Evaluation of Retinoids as Therapeutic Agents in Dermatology

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Evaluation of 13-*cis*-12-substituted analogues of retinoic acid in a series of dermatologic screens has revealed that structural modifications can lead to selectivity and specificity. An analogue, 11-*cis*,13-*cis*-12-hydroxymethylretinoic acid, δ-lactone, has been found to have good activity and to be devoid of topical and systemic toxicity.

KEY WORDS: retinoids; antihyperkeratinization; antihyperproliferation; antihyperactive sebaceous gland; hypervitaminosis A.

INTRODUCTION

Since vitamin A and its analogues, in particular retinoic acid, appear to be involved in the proliferation and differentiation of epithelial tissues, these compounds have been, and continue to be, used in the treatment of dermatological disorders such as acne, psoriasis, and hyperkeratosis (1). However, the known toxicity and teratogenicity of this class of compounds (1) have prompted a continuing search for retinoids with decreased undesirable side effects.

We have been interested in identifying retinoids effective topically against various cutaneous disorders. To this end we examined a group of 12-substituted retinoids (2–5). This report describes the synthesis and biological evaluation of 11 derivatives of 12-carboxyretinoic acid (**1–11**) and their comparison to retinoic acid (**16**) and its 13-*cis*-isomer (**17**).

MATERIALS AND METHODS

Chemistry

All syntheses were performed under dim red light. Melting points were determined on a Thomas Hoover capillary tube apparatus. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WM-250 spectrometer using tetramethylsilane as internal standard. Thin-layer chromatography (TLC) was carried out on Whatman silica gel (SiO_2) 60 plates using 1:1 ethyl acetate (EtOAc) and hexane, with visualization by ultraviolet (UV) and/or iodine. High-performance liquid chromatography (HPLC) was carried out using a system equipped with two M6000 Waters reciprocating pumps, a septumless Waters U6K injector, an automated gradient controller, and a Waters 490 UV detector in the maxplot mode recording at 20-nm intervals between 220 and 440 nm. The HPLC conditions are summarized in Table I. UV spectra were recorded using a Varian 2290 spectrophotometer, and mass spectrometry was performed in the electron impact (EI) mode on a MS9 spectrometer. Ethanol solutions of all the compounds were stable under dim red light (or in the dark) and under argon for at least 24 hr at room temperature and for at least 1 week when refrigerated (~10°C).

N-*Ethyl-11*-cis,*13*-cis-*12-carbomethoxyretinoylamide* (*5*). A solution of 11-*cis*,13-*cis*-12-carbomethoxyretinoic acid (**2**) (3) (8.000 g, 0.0223 mol) in freshly distilled toluene (75 mL) containing dry triethylamine (3.16 mL, 0.0223 mol) in a round-bottomed flask sealed with a septum equipped with a syringe needle was cooled to 0°C, and isobutyl chloroformate (2.90 mL, 0.023 mol) was added. Copious amounts of triethylamine hydrochloride fell out of solution. After stirring at 0°C for 35 min, ethylamine (1.46 mL, 0.023 mol) was added, resulting in an immediate pressure buildup. HPLC analysis using a C_{18} Radial Pak cartridge and a linear gradient from 60% acetonitrile (CH_3CN)/40% [1% aq ammonium acetate (NH_4OAc)] to 100% CH_3CN over 10 min at 2 mL/min and monitoring at 350 nm showed the presence of unreacted starting material **2** and of a less polar substance. After storage overnight in the freezer, there was no apparent change in the product mixture. Removal of the volatiles under vacuum left a residue (10 g) which was subjected to low-pressure chromatography for purification. Using 300 g SiO_2 (470 mesh) in hexane, the column was eluted with a slow gradient of hexane to EtOAc; 250-mL fractions were collected. Fractions 25–27, which contained the more polar material in reasonably pure form were combined, concentrated, and refrigerated overnight to yield 3.35 g of crystals. The mother liquor was evaporated to give 2.42 g of impure product as an oil. The solid was dissolved in EtOAc by sonication at 45°C and hexane was added to induce crystallization. Filtration and drying afforded 2.62 g of a pale yellow powder which was 100% pure by HPLC. Additional crops were obtained from the mother liquors, for a total of 3.93 g (46% yield). The compound had mp 116°C and λ_{max} 339 nm [ε 26,000 methanol (CH_3OH)]. ^1H NMR (dioxane-d_8) δ: 0.98 (t, J = 7.2 Hz, 3, CH_2CH_3), 1.02 (s, 6, H-1a), 1.47 (m, 2, H-2 or H-3), 1.61 (m, 2, H-3 or H-2), 1.69 (s, 3, H-5a), 1.92 (s, 3, H-13a), 1.98 (m, 2, H-4), 2.01 (s, 3, H-9a), 3.12 (dq, J = 7.2 Hz, 5.5, CH_2CH_3), 5.79 (s, 1, H-14), 6.00 (d, J = 12.3 Hz, 1, H-10), 6.14 (d, J = 16.1 Hz, 1, H-8), 6.35 (d, J = 16.1 Hz, 1, H-7), 6.44 (t, J = 5.5 Hz, 1, NH), 7.43 (d, J = 12.3 Hz, 1, H-11): *m/z* calcd for $C_{24}H_{35}NO_3$, 385.2617; found, 385.2614.

