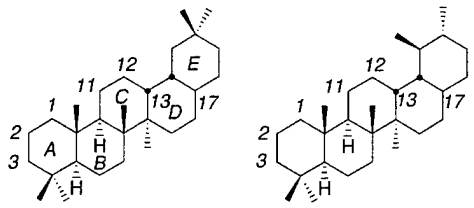
Table 1. Preliminary Screening Results of Synthetic Oleanane and Ursane Triterpenoids

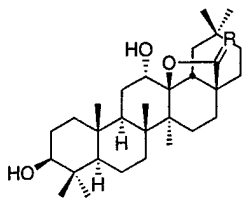


compd	skeleton	C-3	C-12	C-13	C-17	inhibition (%) at 10 μM[b]	ref
1	olean-12-ene	β-OH	H		CO_2H	38	10
2	urs-12-ene	β-OH	H		CO_2H	0	15
A-1	olean-12-ene	β-OH	H		CO_2Me	0	10
A-2	urs-12-ene	β-OH	H		CO_2Me	0	15
A-3	olean-12-ene	β-OAc	H		CO_2Me	10	10
A-4	urs-12-ene	β-OAc	H		CO_2Me	15	15
A-5	olean-12-ene	β-OAc	H		CO_2H	0	10
A-6	urs-12-ene	β-OAc	H		CO_2H	0	15
A-7	olean-12-ene	β-OH	H		CH_2OH	0	28
A-8	urs-12-ene	β-OH	H		CH_2OH	8	29
A-9	olean-12-ene	β-OAc	H		CH_2OAc	4	28
A-10	urs-12-ene	β-OAc	H		CH_2OAc	0	29
A-11	oleanane	β-OAc	α-OH	β-H	CO_2Me	0	*c*
A-12	oleanane	β-OAc	β-OH	β-H	CO_2Me	0	*c*
A-13	oleanane	β-OAc	β-OAc	β-H	CH_2OAc	0	*c*
A-14	oleanane	β-OAc	α-OH	β-H	CH_2OAc	0	*c*
A-15	oleanane	β-OH	α-OH	β-H	CH_2OH	48	*c*
A-16	oleanane	β-OH	β-OH	β-H	CH_2OH	20	*c*
A-17	oleanane	β-OH	=O	β-H	CO_2Me	0	10
A-18	oleanane	β-OAc	=O	β-H	CO_2Me	0	10
A-19	olean-12-ene	α-OH	H		CO_2H	18	30
A-20	urs-12-ene	α-OH	H		CO_2H	48	31
A-21[a]	oleanane	β-OH	α-OH	$-O-$	$-CH(OH)-$	21	32
A-22[a]	oleanane	β-OH	α-OH	$-O-$	$-CO-$	13	32
A-23	oleanane	β-OAc	12α,13α-epoxy-		CO_2Me	0	10
A-24	oleanane	β-OH	α-OH	β-OH	CH_2OH	22	32
B-1	olean-12-ene	=O	H		CO_2H	16	10
B-2	urs-12-ene	=O	H		CO_2H	22	33
B-3	olean-12-ene	=O	H		CO_2Me	24	10
B-4	urs-12-ene	=O	H		CO_2Me	16	15
B-5	olean-12-ene	=O	H		CHO	11	34
B-6	urs-12-ene	=O	H		CHO	21	34
B-7	oleana-11,13(18)-diene	=O	H		CO_2H	47	*c*
B-8	oleanane	=O	=O	β-H	CO_2Me	3	10
B-9	oleanane	=O	=O	β-H	CO_2H	37	*c*
B-10	oleanane	=O	=O	β-H	CHO	38	*c*
B-11[a]	oleanane	=O	α-Br	$-O-$	$-CO-$	4	10
B-12[a]	oleanane	=O	=O	$-O-$	$-CO-$	0	10
B-13	oleana-1,12-diene	=O	H		CO_2Me	19	9
B-14	ursa-1,12-diene	=O	H		CO_2Me	0	*d*
B-15	oleana-1,12-diene	=O	H		CO_2H	85	*d*
B-16	ursa-1,12-diene	=O	H		CO_2H	41	14
C-1[a]	urs-12-ene	=O	H		CO_2H	55	*c*
C-2	olean-12-ene	α-Cl	H		CO_2Me	2	*c*
C-3	olean-12-ene	α-Cl	H		CO_2H	0	*c*
D-1	oleana-2,12-diene	H	H		CO_2Me	3	35
D-2	oleana-2,12-diene	H	H		CO_2H	0	*c*
D-3[a]	olean-12-ene		H		CO_2H	0	*c*
E-1[a]	A-ring cleaved olean-12-ene		H		CO_2Me	21	36
E-2[a]	A-ring cleaved olean-12-ene		H		CO_2H	33	37
E-3[a]	A-ring cleaved urs-12-ene		H		CO_2H	39	37
E-4[a]	A-ring cleaved olean-12-ene		H		CO_2H	22	37
E-5[a]	A-ring cleaved urs-12-ene		H		CO_2H	55	37
E-6[a]	A-ring cleaved urs-12-ene		H		CO_2H	10	37
F-1[a]	C-ring cleaved oleanane	β-OAc			CH_2OAc	52	*c*
F-2[a]	C-ring cleaved oleanane	β-OAc			CH_2OAc	12	*c*
F-3[a]	C-ring cleaved oleanane	β-OAc			CH_2OAc	52	*c*
F-4[a]	C-ring cleaved oleanane	=O			CO_2H	28	*c*

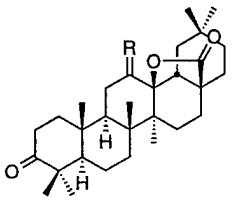
Table 1 (Continued)

compd	skeleton	C-3	C-12	C-13	C-17	inhibition (%) at 10 μM[b]	ref
G-1[a]	olean-12-ene		H		CO_2Me	0	36
G-2[a]	olean-12-ene		H		CO_2H	51	37
hydrocortisone						80	

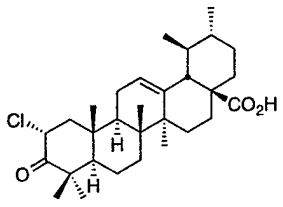
[a] Structure shown below this table. [b] Details of the evaluation method are described in the Experimental Section. [c] Unknown compound (synthesis and spectral data will be published elsewhere). [d] Unknown compound (synthesis and spectral data are shown in this paper).



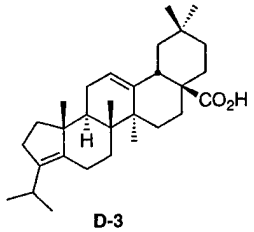
A-21: R = OH, H
A-22: R = O



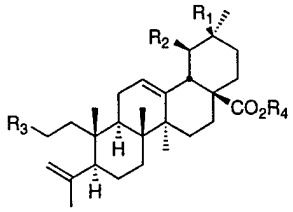
B-11: R = α-Br, β-H
B-12: R = O



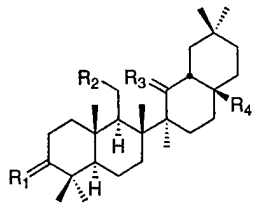
C-1



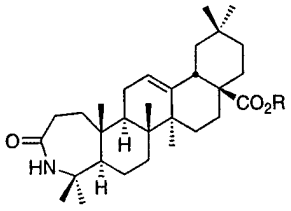
D-3



E-1: $R_1 = R_4 = Me$, $R_2 = H$, $R_3 = CN$
E-2: $R_1 = Me$, $R_2 = R_4 = H$, $R_3 = CN$
E-3: $R_1 = R_4 = H$, $R_2 = Me$, $R_3 = CN$
E-4: $R_1 = Me$, $R_2 = R_4 = H$, $R_3 = CHO$
E-5: $R_1 = R_4 = H$, $R_2 = Me$, $R_3 = CHO$
E-6: $R_1 = R_4 = H$, $R_2 = Me$, $R_3 = CO_2H$

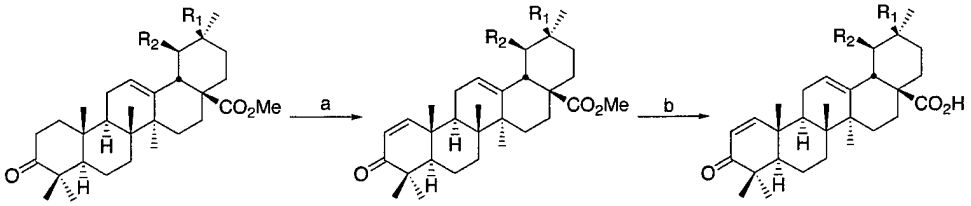


F-1: R_1 = β-OAc, R_2 = CHO, R_3 = O, $R_4 = CH_2OAc$
F-2: R_1 = β-OAc, $R_2 = CH_2OH$, R_3 = OH, $R_4 = CH_2OAc$
F-3: R_1 = β-OAc, $R_2 = CO_2H$, R_3 = O, $R_4 = CH_2OAc$
F-4: $R_1 = R_3$ = O, $R_2 = CO_2H$, $R_4 = CO_2H$



