JOURNAL OF PHARMACEUTICAL SCIENCES

MAY 1979
VOLUME 68 NUMBER 5

RESEARCH ARTICLES

Anti-Inflammatory Activity of Sesquiterpene Lactones and Related Compounds

I. H. HALL *x, K. H. LEE *, C. O. STARNES *, Y. SUMIDA *, R. Y. WU *, T. G. WADDELL ‡, J. W. COCHRAN ‡, and K. G. GERHART ‡

Received August 11, 1978, from the *Division of Medicinal Chemistry, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27514, and the ‡Department of Chemistry, University of Tennessee at Chattanooga, Chattanooga, TN 37401. Accepted for publication October 23, 1978.

Abstract □ Some sesquiterpene lactones and related compounds were tested for anti-inflammatory activity in rodents. In the edema-induced carrageenan inflammation screen, the α-methylene-γ-lactone moiety of the sesquiterpene lactones was required for inhibitory activity. The 6-hydroxy group of helenalin also was required for potency. In the tenulin series, the 2,3-epoxy derivatives were marginally active. The same structure was required for inhibition of the writhing reflex. In the chronic adjuvant arthritic screen, compounds containing the α-methylene-γ-lactone moiety, the β-unsubstituted cyclopentenone ring, and the α-epoxy cyclopentenone system afforded significant inhibition at 2.5 mg/kg/day. The sesquiterpene lactones were marginally effective against induced pleurisy. The delayed hypersensitivity was suppressed by these agents whereas immunoglobulin synthesis was slightly stimulated. No deleterious side effects were observed with these agents from the limited tests performed.

Keyphrases □ Lactones, sesquiterpene—anti-inflammatory activity, structure-activity relationships, edema, arthritis, pleurisy, immunoglobulin synthesis, rats □ Anti-inflammatory agents—sesquiterpene lactones, structure-activity relationships, edema, arthritis, pleurisy, immunoglobulin synthesis, rats □ Structure-activity relationships—sesquiterpene lactones, anti-inflammatory activity

The anti-inflammatory drugs ethacrynic acid and *N*-ethylmaleimide have been shown to bind sulfhydryl groups (1). Cysteine, a sulfhydryl-donating compound, reversed the anti-inflammatory activity of these agents, indicating that sulfhydryl groups participate in the inflammatory process. In addition, the sulfhydryl-binding properties of drugs, *e.g.*, salicylates and indomethacin, were related to their anti-inflammatory activity (1). Other researchers (2–5) showed that sulfhydryl and disulfide interactions are altered in rat adjuvant arthritis, human connective tissue disease, and anti-inflammatory drug therapy.

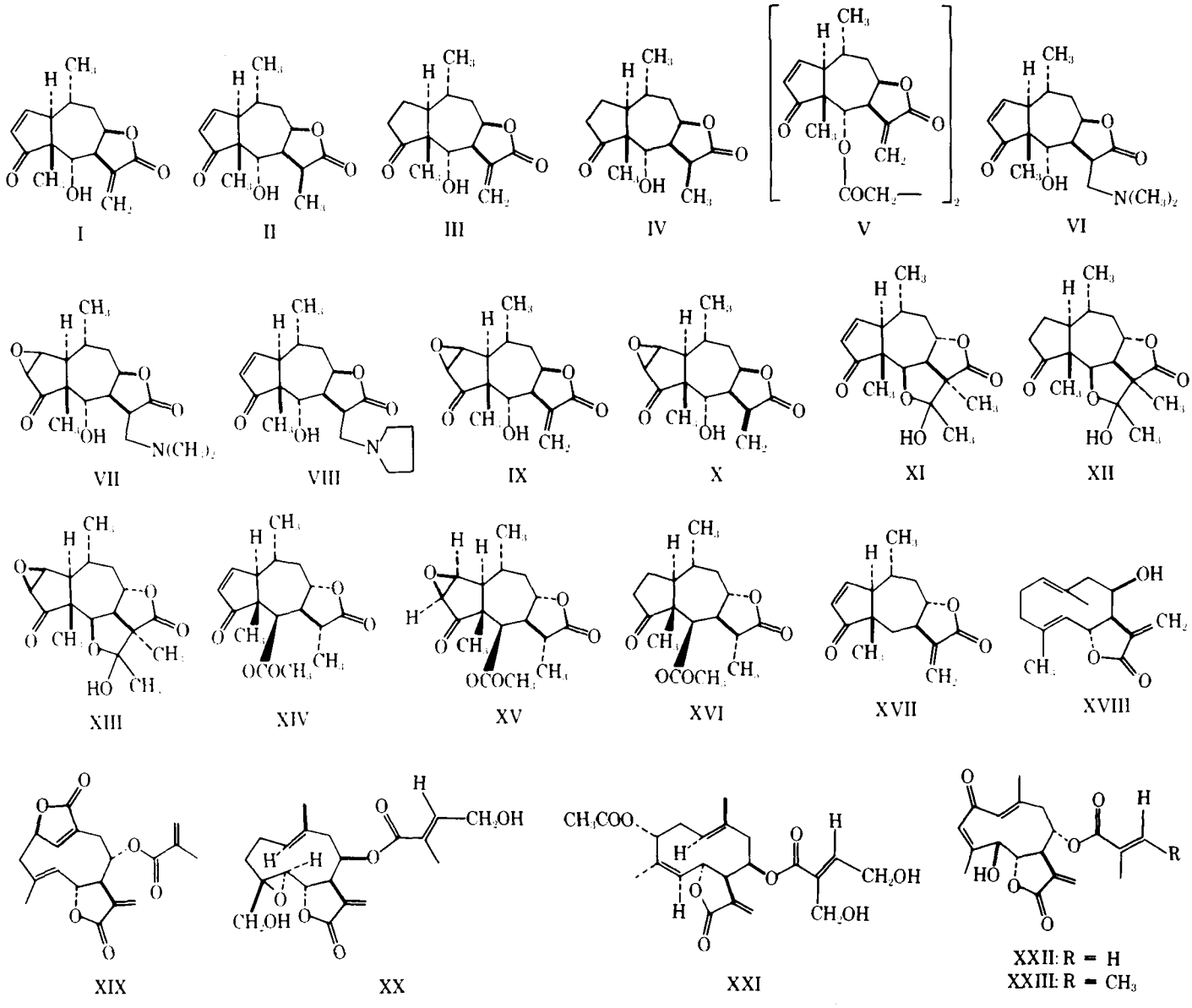
Mechanism of action studies with sesquiterpene lactones showed that the α-methylene-γ-lactone and cyclopentenone moieties undergo Michael-type addition with L-cysteine, glutathionine, and a number of sulfhydryl-bearing cell enzymes (6–10). Metabolic studies with sesquiterpene lactones in mammalian cancer cells demonstrated that these agents are potent inhibitors of the lysosomal enzymes, oxidative phosphorylation, and protein synthesis and that they significantly elevate cellular cyclic AMP levels (7, 11, 12). A number of clinically useful anti-inflammatory agents have similar effects on cell metabolism. Moreover, certain sesquiterpene lactone-producing plants, such as *Eupatorium formosanum*, have been used as anti-inflammatory herbal remedies as well as antipyretic drugs (13). Thus, it was decided to test these agents for anti-inflammatory activity.