N-*Phenyl-11*-cis,*13*-cis-*12-carbomethoxyretinoylamide* (*6*). In a manner analogous to the preparation of **5**, the monoester **2** (5.99 g, 0.0167 mol) was treated with aniline (distilled from zinc dust, 0.974 mL, 0.0166 mol). Low-pressure chromatography on SiO_2 (330 g, 470 mesh) at 70 mL/min using a gradient of hexane to 20% EtOAc in hexane and collection of 150-mL fractions showed pure product in fractions 24–29 (2.55 g). Recrystallization from EtOAc gave

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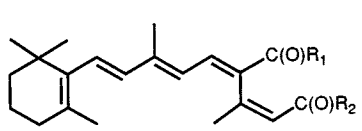

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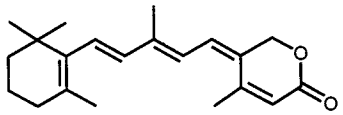
3 $R_1 = R_2 = OCH_3$
4 $R_1 = OH$ $R_2 = OCH_3$
5 $R_1 = OCH_3$ $R_2 = NHCH_2CH_3$
6 $R_1 = OCH_3$ $R_2 = NHC_6H_5$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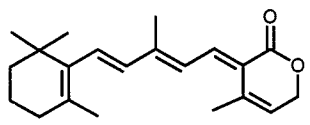
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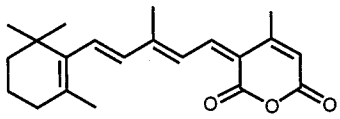
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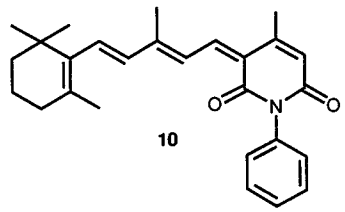
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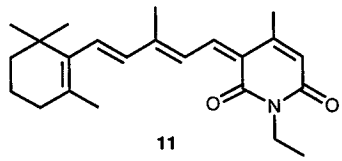
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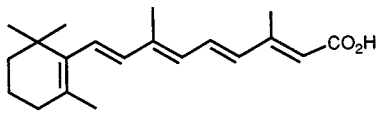
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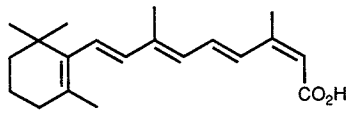
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2.15 g (30%) pure material with mp 110–113°C. ^{1}H NMR (dioxane-d_8) δ: 1.01 (s, 6, H-1a), 1.47 (m, 2, H-2 or H-3), 1.59 (m, 2, H-3 or H-2), 1.67 (s, 3, H-5a), 1.99 (m, 2, H-4), 2.00 (s, 3, H-13a), 2.01 (s, 3, H-9a), 5.99 (s, 1, H-14), 6.01 (d, J = 11.1 Hz, 1, H-10), 6.14 (d, J = 16.0 Hz, 1, H-8), 6.36 (d, J = 16.0 Hz, 1, H-7), 6.97 (t, J = 7.5 Hz, 1, p-Ar), 7.21 (t, J = 7.6 Hz, 2, m-Ar), 7.43 (d, J = 12.0 Hz, 1, H-11), 7.49 (d, J = 8.0 Hz, 2, o-Ar), 8.39 (s, 1, NH): m/z calcd for $C_{28}H_{35}NO_3$, 433.2617; found, 433.2613.

N-Phenyl-3-methylglutaconimide (14). A mixture of 3-methylglutaconic acid (6.00 g, 0.042 mol) and aniline (4.00 g, 0.043 mol) was heated under nitrogen (N_2) at 150°C for 40 min. Treatment of the resulting yellow resin with diethyl ether (Et_2O) led to the precipitation of a white solid which was washed copiously with Et_2O (150 mL) and dried to give 3.71 g (44%) of **14**, mp 160–163°C [lit. (6), 164°C]. ^{1}H NMR (90 MHz, CD_3OD) δ: 2.1 (s, 3, CH_3), 3.5 (s, 2, CH_2), 6.1 (s, 1, CH), 7.0–7.6 (m, 5, Ar).

N-Ethyl-3-methylglutaconimide (15). Following the procedure reported for the *N*-phenyl analogue (**6**), a solution

Table I. HPLC Conditions

Compound	Column	Eluant A	Eluant B	Program	Flow rate (ml/min)
1	C-18	H_2O	75% CH_3CN/25% (1% NH_4OAc in H_2O)	37 → 50% B over 4 min	2
2	C-18	H_2O	75% CH_3CN/25% (1% NH_4OAc in H_2O)	37 → 50% B over 4 min; hold 8 min; →80% B over 1 min	2
3	C-18	H_2O	75% CH_3CN/25% (1% NH_4OAc in H_2O)	37 → 50% B over 4 min; hold 8 min; →80% B over 1 min; hold 7 min; →100% B over 1 min	2
	SiO_2	Hexane	Et_2O	Isocratic 5% B	2
4	C-18	H_2O	75% CH_3CN/25% (1% NH_4OAc in H_2O)	37% → 50% B over 4 min; hold 8 min; →80% B over 1 min	2
5	C-18	CH_3CN	60% CH_3CN/40% (1% NH_4OAc in H_2O)	0–100% A over 10 min	2
	SiO_2	MeOH	1:9 Et_2O/hexane	Isocratic 95% B	
6	C-18	CH_3CN	60% CH_3CN/40% (1% NH_4OAc in H_2O)	0–100% A over 10 min	2
7	SiO_2	Hexane	t-Butyl methyl ether	Isocratic 20% B	2
8	SiO_2	MeOH	1:9 Et_2O/hexane	Isocratic 99% B	2
9	SiO_2	MeOH	1:9 Et_2O/hexane	Isocratic 95% B	2
10	SiO_2	Hexane	t-Butyl methyl ether	25 → 50% B over 5 min	2
11	SiO_2	Hexane	t-Butyl methyl ether	25 → 50% B over 5 min	2