G-1: R = Me
G-2: R = H

Scheme 1[a]



B-3: $R_1 = Me$, $R_2 = H$
B-4: $R_1 = H$, $R_2 = Me$

B-13: $R_1 = Me$, $R_2 = H$
B-14: $R_1 = H$, $R_2 = Me$

B-15: $R_1 = Me$, $R_2 = H$
B-16: $R_1 = H$, $R_2 = Me$

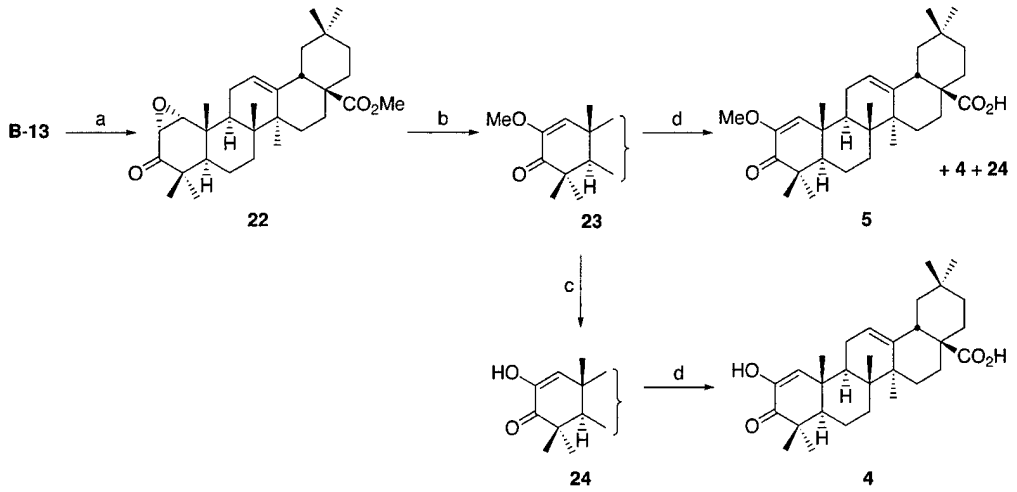
[a] Reagents: (a) PhSeCl, EtOAc; *m*CPBA, pyr, EtOAc; (b) LiI, DMF.

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, IC_{50} = 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**[8] (see Table 2). The syntheses of these newly designed derivatives and compounds **B-13**–**B-16** are illustrated in Schemes 1–6.

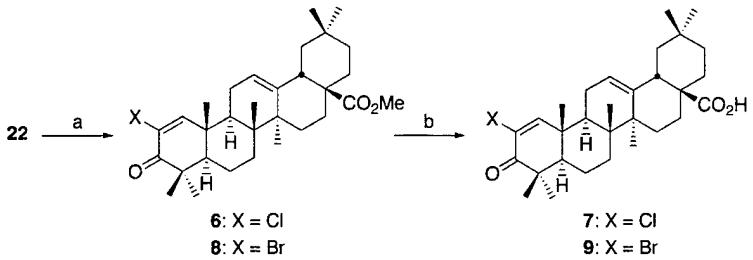
Ester **B-13**[9] was synthesized in 62% yield by introduction of a double bond at C-1 of methyl oleanonate (**B-3**)[10] with phenylselenenyl chloride (PhSeCl) in ethyl acetate and sequential addition of pyridine and *m*-chloroperbenzoic acid.[11,12] Acid **B-15** was synthesized in 85% yield by halogenolysis of **B-13** with lithium iodide in *N*,*N*-dimethylformamide (DMF).[13] Similarly, acid **B-16**[14] was synthesized in 58% yield via ester **B-14** from methyl ursonate (**B-4**).[15] Epoxide **22**[9] was prepared in 99% yield by epoxidation of **B-13** with alkaline hydrogen peroxide. Treatment of **22** with sodium methoxide[16] 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 28% (41% based on recovered

Scheme 2[a]



[a] Reagents: (a) 30% H_2O_2, NaOH(aq), THF; (b) NaOMe, MeOH; (c) HCl, AcOH; (d) LiI, DMF.

Scheme 3[a]



[a] Reagents: (a) HX, AcOH, $CHCl_3$; (b) LiI, DMF.

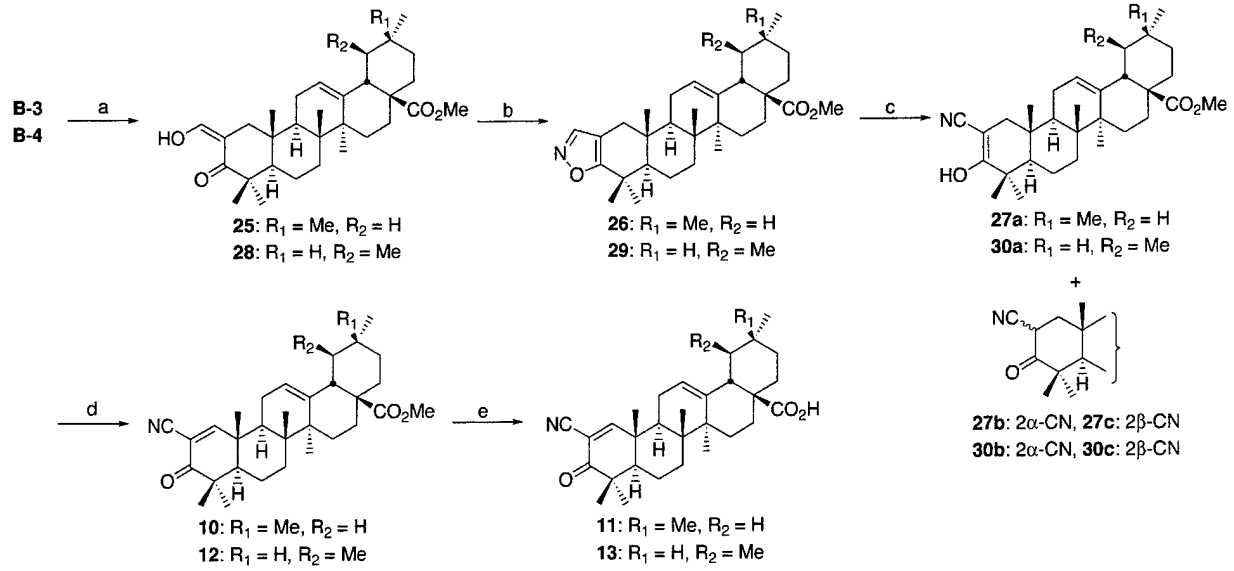
23) yield.[17] Chloride **6** was synthesized in 81% yield from **22** with hydrogen chloride in acetic acid and chloroform.[18] Halogenolysis of **6** gave chloride **7** in 77% yield. Similarly, bromides **8** and **9** were prepared from **22** and **8** (yield, 96% and 76%), respectively. Hydroxymethylene **25**[19,20] was prepared in 95% yield by formylation of **B-3** with ethyl formate in the presence of sodium methoxide in benzene.[21] Isoxazole **26** was prepared in 86% yield by condensation of **25** with hydroxylamine.[22] Cleavage of the isoxazole moiety of **26** with sodium methoxide gave nitrile **27** in 99% yield.[22] ^{1}H NMR showed that **27** is a mixture of three tautomers [**27a**, **27b** (2α-cyano), and **27c** (2β-cyano)] and that **27a** is the major one in $CDCl_3$. 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] **29**, and **30** from **B-4**. Acid **13** was prepared in 74% yield by halogenolysis of **12**. Enal **14** was prepared from **25** by PhSeCl–pyridine in methylene chloride and sequential addition of 30% hydrogen peroxide[24] (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 oleanonic acid (**B-1**)[10] was attempted. Formylation of **B-1** with ethyl formate in the presence of sodium methoxide in tetrahydrofuran gave **32**[20] (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. ^{1}H NMR showed that **31** is the single tautomer in $CDCl_3$ 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 [IC_{50} (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* values[26] of substituents at C-2. These results provide the following interesting structure–activity relationships:

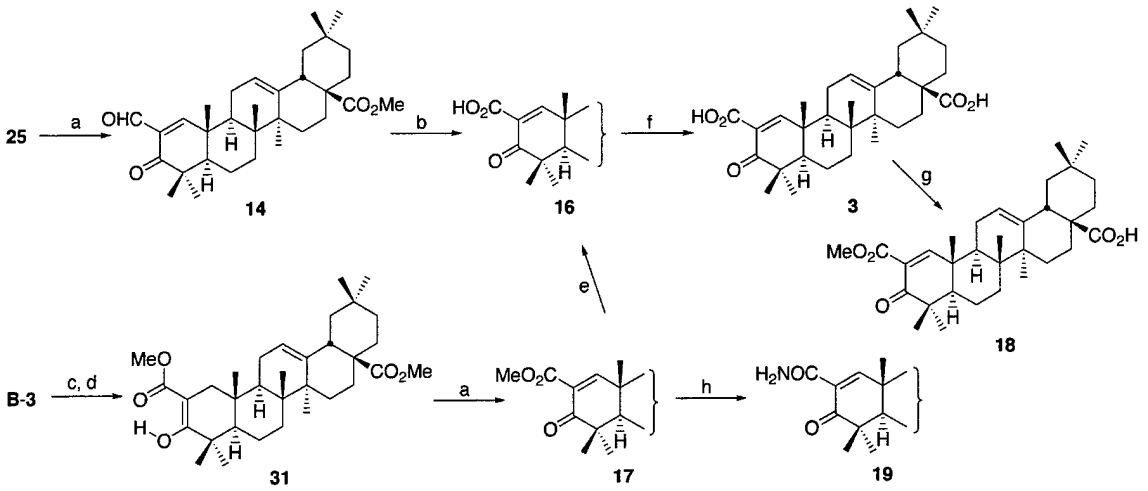
(1) In the A ring, a 1-en-3-one functionality is important for significant activity. The lead compound **B-15** is much more potent than the C-3 ketone **B-1** and the

