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Short communication

Synthesis of some isoxazole analogues of retinoids: biological effects toward tumor cell lines

PG Baraldi[1]*, M Guarneri[1], S Manfredini[1], D Simoni[1]*, M Aghazade Tabrizi[1], R Barbieri[2], R Gambari[2], C Nastruzzi[1,2]

[1]*Dipartimento di Scienze Farmaceutiche, via Scandiana 21, Università di Ferrara;* [2]*Istituto di Chimica Biologica, via L Borsari 46, Università di Ferrara, Italy*

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Summary — A series of isoxazole analogues of retinoids have been prepared. The *in vitro* biological activity of these compounds on different lineages of tumor cells is reported. Some of the prepared derivatives exibited good antiproliferative activity and capability to induce differentiation of tumor cells.

Résumé — Synthèse de quelques dérivés isoxazoliques analogues des rétinoides. Effets biologiques vis-à-vis des cellules tumorales. *Une série de dérivés isoxazoliques analogues aux rétinoides a été préparée. L'activité in vitro de ces composés vis-à-vis d'une variété de cellules cancéreuses a été évaluée. Certains d'entre eux ont révélé une bonne activité anti-proliférante et la capacité d'induire une différenciation des cellules cancéreuses.*

synthetic isoxazole retinoids / FL cells / tumor cell growth / differentiation

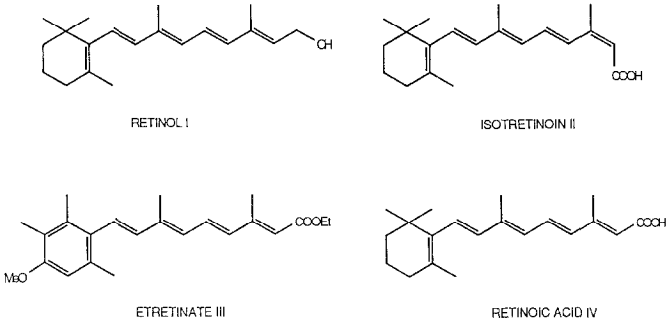
Introduction

Retinoids (retinol **I** and its natural and synthetic analogues such as isotretinoin **II** and etretinate **III**) constitue a group of compounds of enormous current interest [1, 2].

This interest is mainly due to the fact that they seem to offer a new approach to the therapy of some neoplastic diseases. The reasons of this interest derive from observations showing that these compounds exhibit ability to regulate epithelial cell differentiation, have therapeutic potential for the treatment of proliferative skin diseases [3–5], and chemoprevention of cancer [6–10].

Furthermore, the demonstration that percutaneus absorption of vitamin A has striking effects on variety of skin diseases, has opened a new era in the therapeutic dermatology and cosmetology. Unfortunately, the use of natural and synthetic retinoids in these fields has some disadvantages, such as low distribution, high toxicity and poor stability [11, 12].

In order to overcome these limitations and obtain more active retinoids we have designed a series of 'heteroretinoids' **4–9** which display the following structural modifications with respect to the natural ones: a) an isoxazole ring placed between the C10 and C12 atoms of the side chain; b) hydroxylic or ketonic functions on the C7 atom; c) saturation of the double bond in C7–C8.



In these compounds, certain bonds (C10–C12) of the tetraene chain of natural retinoids are included in the isoxazole scaffold, producing a series of conformationally restricted retinoids.

These structural modifications should increase hydrophilicity and lower lipophilicity with, hopefully, concomitant lower toxicity.

In this paper we report the synthesis and the biological activity of **4–9** on tumor cells of different lineages.

