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Short communication

Synthesis of some retinoids bearing different heterocyclic rings with anticancer activity

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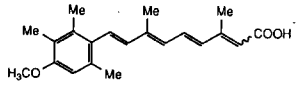
Summary — The synthesis of a series of pyrrole and imidazole retinoids is reported. The compounds were obtained by means of reactions of phosphoranes or phosphonates with aldehydes. Several of these retinoids were evaluated against different tumor systems and some were found to have an activity comparable to that of all-*trans* retinoic acid.

retinoid / heterocycle / antitumoral / cellular differentiation / retinoic acid

Introduction

Retinoids show striking activity in the control of cell differentiation and proliferation for various malignant cells [1]. Recent clinical studies [2–4] revealed complete remission in patients with acute promyelocytic leukemia upon treatment with retinoic acid (RA). However, intolerable side effects for relatively high doses underscores the necessity of developing better-tolerated retinoids as anticancer drugs. Different retinoids with an unsubstituted aromatic ring (phenyl, furyl, pyridyl and thienyl analogues of RA) proved to be less active than the natural acid in all the biological systems tested. On the other hand, the TMMP analogue **1**, with a substituted phenyl ring, has an activity that is even better than the parent RA in the *in vivo* papilloma test [5]. Moreover, a patent has been issued for several β-furylretinoids containing an alkyl-substituted ring which, in regard to the collateral toxicity, are advantageous over the benzene ring since they produce a diminished hypervitaminosis-A effect [6].



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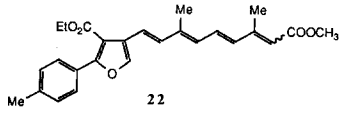

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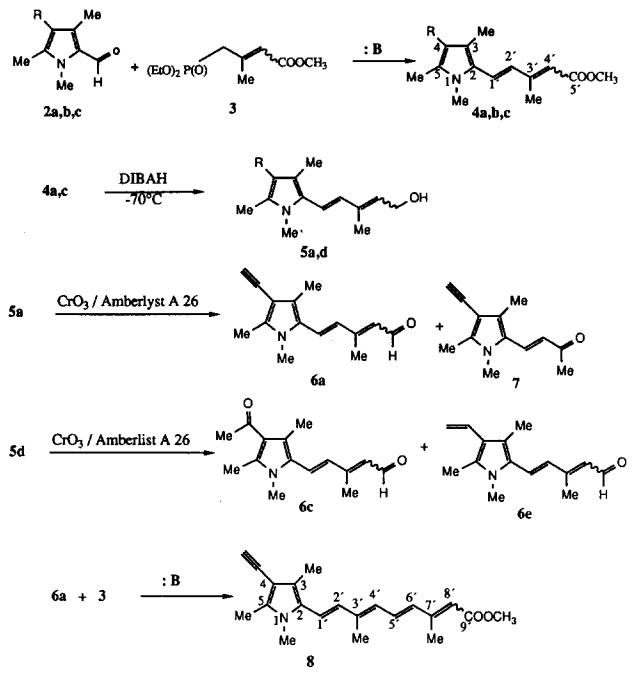
In a previous report [7] we described the synthesis of new retinoids with different substituents on the furan ring in order to achieve a structure which was a potentially active antitumor agent and had tolerable collateral effects. In this sense, we report here the preparation of related compounds bearing differently substituted pyrrole or imidazole rings and the *in vitro* antitumor activities of several of them.

Chemistry

The synthesis of the pyrrole substances was achieved in 4 steps (scheme 1). The starting materials, aldehydes **2a**, **2b** and **2c**, were synthesized following a procedure previously reported by us [8].

Attempts to obtain aldehydes **6** by selective reduction of esters **4** with DIBAH did not proceed in the desired way but gave the alcohols **5a** and **5d** with retained stereochemistry; **5d** was formed by reduction of both carbonyl groups. It is noticeable that in the ^{1}H-NMR spectra of the 3'*E* isomers of these alcohols, H-1' and H-2' appear as singlets with identical chemical shifts. Compounds **6c** and **6e** were identified in the ^{1}H-NMR spectrum of the mixture, but attempts to separate them were unsuccessful due to their high instability.



Scheme 1. **a** R = HC≡C-, **b** R = CH_3CH_2-, **c** R = $CH_3C(O)$-, **d** R = CH_3CHOH-, **e** R = CH_2=CH-.

Condensation of **3** [9] with a mixture of geometric isomers of **6a**, followed by column chromatography and recrystallization, gave the all-*trans* isomer of **8** in 21% yield. Assignment of the signals in its ^{1}H-NMR spectrum (table I) was achieved by comparison with those for etretinate [10] and some furan retinoids [7]. C/H correlated spectra were also used to assign the ^{13}C-NMR signals.

Imidazole retinoids were prepared through 2 alternative routes. One was analogous to that shown above for the synthesis of **8**, *ie* a 4-step procedure starting from **9** through to **13** [11] (scheme 2, table II). The other route introduces the polyene chain through a Wittig reaction between the imidazole aldehyde **14** [12] and the ilide of (2,6-dimethyl-7-methoxycarbonylhepta-2,4,6-trienyl) triphenyl phosphonium bromide **15** [13] (scheme 3, table II) giving the desired product **16** in addition to small amounts of **17** and **18**. This one-step procedure failed for the synthesis of **8** by reaction between **2a** and **15**. In this case, only compounds **17** and **18** were obtained, resulting from the solvolysis of the ilide of **15**.

Finally, reduction of **19** to give **20**, an intermediate in the synthesis of **16** (scheme 3), was unsuitable. Poor yields of **20** were found due to the formation of **21** (13% yield) and to the difficulty in the work-up as a result of the high water solubility of **20**.

Table I. ^{1}H-NMR and ^{13}C-NMR spectral data for **8** (1'*E*, 3'*E*, 5'*E*, 7'*E*) (300 MHz for ^{1}H and 75.47 MHz for ^{13}C, $CDCl_3$, TMS)[a].