EXPERIMENTAL

Test Compounds—Twenty-nine sesquiterpene lactones and related compounds were studied. Some were natural products isolated from plant species by literature techniques (14): helenalin (I) from *Balduina angustifolia* (15), tenulin (XI) (6) and aromaticin (XVII) (6, 16) from *Helenium amarum*, eupatolide (XVIII) from *Eupatorium formosanum* (17), deoxyelephantopin (XIX) from *Elephantopus carolinianus* (18), eupahyssopin (XX) from *Eupatorium hyssopifolium* (19), eupaformosanin (XXI) from *Eupatorium formosanum* (20), and molephantin (XXII) (21), molephantinin (XXIII) (22), and phantomolin (XXIV) (23) from *Elephantopus mollis*.

Plenolin (II) was obtained by hydrogenation of helenalin to give a compound identical to the naturally occurring product (24). 2,3-Dihydrohelenalin (III), 2,3,11,13-tetrahydrohelenalin (IV), 2,3-epoxyhelenalin (IX), and 2,3-epoxyplenolin (X) were chemically modified from helenalin (24), as was dimeric helenalin (V) (12), helenalin dimethylamine adduct (VI) (25), 2,3-epoxyhelenalin dimethylamine adduct (VII) (26), and helenalin pyrrolidine adduct (VIII) (25). 2,3-Dihydrotenulin (XII), 2,3-epoxytenulin (XIII), isotenulin (XIV), 2,3-epoxyisotenulin (XV), and dihydroisotenulin (XVI) were prepared by a literature method (27). Thymine α-methylene-γ-lactone (XXV) and its corresponding trimethoxybenzoyl ester (XXVI) were prepared by literature techniques (28), as was α-methylene-β,β-dicarbethoxy-γ-butyrolactone (XXVIII) (29). α-Methylene-γ-lactone (XXVII) was prepared by the published method (30).

0022-3549/79/0500-0537$01.00/0



The following were purchased from commercial sources: 2-cyclopentenone[1] (XXIX), indomethacin[2] (XXX), phenylbutazone[3] (XXI), diethylstilbestrol[4], and ethynyl estradiol[4].

Anti-Inflammatory Screen—Sprague-Dawley male rats, ~160 g, were administered test drugs at 2.5 mg/kg ip in 0.05% polysorbate 80, 3 hr and 30 min prior to the injection of 0.2 ml of 1% carrageenan in 0.9% saline into the plantar surface of the right hindfoot. Isotonic saline was injected into the left hindfoot, which served as a baseline. After 3 hr, both feet were excised at the tibiotarsal (ankle) joint according to a modified method (31, 32), resulting in an average 0.655-g foot weight increase for controls.

Antipyretic Screen—Sprague-Dawley rats, ~200 g, were administered 2 ml of 44% baker's yeast solution subcutaneously (31) 18 hr prior to the injection of drugs at 2.5 or 5 mg/kg ip, resulting in an average body temperature elevation of 3.46 °F. Rats were also administered 0.25 mg of *Mycobacterium butyricum*[5] (33) subcutaneously prior to drug administration. Rectal temperatures were taken at 0, 2, 4, and 6 hr.

Analgesic Screen—The hot-plate-tail flick method (34) was employed with CF_1 male mice, ~30 g, who were administered test drugs at 20 mg/kg ip 5 min prior to the analgesic test. Normal reaction time was 9.3 sec. Morphine, 10 mg/kg, increased the reaction time to over 1 min.

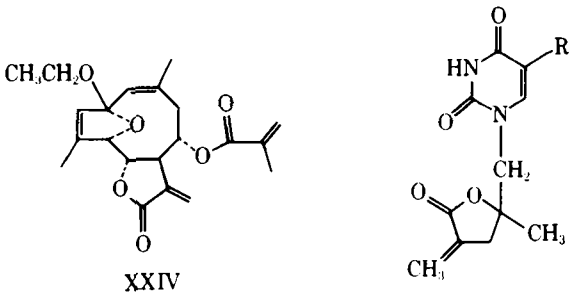
The writhing reflex was also utilized. Mice were administered test drugs at 20 mg/kg ip 20 min (35) prior to the administration of 0.5 ml of 0.6% acetic acid (36). After 5 min, the number of stretches, characterized by repeated contractures of the abdominal musculature accompanied by extension of the hindlimbs, was counted for the next 10 min. The control mice averaged 78 stretch reflexes/10 min.

