

## Antiproliferative and Proapoptotic Activities of a New Class of Pyrazole Derivatives in HL-60 Cells

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A series of *N*-substituted pyrazole derivatives have been synthesized and tested for their anticancer effect on the HL-60 leukaemia cell line. Four were active both in cell-growth inhibition and in inducing apoptosis. The inhibition of cell growth mainly reflects a compound-induced reduction in the number of cells in phases from S to M, whereas the induction of apoptosis involves inhibition of expression of Bcl-2 and enhanced expression of Bax with consequent reduced activation of the proapoptotic caspase 3. Finally, preliminary experiments carried out with tumor cells from myelogenous leukaemic patients showed that the compounds **4c**, **4l**, **4m**, and **4n** are indeed capable of inducing apoptosis.

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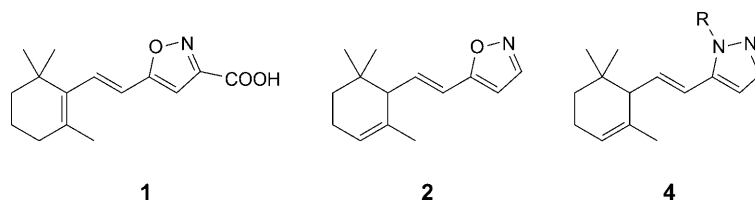
**Introduction.** – The pyrazole framework plays an essential role in numerous pharmaceutically active compounds which indeed occupy a remarkable position in vast areas of medicinal chemistry. From an important past, exemplified in the analgesic and anti-inflammatory pyrazolones and pyrazolinediones, to the present with some of the most recent important drugs, such as COX-2 inhibitors, which are not lacking severe complications, pyrazole derivatives are synthesized and studied as antibacterial compounds [1] [2], p38 MAP kinase inhibitors [3], antiangiogenic agent [4], and Hsp90 and CDK2/Cyclin A inhibitors [5–8].

While the antitumor activity of pyrazole derivatives is widely discussed in literature [4–7][9–13], only few works are focused both to antiproliferative and proapoptotic activity [14][15].

We have already reported [16][17] that compounds which can be linked to the so-called ‘short heteroretinoids’, formed by a cyclohexenyl group linked to a heterocyclic moiety by a short ethenyl chain, possesses antimicrobial [16], anti-inflammatory, and histoprotective properties [17]. The heterocyclic moiety (pyrazole, isoxazole, pyrimidine) and the substituents present in them deeply affect the biological activities. In particular, pyrimidine and isoxazole derivatives showed good antimicrobial activities, while pyrazole derivatives were found to be potent inhibitors of neutrophil chemotactic responsiveness [17].

Taking into account the above considerations and the significant differentiating activity of the ‘isoxazole heteroretinoid’ **1** [18], we started our study by evaluating our

corresponding isoxazole and pyrazole derivatives **2** and **4**, respectively. Since compound **2** showed only antimicrobial activity [16], we focused our attention on pyrazoles **3** (see the *Scheme*, below) expanding this class of compounds which exhibited promising antiproliferative properties in our preliminary experiments.



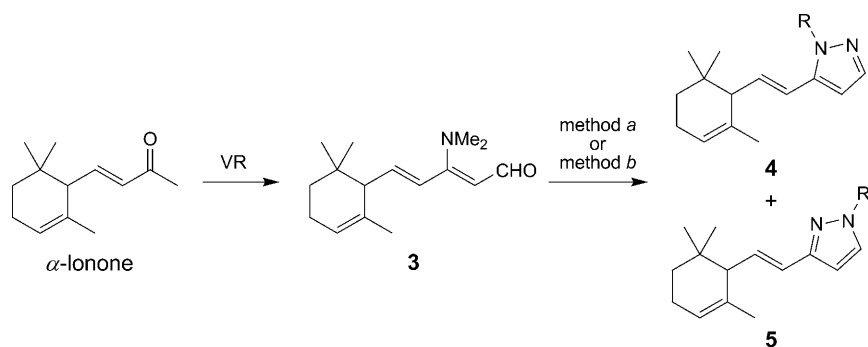
This idea proved to be a winning strategy: in fact, most of **4**, to different extents, showed antiproliferative and/or apoptotic activities. These findings, further supported by the fact that the present compounds **4c**, **4m**, and **4n** are indeed capable of inducing apoptosis in tumor cells from myelogenous leukaemic patients, could pave the way to a new class of drugs for cancer therapy. Moreover, in an attempt to understand the mechanism of action, we found that some of them inhibit Bcl-2 and induce BAX expression.

