Terminal Bifunctional Retinoids. Synthesis and Evaluations Related to Cancer Chemopreventive Activity

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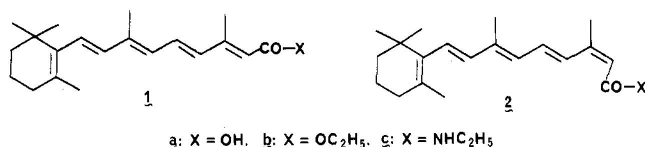
Retinoids that have two functional groups at the side-chain terminus have been synthesized. The two terminal functional groups are combinations of the carboxyl, carbethoxy, and *N*-(ethylamino)carbonyl groups. The synthesis route is based on the sodium amide catalyzed condensation of (*E,E*)-β-ionylideneacetaldehyde with diethyl isopropylidenemalonate. Ethyl 14-carboxyretinoate (6), the initial bifunctional analogue, undergoes isomerization in unbuffered aqueous ethanol and reaches a state of equilibrium with ethyl 14-carboxy-13-*cis*-retinoate. Both of the possible amide–esters and amide–acids were obtained. The structures of the isomeric bifunctional analogues were established by studies of nuclear Overhauser effects. The bifunctional analogues induce differentiation of mouse embryonal carcinoma cells, and those analogues that have a free carboxyl group bind to cellular retinoic acid binding protein.

Numerous studies have demonstrated that *all-trans*-retinoic acid (RA, **1a**), 13-*cis*-retinoic acid (13-*cis*-RA, **2a**), and many of their derivatives (e.g., **1b**, **1c**, **2b**, **2c**) and analogues produce effects in various biological systems and bioassays that may be predictive for the capacity of retinoids to prevent or reverse the neoplastic transformation process.[1] Such active retinoids prevent, reverse, or alter lesions induced in certain cultured organs by carcinogens or by vitamin A deficiency.[3-8] Furthermore, cancer chemopreventive activity[1] in vivo by retinoic acids and their derivatives has been reported. For example, 13-*cis*-RA suppresses carcinogen-induced bladder cancer,[9,10] and RA inhibits carcinogen-induced skin papillomas and carcinomas.[11] Retinamides, both all-trans and 13-cis, are prominent among the retinoids that have chemopreventive activity in vivo as well as in vitro. Certain retinamides suppress carcinogenesis in the bladder,[12-14] breast,[14] or pancreas[15,16] in animal models. Both *all-trans-N*-ethylretinamide (**1c**) and 13-*cis*-*N*-ethylretinamide (**2c**) are active in vivo[12-14] and are much less toxic[17] and teratogenic[18] than RA and 13-*cis*-RA. We describe herein a modification of the terminal, polar end of the retinoid structure that combines the functional groups of the retinoic acids (**1a**, **2a**) and the ethylretinamides (**1c**, **2c**) into a single structure. Such bifunctional retinoids may be regarded as hybrids of *all-trans*- and 13-*cis*-retinoic acids and retinamides. 12-Carboxyretinoic acids have been reported previously.[19]



Chemistry. Synthesis of these hybrid analogues begins with a sodium amide catalyzed reaction of (*E,E*)-ionylideneacetaldehyde (**3**) with diethyl isopropylidenemalonate (**4**). This condensation is analogous to the method of Matsui et al.[20] for preparing 13-*cis*-RA from **3** and ethyl isopropylideneacetate. We postulated that a lactone intermediate[21] (**5**) would be formed and that the lactone would open to give a 13-*cis*-carboxyl group (**6**). The intact ethyl ester group would then be positioned analogously to the ester group of ethyl *all-trans*-retinoate (**1b**). Therefore, ester–acid **6**, rather that its isomer **7**, would be obtained, and the two different functional groups could be modified separately.

A monoacid–monoester (**6**) was, indeed, isolated after reaction of **3** and **4** and was converted conventionally to dicarboxylic acid[22] **8** (a hybrid of RA and 13-*cis*-RA) and also, via an ester–acid chloride (**10**), to the corresponding

(1) The use of chemicals to arrest or reverse the progression of a carcinogenic event to full malignancy has been designated *chemoprevention* by Sporn et al.[2] The term *retinoids* was employed to encompass members of the vitamin A group of compounds, their derivatives, and their analogues.[2]

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(21) The isolation of lactones following lithium diisopropylamide catalyzed reactions of methyl isopropylideneacetate with ketones and aldehydes, including 3, has been reported: Dugger, R. W.; Heathcock, C. H. *J. Org. Chem.* 1980, *45*, 1181–1185.
(22) The preparation of dicarboxylic acid 8 from 3 and 4 in an ethanol solution of potassium hydroxide was claimed.[23] Geometric isomerism was not investigated, and the only physical data reported (the melting point and ultraviolet absorption maximum) differ from the data obtained from our specimen of 8.
(23) Robeson, C. D. U.S. Pat. 2662914, December 15, 1953.