of ethylamine (13.0 mL, 0.04 mol) in toluene (15 mL) was added to a slurry of 3-methylglutaconic anhydride (5.0 g, 0.04 mol) in toluene at 0°C. Immediately after the addition, the ice bath was removed; some solid was still observed in the flask. After a few minutes, two distinct layers appeared. The addition funnel was replaced by a Dean-Stark trap and the reaction was brought to reflux. After 2 hr an additional portion (1 mL) of ethylamine was added to replace the material lost by evaporation. After an additional 45 min, the reaction appeared to be complete (TLC: SiO_2, 1:1 acetone/hexane). After dilution with EtOAc to dissolve the gummy solid that had formed, the reaction mixture was washed with 5% hydrochloric acid (HCl) (2 × 150 mL) and water (100 mL) and evaporated. The solid residue was treated with CH_3OH, resulting in a yellow solution and a white solid. The solid (3.69 g, 60%) had mp 133.8–135.8°C. ^1H NMR (dimethyl sulfoxide-d_6) showed the presence of keto-enol tautomers. The keto tautomer (70%) had δ: 1.02 (t, J = 7 Hz, 3, CH_2CH_3), 1.93 (s, 3, CH_3), 3.47 (s, 2, CH_2), 3.71 (q, J = 7 Hz, 2, CH_2CH_3), 5.99 (s, 1, CH). The enol form (30%) had δ: 1.11 (t, J = 7 Hz, 3, CH_2CH_3), 2.02 (s, 3, CH_3), 3.93 (q, J = 7 Hz, 2, CH_2CH_3), 5.53 (s, 1, CH), 6.00 (s, 1, CH): *m/z* calcd for $C_8H_{11}NO$, 153.0790; found, 153.0792.

N-*Phenyl-13*-cis-*12-carboxyretinimide (10).* To an ice-cooled solution of *N*-phenyl-3-methylglutaconimide (**14**) (6.02 g, 0.03 mol) in dry tetrahydrofuran (THF) (25 mL) under argon was added, through a septum, *trans*-β-ionylidene-acetaldehyde (**13**) (6.5 g, 0.03 mol) in dry THF (30 mL), followed by the dropwise addition of pyridine (0.57 mL). The ice bath was removed and TLC (SiO_2, 1:1 EtOAc/hexane) after 15 min showed the formation of product. Continued monitoring showed no change after an additional 40 min so the reaction mixture was placed in a warm water bath and 1 mL pyridine was added. After 5 hr, starting material was still observed but the reaction was worked up by twofold dilution with Et_2O, washing with 1 *N* HCl (2 × 50 mL), washing with water, and drying over sodium sulfate (Na_2SO_4). After removal of the drying agent, the solution was evaporated to leave a gum. This material was chromatographed on SiO_2, eluting with 5% t-butyl methyl ether in hexane to remove unreacted starting materials. The fractions containing fast-eluting material (TLC) were combined and evaporated. The dark oil which resulted was dissolved in hexane and the solution was refrigerated overnight. The solid which was formed appeared to consist mainly of one component. It was recrystallized from hexane/EtOAc to give **10** as a light powder, mp 115°C and λ_{max} 434.5 nm (ε 40,000, CH_3OH). ^1H NMR (dioxane-d_8) δ: 1.05 (s, 6, H-1a), 1.48 (m, 2, H-2 or H-3), 1.60 (m, 2, H-3 or H-2), 1.73 (s, 3, H-5a), 2.00 (m, 2, H-4), 2.15 (s, 3, H-9a), 2.25 (s, 3, H-13a), 6.12 (s, 1, H-14), 6.41 (d, J = 16.0 Hz, 1, H-8), 6.63 (d, J = 16.0 Hz, 1, H-7), 7.10 (d, J = 6.5 Hz, 2, o-Ar), 7.38 (m, 3, m-, p-Ar), 7.58 (d, J = 12.3 Hz, 1, H-10), 7.95 (d, J = 12.4 Hz, 1, H-11): *m/z* calcd for $C_{27}H_{31}NO_2$, 401.2355; found, 401.2353.

N-*Ethyl-13*-cis-*12-carboxyretinimide (11).* The procedure described for **10** was followed starting with *N*-ethyl-3-methylglutaconimide (**15**) (3.69 g, 0.024 mol), *trans*-β-ionylideneacetaldehyde (**13**) (5.25 g, 0.024 mol), and pyridine (0.51 mL). After 4 days, during which 1 mL additional pyridine was added, the reaction was worked up by twofold dilution with Et_2O, washing with sat. sodium carbonate (Na_2CO_3) (emulsion), 1 *N* HCl (2 × 50 mL), and water, and drying over Na_2SO_4. After removal of the drying agent, the solution was evaporated to leave a sludge. This material was azeotroped twice with cyclohexane to remove any water, leaving an oil which was dissolved in EtOAc with a small amount of hexane and left in the freezer overnight. The resulting crystals appeared pure by TLC and HPLC and had mp 101°C and λ_{max} 429 nm (ε 28,000, CH_3OH). ^1H NMR (dioxane-d_8) δ: 1.07 (s, 6, H-1a), 1.08 (t, J = 7.0 Hz, 3, CH_2CH_3), 1.50 (m, 2, H-2 or H-3), 1.66 (m, 2, H-2 or H-3), 1.77 (s, 3, H-5a), 2.07 (m, 2, H-4), 2.14 (s, 3, H-9a), 2.17 (s, 3, H-13a), 3.90 (q, J = 7.0 Hz, 2, CH_2CH_3), 5.99 (s, 1, H-14), 6.46 (d, J = 16.1 Hz, 1, H-8), 6.64 (d, J = 16.1 Hz, 1, H-7), 7.50 (d, J = 12.2 Hz, 1, H-10), 8.02 (d, J = 12.2 Hz, 1, H-11): *m/z* calcd for $C_{23}H_{31}NO_2$, 353.2355; found, 353.2352.