Chronic Adjuvant Arthritic Screen—Male Sprague-Dawley rats, ~160 g, were injected at the base of the tail with 0.2 ml of 100 mg of dried *M. butyricum* solution and 40 mg of digitonin in 20 ml of light mineral oil (37). Test drugs were administered on Day 3 through Day 30 at 2.5 mg/kg/day ip. Animals were sacrificed on Day 21, and the feet were excised and weighed. Control animals achieved a 0.830-g average net weight gain.

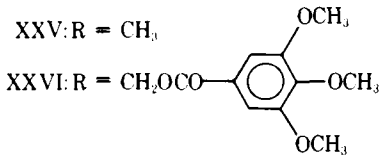
Pleurisy Anti-Inflammatory Screen—Sprague-Dawley rats were administered test drugs at 2.5 mg/kg ip 1 hr before and 3 hr after injection into the pleural cavity of 0.05 ml of 0.316% Evan's Blue and carrageenan (38). Six hours later, the rats were sacrificed, and the fluid was collected from the pleural cavity. Control rats produced an average 2.5 ml of fluid.

Passive Cutaneous Anaphylaxis Screen (Antiallergy)—Egg albumin (10 mg/kg) with 1.5 ml of *Bordetella pertussis* vaccine (8 protective units/ml) was used to induce homocytotropic antibody production in Sprague-Dawley male rats (39). Blood was collected by tail vein bleeding, and the serum was separated and frozen. New rats were shaved,

[1] Aldrich.
[2] Merck Sharp and Dohme
[3] Geigy.
[4] Sigma.
[5] Difco.

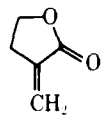


XXIV

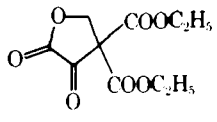


XXV: R = CH_3

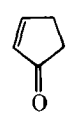
XXVI: R = CH_2OCO



XXVII



XXVIII



XXIX

and 0.1 ml of serum antibodies (diluted 1:2 with isotonic phosphate saline buffer) was injected intradermally at three sites. Forty-eight hours later, the rats were given test drugs at 2.5 mg/kg ip. The rats were challenged 45 min later intravenously with 0.3 ml of 1% egg albumin in pH 7.2 isotonic saline and 0.25% solution of Evan's Blue. The animals were sacrificed 30 min later, and the blue wheals were measured at the serum antibody injection sites (40, 41). Control animals gave an average response of 22.4 mm (diameter).

Histamine-Induced Anaphylaxis or Hypersensitivity—Female HLA-SW mice, ~35 g, received 0.15 ml of reconstituted *B. pertussis* vaccine in 0.9% saline intravenously 4 days prior to the administration of test drugs at 20 mg/kg. Histamine base (1 mg) was injected intraperitoneally 30 min later (42). Histamine anaphylaxis resulted in 88% mortality for the control animals.

Antibody Production—CF_1 male mice, ~30 g, were administered sheep red blood cells as an antigen intraperitoneally. On Days 2–4, test compounds were administered at 5 and 10 mg/kg/day. On Day 5, the spleens were removed and the lymphocytes were harvested. Lymphocytes were incubated with sheep red blood cells and guinea pig complement on 1% agarose slides according to the Jerne plaque microassay technique (43), and the plaques were counted. The control animals produced 275 plaques/10^6 lymphocytes. Animals were treated similarly with chicken red blood cells, blood was collected by tail vein bleeding on Day 5, and the serum was separated. Chromium 51 was incubated with, and taken up by, chicken nucleated red blood cells (44). Labeled cells were then incubated with serum antibodies from the mice. Normal mouse serum resulted in an average 22% release of chromium 51 from the chicken red blood cells and a maximum 95% release with 3% acetic acid.

Mitogenic Test—CF_1 male mice were given helenalin (I) at 5 mg/kg 2 hr prior to sacrifice or on Days 1–3 and 10 μCi of [^{14}C-*methyl*]thymidine (49.2 mCi/mmole) was injected intraperitoneally on Day 4 (6). After 1 hr, the mice were sacrificed, the spleen was excised, and lymphocytes were prepared. The ^{14}C-thymidine incorporation into lymphocyte deoxyribonucleic acid was determined. In an analogous manner, spleen lymphocytes from 2 hr-treated mice were analyzed by radioimmunoassay for cyclic AMP.

Delayed Hypersensitivity Screen—Sprague–Dawley rats, ~160 g, were administered methylated bovine serum albumin as antigen subcutaneously on Days 0 and 7 (45–47). Test drugs were administered daily at 2.5 mg/kg ip. On Day 21, the rats were challenged with antigen injected into the right plantar footpad. The left hindfoot acted as the control. Animals were sacrificed 24 hr later, and the paw weight was obtained.

In the second experiment, rats were administered chicken egg albumin as antigen on Days 0 and 7. However, dosing with test compounds at 2.5 mg/kg only occurred on Days 18–20. The methylated bovine serum albumin resulted in a net increase of 119 mg whereas the egg albumin resulted in a net increase of only 46 mg upon challenge with the respective antigens.

Antifertility and Teratogenic Effects of Drugs—CF_1 female mice, ~30 g, were administered test compounds at 6 mg/kg/day for 28 days. On Day 10, they were exposed to males (two females per male) for the remainder of the experiment. On Days 27–30, pregnant females were sacrificed and the number of viable fetuses and intrauterine deaths were tabulated (48, 49). Fetuses were examined for teratogenic effects by literature methods (50). Uterotropic effects were determined in female rats, ~50 g, by published methods (51).