H	*δ (ppm)*	*C*[b]	*δ (ppm)*
8'	5.77 br s	9'	167.66
6'	6.29 d	8'	118.13
	(15)	7'	153.36
5'	7.03 dd	6'	135.14
	(12) (15)	5'	131.08
4'	6.26 d	4'	130.07
	(12)	3'	139.70
2'	6.51 s	2'	131.40
1'	6.51 s	1'	117.96
$CH_3C_{7'}$	2.37 d	5	135.36
	(0.9)	4	103.67
$CH_3C_{3'}$	2.06 br s	3	120.95
CH_3C_3	2.32 s	2	127.07
CH_3C_5	2.27 s	$CH_3C_{7'}$	13.91
CH_3N	3.51 s	$CH_3C_{3'}$	12.75
HC≡C-	3.20 s	CH_3C_5	12.14
CH_3-O	3.72 s	CH_3C_3	11.56
		HC≡C-	78.91
		HC≡*C*-	79.84
		CH_3N	31.38
		CH_3O	51.03

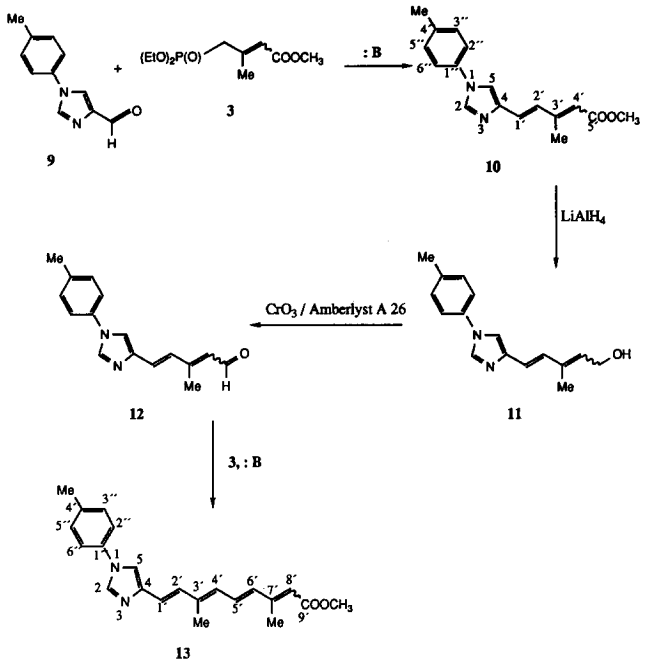
[a]Coupling constants in parentheses; [b]assignments from C/H correlation experiments.

Pharmacological results and discussion

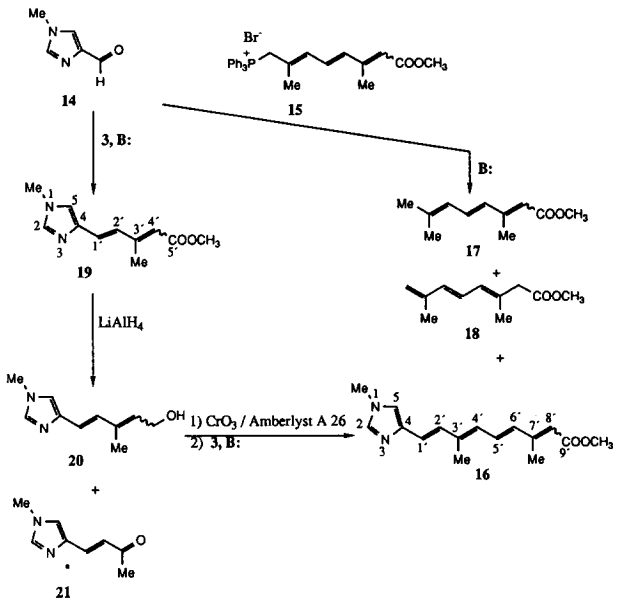
Effects on cellular proliferation

Compounds **8**, **13** and **22** were tested in a dose–response assay for antiproliferative activity against several cell lines and their effects compared with those of RA. The results are summarized as IC_{50} values in table III. It is interesting to remark that the activity of compounds **8** and **13** towards human-tumor cell lines MCF-7 cells and SK Mel-5 cells was higher than RA ($p < 0.001$).

The results on the MCF-7 cell line in cell-cycle analysis are presented in table IV. After 24 h, compounds **8** and **13** showed increases in the GoG1 compartment, in contrast to RA. Analysis after 72 h showed increases in the GoG1 cells upon RA-treatment ($p < 0.01$) and treatment with compounds **8**



Scheme 2.



Scheme 3.

Table II. ^{1}H-NMR and ^{13}C-NMR spectral data for compounds **13** (1'*E*, 3'*E*, 5'*E*, 7'*E*) and **16** (1'*E*, 3'*E*, 5'*E*, 7'*E*) (300 MHz for ^{1}H and 75.47 MHz for ^{13}C, $CDCl_3$, TMS)[a].

H	*13*	*16*	*C*	*13*	*16*
8'	5.88 br s	5.80 br s	9'	167.63	167.66
6'	6.34 d	6.32 d	8'	118.55	118.22
	(15)	(15)	7'	152.66	151.78
5'	7.04 dd	7.00 dd	6'	135.59	135.36
	(15) (12)	(15) (12)	5'	130.92[b]	131.55[b]
4'	6.27 d	6.12 d	4'	130.86[b]	131.45[b]
	(12)	(12)	3'	139.23	139.37
2'	6.87 d	6.60 d	2'	132.28	132.47
	(15)	(16)	1'	116.33	116.05
1'	7.03 d	7.00 d	2	136.41	138.52
	(15)	(16)	4	134.56	140.99
$CH_3C_{7'}$	2.36 br s	2.38 br s	5	130.92	130.21
$CH_3C_{3'}$	2.03 br s	2.06 s	$CH_3C_{7'}$	13.69	13.87
CH_3O	3.70 s	3.73 s	$CH_3C_{3'}$	12.95	12.94
CH_3N	–	3.69 s	CH_3O	50.99	50.99
2	7.78 br s	7.41 br s	CH_3N	–	33.52
5	6.63 br s	6.93 br s	1"	137.85	–
2", 6"	7.26 dd	–	2", 6"	121.22	–
	(8) (1.5)	–	3", 5"	130.48	–
3", 5"	7.24 dd	–	4"	136.05	–
	(8) (1.5)	–	$CH_3C_{4"}$	21.01	–
$CH_3C_{4"}$	2.37 s	–			

[a]Coupling constants (Hz) in parentheses; [b]interchangeable values.

Table III. IC_{50} values (μM) after 6 d treatment[a].

Cell lines	*RA*	*Compounds*		
		8	***13***	***22***
MCF-7	13.1	7.6	7.4	15.7
HeLa	11.5	10.2	11.1	13.3
SK Mel-5	16.1	14.1	14.1	19.8
B16	0.9	7.8	13.3	13.3
MDCK	7.2	11.8	18.5	16.5
VERO	10.6	11.5	15.9	16.1

[a]The results were obtained from means of one experiment with 6 replicates (standard deviations were less than 10%).

Table IV. Cell-cycle analysis on the MCF-7 cell line after 24–72 h of treatment with 1 μM solutions of retinoids[a].