Scheme 4[a]



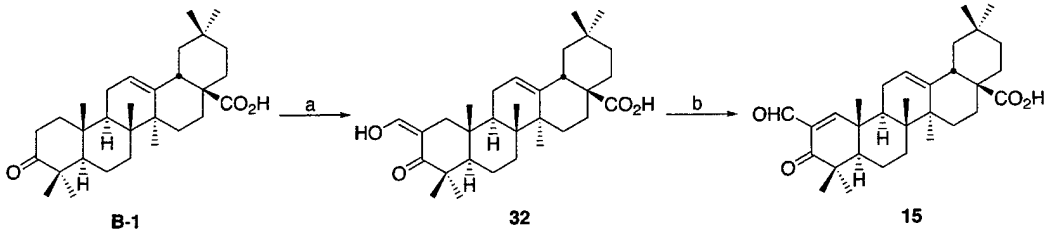
[a] Reagents: (a) HCO_2Et, NaOMe, PhH; (b) NH_2OH·HCl, aq EtOH; (c) NaOMe, Et_2O, MeOH; (d) DDQ, PhH; (e) LiI, DMF.

Scheme 5[a]



[a] Reagents: (a) PhSeCl, pyr, CH_2Cl_2; 30% H_2O_2, CH_2Cl_2; (b) Jones; (c) Stiles' reagent, DMF; (d) CH_2N_2, Et_2O, THF; (e) KOH, aq MeOH; (f) LiI, DMF; (g) H_2SO_4, MeOH; (h) NH_3, MeOH.

Scheme 6[a]



[a] Reagents: (a) HCO_2Et, NaOMe, THF; (b) PhSeCl, pyr, CH_2Cl_2; 30% H_2O_2, CH_2Cl_2.

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

(2) A correlation between Taft's σ^* 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.

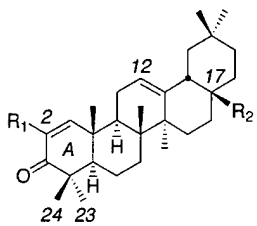
(3) Carboxyl, methoxycarbonyl, and nitrile groups at C-2 enhance activity. Compounds **3**, **10**, **11**, **16**, and **17** are about 10−100 times more potent than **B-15**. In particular, **3** showed the highest activity (IC_{50} = 0.07 μM) in this series of compounds. The potency of **3** was similar to that of hydrocortisone (IC_{50} = 0.01 μM).

(4) Hydroxyl, aminocarbonyl, methoxy, chloride, and bromide groups decrease activity. Compounds **4**−**9** and **19** are much less potent than **B-15**.

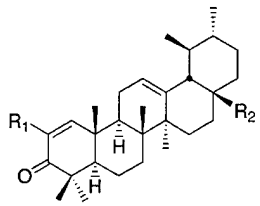
(5) A formyl group does not confer activity but only toxicity.

(6) 23,24-Dimethyl groups are important for signifi-

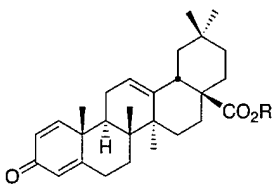
Table 2. Activity of Olean- and Urs-12-ene Triterpenoids with Various 1-En-3-one Functionalities



3-oxooleana-1,12-diene



3-oxoursa-1,12-diene



20: R = Me
21: R = H

compd	skeleton[a]	R_1 at C-2	R_2 at C-17	Taft's σ^* value of R_1	formula	analyses[b]	activity[c] IC_{50} (μM)
B-13	O	H	CO_2Me		$C_{31}H_{46}O_3$	ref 9	31
B-15	O	H	CO_2H		$C_{30}H_{44}O_3 \cdot 3/4H_2O$	C, H	5.6
20	D	H	CO_2Me		$C_{29}H_{40}O_3 \cdot 1/4H_2O$	C, H	>40
21	D	H	CO_2H		$C_{28}H_{38}O_3 \cdot 1/3H_2O$	C, H	13
B-16	U	H	CO_2H		$C_{30}H_{44}O_3$	ref 14	13
5	O	OH	CO_2H	1.34	$C_{30}H_{44}O_4 \cdot 1/2H_2O$	C, H	27
19	O	$CONH_2$	CO_2Me	1.68	$C_{32}H_{47}O_4N \cdot 3/4H_2O$	C, H, N	14
4	O	OMe	CO_2H	1.81	$C_{31}H_{46}O_4 \cdot 1/2H_2O$	C, H	30
17	O	CO_2Me	CO_2Me	2.00	$C_{33}H_{48}O_5$	C, H	0.9
18	O	CO_2Me	CO_2H	2.00	$C_{32}H_{46}O_5$	C, H	2.2
16	O	CO_2H	CO_2Me	2.08	$C_{32}H_{46}O_5 \cdot 1/2H_2O$	C, H	0.8
3	O	CO_2H	CO_2H	2.08	$C_{31}H_{44}O_5$	C, H	0.07
14	O	CHO	CO_2Me	2.15	$C_{32}H_{46}O_4$	C, H	toxic[d]
15	O	CHO	CO_2H	2.15	$C_{31}H_{44}O_4 \cdot 1/2H_2O$	C, H	toxic[d]
8	O	Br	CO_2Me	2.84	$C_{31}H_{45}O_3Br$	C, H	>40
9	O	Br	CO_2H	2.84	$C_{30}H_{43}O_3Br \cdot H_2O$	C, H	7.3
6	O	Cl	CO_2Me	2.96	$C_{31}H_{45}O_3Cl$	C, H	>40
7	O	Cl	CO_2H	2.96	$C_{30}H_{43}O_3Cl \cdot 1/4H_2O$	C, H	>40
10	O	CN	CO_2Me	3.30	$C_{32}H_{45}O_3N \cdot 1/4H_2O$	C, H, N	0.7
11	O	CN	CO_2H	3.30	$C_{31}H_{43}O_3N \cdot 1/2H_2O$	C, H, N	0.6
12	U	CN	CO_2Me	3.30	$C_{32}H_{45}O_3N \cdot 3/4H_2O$	C, H, N	5.1
13	U	CN	CO_2H	3.30	$C_{31}H_{43}O_3N \cdot H_2O$	C, H, N	6.2
B-1		oleanonic acid			$C_{30}H_{46}O_3$	ref 10	37
1		oleanolic acid			$C_{30}H_{48}O_3$	ref 10	>40
2		ursolic acid			$C_{30}H_{48}O_3$	ref 15	toxic[e]
		hydrocortisone					0.01

[a] O, 3-oxooleana-1,12-diene; D, 23,24-dinor-3-oxooleana-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. IC_{50} values of **3** and hydrocortisone were determined in the range of 0.1 pM−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.

cant activity. **B-15** is more potent than 23,24-dinor-olean-1-en-3-one derivative **21**.

(7) The oleanane skeleton is more potent than the ursane skeleton. **B-15**, **10**, and **11** are more potent than **B-16**, **12**, and **13**, respectively.

(8) The role of methoxycarbonyl and carboxyl groups at C-17 is ambiguous. In some analogues, the carboxyl group is more potent than the methoxycarbonyl group: acids **B-15**, **3**, **9**, and **21** are more potent than esters **B-13**, **16**, **8**, and **20**, respectively. For other analogues, the carboxyl and methoxycarbonyl groups show similar activity: acids **11** and **13** show similar activity to esters **10** and **12**, respectively. Lastly, acid **18** is less potent than ester **17**.

The inhibitory activity of new triterpenoids **3** and **11** was not blocked by the glucocorticoid antagonist, RU-486,[27] which reverses the action of hydrocortisone (see Figure 1). These data strongly suggest that the actions of these triterpenoids on the iNOS system are not mediated by their interaction with the glucocorticoid receptor.

On the basis of these structure−activity relationships, further lead optimization is in progress. Further biological evaluation and studies on the mechanism of action of **3** are also in progress.

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 ^{13}C (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 $CHCl_3$ (^1H NMR) and the δ 77.23 signal of $CDCl_3$ (^{13}C 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 F_{254}. 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 $NaHCO_3$ solution (three times) followed by saturated aqueous NaCl solution (three times), then dried over anhydrous $MgSO_4$, and filtered. The filtrate was evaporated in vacuo.