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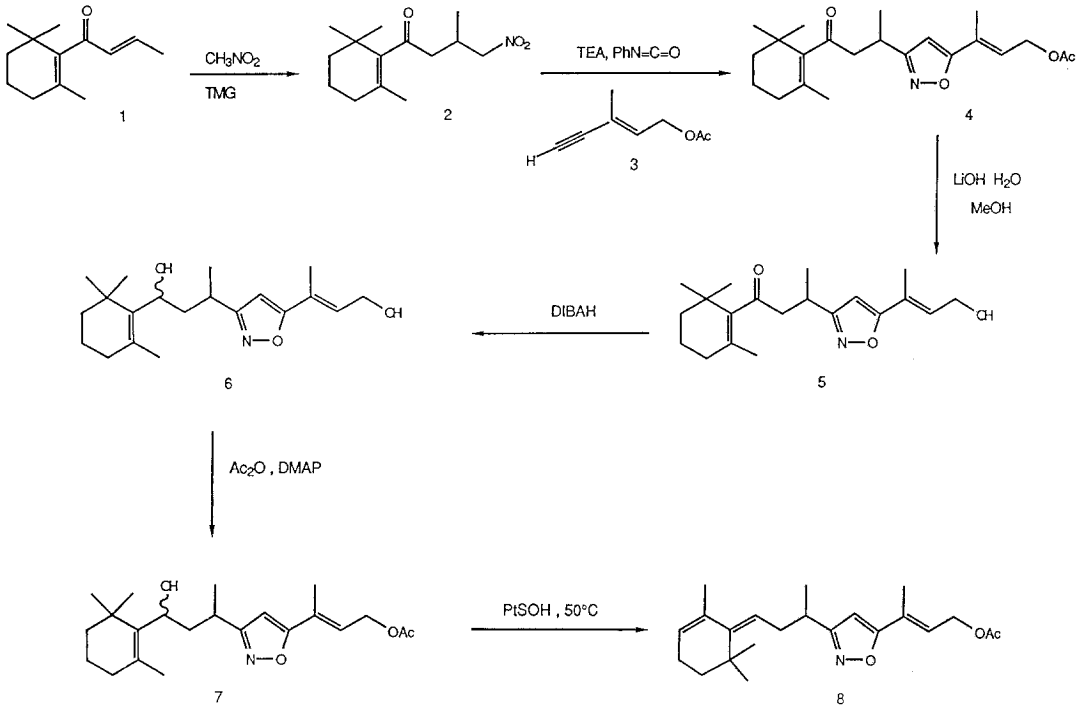
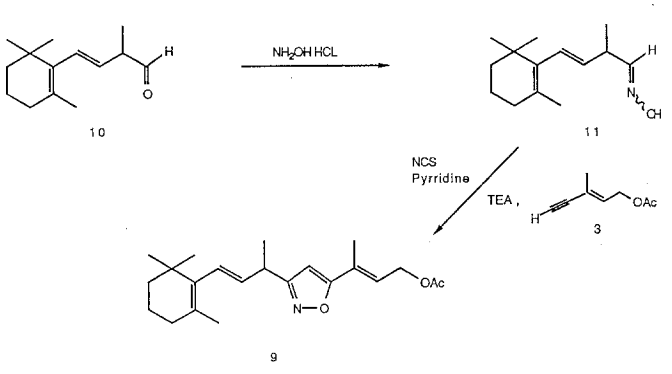


Scheme 1.



Scheme 2.

Chemistry

The synthetic strategy employed to synthesize the C-20 retinoid carbon skeleton of **4–9**, depicted in scheme I, is based on an assemble of two components C(14) and C(6) through 1,3-dipolar cycloaddition, utilizing an approach very similar to that used in our recent synthesis of vitamin A and its 9-*cis* isomer [13, 14].

The preparation of the isoxazole intermediate **4** was obtained in high yield *in regio* and stereospecific manner through cycloaddition of the nitrile oxide generated from the nitroderivative **2**, in turn prepared by an easy 1,1,3,3-tetramethylguanidine catalyzed Michael addition of nitromethane [15] to the commercially available β-damascone **1**, into the acetyl derivative of *trans*-3-methyl-2-penten-4-yn-1-ol [3].

Removal of the acetyl protective group of **4** by treatment at –20°C with lithium hydroxide produced **5** in high yield.

Reduction of the ketone group of **5** with DIBAH in toluene solution at –60°C afforded the diol **6** as an inseparable mixture of diastereoisomers, in turn acetylated selectively with acetic anhydride in the presence of triethylamine and 4-dimethylamino-pyridine to give rise to the acetyl derivative **7** in nearly quantitative yield.