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Scheme I

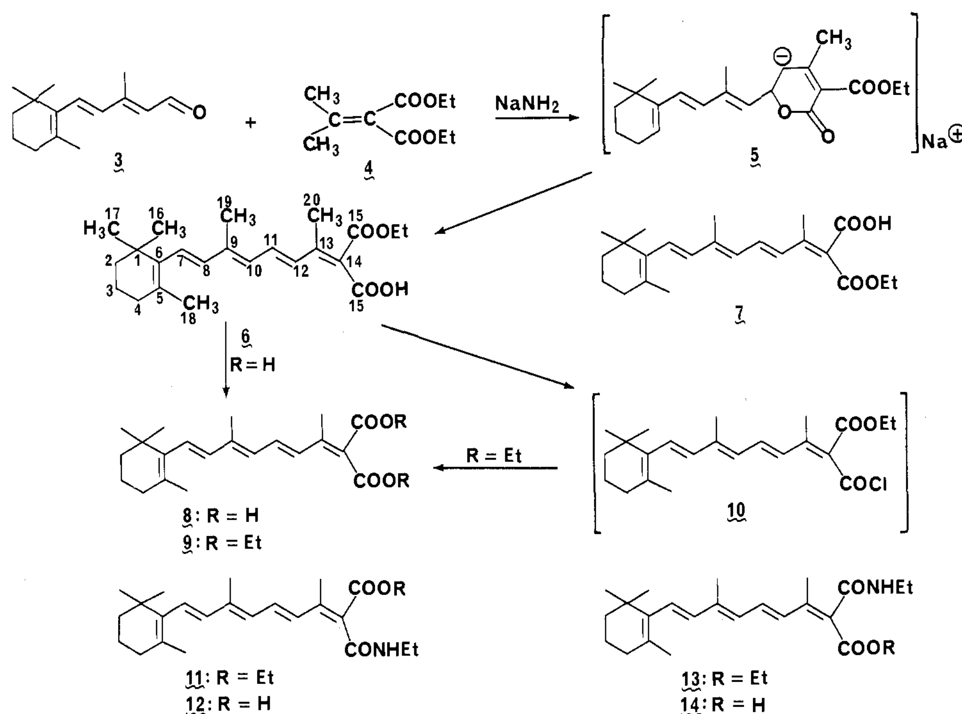

Table I. Proton Chemical Shift Assignments[a,b] and Nuclear Overhauser Enhancements

compd	chemical shift, ppm, of proton on carbon no. and NOE[c] (%)							
	10	11	12	19	20	CH_2 amide	NH amide	CH_2 ester
6	6.25	7.16 (7.1)	7.37 (2.4)	2.01	2.20			4.30 (0.5)
7	6.17	7.18 (7.0)	6.73	2.02	2.36			4.37
8[d]	6.32	7.14	6.98	2.00	2.17			
9	6.19	7.11	6.99	2.01	2.24			4.27, 4.26
11	6.16	7.06 (11.9)	6.62 (1.4)	2.00	2.29	3.42	5.82	4.22 (0.5)
13	6.20	7.04 (7.2)	7.37 (1.1)	2.00	2.13	3.40	5.87 (1.1)	4.23
12	6.15	7.13 (7.0)	6.60	2.02	2.35	3.45	6.07	
14	6.23	7.09 (9.2)	7.46 (2.0)	2.00	2.17	3.43 (0.5)	5.97 (1.6)	

[a] Nondegassed solutions in $CDCl_3$ with TMS as an internal reference. [b] Assignments were made by selective decoupling. [c] NOE enhancements in parentheses. NOE experiments were carried out by irradiation of the C-20 methyl hydrogens in compounds **6**, **7**, **11**–**14**. [d] Me_2SO-d_6 with TMS as an internal reference.

diester (**9**). The ester–acid chloride (**10**) was treated in situ with ethylamine to obtain the hybrid of *all-trans*-RA ethyl ester and 13-*cis*-ethylamide (**11**). However, two major products, the isomeric amide–esters **11** and **13**, were obtained rather than only the expected amide–ester (**11**). Therefore, partial isomerization had occurred either during formation of the acid chloride or during the subsequent amidation step. Amide–esters **11** and **13** were separated from each other, and from minor components of the total product, by flash chromatography. Alkaline hydrolysis of each purified amide–ester produced an amide–acid as the major product, but each amide–acid (**12** or **14**) was contaminated with the other; therefore, some isomerization had occurred again. Pure specimens of each amide–acid (**12** or **14**) were obtained by semipreparative HPLC.

Originally, the synthesis of retinamides **13** and **14** by a different route had been planned. However, the synthesis route (Scheme I) that had been expected to furnish one set of isomers (**11**, **12**) produced both sets. Moreover, we observed during NMR determinations that the ester–acid (assumed to be **6**) would undergo partial isomerization to the other ester–acid (assumed to be **7**) during several days in unstabilized deuteriated chloroform.[24] Observations by HPLC of the isolated ester–acid in an ethanol–water solution (1:1) then showed that it isomerizes in the unbuffered solution and that an equilibrium concentration of about 49:51 is attained in less than 24 h (below). Consequently, it became necessary to confirm the structure of the initially isolated bifunctional retinoid, the ester–acid, and to assign structures to the pairs of amide–esters (**11**, **13**) and amide–acids (**12**, **14**).

Proton NMR analyses and studies of nuclear Overhauser effects (NOE) of the initially isolated ester–acid, the pair of amide–esters, and the pair of amide–acids were carried out. The chemical shifts (Table I) of the protons at positions C-1 through C-11 and the coupling constants are in good agreement with the extended transoid conformation of the polyene chain as in RA (**1a**), 13-*cis*-RA (**2a**), and the corresponding *all-trans*-RA and 13-*cis*-RA ethyl esters (**1b**, **2b**) and *N*-ethylamides (**1c**, **2c**). The chemical shifts arising from the C-12 proton of the three monofunctional *all-trans*-retinoids (**1a–c**) appear at δ 6.22–6.31, whereas the shifts of the corresponding 13-*cis*-retinoids (**2a–c**) appear at δ 7.77–7.84.[25,26] The chemical shifts of the C-12 proton of the diacid (**8**) and the diethyl ester (**9**) are near

(24) There was no evidence of isomerization of any of the other bifunctional analogues during determinations of their NMR spectra or during the NOE studies.