Biology

Animals. Nine- to 14-week-old female hairless rhino mice ($hr^{rh}hr^{rh}$) were purchased from the Skin and Cancer Hospital, Temple University Health Sciences Center (Philadelphia, PA). Female hairless mice (HRS/J), age 6 to 8 weeks, were purchased from Jackson Laboratories (Bar Harbor, ME). Golden Syrian male hamsters, age 10 weeks, were purchased from Charles River Labs (Lakeview, NJ). Female albino CD-1 mice, 5 to 6 weeks of age, were purchased from Charles River Breeding Laboratories (Wilmington, MA). Male New Zealand white rabbits, 10 to 12 weeks of age, were purchased from Beckens Farm (Sanborn, NY). Male albino Harley guinea pigs weighing 350–400 g were purchased from Elm Hill Breeding Laboratory (Chelmsford, MA).

Animals were housed in accordance with the National Institutes of Health guidelines (U.S. Department of Health and Human Services, 1985). Animals had free access to food and water. The quarantine period was at least 7 days, except for hairless mice, for which it was at least 6 days.

Test Materials. Trans- and 13-*cis* retinoid acids were obtained from the Eastman Kodak Company (Rochester, NY); 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and L-[^{14}C]ornithine hydrochloride (sp act, 57 mCi/mmol) were purchased, respectively, from Sigma Chemical Company (St. Louis, MO) and Amersham (Arlington Heights, IL). Retinoid solutions in ethanol were prepared weekly and stored in amber vials under refrigeration. The vials were topped with argon gas to retard oxidation.

Retinoid Treatments. Retinoids were applied topically in an ethanol vehicle in all experiments unless otherwise indicated. The retinoid solutions were applied evenly to the dorsal skin of the animals at a dose of 2 μl/cm^2. All of the procedures were under yellow light to minimize photodegradation of the retinoids. During treatment, all animals were housed individually.

Rhino Mouse Utricle Reduction Model. The test solutions were applied to the dorsal skin of each mouse once daily, 5 days/week, for 4 weeks. Intraperitoneal (ip) administration was made once daily, 5 days/week, for 2 weeks.

Two days after the final topical or ip treatment, the mice were sacrificed by carbon dioxide (CO_2) gas. Utricle diameter of rhino mouse skin was assessed in horizontal epidermal sheets (7). At sacrifice, a ⅞-in.-diameter circular area of

the dorsal skin was removed by arch punch and bisected. The epidermal sheet from one-half of the biopsy was separated from the dermis after incubation of skin in 0.5% acetic acid for 10 to 20 hr at 4°C. These epidermal sheets were fixed in formalin, dehydrated with ethanol, and cleared with xylene.

To assess utricle diameter, each epidermal sheet was placed on a glass slide in a few drops of xylene. The diameter of 40 utricles was measured in each epidermal specimen with an image analyzer (Image Measure, Microscience, Federal Way, WA). The data are expressed as percentage reduction of mean utricle diameter relative to vehicle control.

The other half of the biopsy was placed in 10% buffered formalin immediately after sacrifice and was processed for preparation of H&E-stained, 5-μm-thick vertical sections.

Hamster Flank Test. The hair over the region of the mature flank organs of Golden Syrian male hamsters was close clipped as needed to expose the region for topical treatment and evaluation.

The flank organs of each hamster were treated topically once daily, 7 days/week, for 3 weeks with 20 μl of either acetone or retinoids in acetone. One day following the last treatment, the animals were sacrificed with CO_2 gas, and a 5/16-in. punch biopsy of each flask organ was taken from the center of the organ, weighed, and fixed in 10% buffered formalin for histological evaluation. The sections were evaluated for sebaceous gland size and activity as determined by the presence of lipid content of the glands (8).

Epidermal Ornithine Decarboxylase (ODC) Activity in Hairless Mice. Retinoids (0.1 mL) in acetone were applied topically to the dorsal skin of hairless mice 1 hr before topical application of 17 nmol of TPA. Mice were sacrificed by CO_2 gas inhalation 4 hr after treatment with TPA and epidermal ODC activity was measured as described previously (9). ODC activity was determined by the measurement of release of $^{14}CO_2$ from L-[^{14}C]ornithine (results expressed as CO_2 release per milligram of protein per hour) incubated with the epidermal samples.

Hypervitaminosis A (HVA) in CD-1 Mice. HVA signs in CD-1 mice were evaluated after ip administration of retinoids. Retinoids were suspended in peanut oil and injected at 8 mL/kg, once daily, 5 days/week, for 2 consecutive weeks.

The severity of the toxicity signs was evaluated (10) on a 0 to 4 scale (Table II). Mice were graded daily during treatment using the criteria given in Table II. At the end of the experiment, an animal was defined as having HVA syndrome when the addition of the grades from all four of the individually graded signs totaled at least 3.0.

On the third day after the last treatment, mice were sacrificed and necropsy performed. Portions of the major organs including skin, sternum, and hindlimbs were placed in 10% buffered formalin for histopathological examination.

Repeat Application Skin Irritation in Rabbit and Guinea Pig. The hair of rabbits or guinea pigs was clipped closely at four sites on the back with an electric hair clipper. Each site was a 2 × 2-cm square.

The retinoids in 0.2 mL of ethanol were applied once daily for 14 or 28 days. Each day before applying drug solutions, the degree of erythema, scaling, and edemas was assessed visually using the Draize 0 to 4 grading method (11). The results were also expressed as average daily Draize scores to allow statistical comparisons of the treatments. The average daily Draize scores were calculated by taking cumulative scores over 14 or 28 days for each parameter and dividing by 14 or 28.