On exposure of **7** to *p*-toluensulphonic acid in benzene solution the dehydrated product **8** was obtained as an oil in 55% yield. The structural assignment of **4–9** was made by proton NMR and elemental analysis. The stereochemistry of the exocyclic C6–C7 double bond was unambigously determined by difference NOE: irradiation of the C7 vinylic proton of **8** resulted in an enhancement of C5 methyl protons, but no enhancement of C1 methyl protons.

The preparation of **9** depicted in scheme 2 was performed in high chemo and regioselective manner through cycloaddition between the nitrile oxide generated from the oxime of the 2-methyl-4-(2,6,6-trimethyl-1-ciclohexen-1-yl)-3-butenal (**10**) [16], into to the acetyl derivative *trans*-3-methyl-2-penten-4-yn-1-ol.

In vitro antiproliferative activity on tumor cell lines

The effects of natural and synthetic retinoids on *in vitro* cell proliferation of tumor cell lines of different histotype were analysed. The results obtained are shown in table I, which demonstrates that both natural and synthetic retinoids retain differential inhibitory effects on tumor cell proliferation. This differential efficiency on growth inhibition deserves further comments. Among the compounds tested, **9** is the most active (table I). Accordingly, figure 1 shows the growth kinetics of murine Friend erythroleukemic cells (FLC) cultured for 3, 4 and 5 days without chemicals or with 20 μM of retinoic acid or **9**. This experiment gives evidences for an higher efficiency of **9** in inhibiting *in vitro* cell proliferation. The inhibitory effects of the synthetic retinoids are accompained by activation of differentiated functions, as shown in figure 2 for the FH06N1-1 cell line, carrying the human *Ha-ras*-1 protooncogene. After treatment with natural and synthetic retinoids, adipogenic conversion of FH06N1-1 cells is clearly detectable (fig 2).

Table I. Inhibitory effect, on tumor cell proliferation, of compounds **4–9** and **I–IV**.

Compound	tumor cell lines			
	FLC	*K562*	*FH06N1-1*	*WI-L2*
4	28	6	35	50
5	18	3.5	20	30
6	18	2.5	28	28
7	38	2.8	21	30
8	35	4.6	26	25
9	12	2	12	15
I	50	18	55	24
II	30	15	25	20
III	4	18	25	8
IV	>150	>250	60	>100

Results respresent μM concentrations of the compound tested which cause a 50% inhibition of cell proliferation with respect to untreated control cells. Determinations were performed after 4 days of cell culture. At this stage the untreated cells are in the phase of cell growth (see also fig 1).

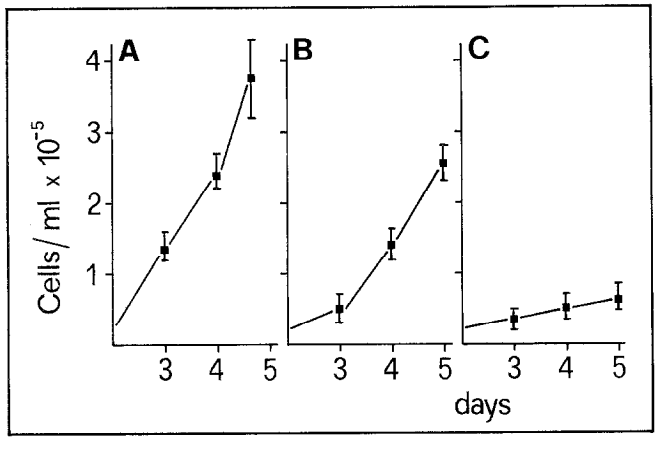


Fig 1. Effects of retinoic acid and 9 on cell proliferation of murine erythroleukemic FL cells. Cells were cultured for the indicated length of time without additions (A) or in the presence of 20 μM retinoic acid (B) or 9 (C). Each value is the mean of 3 independent experiments ±SEM.

Discussion and Conclusions

This paper demonstrates that retinoids inhibit the *in vitro*, proliferation of tumor cells of different lineages, including erythromyeloid, erythroleukemic and B-lymphoid cells. We would like to point out that the synthetic retinoids here described are able to induce differentiated functions in tumor cells including erythroid differentiation of murine erythroleukemic FLC cells [Nastruzzi *et al*; submitted], increase of melanine content in melanoma cells [Nastruzzi *et al*; submitted] and adipogenic conversion of FH06N1-1 fibroblast, transfected with the human *Ha-ras*-1 protoncogene (fig 2). These findings are in agreement with the well described capacity of retinoic acid to induce differentiation.