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the midpoint between these ranges (e.g., δ 6.98), but there are large differences in the C-12 proton chemical shifts when there are two different carbonyl groups at C-14. It is apparent that the chemical shifts, summarized in Table I, of the proximal protons do not permit unambiguous assignments of the configuration of the 13,14 double bond; that is, the chemical-shift data do not show unequivocally which carbonyl group at C-14 is trans to the polyene side chain in the pair of ester–acids (**6**, **7**), in the pair of amide–esters (**11**, **13**), or in the pair of amide–acids (**12**, **14**).

Nuclear Overhauser effect experiments were carried out to resolve the 13,14 double bond configuration. The relaxation of a proton may depend significantly on through-space dipolar–dipolar coupling with a second set of protons, and its NMR signal intensity may be enhanced when the set of protons that assists in relaxation is saturated by irradiation.[27] The phenomenon, termed NOE, is highly dependent on the distance between protons. The NOE experiments (Table I) in conjunction with Dreiding models allowed us to make unambiguous assignments of which carbonyl group is cis to the protons on C-20 in each pair of bifunctional retinoids. Irradiation of the protons on C-20 of the ester–amide (**13**) gave a 1.1% enhancement of the NH at 5.87 ppm. Therefore, the ethylamide is closer to the protons on C-20, and the 13,14 double bond of **13** has a trans amide and a cis ester relative to the polyene chain. Irradiation of the protons on C-20 of ester–amide **11** gave a 0.5% enhancement of the CH_2 of the ethyl ester at 4.22 ppm.[28] Therefore, the ester is closer to the protons on C-20, and the 13,14 double bond in **11** has a trans ester and a cis amide configuration relative to the polyene chain.

The enhancements observed for the protons H-11 and H-12 upon irradiation of the protons on C-20 in the amide–esters (**11**, **13**) can also provide information about the orientation of the C-11=C-12—C-13=C-14 carbons of the polyene chain. If the molecule adopted a planar 12-s-trans conformation around the 12,13 single bond, one would expect no enhancement of H-12 and only enhancement of H-11 on irradiation of protons on C-20. The observed NOE of 1.4% for H-12 and 11.9% for H-11 in **11** and 1.1% for H-12 and 7.2% for H-11 in **13** may indicate an equilibrium between a planar 12-s-cis and a planar 12-s-trans conformation around the 12,13 single bond,[29] or, more probably, it may indicate that **11** and **13** prefer a nonplanar 12-s-trans conformation.[30] A twisting of both the 11,12 and 13,14 double bonds around the 12,13 single bond would relieve steric crowding caused by the highly substituted 13,14 double bond.

Similar NOE experiments carried out on the other two pairs of bifunctional retinoids, the ester–acids (**6**, **7**) and the amide–acids (**12**, **14**) allowed us to determine their configuration (Table I).

Because of the evidence, outlined above, of isomerization of these bifunctional analogues, it was desirable to determine whether they might isomerize or equilibrate during bioassays. Aliquot portions of solutions of the bifunctional analogues were analyzed by HPLC. As mentioned above, ester–acid **6** began to isomerize in 50% ethanol within several minutes to ester–acid **7**. Equilibrium was reached between 7 and 24 h, and the relative amounts of **6** and **7** at equilibrium were about 49% and 51%, respectively. If equilibration results from an acid-catalyzed (free carboxyl group) hydrolysis of the trans ethyl ester and esterification of the 13-*cis*-carboxyl in 50% ethanol, some of the dicarboxylic acid (**8**) would be expected in the equilibrium mixture. The dicarboxylic acid was not detected in an aliquot portion analyzed (at 73–74 h) well after equilibrium had been reached. In addition, the sums of the relative amounts of **6** and **7** and the sums of the integrated areas were consistent with the presence of only **6** and **7**. It seemed likely, therefore, that the equilibration of **6** and **7** is the result of an acid–base catalyzed transesterification in which the ethoxy group does not separate completely from the two carbonyl groups. If so, reduction of the proton concentration, as in a buffered solution, should stabilize **6**. Ester–acid **6** was, indeed, stabilized in 1:1 ethanol–phosphate buffer (pH 7); there was no significant change within 53 h and little, if any, during 5 days. Amide–esters **11** and **13** changed only slightly, if any, during periods of 1–2 weeks in 50% ethanol or 1:1 ethanol–phosphate buffer (pH 7). Amide–acids **12** and **14** were essentially unchanged during 60 days, or longer, in 1:1 ethanol–phosphate buffer (pH 7) or in DMSO. These determinations indicated that the bifunctional analogues would remain essentially unchanged in bioassay media.

Table II. Bioassays of Bifunctional Analogues of Retinoic Acid

compound	relative CRABP binding:[a] % of RA	differentiation of F9 cells:[b] ED_{50}, M
14-carboxyretinoic acid 15-ethyl ester (**6**)	90	10×10^{-10}
14-carboxyretinoic acid (**8**)	65	5×10^{-10}
14-(ethoxycarbonyl)retinoic acid ethyl ester (**9**)	0	45×10^{-10}
14-[(ethylamino)carbonyl]retinoic acid ethyl ester (**11**)	0	60×10^{-10}
14-[(ethylamino)carbonyl]-13-*cis*-retinoic acid ethyl ester (**13**)	0	60×10^{-10}
14-[(ethylamino)carbonyl]retinoic acid (**12**)	55	6×10^{-10}
14-[(ethylamino)carbonyl]-13-*cis*-retinoic acid (**14**)	55	5×10^{-10}
retinoic acid (**1a**)	100	1×10^{-10}
13-*cis*-retinoic acid (**2a**)	100	3×10^{-10}

[a] CRABP from chick embryo skin. [b] The ED_{50} values were determined from averages of triplicate experiments (Experimental Section); the standard deviation of error was less than 5%.