Treatment	*GoG1*	*Synthesis*	*G2M*
24 h			
Control	47.8 ± 4.9	37.9 ± 3.0	14.4 ± 2.0
RA	47.7 ± 4.3	37.8 ± 3.7	14.5 ± 1.1
8	54.7 ± 2.4[b]	38.8 ± 10.8	11.4 ± 1.8[b]
13	52.2 ± 3.9	35.6 ± 3.9	12.3 ± 0.9[b]
72 h			
Control	41.4 ± 8.0	42.6 ± 0.9	16.0 ± 7.7
RA	58.8 ± 3.3[c]	29.0 ± 2.6[d]	12.1 ± 1.4
8	52.0 ± 5.9[b]	34.7 ± 2.7[d]	13.3 ± 3.3
13	52.2 ± 6.9[b]	35.2 ± 4.6[c]	12.5 ± 2.6

[a]The results are presented as the means ± SD of 2 independent experiments in triplicate and the probability *vs* control using the Student's *t*-test; [b]$p < 0.05$; [c]$p < 0.01$; [d]$p < 0.001$.

and **13** ($p < 0.05$). Similar results for 1 μM RA on breast cancer MCF-7 cells were obtained by Guilbaud *et al* [14] after 24–48 h. Zheng *et al* [15] reported no effect on cell cycle after 24 h, whereas an effect was observed when a 10 μM solution of RA was used on human squamous carcinoma ME180 cells. Both of these studies were evaluated by flow cytometry.

Effects on cellular differentiation

MCF-7 breast cancer cells exhibited morphological changes after exposure to 1 μM RA for longer than 4–5 d. By this time, the cells had spread and assumed a more flattened morphology, including cytoplasmatic projections. This effect was slower for compounds **8** and **13**.

The MCF-7 cell line is known to express 3 distinct keratins designated K8 (52 kDa), K18 (45 kDa) and K19 (40 kDa) [16], which are those recognized by CAM 5.2 monoclonal antibodies (mAb) [17]. Numerous studies have shown the activity of retinoids in the synthesis and expression of keratins. Using a head-and-neck squamous carcinoma cell line, 1483 HNSCC cells, Poddar *et al* [18] noticed that the levels of the keratins 52 kDa and 40 kDa after 7 d were higher in 1 μM RA-treated cells than in control cells. We detected keratins 52 kDa, 45 kDa and 40 kDa with the above-mentioned mAb. The results are summarized as SMFU values in table V. The positivity of keratins in the MCF-7 cell line was higher than 99% for all treatments. The activity of RA on the expression of keratins was very small, with specific mean fluorescence uptake (SMFU) values similar to those of the control cells. On the other hand, compounds **8** and **13** increased the expression of keratins after 4 d treatment, $p < 0.001$ and $p < 0.01$, respectively.

An increased expression of epithelial membrane antigen (EMA) monitored by flow cytometry was used as a marker for the acquisition of a more mature phenotype after the treatment with a variety of substances. Nevertheless, RA did not show this effect on the MCF-7 cell line [14].

Because the cells are permeable, we detected EMA antigen both in the cell surface and inside the cells. The positivity of MCF-7 cells was higher than 99% for all treatments. As show in table V, we detected a slight decrease in SMFU but no statistical significance was observed with treatment with RA. Compounds **8** and **13** increased EMA expression in all experiments, and more so after 4 d ($p < 0.05$).

In conclusion, compounds **8** and **13** were effective cell-growth inhibitors, particularly on human cell lines (tables III and IV), and seem to give more mature phenotypes on MCF-7 cells than RA in this assay system (table V). Further studies to discover their side effects in the whole animal are needed.

Experimental protocols

Chemistry

*General procedure for condensation of phosphonate **3** with aldehydes*

Sodium methoxide (27 mmol) in DMF (10 ml) was added dropwise to a stirred solution of **3** (27 mmol) in DMF (50 ml) under an N_2 atmosphere. After completion of the addition, stirring was continued for 30 min, and then 27 mmol aldehyde in 20 ml DMF was added dropwise; stirring was continued for 1 h further. The mixture was neutralized with 10% acetic acid, diluted with water (70 ml) and extracted with chloroform (3 x 30 ml). The extract was washed with water and dried (Na_2SO_4), the solvent was removed *in vacuo* and the residue was chromatographed on a silica-gel column (hexane/Et_2O mixtures) or crystallized.

Table V. SMFU for differentiation markers on MCF-7 cell line after 4–6 d of treatment with 1 μM solutions of retinoids[a].

Treatment	*CAM 5.2*	*EMA*
4 d		
Control	110.6 ± 2.4	120.8 ± 2.6
RA	111.3 ± 2.1	119.2 ± 2.8
8	124.0 ± 4.4[d]	128.8 ± 6.1[b]
13	120.0 ± 4.5[c]	126.0 ± 2.7[b]
6 d		
Control	121.2 ± 7.5	125.5 ± 5.2
RA	124.2 ± 19.5	123.0 ± 10.8
8	125.9 ± 6.2	131.8 ± 5.3[b]
13	124.5 ± 7.5	131.1 ± 7.1

[a]The results are presented as the means ± SD of 3 independent experiments in duplicates and the probability *vs* control using the Student's *t*-test; [b]$p < 0.05$; [c]$p < 0.01$, [d]$p < 0.001$.

Compound ***4a***. Mixture (1:1) of *E/Z* isomers in 96% yield.

1'*E*, 3'*Z* Isomer, mp, 140-141°C. ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.10 (d, *J* = 0.88 Hz, CH_3-$C_{3'}$), 2.29 (s, CH_3-C_5), 2.32 (s, CH_3-C_3), 3.18 (s, HC≡C-), 3.57 (s, CH_3-N), 3.71 (s, OCH_3), 5.63 (br s, $H_{4'}$), 6.79 (d, *J* = 16 Hz, $H_{1'}$), 8.05 (d, *J* = 16 Hz, $H_{2'}$). ^{13}C-NMR (75 MHz, $CDCl_3$, TMS) δ (ppm): 11.57 (CH_3-C_3), 11.98 (CH_3-C_5), 20.43 (CH_3-$C_{3'}$), 31.85 (N-CH_3), 50.93 (O-CH_3), 78.61 (*HC*≡C-), 80.04 (HC≡*C*-), 104.14 (C-4), 114.87 (C-4'), 122.54 (C-1'), 123.56 (C-2'), 124.09 (C-3), 126.60 (C-2), 136.87 (C-5), 152.02 (C-3'), 167.15 (C-5'). IR (KBr): 3240 (s, H-C≡), 2098 (m, C≡C), 1693 (s, C=O) cm^{-1}. MS: m/e (rel int): 258 (15) $[M + 1]^+$, 257 (100) $[M]^+$, 256 (41) $[M - 1]^+$, 242 (63) $[M\text{-}CH_3]$+, 226 (15) $[M\text{-}CH_3O]^+$, 198 (38) $[M\text{-}COOCH_3]^+$, 183 (89) $[M\text{-}CH_3\text{-}COOCH_3]^+$. Anal $C_{16}H_{19}O_2N$: (Calc C: 74.71, H: 7.39, N: 5.45; found, C: 75.08, H: 7.56, N: 5.65).