Adverse Side Effects—Central nervous system (CNS) effects such as drowsiness, convulsions, hyperexcitability, and reflex activity loss were determined by observation of all animal species used in the screens at various doses. Ulcerogenic (estrogenic side effects) tests were carried out in Sprague–Dawley rats, ~160 g, who were administered test drugs at 2.5 mg/kg/day for 3 weeks. After food had been removed for 18 hr, the last dose was administered on Day 21. Four hours later, the rats were sacrificed and the gastric and duodenal linings were examined for bleeding and/or ulcers (52).

In an analogous experiment, animals were bled on Day 21 by tail vein and the red and white blood cells were counted in a hemocytometer and expressed as numbers of cells × $10^6/cm^3$. Hematocrits were also obtained (53). *In vitro* effects of test drugs on aerobic respiration of rat liver homogenates were also determined at 0.75 μmole as previously outlined (12).

RESULTS

In the edema-induced inflammation test, at 2.5 mg/kg twice, III, IX, X, XIII, XIX, XX, XXIV, and XXV caused at least 40% inhibition, while I caused 72% inhibition (Table I). At 2.5 or 5 mg/kg, the sesquiterpene

Table I—Anti-Inflammatory Activity of Sesquiterpene Lactones and Germacranolides

Compound (n = 8)	Percent Control: Anti-Inflammatory Screen, Sprague–Dawley Rats, 2.5 mg/kg twice, $\bar{x} \pm SD$	Percent Control: Writhing Reflex, CF_1 Mice, 20 mg/kg, $\bar{x} \pm SD$
I Helenalin	28 ± 15[a]	7 ± 3[a]
II Plenolin	89 ± 16	83 ± 8[b]
III 2,3-Dihydrohelenalin	51 ± 19[a]	50 ± 11[a]
IV 2,3,11,13-Tetrahydrohelenalin	96 ± 15	93 ± 13
V Dimeric helenalin	68 ± 11[a]	64 ± 5[a]
VI Helenalin dimethylamine adduct	74 ± 16[b]	79 ± 8[a]
VII 2,3-Epoxyhelenalin dimethylamine adduct	75 ± 13[b]	86 ± 7[c]
VIII Helenalin pyrrolidine adduct	72 ± 16[b]	73 ± 6[a]
IX 2,3-Epoxyhelenalin	54 ± 3[a]	47 ± 5[a]
X 2,3-Epoxyplenolin	49 ± 7	77 ± 12
XI Tenulin	84 ± 18	95 ± 7
XII 2,3-Dihydrotenulin	93 ± 8	81 ± 8[a]
XIII 2,3-Epoxytenulin	57 ± 19[a]	28 ± 6[a]
XIV Isotenulin	84 ± 17	87 ± 12
XV 2,3-Epoxyisotenulin	69 ± 9[a]	64 ± 9[a]
XVI 2,3-Dihydroisotenulin	92 ± 15	101 ± 16
XVII Aromaticin	65 ± 6[a]	77 ± 11[a]
XVIII Eupatolide	70 ± 12[a]	64 ± 9[a]
XIX Deoxyelephantopin	49 ± 11[a]	47 ± 7[a]
XX Eupahyssopin	43 ± 6[a]	32 ± 5[a]
XXI Eupaformosanin	69 ± 8[a]	62 ± 5[a]
XXII Molephantin	67 ± 8[a]	46 ± 8[a]
XXIII Molephantinin	68 ± 17[b]	53 ± 6[a]
XXIV Phantomolin	54 ± 19[a]	53 ± 9[a]
XXV Thymine α-methylene-γ-lactone	57 ± 7[a]	59 ± 7[a]
XXVI Trimethoxybenzoyl ester of thymine lactone	83 ± 9[c]	69 ± 7[a]
XXVII α-Methylene-γ-lactone	64 ± 13[a]	78 ± 5[a]
XXVIII α-Methylene-β,β-dicarbethoxy-γ-butyrolactone	75 ± 11[b]	—
XXIX 2-Cyclopentenone	77 ± 13[b]	79 ± 8[a]
XXX Indomethacin (10 mg twice)	22 ± 8[a]	43 ± 7[a]
XXXI Phenylbutazone (50 mg twice)	53 ± 13[a]	—
XXXII 0.05% Polysorbate 80	100 ± 12 (0.655 g)	100 ± 9

[a] $p = 0.001$. [b] $p = 0.005$. [c] $p = 0.010$.

Table II—Antiarthritis Screen Test Drugs in Sprague-Dawley Rats

Compound (2.5 mg/kg for 3 weeks) (n = 6)	Percent Control
0.05% Polysorbate 80	100 (0.830 g)
I	23
VI	59
IX	54
XI	47
XII	92
XIII	33
XIV	47
XV	37
XVIII	70
XIX	31
XX	34
XXII	40
XXVII	17
XXX (10 mg/kg/day)	55

Table III—Antipleurisy Activity of Sesquiterpene Lactones in Rats

Compound (2.5 mg/kg/day) (n = 6)	Fluid Increase, ml	Percent Control
0.05% Polysorbate 80	2.50	—
I	2.02	81
XI	1.58	63
XIX	1.50	60
XX	1.60	64

Table IV—Passive Cutaneous Anaphylaxis Test of Antiallergy Effects in Rats

Compound (2.5 mg/kg/day) (n = 8)	Antigen Challenge, Average Diameter, cm	Percent Control
0.05% Polysorbate 80	2.24	—
I	1.67	74
XI	1.65	73
XIX	2.04	91
XX	1.78	79

lactones afforded no activity against induced hyperpyrexia in rats. In the hot-plate test, which more closely relates to narcotic analgesic activity, these analogs were inactive at 20 mg/kg. The writhing reflex text (Table I), which is more closely related to inflammation pain, showed that III, IX, XIII, XIX, XX, and XXII–XXV caused greater than 40% inhibition; I caused 93% reflex inhibition; XIII caused 72% inhibition; and XX caused 68% inhibition. Compounds I, XII, XV, XIX, XX, and XXVII at 2.5 mg/kg/day caused greater than 60% inhibition of induced adjuvant arthritis in rats (Table II). Compounds VI, IX, XI, XIV, and XXII showed greater than 40% inhibition.