It could be of interest to analyse the relationship between treatment of tumor cells with retinoids and expression of cell-cycle related genes (including for instance the c-*myc* and c-*myb* oncogenes) in order to further characterize the molecular mechanism leading to the alterations here described of the cell growth and differentiation. In addition, it will be of interest to determine whether the new synthetic retinoids described in this paper do interact with the retinoids receptors [17].

Experimental protocols

Chemistry

Reaction courses and product mixture routinely monitored by thin-layer chromatography (TLC) on silica gel precoated F_{254} Merck plates. Infrared spectra (IR) were measured on a Perkin–Elmer 257 instrument. Nuclear magnetic resonance (^1H NMR) spectra were determined for solution in $CDCl_3$ with a Brucker AC 200 spectrometer and peak positions are given in ppm downfield from tetramethylsilane as internal standard.

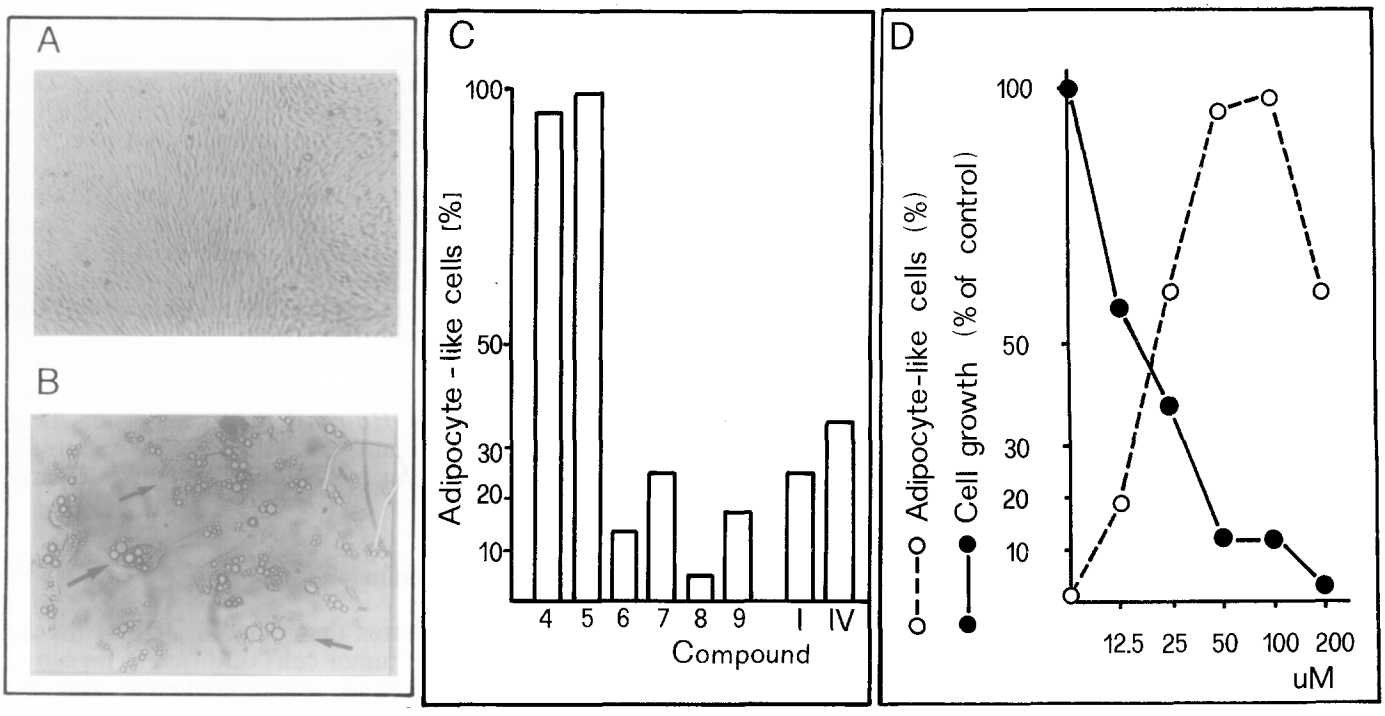


Fig 2. Effects of natural and synthetic retinoids on adipogenic conversion of chinese hamster FH06N1-1 cells A, B: morphology of control FH06N1-1 cells (A) and adipocyte-like FH06N1-1 cell (B). Arrows indicate large accumulation of lipid in adipocyte-like cells. (C): adipogenic conversion after 4-day treatment of FH06N1-1 cells with compounds **4–9**, **I**, and **IV** (50 μM). Adipocyte cells of control untreated FH06N1-1 cells = 0.2–0.5%. (D): effects of increasing concentration of compound 5 on cell proliferation (●) and adipogenic conversion (°) of FH06N1-1 cells. Determinations were performed after 4 days of cell culture.

Light petroleum refers to the fractions boiling range 40–60°C. Column chromatographies were performed with Merck 60–200 mesh silica gel. All of the products reported showed IR and ^{1}H NMR spectra in agreement with the assigned structures.