Methyl 3-Oxooleana-1,12-dien-28-oate (B-13).[9] A solution of methyl oleanonate (**B-3**)[10] (2.00 g, 4.27 mmol) and phenylselenenyl chloride (98%) (1.00 g, 5.12 mmol) in EtOAc (85 mL) was stirred at room temperature for 3 h. To the stirred

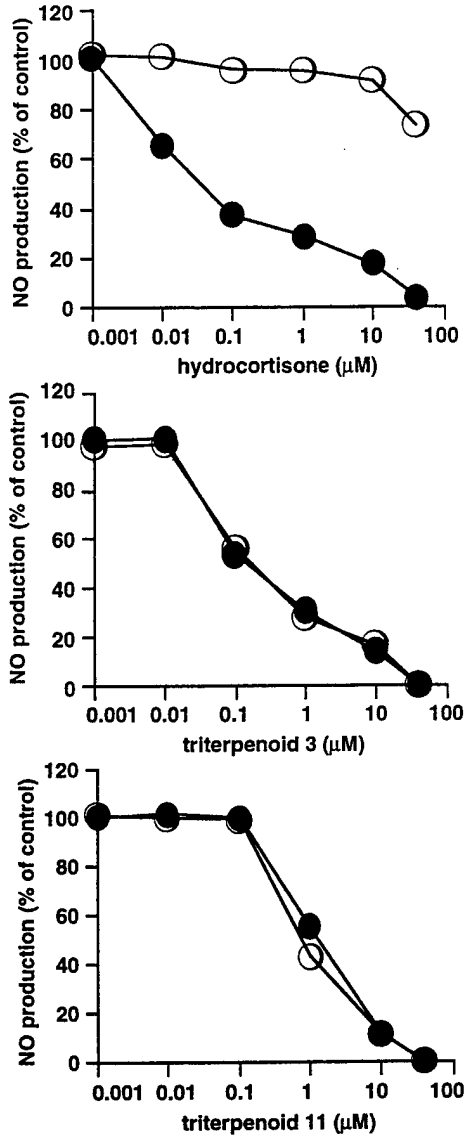


Figure 1. Blockage by glucocorticoid antagonist RU486 of hydrocortisone-inhibited NO production but not of triterpenoid (**3** and **11**) inhibited NO production in primary mouse macrophages. Macrophage cells were incubated with IFN-γ (20 ng/mL) together with hydrocortisone or triterpenoids without RU486 (●); in some cases RU486 (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.

mixture, saturated aqueous $NaHCO_3$ solution was added. After most of the aqueous layer was removed, pyridine (844 mg, 10.7 mmol) and *m*-chloroperbenzoic acid (50–60%) (3.68 g, 10.7 mmol) were added to the organic layer. The mixture was stirred at room temperature for 1 h. The mixture was washed with 5% aqueous NaOH solution (three times), saturated aqueous NH_4Cl solution (three times), and saturated aqueous NaCl solution (three times); dried over anhydrous $MgSO_4$; and filtered. The filtrate was evaporated in vacuo to give a solid. The solid was subjected to flash column chromatography [hexanes–EtOAc (5:1)] to give **B-13** as a crystalline solid (1.23 g, 62%): mp 159–161 °C; $[\alpha]^{25}_D$ +103° (*c* 0.64, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 230 (3.92) nm. IR (KBr): 2946, 2867, 1728, 1660 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.04 (1H, d, J = 10.1 Hz), 5.81 (1H, d, J = 10.1 Hz), 5.36 (1H, t, J = 3.7 Hz), 3.64, (3H, s), 2.90 (1H, dd, J = 4.6, 13.9 Hz), 1.17 (3H, s), 1.16 (6H, s), 1.10, 0.94, 0.91, 0.83 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 205.5, 178.4, 159.3, 144.5, 125.2, 121.9, 53.6, 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, 30.9, 28.0, 27.9, 26.0, 23.8, 23.5, 23.2, 21.8, 19.1, 18.8, 17.5. EIMS (70 eV) *m/z*: 466 $[M]^+$ (73), 451 (11), 407 (31), 262 (57), 203 (100). HREIMS: Calcd for $C_{31}H_{46}O_3$: 466.3447. Found: 466.3446.

Methyl 3-Oxoursa-1,12-dien-28-oate (B-14). **B-14** was prepared from methyl ursonate (**B-4**)[15] according to the same method as for **B-13** to give an amorphous solid (66%): $[\alpha]^{26}_D$ +93° (*c* 0.77, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 232 (3.95) nm. IR (KBr): 2974, 2935, 2871, 1725, 1669 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.06 (1H, d, J = 10.1 Hz), 5.81 (1H, d, J = 10.1 Hz), 5.33 (1H, t, J = 3.8 Hz), 3.63 (3H, s), 2.28 (1H, d, J = 11.5 Hz), 1.17, 1.15 (each 3H, s), 1.10 (6H, s), 0.95 (3H, d, J = 5.4 Hz), 0.87 (3H, d, J = 6.3 Hz), 0.85 (3H, s). ^{13}C NMR ($CDCl_3$): δ 205.5, 178.2, 159.5, 139.0, 125.2, 125.0, 53.7, 53.3, 51.7, 48.4, 44.7, 42.6, 41.9, 40.5, 39.5, 39.2, 39.1, 36.8, 33.0, 30.8, 28.2, 28.1, 24.4, 23.7, 23.5, 21.8, 21.4, 19.1, 19.0, 17.7, 17.2. EIMS (70 eV) *m/z*: 466 $[M]^+$ (14), 406 (12), 262 (74), 203 (100). HREIMS: Calcd for $C_{31}H_{46}O_3$: 466.3447. Found: 466.3442.

3-Oxooleana-1,12-dien-28-oic Acid (B-15). A mixture of **B-13** (100 mg, 0.21 mmol) and LiI (500 mg) in dry DMF (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 CH_2Cl_2 and Et_2O (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%): $[\alpha]^{26}_D$ +103° (*c* 0.45, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 230 (3.75) nm. IR (KBr): 2941, 2866, 1732, 1695, 1671 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.04 (1H, d, J = 10.2 Hz), 5.81 (1H, d, J = 10.2 Hz), 5.35 (1H, t, J = 3.3 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). ^{13}C NMR ($CDCl_3$): 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*: 452 $[M]^+$ (8.8), 437 (3.8), 406 (6.8), 248 (80), 233 (14), 203 (100). HREIMS: Calcd for $C_{30}H_{44}O_3$: 452.3290. Found: 452.3289. Anal. (Table 2).

3-Oxoursa-1,12-dien-28-oic Acid (B-16).[14] **B-16** was prepared from **B-14** according to the same method as for **B-15** to give an amorphous solid (88%): $[\alpha]^{26}_D$ +91° (*c* 0.84, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 230 (3.99) nm. IR (KBr): 3306, 2973, 2930, 2870, 1729, 1695, 1669 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.07 (1H, d, J = 10.1 Hz), 5.82 (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, d, J = 6.4 Hz), 0.88 (3H, s). ^{13}C NMR ($CDCl_3$): δ 205.5, 183.9, 159.4, 138.8, 125.3, 53.6, 52.9, 48.3, 44.7, 42.5, 41.9, 40.5, 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: Calcd for $C_{30}H_{44}O_3$ + H: 453.3369. Found: 453.3335 (by a Micromass 70-SE-4F).

2-Carboxy-3-oxooleana-1,12-dien-28-oic Acid (3). A mixture of **16** (109 mg, 0.21 mmol) and LiI (520 mg) in dry DMF (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 $MgSO_4$, and filtered. The filtrate was evaporated in vacuo to give a residue (108 mg). The residue was subjected to flash column chromatography [CH_2Cl_2–MeOH (15:1) followed by CH_2Cl_2–MeOH (10:1)] to afford **3** as a crystalline solid (61 mg, 58%): mp >250 °C dec; $[\alpha]^{26}_D$ +81° (*c* 0.53, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 234 (3.88) nm. IR (KBr): 3389, 2943, 2872, 1752, 1696, 1637 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.43 (1H, s), 5.37 (1H, t, J = 3.5 Hz), 2.87 (1H, dd, J = 3.8, 13.9 Hz), 1.25, 1.22, 1.18, 1.15, 0.95, 0.93, 0.88 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 209.0, 183.9, 173.2, 165.2, 144.2, 123.4, 121.7, 52.4, 46.8, 45.7, 45.5, 42.3, 41.4, 41.1, 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 (35), 203 (100). HREIMS: Calcd for $C_{31}H_{44}O_5$: 496.3189. Found: 496.3196. Anal. (Table 2).

2-Hydroxy-3-oxooleana-1,12-dien-28-oic Acid (4). **4** was prepared from **24** according to the same method as for **B-15**

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%): $[\alpha]^{25}_D$ +99° (*c* 0.46, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 272 (3.71) nm. IR (KBr): 3434, 2938, 1698, 1667, 1649 cm^{-1}. ^1H NMR ($CDCl_3$): δ 6.35 (1H, s), 5.96 (1H, brs), 5.34 (1H, t, J = 3.5 Hz), 2.86 (1H, dd, J = 3.8, 13.9 Hz), 1.23, 1.22, 1.14, 1.11, 0.94, 0.92, 0.83 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 201.2, 184.0, 144.1, 143.9, 128.4, 122.3, 54.0, 46.8, 45.8, 44.1, 43.3, 42.2, 41.3, 40.2, 38.7, 34.0, 33.3, 32.6, 30.9, 27.8, 27.4, 26.1, 23.8, 23.6, 23.0, 22.0, 19.9, 18.9, 17.7. EIMS (70 eV) *m/z*: 468 $[M]^+$ (3.2), 248 (13), 203 (23), 149 (42), 84 (100). HREIMS: Calcd for $C_{30}H_{44}O_4$: 468.3240. Found: 468.3222. Anal. (Table 2).