**Results and Discussion.** – *Syntheses.* The synthesis of pyrazole derivatives **4a–4r** are outlined in the *Scheme*. The key intermediate **3**, which was synthesized according to our well-tested procedure, was reacted with the hydrazine derivatives modifying the cyclization reaction used in the past [16][17]. When bifunctional reagents were present as hydrochlorides, they were reacted as such (*Method b*), while all the other hydrazine derivatives were reacted with equimolar quantities of HCl (*Method a*). We were then able to obtain the desired pyrazole derivatives in high yields and in a very short period of time. Moreover, in nearly half of the cases, we isolated the 1,3-pyrazole derivative **5** with the predominant 1,5-pyrazoles **4**. It is worth noting that compounds **4p–4q** and all the compounds **5** can only be prepared by this new procedure.

*Structure Elucidation.* The structural isomerism of the compounds was easily determined by detailed examination of the <sup>1</sup>H- and <sup>13</sup>C-NMR spectra of the pyrazole moiety of compounds **4r** and **5r**, which were chosen as an example, because the signals the R moiety do not interfere with those needed to elucidate the structures. The pyrazole CH signals have been assigned in both compounds on the basis of their HECTOR (300 MHz) spectra (*Table 1*) and the literature data [19][20]. In particular, the spin coupling constant (<sup>2</sup>J (H,H) of pyrazole ring) of compounds **4** is lower than that of compounds **5**. Moreover, the chemical shift of the quaternary C(5)-atom of compounds **4** (ca. 141 ppm) is lower than that of the quaternary C(3)-atom of compounds **5** (ca. 150 ppm), and the chemical shift of the H–C(5) of compounds **5** (ca. 130 ppm) is lower than that of the H–C(3) of compounds **4** (ca. 139 ppm). This behavior is consistent with literature data [19][20] and is common to all the isomers we synthesized.

*Biological Results.* Experiments were carried out to test the effects of different compounds on the proliferation and apoptosis of HL60 cells. The results obtained are collected in *Table 2*, showing the concentrations required for 50% inhibition of cell

## Scheme. Synthesis of Pyrazole Derivatives



	R	
<b>4a</b>	H	
<b>4b, 5b</b>	HO-CH <sub>2</sub> -CH <sub>2</sub>	
<b>4c</b>	Me <sub>2</sub> N-C(S)	
<b>4d</b>	<b>d</b>	
<b>4e, 5e</b>	Ph	
<b>4f, 5f</b>	2-Cl-C <sub>6</sub> H <sub>4</sub>	
<b>4g, 5g</b>	4-Cl-C <sub>6</sub> H <sub>4</sub>	
<b>4h, 5h</b>	4-Br-C <sub>6</sub> H <sub>4</sub>	
<b>4i</b>	4-HOOC-C <sub>6</sub> H <sub>4</sub>	
<b>4l, 5l</b>	4-EtOOC-C <sub>6</sub> H <sub>4</sub>	
<b>4m, 5m</b>	3-OH-C <sub>6</sub> H <sub>4</sub> -CH <sub>2</sub>	
<b>4n, 5n</b>	<b>n</b>	
<b>4o, 5o</b>	4-HO <sub>3</sub> S-C <sub>6</sub> H <sub>4</sub>	
<b>4p</b>	4-NC-C <sub>6</sub> H <sub>4</sub>	
<b>4q</b>	4-H <sub>2</sub> NO <sub>2</sub> S-C <sub>6</sub> H <sub>4</sub>	
<b>4r, 5r</b>	EtOOC-CH <sub>2</sub>	