Biological Evaluation. Retinoic acid binds intracellularly to cellular retinoic acid binding protein (CRABP)[31-35] and may be translocated within the cell as the RA-CRABP complex.[34] It is believed that CRABP may have a major role in mediating some of the biological effects of retinoic acid.[33,35-37] Analogues of RA also bind;

(27) Noggle, J. H.; Schirmer, R. E. *The Nuclear Overhauser Effect*; Academic: New York, 1971.

(28) Because the average distances between C-20 and the NH or CH_2 parts of the amide or ester groups are large plus the fact that rotation about the C-14–C-15 bond is not completely restricted, irradiation of C-20 should not produce large nuclear Overhauser enhancements at the NH or CH_2 parts.

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(33) Chytil, F.; Ong, D. E. *Nature (London)* **1976**, *260*, 49–51.

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evidently, the presence of a free carboxyl group is a requirement for binding to avian and mammalian CRABP.[35-38] One of the purposes of this work a priori was to investigate the binding of terminal bifunctional retinoids to retinoid-binding proteins. Since monofunctional retinamides do not bind, it was thought that bifunctional analogues **12** and **14** might constitute a means of achieving binding of retinamides to CRABP. On the other hand, the presence of bulky substituents α to the carboxyl group in **6**, **8**, **12**, and **14** might interfere with binding. The results listed in Table II show that those analogues (**6**, **8**, **12**, and **14**) having a free carboxyl group do, indeed, bind to CRABP, the ester–acid (**6**) being a very effective binder. In keeping with earlier observations, analogues (**9**, **11**, **13**) that lack a free carboxyl group do not bind.

Induction of differentiation of cancer cells is a potential mode of cancer chemoprevention. It has been reported that RA, 13-*cis*-RA, and some other retinoids are highly active inducers of differentiation of certain neoplastic cells to terminally differentiated cells.[38-40] Activity varies with the structure of the retinoid, RA being one of the most active retinoids. Strickland and co-workers[39,41] showed that a mouse embryonal carcinoma cell line, F9 cells, may be induced by certain retinoids to differentiate into parietal endoderm. Elevated release of plasminogen activator by F9 cells in the presence of retinoids is regarded as a marker for differentiation.[39,41] Induction of differentiation of F9 cells by the bifunctional analogues was evaluated by spectrophotometric determination of the release of plasminogen activator. These results are summarized in Table II as values of ED_{50}, and the values of ED_{50} for RA and 13-*cis*-RA are listed for comparison. All of the bifunctional analogues induce differentiation at low concentrations ($ED_{50} = 10^{-9}$–10^{-10} M) and, therefore, are very effective inducers of differentiation of mouse F9 embryonal carcinoma cells, although they are somewhat less active than is RA. The most active analogues have a free carboxyl group. (Under the conditions of this assay, ester derivatives might be hydrolyzed partially to the carboxylic acids.)

Experimental Section

General Methods. All operations involved in the preparation, isolation, purification, and transfer of retinoids were performed in an atmosphere, or under a current, of nitrogen or argon. All such operations were also performed in dim light or in photographic darkroom light and, insofar as possible, with containers wrapped with aluminum foil or with black cloths. All retinoids were stored in an atmosphere of argon or nitrogen in hermetically sealed containers at −20 or −80 °C.

Decomposition and melting temperatures (mp) were determined in capillary tubes heated in a Mel-Temp apparatus. Ultraviolet spectra (UV) were determined with ethanol solutions, unless indicated otherwise, and were recorded with a Cary Model 17 spectrophotometer; absorption maxima are reported in nanometers; sh = shoulder. Infrared spectra (IR) were recorded with a Nicolet 10MXE Fourier transform spectrometer from samples in pressed potassium bromide disks. Only prominent bands are listed, and positions are given at the nearest 5 cm^{-1}; vs = very strong, br = broad, sh = shoulder. Mass spectral data (MS) were taken from low-resolution, electron-impact (EI) spectra determined at 70 eV with a Varian/MAT 311A spectrometer. The peaks listed are those arising from the molecular ion (M), those attributable to the loss of certain fragments (M minus a fragment), and some other prominent peaks. The proton NMR spectra in $CDCl_3$ with tetramethylsilane as an internal reference were determined on a Nicolet NT 300NB spectrometer operating at 300.635 MHz. Chemical shifts (δ) quoted in the case of multiplets are measured from the approximate center. The NOE experiments were conducted on nondegassed solutions in $CDCl_3$. To minimize the effects of magnetic perturbations with the sample nonspinning, eight FID's (free-induction decays) were acquired with the decoupler set at a desired frequency, and eight FID's were recorded with the decoupler off-resonance. The process was repeated until 3600 FID's had been accumulated. Subsequent subtraction of the two spectra afforded the net enhancement. Thin-layer chromatography (TLC) was performed on plates of fluorescing silica gel (silica gel GF), and developed plates were examined with UV lamps (254 and 365 nm).

Flash chromatography[42] was carried out with commercially manufactured columns. High-pressure liquid chromatography (HPLC) was performed with an apparatus comprised of Waters Associates components and a Hewlett-Packard Model 3380-S integrator or with a Hewlett-Packard Model 1084B system. Unless indicated otherwise, HPLC was the reverse-phase kind and was performed on columns of 5-μm particles of octadecylsilyl silica (octadecylsilane chemically bonded to silica; Spherisorb ODS, 5 μm). The flow rate was 1 mL/min, and the elution pattern of retinoids was monitored continuously by UV at 340 nm; retention times are in minutes. The injection solvent and the mobile phase are specified at appropriate places in the procedures described below. Normal-phase HPLC was performed in the same way on columns of spherical silica gel (μPorasil).

Ethyl 14-Carboxyretinoate (6). Diethyl isopropylidenemalonate (**4**) was prepared by the procedure of Cope and Hancock;[43] bp 122–123 °C (7–8 mm) [lit. bp 111–113 °C (9 mm)].