1'*E*, 3'*E* Isomer, mp 148–150°C. ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.24 (s, CH_3-C_5), 2.30 (s, CH_3-C_3), 2.38 (d, *J* = 0.88 Hz, CH_3-$C_{3'}$), 3.18 (s, HC≡C-), 3.49 (s, CH_3-N), 3.71 (s, OCH_3), 5.81 (br s, $H_{4'}$), 6.41 (d, *J* = 16 Hz, $H_{2'}$), 6.82 (d, *J* = 16 Hz, $H_{1'}$). ^{13}C-NMR (75 MHz, $CDCl_3$, TMS) δ (ppm): 11.55 (CH_3-C_3), 12.19 (CH_3-C_5), 13.46 (CH_3-$C_{3'}$), 31.46 (N-CH_3), 50.96 (O-CH_3), 78.52 (*HC*≡C-), 80.10 (HC≡*C*-), 104.19 (C-4), 117.15 (C-4'), 122.65 (C-1'), 122.85 (C-3), 126.23 (C-2), 129.08 (C-2'), 136.34 (C-5), 153.36 (C-3'), 167.68 (C-5'). IR (KBr): 3251 (s, H-C≡), 2094 (m, -C≡C-), 1687 (s, C=O) cm^{-1}. MS: m/e (rel int): 258 (20) $[M + 1]^+$, 257 (100) $[M]^+$, 256 (35) $[M - 1]^+$, 242 (64) $[M\text{-}CH_3]^+$, 226 (17) $[M\text{-}CH_3O]^+$, 198 (38) $[M\text{-}COOCH_3]^+$, 183 (84) $[M\text{-}CH_3\text{-}COOCH_3]^+$.

Compound ***4b***. Mixture (3:2) of 1'*E*, 3'*E*; 1'*E*, 3'*Z* isomers in 88% yield.

1'*E*, 3'*Z* Isomer. ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 1.04 (t, *J* = 8 Hz, CH_3-CH_2-), 2.11 (d, *J* = 0.5 Hz, CH_3-$C_{3'}$), 2.17 (s, CH_3-C_5), 2.21 (s, CH_3-C_3), 2.42 (q, *J* = 8 Hz, CH_3-CH_2), 3.57 (s, CH_3-N), 3.70 (s, OCH_3), 5.57 (br s, $H_{4'}$), 6.89 (d, *J* = 16 Hz, $H_{1'}$), 8.01 (d, *J* = 16 Hz, $H_{2'}$).

1'*E*, 3'*E* isomer. ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 1.04 (t, *J* = 8 Hz, CH_3-CH_2-), 2.17 (s, CH_3-C_5), 2.17 (s, CH_3-C_3), 2.41 (d, *J* = 0.88 Hz, CH_3-$C_{3'}$), 2.42 (q, *J* = 8 Hz, CH_3-CH_2-), 3.51 (s, CH_3-N), 3.71 (s, OCH_3), 5.77 (br s, $H_{4'}$), 6.38 (d, *J* = 16 Hz, $H_{2'}$), 6.89 (d, *J* = 16 Hz, $H_{1'}$). IR (KBr): 1696 (s, C=O), 1138 (s, C-O) cm^{-1}. MS, m/e (rel int): 262 (20) $[M + 1]^+$, 261 (100) $[M]^+$, 260 (25) $[M - 1]^+$, 246 (74) $[M\text{-}CH_3]^+$, 230 (30) $[M\text{-}OCH_3]^+$, 214 (32) $[M\text{-}CH_3\text{-}CH_3OH]^+$, 202 (41) $[M\text{-}COOCH_3]^+$.

Compound ***4c***. Mixture (3:1) of 1'*E*, 3'*E*; 1'*E*, 3'*Z* isomers in 55% yield.

1'*E*, 3'*Z* Isomer. ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.13 (d, *J* = 0.88 Hz, CH_3-$C_{3'}$), 2.39 (s, CH_3-C_3), 2.45 (s, CH_3CO), 2.49 (s, CH_3-C_5), 3.60 (s, CH_3-N), 3.71 (s, OCH_3), 5.69 (br s, $H_{4'}$), 6.81 (d, *J* = 16 Hz, $H_{1'}$), 8.06 (d, *J* = 16 Hz, $H_{2'}$).

1'*E*, 3'*E* Isomer, mp: 89–90°C. ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.34 (s, CH_3-C_5), 2.39 (d, *J* = 0.88 Hz, CH_3-$C_{3'}$), 2.44 (s, CH_3-C_3), 2.49 (s, CH_3CO), 3.51 (s, CH_3-N), 3.72 (s, OCH_3), 5.84 (br s, $H_{4'}$), 6.37 (d, *J* = 16 Hz, $H_{2'}$), 6.82 (d, *J* = 16 Hz, $H_{1'}$). ^{13}C-NMR (75 MHz, $CDCl_3$, TMS) δ (ppm): 12.50 (CH_3-C_3), 13.51 (CH_3-C_5), 13.56 (CH_3-$C_{3'}$), 31.42 (O-CH_3), 31.42 (*CH*$_3$-CO), 31.56 (N-CH_3), 118.28 (C-4), 120.28 (C-3), 122.39 (C-2), 123.15 (C-5), 128.10 (C-4'), 132.35 (C-1'), 137.41 (C-2'), 152.59 (C-3'), 167.40 (C-5'), 195.74 (CH_3-*C*O). IR (KBr): 1696 (s, C=O), 1641 (s, C=O) cm^{-1}. MS, m/e (rel int): 276 (12) $[M + 1]^+$, 275 (86) $[M]^+$, 274 (15) $[M - 1]^+$, 260 (30) $[M\text{-}CH_3]^+$, 244 (29) $[M\text{-}OCH_3]^+$, 232 (12) $[M\text{-}CH_3CO]^+$, 228 (100) $[M\text{-}CH_3\text{-}CH_3OH]^+$, 216 (17) $[M\text{-}COOCH_3]^+$.

Compound ***8***. Mixture of diastereoisomers in 84% yield. By recrystallization, 21% of all-*trans* isomer was recovered.

1'*E*, 3'*E*, 5'*E*, 7'*E* Isomer, mp 186–188°C. ^{1}H-NMR and ^{13}C-NMR: table I. IR (KBr): 3257 (s, H-C≡), 2096 (m, C≡C), 1696 (s, C=O), 1150 (s, C-O) cm^{-1}. MS, m/e (rel int): 323 (91) $[M]^+$, 308 (17) $[M\text{-}CH_3]^+$, 292 (4) $[M\text{-}CH_3O]^+$, 276 (5) $[M\text{-}CH_3OH\text{-}CH_3]^+$, 264 (17) $[M\text{-}COOCH_3]^+$, 249 (11) $[M\text{-}COOCH_3\text{-}CH_3]^+$, 133 (100) $[C_9H_{11}N]^+$. Anal $C_{21}H_{25}O_2N$ (calc C: 78.01, H: 7.74, N: 4.33; found C: 77.01, H: 7.71, N: 4.52). UV: λ_1 415 nm (ε_1 67 000), λ_2 313 nm (ε_2 39 000).