Table 1. Selected Chemical Shifts [ppm] of *sp*<sup>2</sup> CH Groups and C-Atoms for Compounds **4r** and **5r**

	<b>4r</b>		<b>5r</b>	
	$\delta(\text{C})$	$\delta(\text{H})$	$\delta(\text{C})$	$\delta(\text{H})$
C(1(ethene))	117.2	6.10	123.2	6.36
C(2(ethene))	137.3	6.00	133.6	6.06
C(3)	139.4	7.46	151.7	—
C(4)	103.3	6.35	103.2	6.38
C(5)	141.9	—	131.5	7.38
C(3')	121.9	5.43	121.2	5.43

Table 2. Antiproliferative Effects ( $IC_{50}$ ) and Apoptosis-Inducing Effects ( $AC_{50}$ ) of Compounds **4a–4q**, **5l–5n**, and ATRA (= 'All Trans Retinoic Acid') on HL60 Cells

	$IC_{50}$ [ $\mu$ M] <sup>a)</sup>	$AC_{50}$ [ $\mu$ M] <sup>a)</sup>
<b>4a</b>	NA <sup>b)</sup>	NA
<b>4b</b>	>40	NA
<b>4c</b>	27 ± 2	53 ± 11
<b>4d</b>	NA	NA
<b>4e</b>	>40	NA
<b>4f</b>	28 ± 5	>100
<b>4g</b>	15 ± 1.9	NA
<b>4h</b>	17 ± 4	NA
<b>4i</b>	NA	NA
<b>4l</b>	32 ± 1	76 ± 19
<b>5l</b>	NA	NA
<b>4m</b>	12 ± 1	82 ± 17
<b>5m</b>	NA	NA
<b>4n</b>	26 ± 8	48 ± 5
<b>5n</b>	>40	NA
<b>4o</b>	>40	57 ± 25
<b>4p</b>	>40	43 ± 14
<b>4q</b>	– <sup>c)</sup>	– <sup>c)</sup>
ATRA	14 ± 3	>100

<sup>a)</sup> Data represent the mean ± SD of three independent experiments. <sup>b)</sup> Not active. <sup>c)</sup> Insoluble under the experimental conditions.

proliferation (± SD)  $IC_{50}$  [ $\mu$ M] and the concentrations required to induce apoptosis in 50% of cells (± SD)  $AC_{50}$  [ $\mu$ M].

Among the compounds able to inhibit cell proliferation, four, *i.e.*, **4c**, **4l**, **4m**, **4n**, were found to induce cell apoptosis as well. As shown in *Fig. 1*, all these compounds induced apoptosis in a dose-dependent manner.

In an attempt to investigate the mechanism of the proapoptotic effects of these compounds, we focused our attention on Bcl-2 and Bax, known to be involved as inhibitor and promoter of apoptosis, respectively. As shown in *Fig. 2*, after 24 h of incubation with HL60 cells, compounds **4c** and **4n** reduced the expression of Bcl-2 (*Fig. 2,b*) and enhanced the expression of Bax (*Fig. 2,a*). Consistent with these findings and suggesting a crucial role of expression Bcl-2 and Bax in mediating the proapoptotic effects of the compounds, the ability of **4c** and **4n** to induce apoptosis was significantly reduced by DEVD-CHO inhibitor of caspase 3 known to be the final effector of apoptosis (*Fig. 3*).

Finally, to gain further insight into the mechanism of action of these compounds, the flow cytometric analysis of the cell cycle was evaluated, measuring propidium iodide uptake. When compared to untreated cells, cell samples treated with **4c** showed a marked increase in the percentage of apoptotic cells, and a sharp decrease in cell-cycling capacity (*Fig. 4*). In particular, after 24 h of treatment the amount of cells in phases from S to M was reduced to half.