A suspension of sodium amide in liquid ammonia was prepared (in a dry, three-necked, round-bottom flask fitted with a motor-driven stirrer, a stopper, and a dry ice–acetone condenser fitted with a drying-agent tube) by adding 6.75 g (0.293 mol) of sodium to about 400 mL of liquid ammonia containing a small amount (about 10 mg) of ferric nitrate nonahydrate.[44] Diethyl isopropylidenemalonate (**4**, 13 g) was added dropwise (from an addition funnel that replaced the stopper[45]) during 10 min to the cold, vigorously stirred mixture. The cold mixture was stirred for 15 min, and then a solution of 16 g (73.4 mmol) of β-ionylideneacetaldehyde[45,46] (**3**) in 131 g of **4** [total amount of **4**, 144 g (0.72 mol)] was added during 0.5 h. The mixture, which changed in color from green to orange during approximately 1 h, was stirred for 2 h with liquid ammonia refluxing in the dry ice–acetone condenser. The dry ice–acetone mixture was then removed from the condenser, most of the ammonia was allowed to evaporate (ca. 4 h), stirring was discontinued, and the mixture (protected from atmospheric moisture) was kept at room temperature overnight. To remove additional ammonia, a current of nitrogen was passed over the stirred, dark-orange oil for 1 h at room temperature and for 0.5 h at 40 °C. Ether (400 mL) was added, the solution was placed in an ice bath, and cold (0 °C) water was added (dropwise initially and then faster) to the stirred mixture. The resulting mixture was stirred for 15 min, the water layer was separated,[48] the ether layer was washed with water (200 mL), and the resulting water layer was cooled in an ice bath and acidified. The acidic aqueous mixture was washed with ether to extract an orange precipitate, and the ether extract was dried ($MgSO_4$) and concentrated to an orange-red, gummy solid; weight, 23.5 g (86% yield as **6**). The residue was triturated with hexane (3 × 60 mL),

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(45) Each time that the flask was opened briefly, a stream of dry argon was passed over the reaction mixture.
(46) Specimens of **3** were prepared by reducing β-ionylideneacetonitrile with diisobutylaluminum hydride.[47]
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(48) Acidification of this water layer produced only cloudiness.

and the resulting orange solid was collected by filtration, washed with hexane, and dried in vacuo over phosphorus pentoxide: yield, 10.1 g (37%); HPLC (retention time), 96.7% (4.51) of **6** + minor components of 0.9% (4.03), 1.2% (5.14), 0.4% (5.41), and 0.23% (6.33) (injection solvent, methanol; mobile phase, 85:15 acetonitrile–1% aqueous ammonium acetate). A solution (under argon) of the solid in hot ethanol (85 mL) was allowed to cool to room temperature and then chilled. The precipitate was collected by filtration, washed with ethanol, and dried in vacuo: weight, 5.3 g (19%); mp 138–140 °C dec (inserted at 126 °C); HPLC as above (retention time), 98.4% (4.56) of **6** + minor components of 0.46% (4.07), 0.1% (5.19), 0.1% (5.45), and 0.66% (6.39). A 1-g portion was recyrstallized from ethanol, as before: recovery of yellow crystals, 670 mg; mp 140–142 °C dec (inserted at 126 °C); HPLC (retention time), 99.4% (9.96) (injection solvent, MeOH; mobile phase 1:1 MeCN–1% aqueous NH_4OAc); MS (direct-probe temperature, 20 °C), *m/z* 372 (M), 354 (M – H_2O), 339 (M – H_2O – CH_3), 326 (M – EtOH), 308 (M – EtOH – H_2O), 293 (M – EtOH – H_2O – CH_3); UV max 358 nm (ϵ 42400) in methanol; IR (strong and medium bands) 2960, 2930, 2910, 2860, 2825, 1735, 1685, 1570, 1545, 1440, 1400, 1260, 1240 (vs), 1225 (sh), 1065, 1050, 970 cm^{-1}. Anal. ($C_{23}H_{32}O_4$) C, H.

14-Carboxyretinoic Acid (8). A solution of 1.0 g (2.69 mmol) of **6**, potassium hydroxide (87%, 519 mg, 8.1 mmol), 3 mL of water, and 12 mL of ethanol was boiled under reflux for 2 h, cooled, and concentrated under reduced pressure to remove most of the ethanol. The aqueous solution was extracted with ether (2 × 30 mL), placed in an ice bath, and acidified with dilute hydrochloric acid. The aqueous mixture, containing a yellow precipitate, was extracted with ether (3 × 75 mL) and with ethyl acetate (40 mL). The extraction solutions were combined, and the resulting solution was washed twice with brine, dried ($MgSO_4$), and concentrated under reduced pressure to an orange solid: yield, 880 mg (95%); HPLC as below, 99.2% (t_R 10.7 min). This material was recrystallized from 1:1 ethanol–water (ca. 30 mL): recovery of orange crystals, 69%; mp 195–198 °C dec (inserted at 120 °C); HPLC 100% (t_R 10.8 min) (injection solvent, MeOH; mobile phase, 80:20 MeOH–1% acetic acid); MS (direct-probe temperature, 20 °C), *m/z* 344 (M), 329 (M – CH_3), 326 (M – H_2O), 300 (M – CO_2), 285 (300 – CH_3), 255, 239; UV max 358 nm (ϵ 42000), 245 (ϵ 7400) in methanol; IR (strong bands) 2960, 2955 (sh), 2930, 2865, 1685 (vs), 1570, 1550, 1280, 1260, 1245 (vs), cm^{-1}. Anal. ($C_{21}H_{28}O_4$) C, H. Robeson[21,22] reported mp 185 °C and UV max 362 nm for material assigned structure **8**.