Compounds **10** and **3** were prepared according to known directions [14–16]; compound **11** was prepared from **10** by standard procedure.

Synthesis procedures

2-Methyl-4-oxo-(2,6,6-trimethyl-1-cyclohexen-1-yl)1-nitrobutane **2**

A solution of **1** (10 g, 52 mmol) in nitromethane (100 ml) containing tetramethylguanidine (0.3 ml) was stirred for 24 h at ambient temperature. After this time diethyl ether (300 ml) was added and the resulted solution washed with brine (5 x 50 ml), dried over Na_2SO_4 anhydrous and concentrated *in vacuo*. The residue was flash-chromatographed on silica gel (ether/light petroleum 1:9) to give **2** as an oil (10.5 g, 80%). IR (neat) 1680, 1550 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.025 (s, 6 H); 1.11 (d, 3 H J = 6.5 Hz), 1.55 (s, 3 H) 1.15–1.95 (comp m 8 H); 2.62 (m, 1 H); 4.35 (m, 2 H).

3-[3-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-3-oxo-1-methyl-1-propyl]-5-[(E)*-3-(acetyloxy)-1-methyl-1-propenyl]-isoxazole* **4**

To a solution of **2** (2 g, 8 mmol) and **1** (2.2 g, 16 mmol) in dry benzene (5 ml) containing triethylamine (0.1 ml), phenyl-isocyanate (2.17 ml, 20 mmol) in benzene (5 ml) was added dropwise at room temperature, and the mixture was allowed to stand overnight. The cooled mixture (5°C) was filtered, the filtrate washed with 2% aqueous ammonia (2 x 50 ml) and brine (3 x 50 ml) was dried over Na_2SO_4 anhydrous and concentrated *in vacuo*. The residue crude oil was flash chromatographed on silica gel (ether/light petroleum 2:8) to give **4** as an oil (2.6 g, 90%). IR (neat) 1740, 1690, 1580 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.06 (s, 6 H); 1.35 (d, 3 H J = 7 Hz); 1.45 (m, 2 H); 1.54 (s, 3 H); 1.65 (m, 2 H); 1.93 (m, 2 H); 2.04 (s, 3 H); 2.08 (s, 3 H); 3.05 (m, 2 H); 3.50 (m, 1 H); 4.78 (d, 2 H J = 6.9 Hz); 6.15 (s, 1 H); 6.32 (brt, 1 H).

3-[3-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-3-oxo-1-methyl-1-propyl]-5-[(E)*-3-hydroxy-1-methyl-1-propenyl]-isoxazole* **5**