In the antipleurisy screen, I, XI, XIX, and XX caused 20–40% inhibition of pleural edema at 2.5 mg/kg twice (Table III). These same compounds offered only minimal protection in the passive cutaneous anaphylaxis text (antiallergy) in rats (Table IV) and offered no protection against histamine-induced anaphylaxis in mice.

The sesquiterpene lactones (I, XI, and XXIX) caused a slight, but significant, increase in serum immunoglobulin production in mice (Table V). Chromium 51 release from nucleated chicken red blood cells also demonstrated the increase in antibody synthesis after I and XI administration. Sesquiterpene lactones suppressed T cell delayed hypersensitivity with both methylated bovine albumin and chicken egg albumin (Table VI). Short-term dosing prior to antigen challenge caused the most suppression of T cell function compared to continued dosing, although different antigens were used. Compound I caused 81% inhibition, XI caused 32% inhibition, XIX caused 68% inhibition, and XX caused 47% inhibition. Compound I demonstrated 46% inhibition of delayed hypersensitivity to methylated bovine serum albumin. Helenalin (I) was judged to have mitogenic effects on spleen lymphocytes since 5 mg/kg/day resulted in a 342% increase in ^{14}C-thymine incorporation into DNA after 2 hr and a 445% increase after 3 days. Cyclic AMP levels were reduced 51% after 2 hr.

The sesquiterpene lactones had no effects on fertility in mice at 6 mg/kg/day (Table VII). The viable fetuses and intrauterine deaths were

Table V—Jerne Plaque: Immunosuppressant/Stimulation Activity of Sesquiterpene Lactones in CF_1 Male Mice Administered 0.2 ml of Sheep Erythrocytes

Compound[a]	Jerne Plaque, % control	Chromium 51, % released
0.05% Polysorbate 80	100 ± 11[b]	22.2[c]
I (0.125 mg/day)	138 ± 11	65.9
XI (0.25 mg/day)	123 ± 11	76.3
XIX (0.25 mg/day)	104 ± 9	—
XXIX (0.25 mg/day)	140 ± 12	—
Melphalan (0.1 mg/day)	51 ± 8	—

[a] Intraperitoneal administration on Days 2–4. [b] 275 plaques/10^6 spleen cells. [c] Maximum release 95%.

Table VI—Effects of Sesquiterpene Lactones on Delayed Hypersensitivity in Rats

Compound (2.5 mg/kg/day) (n = 6)	Egg Albumin		Methylated Bovine Serum Albumin	
	mg Increase	% Control	mg Increase	% Control
0.05% Polysorbate 80	46.1	—	119	—
I	8.6	18.6	64	54
XI	31.2	68.0	112	94
XIX	14.9	32.0	86	72
XX	24.5	53.1	93	78

Table VII—Effects of Sesquiterpene Lactones on Reproduction

Compound (n = 8)	Fertility in CF_1 Female Mice	
	% Pregnant	Viable Fetuses per Litter
I (6 mg/kg/day)	100	11 ± 2
XI (6 mg/kg/day)	100	9 ± 3
Diethylstilbesterol	0[a]	0[a]
1% Carboxymethylcellulose	100	12 ± 3

Compound (n = 6)	Uterotropic Effects in Rats, mg increase
I (20 mg/kg/day)	63 ± 5
XI (20 mg/kg/day)	73 ± 7
XX (20 mg/kg/day)	87 ± 10
Ethynyl estradiol (10 μg/kg/day)	184 ± 9[a]
1% Carboxymethylcellulose	84 ± 8

[a] p = 0.001.

within normal control limits. No teratogenic or congenital abnormalities were noted in the viable fetuses. The sesquiterpene lactones had no estrogenic effects on immature female rats. After 3 weeks of drug administration in rats, no gastric or duodenal bleeding or ulcerogenic effects were noted. White and red blood cell counts were within normal control limits, and hematocrit values were also normal. No abnormal CNS effects were observed in any of the animals while being administered drugs. Sesquiterpene lactones at equivalent doses had no effect on liver aerobic respiration. In Tables I–VII, the number of animals per group is expressed as n; the mean of the percent of control and standard deviation is expressed as $\bar{x} \pm SD$. The significance level p was determined by the Student t test (54).

DISCUSSION

The general anti-inflammatory screen of induced edema by carrageenan demonstrated that the α-methylene-γ-lactone sesquiterpene lactone moiety and related compounds (compare I–XXIX) was required for activity. When the 11,13-methylene group was saturated (II) or otherwise masked (XI), significant activity was lost. Among the compounds studied, helenalin (I), which contains a cyclopentenone ring in addition to this essential α-methylene-γ-lactone moiety, was the most active. The β-unsubstituted cyclopentenone ring (XXIX) demonstrated minimal inhibitory activity and probably is responsible for the slight anti-inflammatory activity of II, XI, and XIV. Saturation of the 2,3-double bond resulted in the loss of this slight activity, as seen with IV, XII, and XVI, and the reduced activity of III.

The 6-hydroxy group of helenalin may play a significant role in receptor binding since esterification (as in V) or elimination of this group (as in XVII, which has a transfused lactone ring) drastically reduced anti-inflammatory activity. The 2,3-epoxy derivatives in the tenulin and isotenulin series (XIII and XV) afforded increased anti-inflammatory ac-

tivity; however, the 2,3-epoxy of helenalin (IX) did not improve the anti-inflammatory activity of helenalin. If the α-methylene-γ-lactone was converted to the dimethylamine adduct (VI) or the pyrrolidine adduct (VIII), activity was reduced. The adducts may be hydrolyzed slowly *in vivo* to give back the α-methylene-γ-lactone, which would account for the observed activity. Here again, the 2,3-epoxy derivative (VII) of the dimethylamine adduct caused no increase in activity.