Ethyl 14-(Ethoxycarbonyl)retinoate (9). Phosphorus trichloride (92 mg, 0.67 mmol) was added to a stirred mixture of 500 mg (1.34 mmol) of **6** in 20 mL of dry benzene. The mixture soon became homogeneous. The orange solution of **10** was stirred at room temperature for 2 h and was then added dropwise during 10 min to an ethanol solution, placed in an ice bath, of sodium ethoxide (prepared from 0.5 g of sodium and 50 mL of ethanol). The reaction mixture was stirred for 0.5 h and then diluted with ether. The solution was washed with saturated aqueous sodium bicarbonate (2 × 20 mL) and with sodium chloride solution (2 × 20 mL). The organic layer, under argon, was dried ($MgSO_4$) overnight in a freezer, filtered, and concentrated under reduced pressure to a yellow oil (493 mg). The oil was dissolved in 9:1 hexane–ethyl acetate and purified by flash chromatography on silica gel (20 g). Fractions containing **9**, identified by TLC, were combined and concentrated under reduced pressure to a yellow oil: yield, 380 mg (71%); HPLC (retention time), 98.3% (10.6) of **9** + minor components of 0.8% (9.4) and 0.9% (10 min) (injection solvent, MeCN; mobile phase, 85:15 MeCN–1% aqueous NH_4OAc); MS (direct probe temperature, 20 °C), *m/z* 400 (M), 385 (M – CH_3), 354 (M – OEt – H), 339 (354 – CH_3), 327 (M – COOEt), 311, 308, 293, 281, 265, 211; UV max 371 nm (ϵ 41200) and 245 (ϵ 7400) in ethanol; IR (strong and medium bands) 2975, 2955, 2925, 2905 (sh), 2860, 1730 (vs), 1715 (vs), 1580, 1555, 1445, 1365, 1300, 1215 (vs, br), 1060, 970 cm^{-1}. Anal. ($C_{25}H_{36}O_4$) C, H.

Ethyl 14-[(Ethylamino)carbonyl]retinoate (11) and Ethyl 14-[(Ethylamino)carbonyl]-13-*cis*-retinoate (13). Phosphorus trichloride (0.81 g, 5.9 mmol) was added to a well-stirred mixture (in an ice bath) of 3.12 g (8.4 mmol) of **6** and 50 mL of dry toluene. The mixture was stirred at 0–5 °C for 1 h, warmed to 30 °C to complete dissolution of **6**, and stirred for 1 h at 25–28 °C. The solution was placed again in an ice bath, a solution of 1.6 g of ethylamine in 50 mL of dry toluene was added, and the mixture was stirred for 0.5 h. Water was added, the mixture was stirred (20 min), and the water layer was separated and washed with ether (2 × 100 mL). The toluene and ether layers were combined, and the organic solution was washed with aqueous sodium chloride (4 × 25 mL), dried ($MgSO_4$), and concentrated under reduced pressure to an orange glass: yield, 3.1 g (93% as an ester–amide). A solution of 2.95 g of the glass in 2:1 heptane–ethyl acetate was poured onto a flash chromatographic column of silica gel (400 g), and the column was developed and eluted with heptane–ethyl acetate (2:1). When the elution of retinoids (monitored by TLC) commenced, eluent fractions (15–20 mL) were collected and were combined, on the basis of TLC patterns, into larger fractions that were concentrated to dryness under reduced pressure (product fraction). The product fractions and their composition, as determined by HPLC, are summarized in Table III. Normal-phase HPLC[49] analyses of fractions A and I were performed by injecting solutions of these fractions in 70:30 hexane–ethyl acetate, which was also the mobile phase; impurity peaks were not observed.

NMR studies of product fraction A and of related compounds (Table I) showed that the compound of fraction A has structure **11**: MS (direct-probe temperature, 20 °C), *m/z* 399 (M), 384 (M – CH_3), 354 (M – OC_2H_5), 339 (M – OC_2H_5 – CH_3), 328, 326, 308, 293; UV max 367 nm (ϵ 43400) and 243 (ϵ 6500) in ethanol; IR (strong and medium bands) 3350 (sharp), 2990, 2975 (sh), 2970, 2960, 2930, 2905, 2865, 1690, 1660 (vs), 1575, 1545, 1535, 1455, 1445, 1305, 1265, 1255, 1230 (vs), 1055, 975, 960 cm^{-1}. Anal. ($C_{25}H_{37}NO_3$) C, H, N.

NMR studies of product fraction I (Table I) and of related compounds showed that the compound of fraction I has structure **13**: MS (direct-probe temperature, 20 °C), *m/z* 399 (M), 384 (M – CH_3), 354 (M – OC_2H_5), 339 (M – OC_2H_5 – CH_3), 328, 326, 308, 293; UV max 367 (ϵ 40900) and 245 (ϵ 9500) in ethanol; IR (strong and medium bands) 3285, 2970, 2960, 2955, 2930, 2900, 2865, 1710 (vs), 1635, 1600, 1595, 1575, 1555, 1445, 1435, 1320, 1275, 1215 (vs), 1200, 1060, 975 cm^{-1}. Anal. ($C_{25}H_{37}NO_3$) C, H, N.

Initially, methanol was the injection solvent for HPLC analyses of all of the ester–amide fractions. Analyses of methanol solutions of fractions A–I (Table III) by normal-phase HPLC[49] were performed under the same conditions. These analyses indicated that fraction A was 99.5% **11**, that fraction B was 97.3% **11**, and that the remaining fractions also contained **11**, the amounts declining from about 84% in fraction C to about 0.5% in the last three product fractions (G–I). The small amounts of **11** detected in the later fractions might have resulted from isomerization of **13** to **11** in the methanol solutions that were injected. Product fractions F–H contained 93–96% of a component that was identical with the ester–amide in fraction I, which was shown subsequently by NMR analysis to be **13**. With methanol as the injection solvent, fraction I assayed 94.8% **13**.