Lithium hydroxide monohydrate (2.1 g, 50 mmol) was added at –10°C to a stirred solution of **4** (15.2 g, 41 mmol) in methanol (200 ml) containing 30 ml of water. After 10 min at 0°C the solution was concentrated *in vacuo*, the residue was poured into brine (200 ml) and extracted with ethyl acetate (3 x 100 ml). The dried (Na_2SO_4) organic extracts were evaporated *in vacuo* and the residue flashed-chromatographed on silica gel (ethyl acetate/light petroleum, 4:6) to give **5** as an oil (1.58 g, 85%). IR (neat) 3400, 1690, 1590 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.05 (s, 3 H); 1.3 (d, 3 H J = 7 Hz); 1.45 (m, 2 H); 1.54 (s, 3 H); 1.60 (m, 2 H); 1.91 (m, 2 H); 1.98 (s, 3 H); 2.28 (br, 1 H); 2.85 (dd, 1 H J = 7.7 Hz and 16 Hz); 3.1 (dd, 1 H J = 7.7 and 16 Hz); 3.5 (m, 1 H); 4.39 (d, 2 H J = 6.5 Hz); 6.12 (s, 1 H); 6.48 (brt, 1 H).

3-[3-(2,6,6-Trimethyl-1-cycloexen-1-yl)-3-hydroxy-1-methyl-1-propyl]-5-[(E)-3-hydroxy-1-methyl-1-propenyl]-isoxazole ***6***
To a stirred solution of **5** (1.65 g, 5 mmol) in toluene (30 ml) cooled at —78°C under an atmosphere of nitrogen a toluene solution (25%) of diisobutyl—aluminium hydride (6 ml) was added dropwise, while stirring was continued for 2 h at the same temperature. When the reaction was complete, as judged by TLC, methanol (0.2 ml) and water (0.6 ml) were added cautiously. Anhydrous $MgSO_4$ was added and the mixture filtered through Celite. Evaporation of the solvent *in vacuo* afforded **6** as an oil after chromatography on silica gel (ethyl acetate/light petroleum 3:7); (1.1 g, 66.6%). IR (neat) 3400, 1580 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.96 (s, 3 H); 1.06 (s, 3 H); 1.38 (d, 3 H J = 7 Hz); 1.45 (m, 2 H); 1.65 (m, 2 H); 1.87 (s, 3 H); 1.98 (m, 4 H); 2.0 (s, 3 H); 2.44 (m, 2 H); 3.2 (m, 1 H); 4.40 (m, 3 H); 6.0 (s, 1 H); 6.55 (brt, 1 H).

3-[3-(2,6,6-Trymethyl-1-cycloexen-1-yl)-3-hydroxy-1-methyl-1-propyl]-5-[(E)-3-(acethyloxy)-1-methyl-1-propenyl]-isoxazole ***7***
To an ice-cooled solution of **6** (5 g, 15 mmol) in methylene chloride (100 ml) containing triethylamine (2.5 ml, 18 mmol) and acetic anhydride (1.7 ml, 18 mmol), 4-dimethyl-amino-pyridine (DMAP) (50 mg) was added with stirring. After 30 min at room temperature the mixture was treated with 2% citric acid (50 ml) and the organic phase separated, dried over Na_2SO_4 anhydrous and concentrated *in vacuo*. The residue was flash-chromatographed on silica gel (ether/light petroleum, 3:7) to give **7** as an oil (3.9, 70%). IR (neat) 3400, 1740, 1640, 1580 cm^{-1}; ^1H NMR δ 0.97 (s, 3 H); 1.10 (s, 3 H); 1.40 (d, 3 H J = 7 Hz); 1.54 (m, 2 H); 1.65 (m, 2 H); 1.88 (s, 3 H); 1.95 (m, 3 H); 2.92 (s, 3 H); 2.12 (s, 3 H); 2.22 (m, 2 H); 3.25 (m, 1 H); 4.45 (m, 1 H); 4.85 (d, 2 H J = 6.5 Hz); 6.10 (s, 1 H); 3.35 (brt, 1 H).

3-[(E)-3-(2,6,6-Trimethyl-2-cyclohexen-1-ylidene)-1-methyl-1-propyl]-5-[(E)-3-(acetyloxy)-1-methyl-1-propenyl]-isoxazole ***8***
A solution of **7** (0.5 g, 1.3 mmol) in benzene containing p-toluensulfonic acid (50 mg) was heated for 1.5 h at 50°C. The cooled solution was washed with 5% sodium hydrogen carbonate (20 ml) and brine (3 x 30 ml), dried over Na_2SO_4 anhydrous and concentrated *in vacuo*.