The structural types of sesquiterpene lactones might affect their anti-inflammatory potency; thus, the pseudoguaianolide 2,3-dihydrohelenalin (III, 49% inhibition) is more active than the germacranolide, eupatolide (XVIII, 30% inhibition). The difference in the degree of potency (57–30%) among the germacranolides tested (XVIII–XXIV) might be due to their dissimilar conformations as well as to functional groups, *e.g.*, eupahyssopin (XX) which, in addition to the essential α-methylene-γ-lactone moiety, contains an allylic ester, an epoxy ring, and a primary hydroxyl group, any one of which may be responsible for the additional activity. The simple α-methylene-γ-lactones (XXVII and XXVIII) caused minimal anti-inflammatory activity, indicating again that there are other steric requirements for potent pharmacological activity.

The writhing reflex test demonstrated the same structural requirements as did the general anti-inflammatory screen; *i.e.*, the α-methylene-γ-lactone in the *cis*-position with relation to the central cycloheptane ring resulted in the best activity. Here again the 6-hydroxy group of the cycloheptane ring appeared to play a functional role and the β-unsubstituted cyclopentenone ring resulted in only minimal activity. Of the germacranolides tested, eupahyssopin and deoxyelephantopin again showed the best inhibitory effects. The simple α-methylene-γ-lactones possessed only minimal activity. In the chronic adjuvant arthritic screen, at a relatively low dose, the simple α-methylene-γ-lactone (XXVII) possessed the best inhibitory activity against arthritis.

Helenalin (I), which contains both the α-methylene-γ-lactone moiety and β-unsubstituted cyclopentenone ring, was very potent. The dimethylamine adduct (VI) of helenalin showed less activity, as did the 2,3-epoxy derivative (IX). In the tenulin series (XI and XIV), the β-unsubstituted cyclopentenone appeared to play a major role in inhibitory activity against arthritis. Saturation of the ring led to loss of activity (XII). The tenulin epoxy derivatives (XIII and XV) had improved activity. Of the germacranolides tested, the allylic ester-bearing deoxyelephantopin (XIV), eupahyssopin (XX), and molephantin (XXII) all were more active than the simple germacranolide, eupatolide (XVIII). Thus, the structural requirements for chronic arthritic inhibition were not as rigid as those for general anti-inflammatory activity.

The sesquiterpene lactones at 2.5 mg/kg had only marginal inhibitory effects in the pleurisy screen, with helenalin (I) having less effect than the germacranolides. As antiallergy agents, the sesquiterpene lactones were essentially inactive. The effects of these agents on the immunological system were twofold. First, the serum antibody production seemed to be elevated with agents containing the β-substituted cyclopentenone ring. Second, T lymphocyte production seemed to be depressed, with helenalin (I) having the best effects. The mitogenic effects of helenalin would explain the immunostimulant effects on B cells.

Chronic arthritis in humans has been treated with BCG vaccine, levamisole, tilorone, and various immunological fractions like transfer factor, which are immunostimulants (55, 56). Complement, T cells, and IgM of rheumatoid factor have been found in the joint synovium or pannus (57–59). The exact etiology of rheumatoid arthritis is unknown. It is believed to be related to persistent viral (60, 61), mycoplasma, or bacterial infection or to an autoimmune disease due to uncoiled IgG autoantibody formation and a deficiency of suppressor T cell function (62). Substances of T cells, *e.g.*, lymphotoxin, migration inhibition factor (MIF), and blastogenic factor, were found in the synovial fluid of patients with rheumatoid arthritis (63, 64). For these reasons, immunosuppressors, *e.g.*, azathioprine, cyclophosphamide, D-penicillamine (56), and gold (55), are used. Whether elevated T cell responses are due to the primary pathogenesis of chronic arthritis or to secondary changes in the disease state, *e.g.*, response against phagocytic products or vasoamine release, is not known. Thus, the sesquiterpene lactones offer an alternative to the current anti-inflammatory therapy. The dose required for antiarthritic activity is relatively low compared to marketed drugs. No deleterious side effects were noted in rodents with the sesquiterpene lactones at the doses employed. The exact mode of action as antiarthritic agents is being studied.

REFERENCES

(1) A. L. Oronsky, L. Triner, O. S. Steinsland, and G. G. Nahas, *Nature*, **223**, 619 (1969).

(2) A. Lorber, *Clin. Res.*, **14**, 334 (1966).

(3) M. Butler, T. Giannina, D. Cargill, D. I. Popick, and B. G. Steinitz, *Proc. Soc. Exp. Biol. Med.*, **132**, 484 (1969).

(4) D. A. Gerber, R. Guistra, and N. Cohen, *Arthritis Rheum.*, **9**, 507 (1966).

(5) D. A. Gerber, N. Cohen, and R. Guistra, *Biochem. Pharmacol.*, **16**, 115 (1967).

(6) I. H. Hall, K. H. Lee, E. C. Mar, C. O. Starnes, and T. G. Waddell, *J. Med. Chem.*, **20**, 333 (1977).

(7) K. H. Lee, I. H. Hall, E. C. Mar, C. O. Starnes, S. A. ElGebaly, T. G. Waddell, R. I. Hadgraft, C. G. Ruffner, and I. Weidner, *Science*, **196**, 533 (1977).

(8) C. H. Smith, J. Larner, A. M. Thomas, and S. M. Kupchan, *Biochim. Biophys. Acta*, **276**, 94 (1972).

(9) S. M. Kupchan, D. C. Fessler, M. A. Eakin, and T. J. Giacobbe, *Science*, **168**, 376 (1970).

(10) R. L. Hanson, H. A. Lardy, and S. M. Kupchan, *ibid.*, **168**, 378 (1970).