Compound ***10***. Mixture (4:1) of 1'*E*, 3'*E*; 1'*E*, 3'*Z* isomers in 60% yield.

1'*E*, 3'*E* Isomer: ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.37 (s, CH_3-$C_{4''}$), 2.37 (s, CH_3-$C_{3'}$), 3.71 (s, CH_3-O), 5.90 (br s, $H_{4'}$), 6.90 (d, *J* = 16 Hz, $H_{2'}$), 7.10 (d, *J* = 16 Hz, $H_{1'}$), 7.28 (br s, $H_{2''}$, $H_{6''}$), 7.28 (br s, $H_{3''}$, $H_{5''}$), 7.28 (br s, H_5), 7.81 (br s, H_2). ^{13}C-NMR (20 MHz, $CDCl_3$, TMS) δ (ppm): 13.57 (CH_3-$C_{3'}$), 20.90 (CH_3-$C_{4''}$), 50.85 (CH_3-O), 117.60 (C-1'), 118.58 (C-4'), 121.13 (C-2",C-6"), 125.25 (C-5), 130.44 (C-3", C-5"), 130.95 (C-2'), 134.44 (C-4), 136.21 (C-2), 137.82 (C-4"), 140.62 (C-1"), 158.48 (C-3'), 167.53 (C-5'). IR (film): 1720 (s, C=O), 1150 (s, C-O) cm^{-1}. MS, m/e (rel int): 282 (5) $[M]^+$, 267 (2) $[M\text{-}CH_3]^+$, 251 (6) $[M\text{-}OCH_3]^+$, 223 (100) $[M\text{-}COOCH_3]^+$, 181 (14) $[M\text{-}C_5H_9O_2]^+$, 132 (12) $[M\text{-}COOCH_3\text{-}p\text{-tolyl}]^+$, 91 (24) $[C_7H_7]^+$, 65 (11) $[C_5H_5]^+$.

Compound ***13***. Mixture of diastereoisomers in 88% yield.

^{1}H-NMR and ^{13}C-NMR: table II. IR (film): 1707 (s, C=O), 1605 (s, C=C), 1155 (s, C-O) cm^{-1}. MS, m/e (rel int): 349 (8) $[M + 1]^+$, 348 (30) $[M]^+$, 333 (21) $[M\text{-}CH_3]^+$, 317 (6) $[M\text{-}OCH_3]^+$, 316 (6) $[M\text{-}CH_3OH]^+$, 289 (100) $[M\text{-}COOCH_3]^+$, 249 (21) $[M\text{-}C_5H_7O_2]^+$, 223 (84) $[M\text{-}C_7H_9O_2]^+$, 118 (26) [*p*-tolyl-NCH]$^+$, 91 (80) $[C_7H_7]^+$, 65 (53) $[C_5H_5]^+$.

Compound ***19***. Mixture (4:1) of 1'*E*, 3'*E*; 1'*E*, 3'*Z* isomers in 63% yield.

1'*E*, 3'*E* Isomer: ^{1}H-NMR (300 MHz, $CDCl_3$, TMS) δ (ppm): 2.33 (br s, CH_3-$C_{3'}$), 3.65 (s, CH_3-N), 3.68 (s, CH_3-O), 5.83 (br s, $H_{4'}$), 6 78 (d, *J* = 15 Hz, $H_{2'}$), 6.91 (br s, H_5), 6.93 (d, *J* =

15 Hz, $H_{1'}$), 7.37 (br s, H_2). ^{13}C-NMR (75 MHz, $CDCl_3$, TMS) δ (ppm): 13.63 (CH_3-$C_{3'}$), 33.48 (CH_3-N), 50.90 (CH_3-O), 118.07 (C-4'), 119.92 (C-2'), 125.93 (C-5), 129.98 (C1'), 138.64 (C-2), 140.24 (C-4), 152.83 (C-3'), 167.68 (C-5'). IR (film): 1697 (s, C=O), 1153 (s, C-O) cm^{-1}. MS, m/e (rel int): 206 (6) $[M]^+$, 191 (2) $[M\text{-}CH_3]^+$, 175 (4) $[M\text{-}OCH_3]^+$, 147 (100) $[M\text{-}COOCH_3]^+$, 132 (8) $[M\text{-}COOCH_3\text{-}CH_3]^+$, 77 (9) $[C_4H_5N_2]^+$.

General procedure for reduction of methyl esters

A suspension of $LiAlH_4$ (2.57 mmol) in Et_2O (4 ml) was added to a solution of ester (3.2 mmol) in Et_2O (7 ml) under an N_2 atmosphere. The mixture was stirred for several hours (TLC monitoring) at 0°C. Two drops of water were added to the suspension and the organic product was extracted with Et_2O. The solvent was removed and the residue was chromatographed on a silica-gel column (hexane/Et_2O mixtures).

The experiment with DIBAH as reducting agent was performed in toluene at −70°C, by adding a solution of 20% DIBAH in hexane.

The isomer **5a** (1'*E*, 3'*E*) was obtained from **4a** (1'*E*, 3'*E*) in quantitative yield. ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 1.80 (d, *J* = 1.8 Hz, CH_3-$C_{3'}$), 2.22 (s, CH_3-C_5), 2.30 (s, CH_3-C_3), 3.18 (s, HC≡C-), 3.46 (s, CH_3-N), 4.32 (d, *J* = 7 Hz, CH_2-OH), 5.73 (t, *J* = 7 Hz, $H_{4'}$), 6.41 (s, $H_{1'}$), 6.41 (s, $H_{2'}$). IR (KBr): 3310 (m, O-H), 3298 (s, H-C≡), 2098 (m, C≡C) cm^{-1}. MS, m/e (rel int): 230 (7) $[M + 1]^+$, 229 (43) $[M]^+$, 228 (10) $[M - 1]^+$, 214 (25) $[M\text{-}CH_3]^+$, 211 (10) $[M\text{-}H_2O]^+$, 198 (28) $[M\text{-}CH_2OH]^+$, 184 (100) $[M\text{-}(CH_2\text{-}CH_2\text{-}OH)]^+$, 133 (28) $[M\text{-}C_9H_{11}N]^+$, 132 (17) $[M\text{-}C_6H_8O]^+$.