2-Methoxy-3-oxooleana-1,12-dien-28-oic Acid (5). A mixture of **23** (230 mg, 0.46 mmol) and LiI (1045 mg) in dry DMF (3.5 mL) was heated under reflux for 4 h. The reaction mixture was worked up according to the same method as for **B-15** to give a solid (230 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (3:1) followed by hexanes–EtOAc (2:1), then hexanes–EtOAc (1:1)] to give **24** (35 mg; 16%, 23% based on recovered **23**), **23** (74 mg), **4** (27 mg; 12%, 18% based on recovered **23**), and **5** as an amorphous solid (63 mg; 28%, 41% based on recovered **23**): $[\alpha]^{26}_D$ +96° (*c* 0.29, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 266 (3.84) nm. IR (KBr): 3307, 2947, 2862, 1732, 1693, 1622 cm^{-1}. ^1H NMR ($CDCl_3$): δ 5.96 (1H, s), 5.36 (1H, t, J = 3.3 Hz), 3.56 (3H, s), 2.87 (1H, dd, J = 4.2, 13.9 Hz), 1.17 (9H, s), 1.11, 0.94, 0.91, 0.84 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 200.0, 184.4, 149.1, 144.4, 126.1, 122.1, 55.0, 53.2, 46.8, 45.9, 45.4, 43.3, 42.2, 41.3, 40.2, 38.5, 34.0, 33.3, 32.5, 30.9, 28.6, 27.8, 26.1, 23.8, 23.0, 22.0, 20.4, 19.2, 17.6. EIMS (70 eV) *m/z*: 482 $[M]^+$ (11), 415 (6.5), 245 (18), 203 (33), 157 (100). HREIMS: Calcd for $C_{31}H_{46}O_4$: 482.3396. Found: 482.3375. Anal. (Table 2).

Methyl 2-Chloro-3-oxooleana-1,12-dien-28-oate (6). A solution of **22** (99 mg, 0.21 mmol) in AcOH including 1 M HCl (2.5 mL) and $CHCl_3$ (2.5 mL) was stirred at room temperature overnight. The mixture was diluted with CH_2Cl_2. After it was washed with water three times, it was worked up according to the standard method to give a solid (96 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (6:1)] to afford **6** as an amorphous solid (84 mg, 81%): $[\alpha]^{26}_D$ +98° (*c* 0.26, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 250 (3.91) nm. IR (KBr): 2943, 2866, 1727, 1689 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.22 (1H, s), 5.34 (1H, t, J = 3.5 Hz), 3.62 (3H, s), 2.89 (1H, dd, J = 4.2, 13.7 Hz), 1.203, 1.197 (each 3H, s), 1.14 (6H, s), 0.93, 0.90, 0.80 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 197.4, 178.3, 155.0, 144.5, 129.8, 121.5, 53.3, 51.8, 46.9, 46.3, 45.8, 42.2, 42.1, 41.64, 41.57, 40.3, 34.0, 33.3, 32.4, 30.9, 28.4, 27.8, 26.0, 23.8, 23.5, 23.1, 22.1, 19.1, 18.8, 17.5. EIMS (70 eV) *m/z*: 500 $[M]^+$ (21), 262 (27), 247 (96), 203 (100). HREIMS: Calcd for $C_{31}H_{45}O_3Cl$: 500.3057. Found: 500.3060. Anal. (Table 2).

2-Chloro-3-oxooleana-1,12-dien-28-oic Acid (7). **7** was prepared from **6** 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 **7** as an amorphous solid (77%): $[\alpha]^{26}_D$ +88° (*c* 0.50, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 252 (3.20) nm. IR (KBr): 3297, 2943, 2870, 1733, 1691, 1601 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.23 (1H, s), 5.35 (1H, t, J = 3.3 Hz), 2.86 (1H, dd, J = 4.3, 13.8 Hz), 1.22, 1.21, 1.16, 1.13, 0.94, 0.92, 0.84 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 197,4, 184.4, 154.9, 144.3, 129.9, 121.8, 53.2, 46.8, 46.4, 45.8, 42.21, 42.16, 41.6, 41.3, 40.3, 34.0, 33.3, 32.5, 32.4, 30.9, 28.5, 27.8, 26.0, 23.7, 23.5, 23.0, 22.1, 19.0, 18.9, 17.7. EIMS (70 eV) *m/z*: 486 $[M]^+$ (25), 248 (100), 203 (96). HREIMS: Calcd for $C_{30}H_{43}O_3Cl$: 486.2901. Found: 486.2898. Anal. (Table 2).

Methyl 2-Bromo-3-oxooleana-1,12-dien-28-oate (8). A solution of **22** (220 mg, 0.46 mmol) in AcOH including 1 M HBr (4.9 mL) and $CHCl_3$ (6.1 mL) was stirred at room temperature for 1 h. The mixture was diluted with CH_2Cl_2. After it was washed with water three times, it was worked up according to the standard method to give a solid (260 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (6:1)] to afford **8** as an amorphous solid (238 mg, 96%): $[\alpha]^{26}_D$ +88° (*c* 0.51, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 260 (3.69) nm. IR (KBr): 2943, 2870, 1733, 1691, 1601 cm^{-1}. ^1H NMR ($CDCl_3$): 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 (each 6H, s), 0.94, 0.91, 0.81 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 197.3, 178.3, 159.5, 144.6, 121.8, 121.5, 53.3, 51.8, 46.9, 46.5, 45.8, 43.1, 42.3, 42.1, 41.7, 40.3, 34.0, 33.3, 32.4, 30.9, 28.7, 27.8, 26.0, 23.8, 23.6, 23.2, 22.3, 19.1, 18.7, 17.5. EIMS (70 eV) *m/z*: 546 (5.0) and 544 (5.2) $[M]^+$, 262 (8.5), 203 (24), 118 (100), 116 (100). HREIMS: Calcd for $C_{31}H_{45}O_3Br$: 544.2552. Found: 544.2553. Anal. (Table 2).

2-Bromo-3-oxooleana-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%): $[\alpha]^{26}_D$ +82° (*c* 0.31, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 260 (3.52) nm. IR (KBr): 3434, 2939, 2870, 1727, 1686, 1601 cm^{-1}. ^1H NMR ($CDCl_3$): 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, 0.92, 0.83 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 197.2, 184.4, 159.3, 144.3, 121.84, 121.79, 53.3, 46.8, 46.5, 45.8, 43.1, 42.2, 42.0, 41.3, 40.3, 34.0, 33.2, 32.5, 32.4, 30.9, 28.7, 27.8, 26.0, 23.7, 23.5, 23.0, 22.2, 19.1, 18.7, 17.7. EIMS (70 eV) *m/z*: 532 (13) and 530 (14) $[M]^+$, 285 (5.6), 283 (6.2), 248 (100), 235 (10), 233 (11), 203 (84). HREIMS: Calcd for $C_{30}H_{43}O_3Br$: 530.2396. Found: 530.2383. Anal. (Table 2).

Methyl 2-Cyano-3-oxooleana-1,12-dien-28-oate (10). A solution of **27** (141 mg, 0.28 mmol) and DDQ (98%) (79 mg, 0.34 mmol) in benzene (10 mL) was heated under reflux for 4 h. After insoluble matter was removed by filtration, the filtrate was evaporated in vacuo to give a solid. The solid was subjected to flash column chromatography [benzene–acetone (20:1)] to give a crystalline solid (123 mg, 88%): mp 201–202 °C; $[\alpha]^{26}_D$ +67° (*c* 0.53, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 240 (3.65) nm. IR (KBr): 2945, 2874, 2232, 1724, 1686 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.75 (1H, s), 5.36 (1H, t, J = 3.5 Hz), 3.64 (3H, s), 2.91 (1H, dd, J = 3.9, 13.9 Hz), 1.22, 1.21, 1.15, 1.14, 0.94, 0.92, 0.83 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 198.3, 178.3, 170.2, 144.8, 121.1, 115.2, 114.0, 52.8, 51.8, 46.9, 45.8, 45.1, 42.3, 41.7, 41.3, 40.8, 40.5, 34.0, 33.3, 32.4, 32.3, 30.9, 27.9, 27.8, 26.0, 23.8, 23.4, 23.1, 21.8, 18.9, 18.1, 17.6. EIMS (70 eV) *m/z*: 491 $[M]^+$ (35), 459 (13), 432 (27), 262 (22), 247 (24), 203 (100). HREIMS: Calcd for $C_{32}H_{45}O_3N$: 491.3399. Found: 491.3391. Anal. (Table 2).