(11) I. H. Hall, K. H. Lee, and S. A. ElGebaly, *J. Pharm. Sci.*, **67**, 552 (1978).

(12) I. H. Hall, K. H. Lee, C. O. Starnes, S. A. ElGebaly, T. Ibuka, Y. S. Wu, T. Kimura, and K. Haruna, *ibid.*, **67**, 1235 (1978).

(13) W. S. Kan, "Pharmaceutical Botany," National Research Institute of Chinese Medicine, Taiwan, Republic of China, 1969, p. 561.

(14) K. H. Lee and T. A. Geissman, *Phytochemistry*, **9**, 403 (1970).

(15) K. H. Lee, D. C. Anuforo, E. S. Huang, and C. Piantadosi, *J. Pharm. Sci.*, **61**, 626 (1972).

(16) R. A. Lucas, S. Rovinski, R. J. Kiesel, L. Dortman, and H. B. MacPhillamy, *J. Org. Chem.*, **29**, 1549 (1964).

(17) K. H. Lee, H. C. Huang, E. S. Huang, and H. Furukawa, *J. Pharm. Sci.*, **61**, 629 (1972).

(18) K. H. Lee, C. M. Cowherd, and M. T. Walo, *ibid.*, **64**, 1572 (1975).

(19) K. H. Lee, T. Kimura, M. Okamoto, C. M. Cowherd, A. T. McPhail, and K. D. Onan, *Tetrahedron Lett.*, **14**, 1051 (1976).

(20) K. H. Lee, T. Kimura, M. Haruna, A. T. McPhail, K. D. Onan, and H. C. Huang, *Phytochemistry*, **16**, 1068 (1977).

(21) K. H. Lee, H. Furukawa, M. Kozuka, H. C. Huang, P. A. Luchan, and A. T. McPhail, *J. Chem. Soc., Chem. Commun.*, **1973**, 476.

(22) K. H. Lee, T. Ibuka, H. C. Huang, and D. L. Harris, *J. Pharm. Sci.*, **64**, 1077 (1975).

(23) A. T. McPhail, K. D. Onan, K. H. Lee, T. Ibuka, M. Kozuka, T. Shingu, and H. C. Huang, *Tetrahedron Lett.*, **1974**, 2739.

(24) K. H. Lee, T. Ibuka, A. T. McPhail, K. D. Onan, T. A. Geissman, and T. G. Waddell, *ibid.*, **1974**, 1149.

(25) K. H. Lee, H. Furukawa, and E. S. Huang, *J. Med. Chem.*, **15**, 609 (1972).

(26) K. H. Lee, S. H. Kim, H. Furukawa, and C. Piantadosi, *ibid.*, **18**, 59 (1975).

(27) T. G. Waddell, A. M. Austin, J. W. Cochran, K. G. Gerhart, I. H. Hall, and K. H. Lee, *J. Pharm. Sci.*, in press.

(28) K. H. Lee, Y. S. Wu, and I. H. Hall, *J. Med. Chem.*, **20**, 911 (1977).

(29) K. H. Lee, T. Ibuka, S. H. Kim, B. R. Vestal, and I. H. Hall, *ibid.*, **18**, 812 (1975).

(30) G. M. Ksander, J. E. McMurry, and M. Johnson, *J. Org. Chem.*, **42**, 1180 (1977).

(31) A. P. Roszkowski, W. H. Rooks, A. Tomolonis, and L. M. Miller, *J. Pharmacol. Exp. Ther.*, **179**, 114 (1971).

(32) C. A. Winter, E. A. Risley, and G. W. Nuss, *Proc. Soc. Exp. Biol. Med.*, **111**, 544 (1962).

(33) L. O. Randall and H. Baruth, *Arch. Int. Pharmacodyn Ther.*, **220**, 94 (1976).

(34) W. L. Dewey, L. S. Harris, J. E. Howes, and J. A. Nuit, *J. Pharmacol. Exp. Ther.*, **175**, 435 (1970). *Ibid.*, **179**, 652 (1971).

(35) L. C. Hendershot and J. Forsaith, *ibid.*, **125**, 237 (1959).

(36) R. Vingar, J. F. Traux, and J. L. Selph, *Eur. J. Pharmacol.*, **37**, 23 (1976).

(37) B. H. Waksman, C. M. Pearson, and J. T. Sharp, *J. Immunol.*, **85**, 403 (1960).

(38) L. F. Sancilio, *Proc. Soc. Exp. Biol. Med.*, **127**, 597 (1968).

(39) I. Mota, *Immunology*, **7**, 681 (1964).

(40) J. Goose and A. M. J. Blair, *ibid.*, **16**, 749 (1969).

(41) D. R. Buckle, N. J. Morgan, J. W. Ross, H. Smith, and B. A. Spicer, *J. Med. Chem.*, **16**, 1334 (1973).

(42) Y. Matsumura, E. M. Tan, and J. H. Vaughan, *J. Allergy Clin. Immunol.*, **58**, 387, 395 (1976).