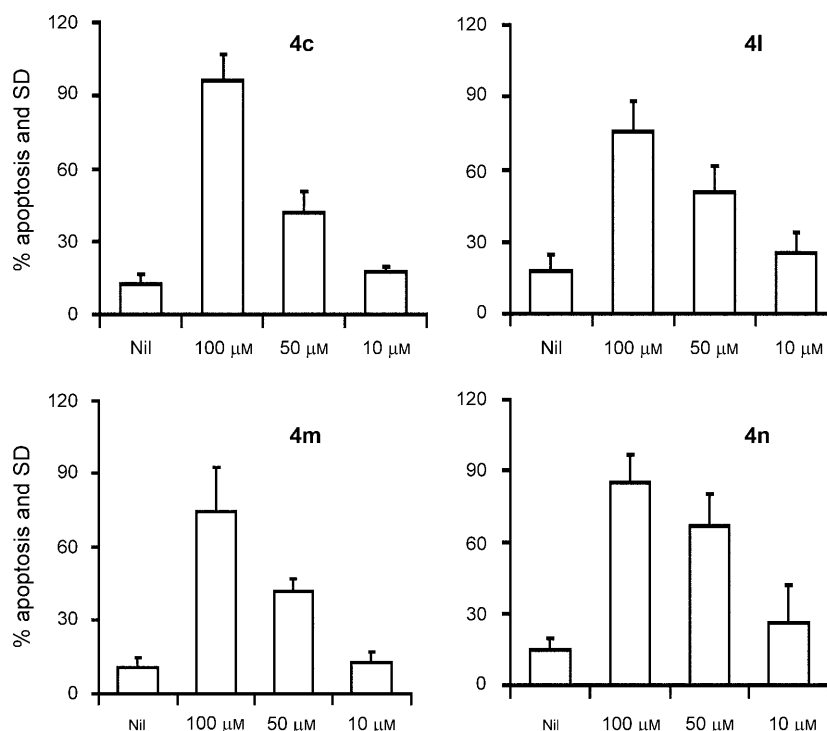


Fig. 1. Effect of different doses of pyrazole derivatives **4c**, **4l**, **4m**, and **4n** on cell apoptosis. Data represent the mean  $\pm$  SD of four independent experiments ( $n=4$ ).

**Conclusions.** – The general aim of the present work was to develop new compounds with potent anticancer activity. In this context, among 17 pyrazole derivatives tested, four, *i.e.*, **4c**, **4l**, **4m**, **4n**, appear to be active both in cell-growth inhibition and in induction of apoptosis using HL-60 cells, while the 1,3-isomers, *i.e.*, **5l**, **5m**, **5n**, were devoid of any activity. Concentrations of the compounds required for 50% cell-growth inhibition ( $IC_{50}$ ) and those required to induce 50% apoptosis ( $AC_{50}$ ) are equimolar. Nevertheless, the possibility that these two effects merely reflect a single molecular mechanism is far from being established. In this regard, the involvement of distinct molecular targets responsible for the antiproliferative and apoptotic effects has indeed suggested to explain the biological activity of synthetic retinoids only partially related to the present compounds [17][21][22]. In the present setting, preliminary experiments indicate that the inhibition of the cell growth mainly reflects a compound-induced reduction in the number of cells in phases from S to M, whereas the induction of apoptosis involves inhibition of expression of Bcl-2 and enhanced expression of Bax with consequently reduced activation of the proapoptotic caspase 3. It is noteworthy that compounds **4e**, **4f**, **4h**, and **4g** display antiproliferative activity but are unable to induce apoptosis. On the contrary **4o** and **4p** show only proapoptotic activity. These results indicate that the phenyl moiety alone or substituted with Cl or Br in *ortho*- or *para*-position lead to compounds without apoptotic activity. It is worth noting that the