2-Cyano-3-oxooleana-1,12-dien-28-oic Acid (11). **11** was prepared from **10** according to the same method as for **B-15** except that the reaction time was 3 h. The reaction mixture was subjected to flash column chromatography [hexanes–EtOAc (3:1) followed by hexanes–EtOAc (2:1), then hexanes–EtOAc (1:1)] to give **11** as an amorphous solid (71%, 91% based on recovered **10**): $[\alpha]^{26}_D$ +61° (*c* 0.66, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 238 (3.87) nm. IR (KBr): 3387, 2947, 2870, 2233, 1729, 1691, 1609 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.75 (1H, s), 5.35 (1H, t, J = 3.3 Hz), 2.86 (1H, dd, J = 4.0, 13.6 Hz), 1.22, 1.21, 1.15, 1.12, 0.94, 0.92, 0.85 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 198.2, 184.3, 170.1, 144.4, 121.4, 115.1, 114.1, 52.7, 46.8, 45.7, 45.0, 42.2, 41.3, 40.8, 40.5, 33.9, 33.2, 32.5, 32.2, 30.9, 27.9, 27.7, 25.9, 23.7, 23.4, 22.9, 21.8, 18.9, 18.1, 17.7. EIMS (70 eV) *m/z*: 477 $[M]^+$ (18), 462 (5.6), 431 (16), 416 (10), 248 (76), 235 (25), 203 (100). HREIMS: Calcd for $C_{31}H_{43}O_3N$: 477.3243. Found: 477.3240. Anal. (Table 2).

Methyl 2-Cyano-3-oxoursa-1,12-dien-28-oate (12). **12** was prepared from **30** according to the same method as for **10** to give an amorphous solid (62%): $[\alpha]^{26}_D$ +53° (*c* 0.35, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 240 (3.74) nm. IR (KBr): 2973, 2926, 2870, 2229, 1723, 1686 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.77 (1H, s), 5.33 (1H, t, J = 3.7 Hz), 3.62 (3H, s), 2.29 (1H, d, J = 11.2 Hz), 1.23, 1.21, 1.14, 1.11 (each 3H, s), 0.96, 0.88 (each 3H, d, J = 6.3 Hz), 0.86 (3H, s). ^{13}C NMR ($CDCl_3$): δ 198.3, 178.1, 170.4, 139.3, 124.2, 115.2, 114.0, 53.2, 52.8, 51.7, 48.3, 45.1, 42.7, 41.2, 40.70, 40.65, 39.1, 39.0, 36.7, 32.6, 30.8, 28.1, 28.0, 24.3, 23.6, 23.4, 21.8, 21.3, 18.9, 18.2, 17.8, 17.2. EIMS (70

eV) *m/z*: 491 $[M]^+$ (38), 431 (35), 262 (46), 249 (82), 203 (65), 84 (100). HREIMS: Calcd for $C_{32}H_{45}O_3N$: 491.3399. Found: 491.3395. Anal. (Table 2).

2-Cyano-3-oxoursa-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 (1.5:1)] to give **13** as an amorphous solid (74%): $[\alpha]^{26}_D$ +48° (*c* 0.50, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 238 (3.86) nm. IR (KBr): 3417, 2973, 2926, 2870, 2233, 1731, 1689 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.77 (1H, s), 5.31 (1H, t, J = 3.2 Hz), 2.24 (1H, d, J = 11.0 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 (3H, s). ^{13}C NMR ($CDCl_3$): δ 198.2, 184.2, 170.2, 139.0, 124.4, 115.1, 114.1, 52.8, 52.7, 48.2, 45.0, 42.6, 41.2, 40.68, 40.65, 39.1, 39.0, 36.7, 32.5, 30.7, 28.1, 28.0, 24.1, 23.6, 23.3, 21.8, 21.3, 18.9, 18.2, 17.7, 17.2. EIMS (70 eV) *m/z*: 477 $[M]^+$ (22), 431 (23), 248 (100), 203 (48). HREIMS: Calcd for $C_{31}H_{43}O_3N$: 477.3243. Found: 477.3240. Anal. (Table 2).

Methyl 2-Formyl-3-oxooleana-1,12-dien-28-oate (14). To a stirred solution of phenylselenenyl chloride (98%) (161 mg, 0.82 mmol) in CH_2Cl_2 (7.2 mL) was added a solution of pyridine (75 mg, 0.95 mmol) in CH_2Cl_2 (1.0 mL) in an ice bath. After 15 min, a solution of **25** (204 mg, 0.41 mmol) in CH_2Cl_2 (2.0 mL) was added, and the mixture was stirred an additional 1 h. After the mixture was washed with 10% aqueous HCl solution (3 mL) twice, 30% H_2O_2 (0.4 mL) was added to the stirred mixture in the ice bath. After an additional 40 min, the mixture was worked up according to the standard method to give a solid (199 mg). The solid was subjected to flash column chromatography [hexanes−EtOAc (5:1)] to afford **25** (20 mg) and **14** as an amorphous solid (144 mg; 71%, 79% based on recovered **25**): $[\alpha]^{26}_D$ +12° (*c* 0.60, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 238 (3.85) nm. IR (KBr): 2946, 2867, 1724, 1703, 1673, 1610 cm^{-1}. ^1H NMR ($CDCl_3$): δ 10.00 (1H, s), 7.79 (1H, s), 5.37 (1H, t, J = 3.6 Hz), 3.63 (3H, s), 2.90 (1H, dd, J = 4.2, 13.9 Hz), 1.18, 1.17, 1.16, 1.14, 0.94, 0.91, 0.85 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 203.7, 190.7, 178.3, 165.2, 144.5, 131.2, 121.6, 52.8, 51.8, 47.0, 45.8, 45.1, 42.3, 41.7, 41.3, 40.5, 39.8, 34.0, 33.3, 32.44, 32.38, 30.9, 28.2, 27.8, 26.0, 23.8, 23.5, 23.2, 21.7, 19.2, 18.2, 17.6. EIMS (70 eV) *m/z*: 494 $[M]^+$ (95), 435 (87), 262 (40), 203 (100). HREIMS: Calcd for $C_{32}H_{46}O_4$: 494.3396. Found: 494.3398. Anal. (Table 2).

2-Formyl-3-oxooleana-1,12-dien-28-oic Acid (15). **15** was prepared from **32** according to the same method as for **14**. The reaction mixture was subjected to flash column chromatography [hexanes−EtOAc (3:1) followed by hexanes−EtOAc (2:1)] to give **15** as an amorphous solid (71%, 84% based on recovered **32**): $[\alpha]^{26}_D$ +26° (*c* 0.95, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 240 (3.82) nm. IR (KBr): 2948, 2866, 1725, 1701, 1674, 1608 cm^{-1}. ^1H NMR ($CDCl_3$): δ 10.00 (1H, s), 7.79 (1H, s), 5.36 (1H, t, J = 3.3 Hz), 2.86 (1H, dd, J = 3.8, 13.9 Hz), 1.18, 1.17, 1.15, 1.14, 0.94, 0.92, 0.87 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 203.7, 190.7, 184.3, 165.0, 144.2, 131.2, 121.8, 52.8, 46.8, 45.7, 45.1, 42.3, 41.4, 41.3, 40.5, 39.8, 34.0, 33.2, 32.5, 32.3, 30.9, 28.2, 27.8, 26.0, 23.7, 23.5, 23.0, 21.6, 19.2, 18.2, 17.8. EIMS (70 eV) *m/z*: 480 $[M]^+$ (5.5), 434 (3.1), 419 (3.4), 248 (56), 233 (27), 203 (100). HREIMS: Calcd for $C_{31}H_{44}O_4$: 480.3240. Found: 480.3237. Anal. (Table 2).

Methyl 2-Carboxy-3-oxooleana-1,12-dien-28-oate (16). **(1) 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. After 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 $MgSO_4$, 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; $[\alpha]^{26}_D$ +85° (*c* 0.61, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 234 (3.78) nm. IR (KBr): 3436, 2946, 2876, 1756, 1722, 1633 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.43 (1H, s), 5.36 (1H, t, J = 3.5 Hz), 3.64 (3H, s), 2.90 (1H, dd, J = 3.9, 13.7 Hz), 1.24, 1.21, 1.19, 1.13, 0.94, 0.91, 0.85 (each 3H, s). ^{13}C NMR ($CDCl_3$): 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, 17.7. EIMS (70 eV) *m/z*: 510 $[M]^+$ (16), 492 (15), 451 (14), 433 (14), 262 (27), 203 (100). HREIMS: Calcd for $C_{32}H_{46}O_5$: 510.3345. Found: 510.3347. Anal. (Table 2).

(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 $MgSO_4$, and filtered. The filtrate gave **16** as a crystalline solid (470 mg, 97%). It was used for the next reaction without further purification.