(43) N. K. Jerne and D. A. Nordin, *Science*, **140**, 405 (1963).
(44) T. Igarashi, M. Okada, S. Kishimoto, and Y. Yamamura, *Immunology*, **28**, 37 (1975).
(45) E. M. Uyeki, R. S. Klassen, and V. Llacer, *Proc. Exp. Biol. Med.*, **132**, 1140 (1969).
(46) A. J. Crowle and C. C. Hu, *Proc. Soc. Exp. Biol.*, **127**, 190 (1968).
(47) A. J. Crowle, C. C. Hu, and A. Patruce, *J. Allergy*, **42**, 140 (1968).
(48) B. Baggett, I. H. Hall, R. G. Boelgi, K. H. Palmer, and M. Wall, *Fertil. Steril.*, **21**, 68 (1970).
(49) I. Hall, G. L. Carlson, G. S. Abernethy, and C. Piantadosi, *J. Med. Chem.*, **17**, 1253 (1974).
(50) J. G. Wilson, in "First Fertility Conference on Congenital Malformations," General Principles in Experimental Teratology, Lippincott, Philadelphia, Pa., 1961, pp. 187–194.
(51) C. W. Emmens, *Methods Horm. Res.*, **2**, 61 (1970).
(52) D. A. Brodie and B. J. Chase, *J. Gastroenterol.*, **53**, 604 (1967).
(53) S. A. Lang, B. D. Johnson, E. Cohen, A. E. Sloboda, and E. Greenblatt, *J. Med. Chem.*, **19**, 1404 (1976).
(54) G. W. Snedecor, "Statistical Methods," Iowa State College Press, Ames, Iowa, 1956.
(55) E. Arrigoni-Mantelli, "Inflammation and Anti-inflammatories," Spectrum, New York, N.Y., 1977.
(56) Y. H. Chang, *Annu. Rep. Med. Chem.*, **11**, 138 (1976).
(57) O. J. Mellbye, J. B. Natvig, T. E. Michaelson, and H. M. Hoyeraal, in "The Immunological Basis of Connective Tissue Disorders," L. G. Silvestri, Ed., North-Holland Publishing, Amsterdam, The Netherlands, 1975.
(58) P. Davies and R. J. Bonney, *Annu. Rep. Med. Chem.*, **12**, 152 (1977).
(59) E. Munthe and J. B. Natvig, *Scand. J. Immunol.*, **1**, 217 (1972).
(60) D. Hamerman, in "The Immunological Basis of Connective Tissue Disorders," L. G. Silvestri, Ed., North-Holland Publishing, Amsterdam, The Netherlands, 1975, p. 17.
(61) D. Hurwitz, N. E. Cremer, F. Quismorio, E. H. Lennette, and G. J. Friou, *J. Rheum. (Suppl.)*, **1**, 120 (1974).
(62) A. C. Allison, *Ann. Rheum. Dis.*, **32**, 283 (1973).
(63) P. Stastny, *Arthritis Rheum.*, **16**, 572 (1972).
(64) J. B. Peter, T. Konen, K. Stenepel, and C. Cardin, *ibid.*, **14**, 179 (1971).

ACKNOWLEDGMENTS

The authors acknowledge financial support from the following sources: University of North Carolina Research Council Grant VF475 awarded to I. H. Hall, NIH Grant CA-17625 and American Cancer Society Grants CH-19A and CH-19B awarded to K. H. Lee, and the Research Corporation of America grant and the American Chemical Society grant awarded to T. G. Waddell.

Simultaneous Solubilization of Steroid Hormones II: Androgens and Estrogens

B. LUNDBERG ˣ, T. LÖVGREN, and B. HEIKIUS

Received March 1, 1978, from the *Department of Biochemistry and Pharmacy, Åbo Akedemi, Porthansgatan 3, 20500 Åbo 50, Finland.* Accepted for publication October 18, 1978.

Abstract □ The simultaneous solubilization of some androgens and estrogens in aqueous polysorbate 40, tetradecyltrimethylammonium bromide, and sodium lauryl sulfate was studied. The solubilizations of estradiol and testosterone were independent of each other in all three association colloids. However, if the estrogen component was ethinyl estradiol, the solubilization was dependent on the addition order. The estrogen precipitates more readily than testosterone in polysorbate 40 and tetradecyltrimethylammonium bromide, but the opposite is true in sodium lauryl sulfate. The simultaneous solubilizations of methyltestosterone or ethisterone with the estrogens tested were different from those of testosterone. The solubilization behavior of the steroids is discussed, starting with the pseudophase model and different solubilization loci. Results indicated that the free energy change of micellar binding, ΔG_b, decreases with increased steroid polarity. The simultaneous solubilization cannot be predicted by ΔG_b but may be explained by differences in the solubilization mechanism.

Keyphrases □ Solubilization—androgens and estrogens in various association colloids □ Androgens—solubilization in various association colloids □ Estrogens—solubilization in various association colloids

The solubilization of poorly soluble drugs is of great pharmaceutical interest. The surfactants used may be important in drug bioavailability (1).

The solubilization of steroid hormones by aqueous solutions of surfactants was reviewed previously (2). More recent reports (3–7) indicated that solubilization continues to be of interest.

A recent report from this laboratory (8) dealt with the simultaneous solubilization of estrogens and C_{21}-steroids in aqueous solutions of association colloids. The poorly soluble estrogen, estradiol, solubilized independently of the C_{21}-steroids, whereas the solubilization of ethinyl estradiol was independent of corticosterone and hydrocortisone but dependent on the presence of progesterone and desoxycorticosterone (21-hydroxyprogesterone).

This report deals with the dissolution behavior of estrogens and androgens simultaneously solubilized in aqueous solutions of three association colloids chosen as representatives of nonionic, cationic, and anionic types.

EXPERIMENTAL

Materials—Purification methods and the tests of purity of the steroid hormones and the association colloids were described previously (8). The association colloids used were sodium lauryl sulfate[1], tetradecyltrimethylammonium bromide[2], and polysorbate 40[3].

Solubilization Experiments—The solubility studies were carried out as previously described (8). The procedures were: saturation of the solution of association colloid with the first steroid and quantitation of solubilized steroid, saturation with the second steroid, and, finally, UV spectroscopic quantitation of both solubilized steroids. Special notice was paid to complete equilibration of the solutions. The solubilization temperatures were 20° for tetradecyltrimethylammonium bromide and polysorbate 40 and 40° for sodium lauryl sulfate.

The UV absorbance of the steroid solutions was recorded at around

[1] Koch-Light Laboratories.
[2] K & K Laboratories.
[3] Tween 40, Atlas Chemical Industries.

0022-3549/79/0500-0542$01.00/0