14-[(Ethylamino)carbonyl]retinoic Acid (12). A solution of 183 mg (2.78 mmol) of potassium hydroxide in 6.5 mL of 80% ethanol was added to a stirred solution of 370 mg of a specimen of impure ester-amide **11** (fraction C, containing about 84% of **11**) in 6 mL of ethanol. The solution was boiled under gentle reflux during 40 min, chilled in an ice bath, and diluted with 15 mL of water. The resulting solution was acidified to pH 3 with dilute hydrochloric acid, most of the ethanol was evaporated under reduced pressure, and the aqueous residue was extracted twice with 30-mL portions of ether. The ether extract was washed with water and with aqueous sodium chloride, dried ($MgSO_4$), and concentrated under reduced pressure to an orange oil; weight, 300 mg (87% yield as an amide–acid). Analysis by HPLC (85:15 acetonitrile–1% NH_4OAc) revealed five components as follows (retention time): 7.6% (4.4), 2.8% (5.6), 23.8% (6.3), 13.4% (7.3), 52.4% (8.2). Subsequent analyses of purified **12** and **14** indicated that the major component was **12** and the third was **14**. A solution

(49) Reverse-phase HPLC of mixtures of ester–amides on columns of Spherisorb ODS (5-μm particle size) and with various proportions of acetonitrile–1% aqueous ammonium acetate as eluting solvents did not give adequate separation of the major peaks. Also, TLC indicated the presence of more components in some of the intermediate product fractions than reverse-phase HPLC indicated.

Table III. Chromatography of Ester–Amide Mixture Obtained from **6**

approx vol of eluent, mL	product fraction	wt, mg	mp	HPLC[a] t_R, min	HPLC[a] %	compd[b]
90	A	340	100–103 °C	5.5[c]	100	11
				4.3	99.5	11
				5.6	0.5	NI
55	B	330	crystallized in freezer	4.3	97.3	11
				4.8	2.1	NI
				5.5	0.5	NI
125	C	370	oil	4.3	83.8	11
				4.8	15.7	NI
				5.6	0.5	NI
230	D	470	oil	4.3	35.4	11
				4.8	15.9	NI
				5.1	48.0	NI
				6.0	0.8	13
70	E	30	oil	4.4	21.1	11
				5.2	32.7	NI
				6.1	46.1	13
50	F	100	crystallized in freezer	4.3	1.9	11
				5.1	4.5	NI
				6.0	93.6	13
90	G	300	crystallized in freezer	4.4	0.6	11
				5.1	4.0	NI
				6.0	95.4	13
70	H	210	crystallized in freezer	4.4	0.5	11
				5.0	4.0	NI
				6.1	95.4	13
420	I	550	132–134 °C	9.2[c]	100	13
				4.4	0.5	11
				5.0	3.7	NI
				6.0	95.8	13

[a] Normal-phase HPLC on a silica gel column (μPorasil); 70:30 hexane–ethyl acetate, isocratic; flow rate, 2 mL/min; monitored by UV at 340 nm. The injection solvent was methanol except where indicated otherwise (footnote *c*). When methanol was the injection solvent, fractions A–I were analyzed on the same day under similar ambient conditions. [b] NI = not identified. [c] Injection solvent: 7:3 hexane–ethyl acetate.

of the oil in acetonitrile (4 mL) was applied in eight equal parts to a semipreparative HPLC column.[50] The column was eluted with 85:15 acetonitrile–1% aqueous ammonium acetate (isocratic), and the course of the elution was monitored by UV absorption at 254 nm. Fractions containing **12** were identified (on the basis of preliminary runs) by analytical HPLC monitored at 340 nm and combined, acetonitrile was evaported under reduced pressure, and the residual aqueous mixture was diluted with water (50 mL) and extracted with ether (2 × 75 mL). The ether extract was washed with water (2 × 30 mL) and with aqueous sodium chloride solution (2 × 30 mL), dried ($MgSO_4$), and concentrated in vacuo to a crystalline solid: weight, 80 mg; mp 52–56 °C; identified as **12** by NMR studies; HPLC (retention time), 99.6% (8.4) of **12**, 0.4% (6.5) of **14** (injection solvent, MeCN; eluting solvent, 85:15 MeCN–1% aqueous NH_4OAc, isocratic); MS (direct-probe temperature, 150 °C), *m/z* 371 (M), 356 (M − CH_3), 327 (M − CO_2 or NHEt), 312 (327 − CH_3); IR (strong and medium bands) 3270 (br), 3055, 2960, 2930 (vs), 2910 (sh), 2865, 2825, 2580 (br), 1700 (sh), 1685 (vs), 1650, 1610, 1575 (vs), 1555 (vs), 1440, 1380, 1360, 1290, 1280, 1245, 1205 (vs), 965 cm^{-1}. Anal. ($C_{23}H_{33}NO_3{\cdot}0.75H_2O$) C, H, N.