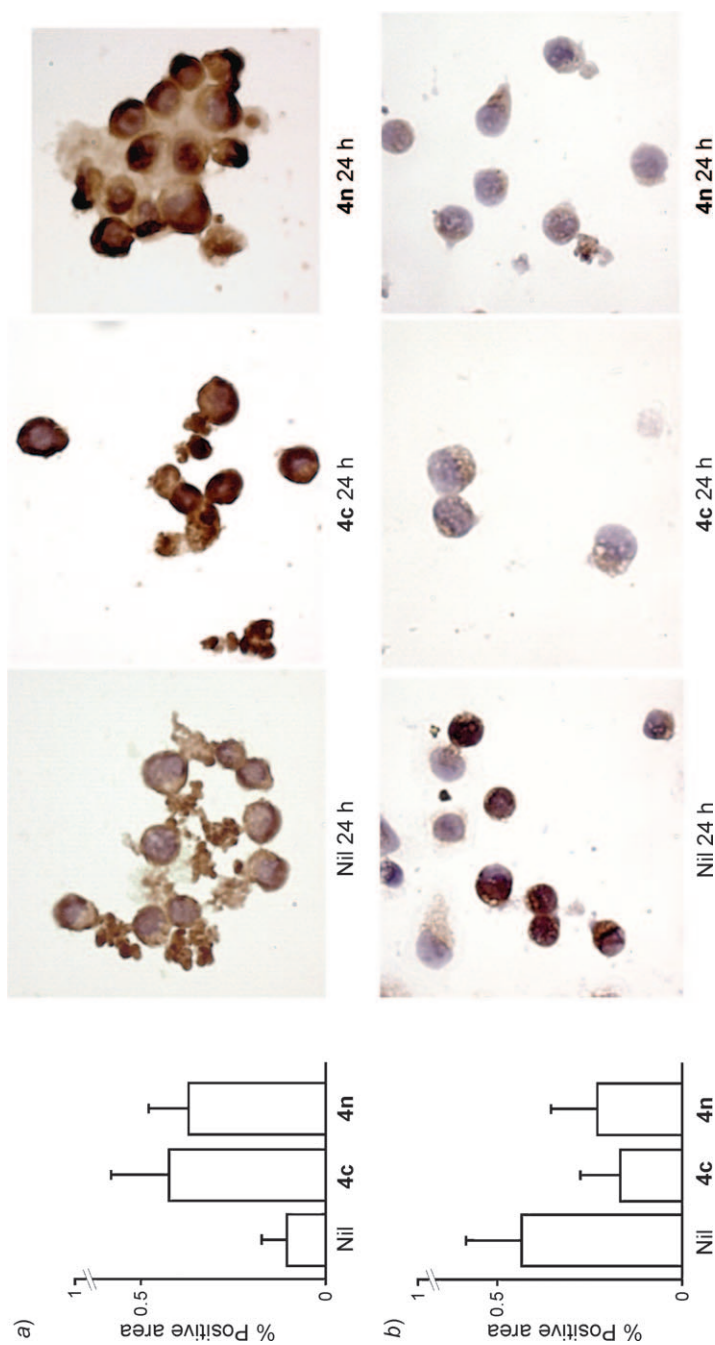


Fig. 2. a) *Modulation of Bax and b) Bcl-2 expression in HL60 cells cultured with or without 50  $\mu$ M 4c and 4n.* HL60 Cells were incubated for 24 h with or without the compounds. Bax and Bcl-2 expressions were determined by immunocytochemistry and digital image analysis. The results are expressed as mean  $\pm$  standard deviation (SD) from three independent experiments