The isomer **5a** (1'*E*, 3'*Z*) was obtained from **4a** (1'*E*, 3'*Z*) in quantitative yield. ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 1.97 (d, *J* = 1.8 Hz, CH_3-$C_{3'}$), 2.24 (s, CH_3-C_5), 2.30 (s, CH_3-C_3), 3.18 (s, HC≡C-), 3.48 (s, CH_3-N), 4.32 (d, *J* = 7 Hz, CH_2-OH), 5.57 (t, *J* = 7 Hz, $H_{4'}$), 6.42 (d, *J* = 16 Hz, $H_{2'}$), 6.77 (d, *J* = 16 Hz, $H_{1'}$). IR (KBr): 3300 (s, O-H), 3270 (s, H-C≡), 2094 (m, C≡C) cm^{-1}. MS, m/e (rel int): 230 (14) $[M + 1]^+$, 229 (86) $[M]^+$, 228 (12) $[M - 1]^+$, 212 (7) $[M\text{-}OH]^+$, 198 (31) $[M\text{-}CH_2OH]^+$, 184 (100) $[M\text{-}(CH_2\text{-}CH_2OH)]^+$, 168 (25) $[M\text{-}CH_2OH\text{-}2CH_3]^+$, 146 (84) $[C_{10}H_{10}N]^+$, 133 (47) $[C_9H_{11}N]^+$, 56 (35) $[CH_3CNCH_3]^+$. Anal $C_{15}H_{19}ON$ (calc C: 78.60, H: 8.30, N: 6.11; found C: 78.76, H: 8.45, N: 6.32).

A 1:1 mixture of 1'*E*, 3'*E* and 1'*E*, 3'*Z* isomers of **5d** was obtained from the same proportion of **4c** in quantitative yield. IR (film): 3372 (s, O-H) cm^{-1}. MS, m/e (rel int): 231 (29) $[M\text{-}H_2O]^+$, 201 (30) $[M\text{-}H_2O\text{-}2CH_3]^+$, 198 (22) $[M\text{-}2H_2O\text{-}CH_3]^+$, 186 (100) $[M\text{-}H_2O\text{-}C_2H_5O]^+$, 171 (67) $[M\text{-}H_2O\text{-}CH_3\text{-}C_2H_5O]^+$, 56 (36) $[CH_3CNCH_3]^+$.

1'*E*, 3'*E* Isomer: ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 1.51 (d, *J* = 6 Hz, CH_3-CH-OH), 1.91 (br s, CH_3-$C_{3'}$), 2.23 (s, CH_3-C_5), 2.35 (s, CH_3-C_3); 3.46 (s, CH_3-N), 4.32 (d, *J* = 7 Hz, CH_2-OH), 4.99 (q, *J* = 6 Hz, CH_3-CH-OH), 5.70 (t, *J* = 7 Hz, $H_{4'}$), 6.39 (s, $H_{1'}$), 6.39 (s, $H_{2'}$).

1'*E*, 3'*Z* Isomer: ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 1.51 (d, *J* = 6 Hz, CH_3-CH-OH), 1.98 (br s, CH_3-$C_{3'}$), 2.26 (s, CH_3-C_5), 2.35 (s, CH_3-C_3), 3.46 (s, CH_3-N), 4.32 (d, *J* = 7 Hz, CH_2-OH), 4.99 (q, *J* = 6 Hz, CH_3-CH-OH), 5.60 (t, *J* = 7 Hz, $H_{4'}$), 6.44 (d, *J* = 16 Hz, $H_{2'}$), 6.72 (d, *J* = 16 Hz, $H_{1'}$).

We obtained *all-trans* **11** from all-*trans* **10** in 84%. ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 1.88 (br s, CH_3-$C_{3'}$), 2.39 (s, CH_3-$C_{4'}$), 4.31 (d, *J* = 7 Hz, $H_{5'}$), 5.83 (br t, *J* = 7 Hz, $H_{4'}$), 6.47 (d, *J* = 16 Hz, $H_{2'}$), 7.01 (d, *J* = 16 Hz, $H_{1'}$), 7.25 (br s, $H_{2''}$, $H_{3''}$, $H_{5''}$, $H_{6''}$), 7.25 (br s, H_5), 7.78 (br s, H_2). ^{13}C-NMR (20 MHz, $CDCl_3$, TMS) δ (ppm): 12.15 (CH_3-$C_{3'}$), 20.29 (CH_3-$C_{4''}$), 57.63 (C-5'), 115.40 (C-1'), 119.16 (C-4'), 119.74 (C-2", C-6"), 119.94 (C-2'), 130.09 (C-3", C-5"), 131.14 (C-5), 132.22 (C-2), 133.33 (C-3'), 134.28 (C-4), 136.08 (C-1"), 140.78 (C-4"). IR (film): 3412 (s, O-H), 1660 (s, C=C), 1159 (s, C-O) cm^{-1}. MS, m/e (rel int): 254 (9) $[M]^+$, 239 (6) $[M\text{-}CH_3]^+$, 223 (100) $[M\text{-}CH_2OH]^+$, 118 (16) [*p*-tolyl-NCH]$^+$, 91 (55) $[C_7H_7]^+$, 65 (34) $[C_5H_5]^+$.

We obtained **20** (1'*E*, 3'*E*: 1'*E*, 3'*Z* (4:1) mixture, 43%) and **21** (13%) from a 1'*E*, 3'*E*/1'*E*, 3'*Z* (4:1) mixture isomers of **19**.

Compound ***20***. 1'*E*, 3'*Z* Isomer: ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 1.94 (br s, CH_3-$C_{3'}$), 3.63 (s, CH_3-N), 4.40 (d, *J* = 7 Hz, $H_{5'}$), 5.60 (t, *J* = 7 Hz, $H_{4'}$), 6.49 (d, *J* = 16 Hz, $H_{2'}$), 6.84 (br s, H_5), 7.26 (d, *J* = 16 Hz, $H_{1'}$), 7.36 (br s, H_2). IR (film): 3318 (s, O-H), 1619 (s, C=C), 1162 (s, C-O) cm^{-1}.

1'*E*, 3'*E* Isomer: ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 1.85 (br s, CH_3-$C_{3'}$), 3.63 (s, CH_3-N), 4.30 (d, *J* = 7 Hz, $H_{5'}$), 5.78 (t, *J* = 7 Hz, $H_{4'}$), 6.43 (d, *J* = 16 Hz, $H_{2'}$), 6.83 (br s, H_5), 6.90 (d, *J* = 16 Hz, $H_{1'}$), 7.37 (br s, H_2).

Compound ***21***. ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.34 (s, 3H, CH_3-C(O)), 3.72 (s, 3H, CH_3-N), 6.85 (d, *J* = 16 Hz, 1H, $H_{2'}$), 7.13 (br s, 1H, H_5), 7.40 (d, *J* = 16 Hz, 1H, $H_{1'}$), 7.47 (br s, 1H, H_2). IR (film): 1661 (s, C=O), 1623 (s, C=C) cm^{-1}.