14-[(Ethylamino)carbonyl]-13-*cis*-retinoic Acid (14). Ester–amide **13** (204 mg, 98% by normal-phase HPLC) was hydrolyzed according to the procedure described for the hydrolysis of **11**. Analysis of the crude product (170 mg, 90% yield as an amide–acid) by reverse-phase HPLC (as described below) gave the following results (retention time): 67.6% (6.6) of **14**, 5.9% (8.2) of an unidentified compound, 22.5% (9.8) of **12**, three lesser components with lower retention times. A solution of the crude product in acetonitrile deposited yellow crystals (**14**): weight 40 mg; mp 159–160 °C dec; HPLC (as described below), 98%. The filtrate was applied in four portions to a semipreparative HPLC column,[50] as described for the isolation of **12**. An orange glass was obtained from the effluent fractions that contained **14** (confirmed by analytical HPLC monitored at 340 nm): weight 26 mg; HPLC, 99.2%. This material crystallized when it was triturated with acetonitrile: weight 18 mg; mp 157–159 °C dec; HPLC, 99.8%. This material was combined with the specimen that crystallized prior to semipreparative HPLC, the solid was slurried with a small amount of acetonitrile at room temperature, and yellow crystals were collected from the chilled mixture: mp 159–160 °C dec; identified as **14** by NMR studies; HPLC, >99.8% (retention time 5.4), trace of **12** (injection solvent Me_2SO; eluting solvent 85:15 Me_2SO–1% aqueous NH_4OAc, isocratic); MS (direct-probe temperature, 200 °C), *m/z* 371 (M), 356 (M − CH_3), 338 (M − CH_3 − H_2O), 327 (M − CO_2 or NHEt), 293; IR (strong and medium bands) 3235, 3075, 2970, 2960, 2950 (sh), 2930, 2910, 2900, 2865, 1675, 1650, 1625, 1590, 1565 (vs), 1540, 1445, 1400, 1320, 1290, 1280, 1260, 1245 (vs), 975 cm^{-1}. Anal. ($C_{23}H_{33}N_3O{\cdot}0.25CH_3CN$) C, H, N.

Bioassays. Binding affinities of retinoids for CRABP from chick embryo skin were determined as described before.[35] The inhibition of binding of [^{3}H]retinoic acid to CRABP caused by a 100-fold molar excess of unlabeled retinoic acid is regarded as 100% inhibition. The inhibition caused by a similar excess of other retinoids is expressed as relative inhibition to the above standard. This type of data provides a simple way of comparing the relative binding affinities of the retinoids as compared to the standard, retinoic acid.

Evaluation of the differentiation potential of retinoids was carried out with an embryonal carcinoma cell line, F9.[51] Elevation of plasminogen activator release by F9 cells in the presence of retinoids is regarded as a marker for inducing differentiation into parietal endoderm.[51] F9 cells were grown at a density of 1×10^5 cells/mL in the presence or absence of 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, and 10^{-11} M retinoids for 4 days. Aliquots (20 μL) of the harvest fluid were mixed with 0.13 μM plasminogen, 0.3 mM H-D-Val-Leu-Lys-*p*-NA (a synthetic substrate of plasmin), 0.1% Tween 80, and 25 μg of fibrinogen fragments in a final volume of 0.2 mL of

(50) A semipreparative HPLC column of Spherisorb ODS, 5-μm particle size, was used; 25 cm × 20 mm i.d.; purchased from Phase Separations, Inc., Norwalk, CT.

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Tris·HCl, pH 7.5.[52] After incubation for 4 h at 25 °C, the generation of p-nitroaniline was measured by absorbance at 405 nm. The average absorbencies from triplicate experiments were plotted (semilog plots) versus concentration (molarity). The ED_{50} values were determined by finding the midpoint between the maximal and minimal absorbance values.

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Central Dopaminergic and 5-Hydroxytryptaminergic Effects of C3-Methylated Derivatives of 8-Hydroxy-2-(di-*n*-propylamino)tetralin

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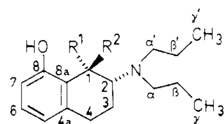
A number of stereochemically well defined C3-methylated derivatives of the potent 5-hydroxytryptamine (5-HT) receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) have been synthesized, and their stereochemical characteristics have been studied by use of NMR spectroscopy, X-ray crystallography, and molecular mechanics calculations. The compounds were tested for activity at central 5-HT and dopamine (DA) receptors, by use of biochemical and behavioral tests in rats. In addition, the ability of the *cis*- and *trans*-8-hydroxy-3-methyl-2-(di-*n*-propylamino)tetralins (15 and 11) to displace [^{3}H]-8-OH-DPAT from 5-HT_{1A} binding sites was evaluated. The stereoselectivity of the interaction of 11 and 15 with 5-HT receptors was much greater than that of 8-OH-DPAT. Observed rank order of potencies in the 5-HT_{1A} binding assay corresponds to that in the in vivo biochemical assay.

8-Hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT, 1)[1] is a potent and highly interesting, centrally active, 5-hydroxytryptamine (serotonin; 5-HT) receptor agonist.[2] Compound 1 appears to lack prominent effects on dopamine (DA)[3] and norepinephrine (NE) receptors[3] and has a pronounced selectivity for 5-HT_{1A} sites.[4,5] Most likely, 1 will prove very useful as a pharmacological tool in the elucidation of central 5-hydroxytryptaminergic mechanisms and as a lead compound in structure–activity relationship studies. In fact, tritiated 1 has already turned out to be useful as a ligand for 5-HT_{1A} sites.[6]

Compound 1 is weakly stereoselective in its interaction with 5-HT receptors.[1] In contrast, *cis*-8-hydroxy-1-methyl-2-(di-*n*-propylamino)tetralin (2), a Cl-methylated derivative of 1, exhibits a pronounced stereoselectivity; the 1*S*,2*R* enantiomer is equipotent to 1 as a 5-HT-receptor agonist while (1*R*,2*S*)-2 appears to be inactive.[7,8] The racemic trans diastereomer 3 has been reported to be inactive as a 5-HT-receptor agonist.[7,8]



(2*R*)-1: $R^1 = R^2 = H$
(1*S*,2*R*)-2: $R^1 = CH_3$; $R^2 = H$
(1*R*,2*R*)-3: $R^1 = H$; $R^2 = CH_3$

In the present study, which is part of a current effort to develop novel and selective 5-HT-receptor agonists and antagonists, we have synthesized a number of stereo-

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