Methyl 2-Methoxycarbonyl-3-oxooleana-1,12-dien-28-oate (17). **17** was prepared from **31** 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**): $[\alpha]^{26}_D$ +63° (*c* 0.78, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 230 (3.97) nm. IR (KBr): 2947, 2866, 1727, 1684, 1624 cm^{-1}. ^1H NMR ($CDCl_3$): 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), 1.15, 1.12, 0.94, 0.91, 0.84 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 201.2, 178.4, 166.0, 164.3, 144.5, 129.2, 121.7, 52.7, 52.4, 51.8, 47.0, 45.9, 45.8, 42.3, 41.8, 41.5, 40.3, 39.5, 34.1, 33.3, 32.4, 32.3, 30.9, 28.7, 27.8, 25.9, 23.8, 23.6, 23.2, 21.5, 19.4, 18.0, 17.5. EIMS (70 eV) *m/z*: 524 $[M]^+$ (24), 492 (23), 465 (13), 262 (35), 203 (100). HREIMS: Calcd for $C_{33}H_{48}O_5$: 524.3502. Found: 524.3494. Anal. (Table 2).

2-Methoxycarbonyl-3-oxooleana-1,12-dien-28-oic Acid (18). A solution of **3** (52 mg, 0.10 mmol) in MeOH (5.2 mL) containing concentrated H_2SO_4 (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%): $[\alpha]^{26}_D$ +61° (*c* 0.56, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 230 (3.83) nm. IR (KBr): 3323, 2947, 2866, 1733, 1695, 1622 cm^{-1}. ^1H NMR ($CDCl_3$): 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, 1.15, 1.14, 1.12, 0.94, 0.92, 0.86 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 201.1, 184.2, 166.0, 164.2, 144.2, 129.2, 122.0, 52.7, 52.4, 46.9, 45.9, 45.8, 42.2, 41.5, 41.4, 40.3, 39.5, 34.0, 33.3, 32.5, 32.3, 30.9, 28.7, 27.8, 26.0, 23.7, 23.6, 23.0, 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: Calcd for $C_{32}H_{46}O_5$: 510.3345. Found: 510.3344. Anal. (Table 2).

Methyl 2-Aminocarbonyl-3-oxooleana-1,12-dien-28-oate (19). A solution of **17** (100 mg, 0.19 mmol) in saturated ammonia MeOH (10 mL) was kept at room temperature overnight. The mixture was evaporated in vacuo to give a solid (108 mg). The solid was subjected to flash column chromatography [hexanes−EtOAc (1.5:1)] to give **19** as an amorphous solid (94 mg, 96%): $[\alpha]^{26}_D$ +77° (*c* 0.60, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 236 (3.91) nm. IR (KBr): 3413, 2943, 2866, 1727, 1689 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.45 (1H, brs), 8.27 (1H, s), 5.72 (1H, brs), 5.37 (1H, t, J = 3.4 Hz), 3.64 (3H, s), 2.90 (1H, dd, J = 4.2, 13.9 Hz), 1.17, 1.16, 1.15, 1.14, 0.94, 0.92, 0.84 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 205.8, 178.4, 169.0, 165.8, 144.3, 121.8, 52.2, 51.8, 47.0, 46.0, 45.7, 42.3, 41.8, 41.2, 40.4, 39.6, 34.1, 33.3, 32.5, 32.3, 30.9, 29.1, 27.8, 26.0, 23.8, 23.6, 23.2, 21.9, 19.4, 18.6, 17.6. EIMS (70 eV) *m/z*: 509 $[M]^+$ (34), 492 (23), 450 (100), 262 (19), 203 (56). HREIMS: Calcd for $C_{32}H_{47}O_4N$: 509.3505. Found: 509.3500. Anal. (Table 2).

Methyl 1α,2α-Epoxy-3-oxoolean-12-en-28-oate (22).[9] To a solution of **B-13** (223 mg, 0.48 mmol) in 2 N aqueous NaOH solution (1.7 mL) and THF (11 mL) was added a solution of

30% H_2O_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 $NaHSO_3$ and 5% aqueous NaOH solutions, successively. After removal of THF and MeOH, the resultant mixture was acidified with 6 M aqueous HCl solution. The acidic layer was extracted with CH_2Cl_2 three times. The extract was worked up according to the standard method to give **22** as a crystalline solid (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; $[\alpha]^{26}_D$ +157° (*c* 0.80, $CHCl_3$). IR (KBr): 2943, 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, 1.01, 0.97, 0.94, 0.92, 0.80 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 213.0, 178.4, 144.5, 121.8, 64.1, 57.1, 51.8, 47.0, 46.3, 45.9, 45.0, 42.1, 41.7, 40.8, 39.7, 38.8, 34.1, 33.3, 32.5, 32.3, 30.9, 28.2, 28.0, 26.0, 24.0, 23.8, 23.3, 21.1, 19.1, 17.4, 15.1. EIMS (70 eV) *m/z*: 482 $[M]^+$ (7.7), 422 (13), 262 (31), 249 (11), 203 (100). HREIMS: Calcd for $C_{31}H_{46}O_4$: 482.3396. Found: 482.3391.

Methyl 2-Methoxy-3-oxooleana-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 mixture was extracted with a mixture of CH_2Cl_2 and Et_2O (1:2) three times. The extract was worked up according to the standard method to give a solid (320 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (4:1)] to afford **22** (31 mg) and **23** as an amorphous solid (270 mg; 87%, 98% based on recovered **22**): UV (EtOH) λ_{max} (log ϵ): 266 (3.77) nm. IR (KBr): 2946, 2866, 1727, 1682, 1621 cm^{-1}. ^1H NMR ($CDCl_3$): δ 5.96 (1H, s), 5.36 (1H, t, J = 3.5 Hz), 3.64, 3.55 (each 3H, s), 2.90 (1H, dd, J = 4.1, 13.7 Hz), 1.17 (6H, s), 1.16, 1.13, 0.93, 0.90, 0.81 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 200.1, 178.4, 149.0, 144.6, 126.3, 121.9, 54.9, 53.2, 51.8, 47.0, 45.9, 45.4, 43.3, 42.3, 41.7, 40.2, 38.4, 34.0, 33.3, 32.6, 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*: 496 $[M]^+$ (80), 436 (21), 328 (19), 262 (36), 203 (100). HREIMS: Calcd for $C_{32}H_{48}O_4$: 496.3553. Found: 496.3544.

Methyl 2-Hydroxy-3-oxooleana-1,12-dien-28-oate (24). A suspension of **23** (100 mg, 0.20 mmol) in 3 M aqueous HCl solution (3 mL) and AcOH (3 mL) was heated under reflux for 5 h. The mixture was neutralized with saturated aqueous Na_2CO_3 solution. The mixture was extracted with CH_2Cl_2 three times. The extract was worked up according to the standard method to give a solid (90 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (5:1)] to afford **24** as an amorphous solid (78 mg, 81%): UV (EtOH) λ_{max} (log ϵ): 272 (3.63) nm. IR (KBr): 3426, 2939, 2870, 1725, 1667, 1648 cm^{-1}. ^1H NMR ($CDCl_3$): δ 6.35 (1H, s), 5.93 (1H, brs), 5.34 (1H, t, J = 3.5 Hz), 3.63 (3H, s), 2.89 (1H, dd, J = 4.0, 13.7 Hz), 1.22 (6H, s), 1.13, 1.12, 0.94, 0.91, 0.80 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 201.3, 178.4, 144.4, 143.9, 128.4, 122.0, 54.1, 51.8, 47.0, 45.9, 44.1, 43.3, 42.2, 41.6, 40.2, 38.7, 34.1, 33.3, 32.7, 32.5, 30.9, 27.8, 27.4, 26.1, 23.8, 23.6, 23.2, 22.0, 19.8, 18.9, 17.5. EIMS (70 eV) *m/z*: 482 $[M]^+$ (26), 446 (68), 422 (25), 262 (35), 203 (100). HREIMS: Calcd for $C_{31}H_{46}O_4$: 482.3396. Found: 482.3387.

Methyl 2-Hydroxymethylene-3-oxoolean-12-en-28-oate (25).[19] To a stirred mixture of **B-3** (1084 mg, 2.31 mmol) and ethyl formate (97%) (707 mg, 9.26 mmol) in benzene (12 mL) was added NaOMe (501 mg, 9.27 mmol). The mixture was stirred at room temperature for 1 h. After the mixture was washed with 5% aqueous HCl solution twice, it was worked up according to the standard method to give **25** as an amorphous solid (1095 mg, 95%). 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 199–201 °C. UV (EtOH) λ_{max} (log ϵ): 296 (3.94) nm. IR (KBr): 3426, 2943, 2862, 1725, 1637, 1588 cm^{-1}. ^1H NMR ($CDCl_3$): δ 14.92 (1H, d, J = 3.1 Hz), 8.58 (1H, d, J = 3.1 Hz), 5.35 (1H, t, J = 3.7 Hz), 3.64 (3H, s), 2.90 (1H, dd, J = 4.2, 13.6 Hz), 2.29 (1H, d, J = 14.4 Hz), 1.92 (1H, d, J = 14.4 Hz), 1.20, 1.16, 1.12, 0.94 (each 3H, s), 0.91 (6H, s), 0.80 (3H, s). ^{13}C NMR ($CDCl_3$): δ 190.9, 188.6, 178.4, 144.0, 122.3, 106.0, 52.3, 51.8, 47.0, 46.0, 45.9, 42.0, 41.6, 40.3, 39.4, 39.3, 36.5, 34.1, 33.3, 32.5, 32.1, 30.9, 28.6, 27.9, 25.9, 23.8, 23.6, 23.3, 21.1, 19.7, 16.8, 14.7. EIMS (70 eV) *m/z*: 496 $[M]^+$ (4.4), 437 (23), 262 (38), 233 (20), 203 (100). HREIMS: Calcd for $C_{32}H_{48}O_4$: 496.3553. Found: 496.3550.