General procedure for oxidations

Alcohols **5a**, **5d** or **11** (12.6 mmol), 60 g Amberlist A-26 treated with CrO_3 [19] and 50 ml absolute THF were heated under reflux until the reaction was completed (TLC monitoring). The resin was filtered, washed with THF and the solvent removed. The reaction products were purified by column chromatography (hexane/Et_2O mixtures).

Compounds **6a** (80%), as a 2:1 mixture of 1'*E*, 3'*E* and 1'*E*, 3'*Z* isomers, and **7** (8%) were obtained from all-*trans* **5a**. The same results were obtained when the 1'*E*, 3'*Z* isomer of **5a** was oxidized. Compound **5d** (1:1 mixture of 1'*E*, 3'*E*/1'*E*, 3'*Z* isomers) gave **6c** (35% yield) and **6e** (35% yield). Both compounds were obtained as a 3:1 mixture of 1'*E*, 3'*E* and 1'*E*, 3'*Z* isomers.

Compound ***6a***. IR (film): 3285 (m, H-C≡), 2099 (w, C≡C), 1650 (s, C=O) cm^{-1}.

1'*E*, 3'*E* Isomer: ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.26 (d, *J* = 1.2 Hz, CH_3-$C_{3'}$), 2.30 (s, CH_3-C_5), 2.30 (s, CH_3-C_3), 3.20 (s, HC≡C-), 3.52 (s, CH_3-N), 6.01 (d, *J* = 8 Hz, $H_{4'}$), 6.95 (d, *J* = 16 Hz, $H_{1'}$), 7.48 (d, *J* = 16 Hz, $H_{2'}$), 10.06 (d, *J* = 8 Hz, CH=O).

1'*E*, 3'*Z* Isomer: ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.16 (d, *J* = 1.2 Hz, CH_3-$C_{3'}$), 2.30 (s, CH_3-C_5), 2.30 (s, CH_3-C_3), 3.20 (s, HC≡C-), 3.52 (s, CH_3-N), 5.84 (d, *J* = 8 Hz, $H_{4'}$), 6.83 (d, *J* = 16 Hz, $H_{1'}$), 7.51 (d, *J* = 16 Hz, $H_{2'}$), 10.12 (d, *J* = 8 Hz, CH=O).

Compound ***6c***. 1'*E*, 3'*E* Isomer: ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.27 (s, CH_3-$C_{3'}$), 2.38 (s, CH_3-C_5), 2.47 (s, CH_3-C_3), 2.51 (s, CH_3CO), 3.54 (s, CH_3-N), 6.01 (d, *J* = 8 Hz, $H_{4'}$), 6.80 (d, *J* = 16 Hz, $H_{1'}$), 7.43 (d, *J* = 16 Hz, $H_{2'}$), 10.05 (d, *J* = 8 Hz, CH=O).

1'*E*, 3'*Z* Isomer: ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.20 (s, CH_3-$C_{3'}$), 2.38 (s, CH_3-C_5), 2.47 (s, CH_3-C_3), 2.51 (s, CH_3CO), 3.54 (s, CH_3-N), 5.82 (d, *J* = 8 Hz, $H_{4'}$), 6.46 (d, *J* = 16 Hz, $H_{2'}$), 6.80 (d, *J* = 16 Hz, $H_{1'}$), 10.05 (d, CH=O).

Compound ***6e***. 1'*E*, 3'*E* Isomer: ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.22 (s, CH_3-C_5), 2.27 (s, CH_3-$C_{3'}$), 2.29 (s, CH_3-C_3), 3.54 (s, CH_3-N), 5.18 (dd, *J* = 2, 11 Hz, CH_2=CH-), 5.23 (dd, *J* = 2, 18 Hz, CH_2=CH-), 6.01 (d, *J* = 8 Hz, $H_{4'}$), 6.60 (dd, *J* = 11, 18 Hz, CH_2=CH-), 6.80 (d, *J* = 16 Hz, $H_{1'}$), 7.43 (d, *J* = 16 Hz, $H_{2'}$), 10.06 (d, *J* = 8 Hz, CH=O).

1'*E*, 3'*Z* Isomer: ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.20 (s, CH_3-$C_{3'}$), 2.22 (s, CH_3-C_5), 2.29 (s, CH_3-C_3), 3.54 (s, CH_3-N), 5.18 (dd, J = 2; 11 Hz, CH_2=CH-), 5.23 (dd, J = 2; 18 Hz, CH_2=CH-), 5.82 (d, J = 8 Hz, $H_{4'}$), 6.46 (d, J = 16 Hz, $H_{2'}$), 6.60 (dd, J = 11, 18 Hz, CH_2=CH-), 6.80 (d, J = 16 Hz, $H_{1'}$), 10.10 (d, J = 8 Hz, CH=O).

Compound **7**. ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.28 (s, CH_3-C_5), 2.29 (s, CH_3-C_3), 2.30 (s, CH_3-$C_{3'}$), 3.19 (s, H-C≡C), 3.57 (s, CH_3-N), 6.33 (d, J = 16 Hz, $H_{2'}$), 7.52 (d, J = 16 Hz, $H_{1'}$). IR (KBr): 3197 (m, H-C≡), 2095 (w, C≡C), 1666 (s, C=O) cm^{-1}.

From all-*trans* **11** we obtained a 3:1 mixture of 1'*E*, 3'*E*/1'*E*, 3'*Z* isomers of **12** in 84% yield. 1'*E*, 3'*E* Isomer: ^{1}H-NMR (80 MHz, $CDCl_3$, TMS) δ (ppm): 2.34 (br s, CH_3-$C_{3'}$), 2.40 (s, CH_3-$C_{4'}$), 6.09 (d, J = 8.5 Hz, $H_{4'}$), 7.15 (d, J = 16 Hz, $H_{2'}$), 7.27 (br s, $H_{2''}$, $H_{3''}$, $H_{5''}$, $H_{6''}$), 7.27 (d, J = 16 Hz, $H_{1'}$), 7.38 (br s, H_5), 7.81 (s, H_2), 10.01 (d, J = 8.5 Hz, $H_{5'}$). ^{13}C-NMR (20 MHz, $CDCl_3$, TMS) δ (ppm): 15.36 (CH_3-$C_{3'}$), 21.06 (CH_3-$C_{4'}$), 104.21 (C-1'), 18.46 (C-4'), 121.32 (C-2'', C-6''), 121.42 (C-2'), 126.89 (C-5), 129.45 (C-2), 130.61 (C-3'', C-5''), 136.78 (C-4), 138.50 (C-1''), 140.50 (C-4''), 154.50 (C-3'), 191.55 (C-5'). IR (film): 1721 (s, C=O) cm^{-1}. MS, m/e (rel int): 252 (21) $[M]^+$, 223 (100) $[M\text{-}CHO]^+$, 118 (5) [*p*-tolyl-NCH]$^+$, 106 (16) [M-CHO-(*p*-tolyl-NC)]$^+$, 91 (14) $[C_7H_7]^+$, 65 (9) $[C_5H_5]^+$.