Table II. HVA Grading System

Sign and degree of severity	Grade
Loss of body weight	
1 g	0
1–3 g	1
4–6 g	2
7–9 g	3
>10 g	4
Skin scaling and hair loss	
None	0
Slight	1
Moderate	2
Severe	3
Very severe	4
Number of bone fractures in extremities	
0	0
1	1
2	2
3	3
4	4

On day 15 or 29, animals were sacrificed by CO_2 inhalation, treated sites were excised, and a strip was placed in 10% buffered formalin. These were processed, stained with H&E, and evaluated for microscopic signs of inflammation.

Statistics

Data were analyzed for significant differences by analysis of variance and Tukey's Studentized range test for multiple comparisons (12).

RESULTS

Chemistry

Compounds **1–4** and **7–9** were prepared as described previously (2–4). The amides **5** and **6** were prepared from the activated ester **12**, which was obtained by treatment of the monomethyl ester **2** with isobutyl chloroformate in the presence of triethylamine (Fig. 1). Treatment of the ester **12** with ethylamine and aniline gave the amido esters **5** and **6**, respectively. The imides **10** and **11** were prepared by condensation of *trans*-β-ionylideneacetaldehyde (**13**) with *N*-phenyl and *N*-ethyl-3-methylglutaconimide (**14** and **15**, respectively) (Figs. 2 and 3).

The configurations of the retinoid amides (**5**, and **6**) and imides (**10** and **11**) were determined by their ^{1}H NMR spectra. The 11-*cis*,13-*cis* configuration of **5** and **6** was confirmed by the close resemblance of their ^{1}H NMR parameters to those of other 11-*cis*,13-*cis*-12-carboxyretinoic acids (2–4). Similarly, assignment of the 13-*cis* configuration to **10** and **11** was based primarily on the 1.5-ppm downfield shift of H-10. We showed previously that the downfield shift of H-10 was associated with the anisotropy of the coplanar 12a-carboxyl group in the 13-*cis* configuration (2,3). In agreement with previous observations on 13-*cis*-12-carboxyretinoic anhy-

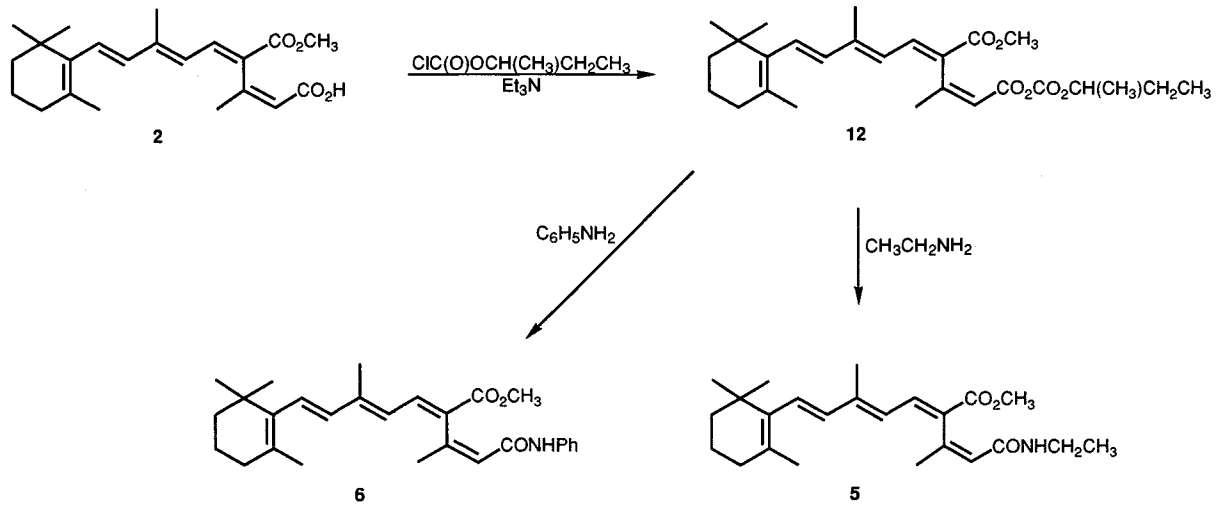

Fig. 1.

dride (3), the imides **10** and **11** were both light sensitive, producing isomeric imides. These transformations were reversible, the initial imide being reformed after several hours in the dark.

Biological

The compounds were evaluated topically for activity against hyperkeratinization (rhino mouse model) (7,13–15), hyperactive sebaceous glands (hamster flank/ear model), and epidermal ODC activity (16–19). Retinoids that were active in these models were evaluated further for local irritation by repetitive application and for systemic toxicity measured by symptoms of HVA.

Rhino Mouse Test. Utricles are hair-derived, keratin-filled, superficial cysts in skin resembling human comedones. Utricle diameter of rhino mouse skin was assessed in whole mounts of the horizontal epidermal sheets (7); the results are shown in Table III. Compounds that reduced the size of the utricles also showed reductions in the size of surface cysts or pseudocomedones. Histological evaluation also showed that these compounds stimulated an increase in epidermal thickness, especially of the granular layer. Only the lactones **6** and **7** and the 12-carbomethoxyanilide **8** had any activity in this assay. The lactone **7** had activity comparable to that of *trans*- and 13-*cis*-retinoic acid (**16** and **17**, respectively). Interestingly, although both **16** and **17** exhibited a 10 to 40% reduction in utricles on the control-treated contralateral site, compound **7** showed little or no effect on the contralateral side.