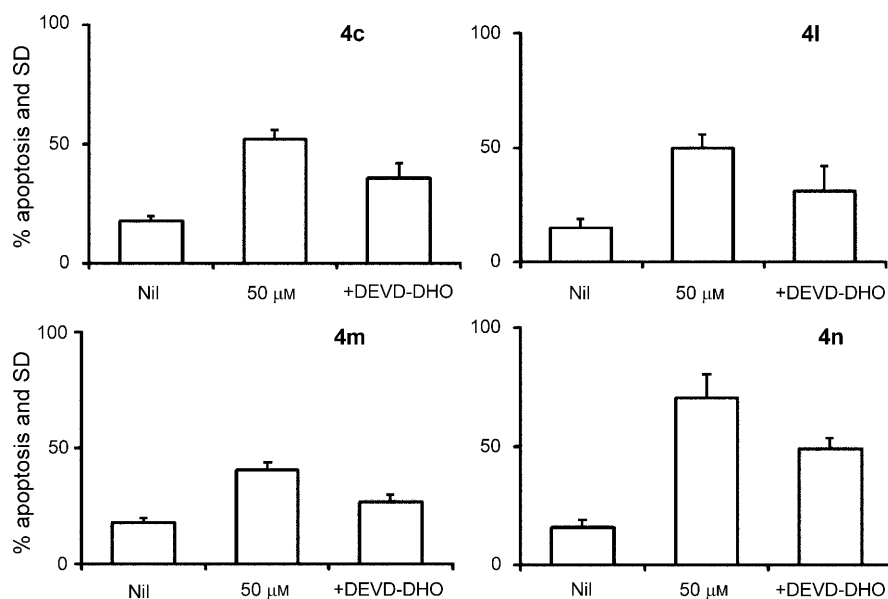


Fig. 3. Effect of pretreatment with DEVD-CHO for 1 h on cell apoptosis of HL60 cells exposed to different doses of compounds **4c**, **4l**, **4m**, and **4n**. The values are presented as mean  $\pm$  SD ( $n=3$ ).

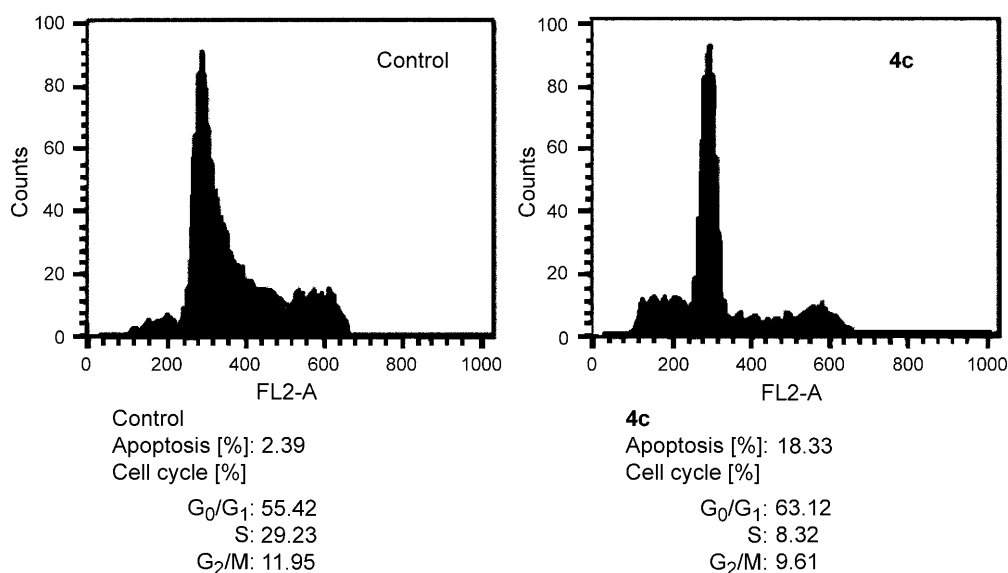


Fig. 4. Compound **4c** promotes apoptosis and inhibits cell-cycle progression. DNA Content and fragmentation were evaluated by measuring propidium iodide uptake by flow cytometry on cells incubated for 24 h with either medium (control), or with medium added with 50  $\mu$ M **4c**. Percentages of cells in apoptosis and of cells in the different phases of cell cycle are indicated. Single representative experiments out of three.

replacement of phenyl with pyridine causes an impressive change in activity. In fact, **4n** presents a remarkable activity both as an apoptosis inducer and as an antiproliferative agent. Compounds **4a**, **4d**, and **4i** are completely ineffective in the present setting. It is relevant that **4a** and **4d** have previously been shown to exert bacterial killing [16] and to interfere with the activation of the respiratory burst in human neutrophils [17], consistent with an anti-inflammatory potential of these molecules.