Reactions of ***15*** *with aldehydes*

In a flask with an N_2 atmosphere, **15** (12 mmol) was suspended in 130 ml anhydrous THF and potassium *t*-butoxide (12 mmol) in 225 ml anhydrous THF was added in small amounts with stirring, turning the mixture deep-red. After completion of the addition, stirring was continued for 15 min and a solution of 12 mmol aldehyde in 25 ml THF was added. The mixture was refluxed for 1 h and stirred at room temperature for 24 h. The solvent was then removed *in vacuo* and the residue dissolved in 30 ml 80% methanol and extracted with *t*-butyl methyl ether. The extract was washed with water, dried (Na_2SO_4) and the solvent removed *in vacuo*. The residue was column chromatographed (hexane /Et_2O mixtures).

We obtained **16** (85%), **17** (1%) and **18** (10%) from **14**.

Compound ***16***. 1'*E*, 3'*E*, 5'*E*, 7'*E* Isomers: ^{1}H-NMR and ^{13}C-NMR: table II. IR (film): 1706 (s, C=O), 1606 (s, C=C), 1158 (s, C-O) cm^{-1}. MS, m/e (rel int): 272 (3) $[M]^+$, 213 (12) $[M\text{-}COOCH_3]^+$, 173 (7) $[M\text{-}C_5H_7O_2]^+$, 147 (22) $[M\text{-}C_7H_9O_2]^+$, 125 (16) $[C_7H_9O_2]^+$, 43 (100).

Compound ***17***. Identified by comparison with a reference sample.

Compound ***18***. ^{1}H-NMR (300 MHz, $CDCl_3$, TMS) δ (ppm): 1.88 (d, J = 1.23 Hz, CH_3-C_3), 1.89 (br s, CH_3-C_7), 3.07 (s, H_2), 4.95 (br s, H_8), 5.97 (dq, J = 10.33, 1.23 Hz, H_4), 6.26 (d, J = 15.35 Hz, H_6), 6.36 (dd, J = 15.35, 10.33 Hz, H_5). ^{1}H-NMR double resonance: irradiation at 1.89 ppm affected the signal width at 4.95 ppm and *vice versa*. NOEDIF experiment: δ irradiated (δ affected): 1.8 (4.95, 6.36). ^{13}C-NMR (75 MHz, $CDCl_3$, TMS) δ (ppm): 17.03 (CH_3-C_3), 18.53 (CH_3-C_7), 45.06 (C-2), 51.80 (C-OCH_3), 116.62 (C-8), 124.76 (C-5), 129.10 (C-4), 131.25 (C-3), 135.18 (C-6), 142.17 (C-7), 171.63 (C-1).

Assignments were performed through DEPT and ^{13}C–^{1}H shift-correlated experiments.

The same reaction with compound **2a** yielded only **17** and **18**.

Biochemistry

Retinoids and cell culture

All-*trans* RA was obtained from Sigma. The concentrations chosen for our studies were within the range cited in previous *in vitro* experiments with RA.

Two human carcinoma cell lines, MCF-7 cells and HeLa cells, 2 melanoma cell lines SK Mel-5 cells (human) and B16 cells (murine), and 2 virally transformed cell lines, VERO monkey fibroblastic cells and MDCK canine epithelial cells were used. All cell lines were routinely grown in Eagle's modified minimun essential medium (Gibco) and supplemented with 5% heat-inactivated fetal bovine serum (Flow), penicillin and streptomycin. Cells were maintained at 37°C in humidified air and 5% CO_2. In all experiments, the supplemented medium was changed every 48 h. The control cultures contained supplemented medium and no more that 0.1% ethanol or DMSO [20].

Doses-response

The colorimetric method based on MTT-formazan salt reduction as an estimate of surviving cell number following exposure to retinoids was used. This has been described by Carmichael *et al* [21]. Initial inoculum was 2500 cells/well in 96-well microtiter plates (Costar). RA, compounds **8**, **13** and **22** solved in DMSO were added at different concentrations (1–35 μM) the next day. After 6 d treatment the cells were incubated with MTT-formazan (1 mg/ml final concentration) (Sigma). The absorbance was recorded in Multiskan PLUS MK II plate reader (Tittertek) at a wavelength of 540 nm. The IC_{50} values were obtained graphically by plotting survival against concentration.

Flow cytometry

For cell-cycle analysis and cellular differentiation, flow cytometry methods were used (Ortho Cytoron Absolute). The laser excitation wavelength was 488 nm at 15 mW. Green and red photomultiplier tubes were guarded by a 518–548 nm band pass filter and 570 nm longpass filter, respectively. Some. 3000–5000 cells for green fluorescence and 10 000 cells for red fluorescence were collected and analyzed using the software ARS (Ortho). Green fluorescence was quantified in 2 ways: SMFU on a log scale defined by the difference in mean channel number for total and non-specific uptake histograms [22]; and the percentage of positive cells reflecting the cells with fluorescence beyond that of the negative control.

Cell-cycle analysis

DNA staining was accomplished through the use of a modified Vindelov's propidium iodide method [23]. One million MCF-7 breast cancer cells were placed in a 75 cm^2 flask culture (Costar) and the following day 1 μM ethanol solutions of RA, **8** and **13** were added. Cells were harvested after 24 and 72 h treatment and then subjected to DNA staining.

Cellular differentiation

The method used has been reported previously [24]. In an indirect immunofluorescence technique the cells coated with mAbs were stained with fluorescein isothiocynate (FITC) conjugate. One million MCF-7 cells were placed in a 75 cm^2 flask culture (Costar). The following day, 1 mM ethanol solutions of RA, **8** and **13** were added. The 4th and 6th days of treatment, the cells were harvested and then labelled with the antibodies. The cells were made permeable with 70% cold ethanol at –20°C. The anti-keratins CAM 5.2 mAb (1 μg/ml) (Becton Dickinson) and E29 mAb against EMA (10 μg/ml) (Dako), both IgG2a isotype, and goat anti-mouse IgG-FITC

(1 μg/ml) (Biomeda) were used. As a negative control for mAbs staining, an isotype-matched IGgG2a antibody (Dako) with identical amount of Ig protein was used in the place of mAbs.

Statistical analysis

Survival percentage of cells in each phase of the cell cycle and SMFU of mAbs in treated cultures were compared with the control cells using the Student's *t*-test.

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