Hamster Flank Test. The effects of subcutaneous administration of compounds **1**–**11** were compared to those of *trans*- and 13-*cis*-retinoic acid (**16** and **17**, respectively) on suppression of the development of the male Golden Syrian hamster flank organ sebaceous gland in terms of size and activity, as determined by histopathological examination of

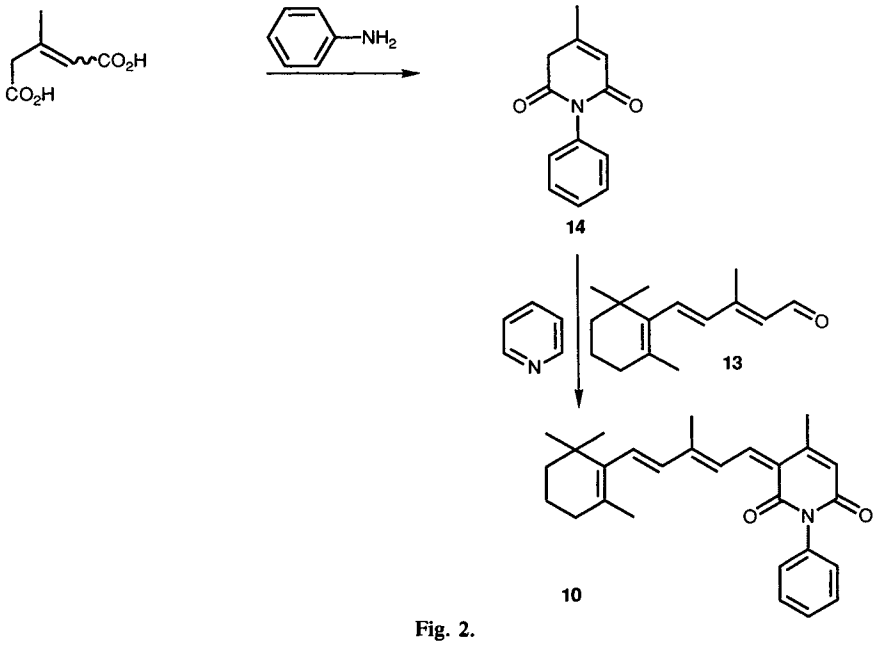

Fig. 2.

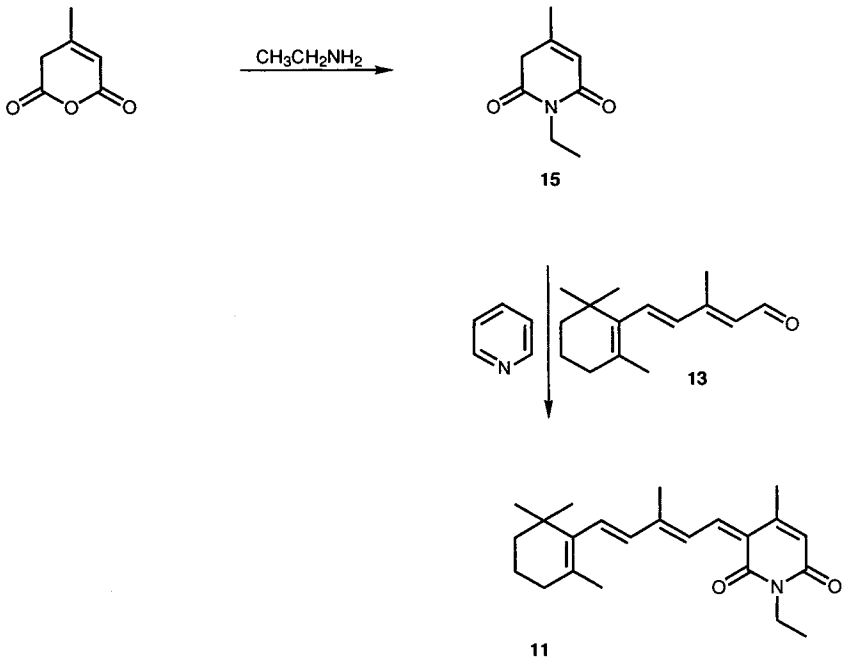

Fig. 3.

the glands (8). For the controls, 13-*cis*-retinoic acid (17) at doses of 5 to 150 mg/kg reduced the size and activity of the sebaceous gland; *trans*-retinoic acid (16) at 10 and 50 mg/kg reduced the size and lipid content of the gland. At doses higher than 50 mg/kg, toxic effects such as weight loss, anal bleeding, and lethargy were noted. In addition, a high incidence of mortality (30–40%) was observed for the 13-*cis*-retinoic acid (17)-treated (75 mg/kg) animals as well as for the animals treated (50 mg/kg) with *trans*-retinoic acid (16) (100%). Compounds 1–11 showed no activity in this screen.

Table III. Effect of Topically Applied Retinoids on hyperkeratotic Activity[a] and TPA-Induced ODC Activity[b] in the Epidermis

	% inhibition of	
Compound	Urticles at 0.1%[c]	ODC activity[d]
1	0	0
2	0	37
3	0	0
4	0	16
5	0	0
6	15	0
7	50	71
8	10	0
9	0	20
10	0	0
11	0	0
16	55–70	97–99
17	33–51	84

[a] Evaluated by the reduction in size of the utricles in the rhino mouse.
[b] In hairless mice.
[c] Topical application to rhino mouse skin (5–10 animals/group) daily for 4 weeks. Percentage reduction based on vehicle control.
[d] Topical application of 50 nmol retinoid to hairless mouse skin (6 animals/group) 1 hr before 17 nmol TPA in acetone. Percentage inhibition based on acetone control.