The residue was flash-chromatographed on silica gel (ether/light petroleum, 1:9) to give **8** as an oil (0.3 g, 65%). NOE difference experiment: irradiation of the olefinic proton at 5.3 ppm resulted in an enhancement of the methyl protons at 1.76 ppm and no enhancement of the methyl protons at 1.18 ppm. IR (neat) 1740, 1580 cm^{-1}; ^1H NMR δ 1.18 (s, 3 H); 1.35 (d, 2 H J = 7 Hz); 1.20—1.52 (m, 4 H); 1.75 (s, 3 H); 2.04 (s, 3 H); 2.09 (s, 3 H); 2.63 (m, 2 H); 2.99 (m, 1 H); 4.80 (d, 2 H J = 7 Hz); 5.35 (brt, 1 H); 5.65 (m, 1 H); 6.0 (s, 1 H); 6.38 (brt, 1 H).

3-[3-(2,6,6-Trimethyl-2-cyclohexen-1-yl)-1-methyl-2-propenyl]-5-[(E)-3-(acetyloxy)-1-methyl-1-propenyl]-isoxazole ***9***
The oxime **11** (4.12 g, 20 mmol) was added at 25°C, in one portion, to a solution of chlorosuccinimide (2.67 g, 20 ml) in dry chloroform (18 ml) containing 0.1 ml of pyridine. After 20 min at 25°C the olefine (3.45 g, 25 mmol) was added and then triethylamine (3.48 ml, 25 mmol) in $CHCl_3$ (3 ml) was added dropwise over 30 min. After 1.5 h the mixture was washed with water, dried over Na_2SO_4 anhydrous and evaporated *in vacuo*. The residue was flash-chromatographed on silica gel (ethyl acetate/light petroleum, 2:8) to give **9** as an oil (4.7 g, 67%). IR (neat) 1740, 1580 cm^{-1}; ^1H NMR δ 0.95 (s, 6 H); 1.40 (d, 3 H J = 7 Hz); 1.42—1.95 (m, 6 H); 1.62 (s, 3 H); 1.93 (s, 3 H); 2.0 (s, 3 H); 3.65 (m, 1 H); 4.78 (d, 2 H J = 6.8 Hz); 5.43 (dd, 1 H J = 16 and 7.5 Hz); 5.92 (d, 1 H J = 16 Hz); 6.05 (s, 1 H); 6.35 (brt, 1 H).

Biological assays

Cell lines and culture conditions
Human leukemic K562 [18] and B-lymphoid WI-L2 [19], murine Friend erythroleukemia [20] and chinese hamster FH06N1-1 [22] cell lines were maintained in α-medium (GIBCO, Grand Island, NY), 50 mg/l streptomycin, 300 mg/l penicillin, supplemented with 10–15% fetal calf serum (Flow Laboratories Inc, McLean, VA) in 5% CO_2, 80% humidity. FH06N1-1 is a chinese hamster cell line obtained after transfection of primary lung fibroblasts with the human Ha-ras-1 proto-oncogene [21]. This cell line retains the capability to undergo adipogenic conversion after treatment with inducers of differentiation, including 5-azacytidine and retinoic acid [22]. Viable cells count were obtained after 0.1% Trypan blue exclusion test.

Studies of in vitro cell growth and differentiation
Cells growing in suspension were usually seeded at the initial densities of 10^5 cells/ml and counted with a Coulter counter (Coulter Electronics Inc., Hialeah, FL, USA). Adherent cells were plated at initial densities of 2–5 x 10^4 cells per 35-mm tissue culture dish. Before determinations of cell growth the unattached cells were usually rinsed from the dishes with phosphate-buffered saline (PSB)–0.5 M NaCl/0.02 M NaH_2PO_4, pH 7.4. The adherent cells were then removed by 0.05% Trypsin/0.05 mM EDTA and counted electronically.

Assays were carried out in triplicate and usually counts differed less than 9%. Solutions of natural retinoids and synthetic analogues were prepared in ethanol (0.1%) under red light and used immediately. Control cell populations were treated with an equivalent amount of ethanol; this treatment has no effect on cell growth. Adipogenic differentiation was analysed on FH06N1-1 cells. Large accumulation of lipid in adipocyte-like cells was detected by staining the cells with Oil red O [23] as described elsewhere [22].

Acknowledgments

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