Methyl Isoxazolo[4,5-*b*]olean-12-en-28-oate (26). A mixture of **25** (994 mg, 2.0 mmol), hydroxylamine hydrochloride (1391 mg, 20 mmol) in water (1.8 mL) and EtOH (48 mL) was heated under reflux for 1 h. After EtOH was removed in vacuo, EtOAc was added to the resultant mixture. The EtOAc layer was washed with water (three times) and saturated aqueous NaCl solution (three times), dried over $MgSO_4$, and filtered. The filtrate gave a solid (1086 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) λ_{max} (log ϵ): 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, 0.88, 0.79 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 178.4, 173.2, 150.4, 144.0, 122.3, 109.0, 53.7, 51.7, 46.9, 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, 21.6, 19.0, 16.7, 15.4. EIMS (70 eV) *m/z*: 493 $[M]^+$ (11), 434 (18), 262 (28), 249 (16), 203 (100). HREIMS: Calcd for $C_{32}H_{47}O_3N$: 493.3556. Found: 493.3556.

Methyl 2-Cyano-3-oxoolean-12-en-28-oate (27). To a stirred solution of **26** (887 mg, 1.80 mmol) in Et_2O (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_2Cl_2 and Et_2O (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 (879 mg, 99%). 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) λ_{max} (log ϵ): 238 (3.88) nm. IR (KBr): 2946, 2870, 2202, 1724, 1633 cm^{-1}. ^1H NMR of major tautomer **27a** ($CDCl_3$): δ 6.15 (1H, brs), 5.31 (1H, t, J = 3.6 Hz), 3.63 (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 $C_{32}H_{47}O_3N$: 493.3556. Found: 493.3548.

Methyl 2-Hydroxymethylene-3-oxours-12-en-28-oate (28).[23] **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) λ_{max} (log ϵ): 294 (3.86) nm. IR (KBr): 3426, 2947, 2921, 2866, 1727, 1637, 1590 cm^{-1}. ^1H NMR ($CDCl_3$): δ 14.91 (1H, brs), 8.57 (1H, s), 5.31 (1H, t, J = 3.7 Hz), 3.62 (3H, s), 2.31 (1H, d, J = 14.4 Hz), 2.27 (1H, d, J = 12.5 Hz), 1.95 (1H, d, J = 14.4 Hz), 1.19, 1.12, 1.10 (each 3H, s), 0.96 (3H, d, J = 6.0 Hz), 0.92 (3H, s), 0.87 (3H, d, J = 6.6 Hz), 0.81 (3H, s). ^{13}C NMR ($CDCl_3$): δ 191.0, 188.5, 178.2, 138.4, 125.6, 106.0, 53.2, 52.3, 51.7, 48.4, 45.7, 42.4, 40.3, 39.7, 39.5, 39.3, 39.1, 36.8, 36.4, 32.4, 30.9, 28.7, 28.2, 24.4, 23.7, 23.6, 21.4, 21.1, 19.7, 17.2, 17.0, 14.8. EIMS (70 eV) *m/z*: 496 $[M]^+$ (11), 437 (15), 262 (80), 233 (41), 203 (100). HREIMS: Calcd for $C_{32}H_{48}O_4$: 496.3553. Found: 496.3547.

Methyl Isoxazolo[4,5-*b*]urs-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) λ_{max} (log ϵ): 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,

1.10 (each 3H, s), 0.96 (3H, d, J = 6.3 Hz), 0.90 (3H, s), 0.88 (3H, d, J = 6.3 Hz), 0.81 (3H, s). ^{13}C NMR ($CDCl_3$): δ 178.2, 173.2, 150.4, 138.4, 125.5, 109.1, 53.7, 53.2, 51.7, 48.3, 46.3, 42.3, 39.7, 39.3, 39.1, 38.8, 36.8, 35.8, 34.9, 32.4, 30.9, 29.1, 28.3, 24.4, 23.7, 23.5, 21.6, 21.4, 19.0, 17.2, 16.9, 15.6. EIMS (70 eV) *m/z*: 493 $[M]^+$ (9.1), 434 (20), 262 (65), 249 (33), 203 (100). HREIMS: Calcd for $C_{32}H_{47}O_3N$: 493.3556. Found: 493.3547.

Methyl 2-Cyano-3-oxours-12-en-28-oate (30). **30** was prepared from **29** according to the same method as for **27** 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 (5:1)] as an amorphous solid: UV (EtOH) λ_{max} (log ϵ): 238 (3.93) nm. IR (KBr): 2947, 2870, 2203, 1724, 1631 cm^{-1}. ^{1}H NMR of major tautomer **30a** ($CDCl_3$): δ 5.92 (1H, brs), 5.28 (1H, t, J = 3.5 Hz), 3.61 (3H, s), 2.26 (1H, d, J = 11.0 Hz), 2.13 (1H, d, J = 15.0 Hz), 1.16, 1.13, 1.08, 1.07, 0.96 (each 3H, s), 0.95, 0.77 (each 3H, d, J = 6.3 Hz). EIMS (70 eV) *m/z*: 493 $[M]^+$ (6.8), 434 (19), 262 (62), 249 (44), 203 (100). HREIMS: Calcd for $C_{32}H_{47}O_3N$: 493.3556. Found: 493.3558.

Methyl 3-Hydroxy-2-methoxycarbonyloleana-2,12-dien-28-oate (31). A mixture of **B-3** (2.0 g, 4.27 mmol) and 1.8 M DMF solution of methoxymagnesium methyl carbonate (Stiles' reagent) (20 mL, 36 mmol) was heated under reflux for 2 h while a slow stream of N_2 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 $MgSO_4$, 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 excessive 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 [hexanes–EtOAc (7:1)] to give **B-3** (330 mg) and **31** as crystals (1.66 g; 74%, 89% based on recovered **B-3**): mp 160–162 °C. UV (EtOH) λ_{max} (log ϵ): 262 (4.01) nm. IR (KBr): 2948, 2858, 1737, 1660, 1615 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 12.51 (1H, s), 5.33 (1H, t, 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). ^{13}C NMR ($CDCl_3$): 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, 23.3, 20.4, 19.8, 16.8, 15.1. EIMS (70 eV) *m/z*: 526 $[M]^+$ (0.6), 494 (5.6), 479 (2.5), 466 (1.6), 435 (13), 262 (28), 203 (100). HREIMS: Calcd for $C_{33}H_{50}O_5$: 526.3658. Found: 526.3658.

2-Hydroxymethylene-3-oxoolean-12-en-28-oic Acid (32). To a stirred mixture of oleanonic acid (**B-1**)[10] (540 mg, 1.19 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 at 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 to give a solid (600 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (5:1) followed by hexanes–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) λ_{max} (log ϵ): 292 (3.93) nm. IR (KBr): 2946, 2654, 1732, 1694, 1644, 1587 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 14.91 (1H, brs), 8.59 (1H, s), 5.34 (1H, t, J = 3.5 Hz), 2.86 (1H, dd, J = 4.5, 13.9 Hz), 2.29 (1H, d, J = 14.6 Hz), 1.93 (1H, d, J = 14.6 Hz), 1.19, 1.16, 1.10, 0.94, 0.92, 0.91, 0.82 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 190.7, 188.8, 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 (3.6), 248 (77), 203 (100). HREIMS: Calcd for $C_{31}H_{46}O_4$: 482.3396. Found: 482.3392.

Evaluation Methods. 1. Reagents. Recombinant mouse IFN-γ (LPS content, <10 pg/mL) was purchased from Genzyme (Cambridge, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Inhibitory test compounds were dissolved in DMSO before addition to cell cultures; final concentrations of DMSO were 0.1% or less. Controls with DMSO alone were run in all cases.

2. Cell Culture. To obtain primary macrophages, female CD-1 mice, 5–10 weeks of age (Charles River Breeding Laboratories, Wilmington, MA), were injected intraperitoneally with 2 mL of 4% thioglycollate broth (Difco Laboratories, Detroit, MI). Four days after injection, peritoneal macrophages were harvested and processed according to Nathan's procedure.[7b] Cells were seeded in 96-well plates at 2 × 10^5 cells/well and incubated for 48 h with 20 ng/mL IFN-γ in the presence or absence of inhibitory test compounds.

3. Measurement of NO Production in Mouse Macrophages. Nitrite accumulation was used as an indicator of NO production in the medium and was assayed by the Griess reaction.[7a] Griess reagent (100 μL) was added to 100 μL of each supernatant from IFN-γ or inhibitory test compound-treated cells in triplicate. The protein determination was performed by Bradford protein assay. The plates were read at 550 nm against a standard curve of sodium nitrite.

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