Epidermal ODC Activity in Hairless Mice. The inhibitory effect of compounds 1–11 against ODC activity, which is a measure of epidermal hyperplasia, was evaluated in TPA-stimulated hairless mouse skin by the topical application of an acetone solution 1 hr before topical application of TPA. After 4 hrs the animals were sacrificed and ODC activity was determined by the measurement of carbon-14-labeled CO_2 released upon incubation of separated epidermal samples with carbon-14-labeled ornithine. Only four of the compounds tested showed any inhibition (Table III); compound 7 had activity close to that of 13-*cis*-retinoic acid (17).

HVA Test. Only compound 7, which was quite active in both the hyperkeratotic activity and the ODC activity screens, was tested for HVA. The following symptoms were graded on a 0–4 scale (10) (Table IV): weight loss, skin scaling, hair loss, bone fractures, and death. Animals were defined as having HVA syndrome when the addition of the grades from the individually graded signs totaled 3.0 or more.

All animals on 13-*cis*-retinoic acid (17) began to show overt signs of toxicity (unkempt appearance with oily or greasy hair, decreased spontaneous activity) by the fifth dose. During the second week 13-*cis*-retinoic acid (17)-treated groups showed definite hair and body weight losses. With the 400 mg/kg dose, four of nine mice were found dead on day 7 and the remainder had died by day 9. In the group dosed with 200 mg/kg of 13-*cis*-retinoic acid (17), 100% mortality was observed after 14 days. Treatment with *trans*-retinoic acid (16) at 100 mg/kg showed marked body weight loss with a 70% mortality rate. In contrast, animals treated with the lactone 7 could not be differentiated from the vehicle-treated or from the untreated control groups.

Table IV. Hypervitaminosis A Test in Swiss Webster Mice: Comparison of the Effects of 13-*cis*-Retinoic Acid (17) and 11-*cis*,13-*cis*-12-Hydroxymethylretinoic Acid, δ-Lactone (7)

Treatment[a]	Dose (mg/kg)	(*N*)	Mean daily hypervitaminosis A grade ± SD[b]									
			1	2	3	4	5	6	7	8	9	10
Vehicle control	NA	(5)	0	0	0	0	0	0	0	0	0	0
Treated control	NA	(5)	0	0	0	0	0	0	0	0	0	0
17	400	(9)	0	0	0.6 ± 0.5	1 ± 0	2.6 ± 0.5	3.7 ± 1.0	7.1 ± 4.7	8.1 ± 4.6	12 ± 0	12 ± 0
17	200	(9)	0	0.1 ± 0.3	0.1 ± 0.3	0.6 ± 0.5	2.0 ± 0.5	2.8 ± 0.7	3.1 ± 0.6	3.4 ± 0.5	3.7 ± 0.7	4.3 ± 0.8
7	200	(9)	0	0.1 ± 0.3	0.2 ± 0.4	0.4 ± 0.5	0.1 ± 0.3	0	0	0.1 ± 0.3	0	0

[a] All test material suspended in peanut oil and administered intraperitoneally once daily, 5 days/week, for 2 weeks. Peanut oil administered at 8 mL/kg.

[b] Hypervitaminosis A = sum of symptom grades (body weight loss, skin scaling, hair loss), yielding at least 3. Each symptom graded on a 0–4 scale, and death graded as a maximum score of 12. (See Materials and Methods for more details.)

Skin Irritation Models. Skin irritation due to repeat skin application in rabbits and guinea pigs was evaluated for compound 7 compared to compounds 16 and 17 (Tables V and VI). Animals were observed daily during the four week treatment period and for one week thereafter. Test sites were assessed visually for signs of irritation and for scaling using the Draize (11) 0–4 grading method. Both *trans*- and 13-*cis*-retinoic acid caused moderate to severe erythema and scaling in rabbits which peaked around the second week and declined throughout the remaining weeks (Tables V and VI). No evidence of irritation was apparent at the untreated or the vehicle-treated sites. The two acids had equivalent edema and scaling scores but the *trans* acid showed slightly higher erythema scores. In guinea pigs the irritation reactions were delayed, peaking at week 3; less erythema was observed. Both scaling and erythema declined rapidly at week 4. Histological changes observed in rabbits and guinea pigs for both acids included a thickening of the epidermal layer to two to three times normal. Inflammatory infiltrate was present in skins, ranging from minimal to moderate, and was more severe in the application sites. This inflammatory response was accompanied by congestion and hemorrhage. In contrast, the lactone 7 caused minimal to slight erythema and scaling in rabbits and guinea pigs. This reaction peaked at the second or third week and then declined slowly. Epidermal thickening to two to three times normal were also observed for 7, however, it was accompanied by very little or no inflammatory infiltration into the skin.

DISCUSSION

Retinoids are known to reduce the size of sebaceous glands as well as sebum secretion, making them attractive agents for the treatment of skin disorders. However, the mechanism involved remains unknown and since the effectiveness of only a small number of compounds has been reported, no conclusions about the relationship of structure to activity and/or toxicity can be drawn. Since 13-*cis*-retinoic acid (17) has a considerably higher therapeutic index than the isomeric *trans*-acid 16, we had undertaken the examination of a series of 13-*cis*-12-substituted retinoids. Among these were compounds constrained to 13-*cis*-stereochemistry by the incorporation of the 13,14 double bond in a six-membered ring.

The models used as tests for antihyperkeratinization (rhino mouse, topical), antihyperproliferation (ODC, topical), antihyperactive sebaceous gland activity (hamster flank organ, subcutaneous), HVA (ip), and repeat-application irritation (topical) were validated by the results obtained with *trans*- and 13-*cis*-retinoic acid (16 and 17, respectively).

Table V. Skin Irritation in Rabbits and Guinea Pigs: Erythema Resulting from Repeat Application of 13-*cis*-Retinoic Acid (17), *trans*-Retinoic Acid (16), and 11-*cis*-12-Hydroxymethylretinoic Acid, δ-Lactone (7)

Treatment[a]	Species (*N*)	Weekly mean cumulative erythema score ± SD[b]				
		1	2	3	4	5
17	Rabbit (4)	15 ± 3.1	24 ± 1.6	14 ± 2.6	12 ± 3.4	9 ± 2.3
16		17 ± 1.7	25 ± 1.3	17 ± 2.4	16 ± 3.6	12 ± 2.6
7		8.9 ± 1.5	12.2 ± 4.4	8.1 ± 3.0	9.0 ± 4.0	2.2 ± 1.8
17	Guinea pig (4)	1 ± 1.3	8 ± 3.6	17 ± 2.8	16 ± 2.9	8 ± 0.7
16		2 ± 1.8	10 ± 4.5	16 ± 3.7	17 ± 3.1	10 ± 2.3
7		0	0	4.0 ± 2.7	8.0 ± 1.5	0

[a] Skin sites (4 × 4 cm) of animals were treated with 0.2 mL/site of a 0.1% (w/v) solution, 7 days/week, for 4 weeks. Week 5 was a recovery week.

[b] Scores were determined by daily grading of the treated site, on a scale of 0–4 erythema, according to the Draize scoring system: 0 = none, 1 = minimal, 2 = slight, 3 = moderate, and 4 = severe. Cumulative weekly scores were obtained and the mean was determined for each site. Maximum cumulative score = 28.

Table VI. Skin Irritation in Rabbits and Guinea Pigs: Scaling Resulting from Repeat Application of 13-*cis*-Retinoic Acid (17), *trans*-Retinoic Acid (16), and 11-*cis*-12-Hydroxymethylretinoic Acid, δ-lactone (7)

		Weekly mean cumulative erythema score ± SD[b]				
Treatment[a]	Species (*N*)	1	2	3	4	5
17	Rabbit (4)	0.2 ± 0.4	21.7 ± 6.4	21.4 ± 9.0	14.1 ± 10.4	7.0 ± 4.3
16		2.5 ± 1.6	24.2 ± 3.4	21.5 ± 7.6	21.4 ± 6.6	14.2 ± 2.7
7		1.6 ± 1.0	6.7 ± 1.4	3.6 ± 1.6	3.5 ± 3.0	0
17	Guinea pig (4)	0	8.6 ± 3.0	24.1 ± 2.8	21.9 ± 5.8	12.2 ± 3.0
16		0	10.9 ± 5.2	24.6 ± 3.9	24.7 ± 3.4	10.2 ± 1.5
7		0	0	4.8 ± 1.7	7.0 ± 0	3.5 ± 0.5

[a] Skin sites (4 × 4 cm) of animals were treated with 0.2 mL/site of a 0.1% (w/v) solution, 7 days/week, for 4 weeks. Week 5 was a recovery week.

[b] Scores were determined by daily grading of the treated site, on a scale of 0–4: 0 = none, 1 = minimal, 2 = slight, 3 = moderate, and 4 = severe. Cumulative weekly scores were obtained and the mean was determined for each site. Maximum cumulative score = 28.

Thus, in agreement with reported results (20), *trans*-retinoic acid (16), with a mean ED_{30} of 0.001%, is more potent than 13-*cis*-retinoic acid (17), with a mean ED_{30} of 0.015%, in reducing the size of utricles in the rhino mouse test. As expected, both 16 and 17 showed activity in the hamster flank organ screen and in ODC inhibition. Similarly, symptoms of both HVA and skin irritation due to repeat applications appeared to be more severe with *trans*-retinoic acid (16) than with the 13-*cis* isomer (17), as expected (1).

Since retinoids 1–11 showed no activity in the flank organ test, the usefulness of this model as a screen for assessing retinoid effects on sebaceous gland size and activity may be questionable. However, it should be borne in mind that as a group, retinoids 1–11 showed little activity in the other assays as well. In fact, only one compound, the lactone 7, was active both in reducing the size of utricles and in inhibiting TPA-induced ODC. Thus, the lactone 8 and the anilide 6, which had some activity in utricles reduction, were devoid of activity in the inhibition of TPA-induced ODC. Conversely, the monomethyl esters 2 and 4 and the anhydride 9 exhibited some activity in ODC inhibition but had no effect on utricle reduction. Interestingly, although the monoesters 2 and 4 had activity, both the diacid 1 and the dimethyl ester 3 were completely inactive in both assays.

The results obtained with the lactone 7 were of particular interest. Although its activity was slightly less than that observed for either *trans*- or 13-*cis*-retinoic acid (16 and 17, respectively) in both utricle reduction and ODC inhibition, it exhibited no systemic toxicity when injected intraperitoneally (200 mg/kg) once daily for 2 weeks to Swiss Webster mice. It is also noteworthy that the apparent systemic effects elicited by 16 and 17 on the control-treated contralateral side in the rhino mouse test were absent in the animals treated with the lactone 7. Similarly, topical application for 4 weeks to rabbits or guinea pigs produced directionally less local irritation than either 16 or 17. These results suggest that it may be possible to separate the beneficial therapeutic effects of retinoids from their toxicity.

CONCLUSIONS

The activities of 12-substituted retinoic acid analogues in animal models suggests that structural modifications may lead to selective activity. Specifically, (a) whereas both isomers of retinoic acid (16 and 17) reduced the size of hyperactive sebaceous glands, none of the 12-substituted analogues did; (b) some compounds active in utricle reduction (6 and 8) were inactive in the inhibition of TPA-induced ODC, while others (2, 4, 9) exhibited the opposite selectivity; and (c) an analogue (7) with good activity in both utricle reduction and ODC inhibition was devoid of topical and systemic toxicity.

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