In conclusion, the biological activity of pyrazole derivatives appears to be strongly dependent both on the isomerism and the substituents present in the heterocyclic moiety. In particular, the substituents present in position 1 of the pyrazole ring play a fundamental role in determining and increasing the activity. The present results also indicate the possibility of developing this class of molecules which could arrest cell cycle at G1/S phase and induce apoptosis of HL-60 cells. Consistently, preliminary experiments carried out on tumor cells from myelogenous leukaemic patients show that the compounds **4c**, **4l**, **4m**, and **4n** are indeed capable of efficiently inducing apoptosis (Table 3).

Table 3. Induction of Apoptosis in Blast Cells from Myelogenous Leukaemia Patients. Results are expressed as (% apoptosis in the presence of the drugs) – (% apoptosis in the absence of the drugs).

	Patient 1	Patient 2
<b>4c</b>	50	52
<b>4l</b>	52	48
<b>4m</b>	60	58
<b>4n</b>	84	75

We would like to thank Prof. *Francesco Lucchesini*, University of Genoa, Italy, for the  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR analyses. Financial support of this research by the *Ateneo of Genoa* is gratefully acknowledged.

### Experimental Part

**General.** The purity of all compounds was checked by TLC ( $\text{SiO}_2$  60-F-254 pre-coated plates; visualization by UV light or by vanillin in  $\text{H}_2\text{SO}_4$ . M.p.: *Fisher-Johns* apparatus; uncorrected. IR Spectra: film or KBr disks, on a *Perkin-Elmer 398* spectrometer.  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra: *Varian Gemini 200* ( $^1\text{H}$ : 200 MHz,  $^{13}\text{C}$ : 50 MHz) or *Bruker DPX 300* (300 MHz) instrument, in  $\text{CDCl}_3$  or ( $\text{D}_6$ )DMSO; chemical shifts ( $\delta$ ) in ppm from the peak for internal TMS; coupling constants ( $J$ ) in Hz. Elemental analyses: *Carlo Erba 1106 Elemental Analyser* in the Microanalysis Laboratory of our Department.

Compound **3** was prepared as described in [16].

**General Procedure (GP) for the Preparation of Compounds 4 and 5.** The hydrazines (*Method a*) or hydrazine hydrochlorides (*Method b*) (2 mmol) were added in one portion to a stirred soln. of compound **3** (0.5 g, 2 mmol) in acidified EtOH (10 ml containing 0.17 ml of HCl 37%) (*Method a*) or EtOH (10 ml) (*Method b*). The resulting soln. was stirred for 1 h at different temps. After cooling, compounds **4i**, **4p**, and **4q** were collected by filtration from the mixture and crystallized. All the other compounds were obtained by  $\text{SiO}_2$  chromatography of the residue after removal of the solvent.

5-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazole (**4a**). From  $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$  at  $50^\circ$  (*Method a*); chromatographic eluent: toluene. Thick oil. 89% Yield. For IR, and  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data, see [17].

2-[5-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]ethanol (**4b**) and 2-[3-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]ethanol (**5b**). From 2-hydrazinoethanol at  $25^\circ$  (*Method a*). After evaporation to dryness, the residue was chromatographed on  $\text{SiO}_2$  eluting first with



toluene/AcOEt 9:1 and then with AcOEt. The first eluate gave **5b** as thick yellow oil in 10% yield. The second eluted fraction gave **4b** in 70% yield as a thick yellow oil.

*Data of 4b.* For IR, and  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data, see [17].

*Data of 5b.* IR (film): 3303, 2956, 1630, 1508, 1375, 1363.  $^1\text{H}$ -NMR (200 MHz,  $\text{CDCl}_3$ ): 0.86 (s, 3 H); 0.92 (s, 3 H); 1.16–1.22 (m, 3 H); 1.41–1.51 (m, 3 H); 1.60 (s, 3 H); 1.99–2.03 (m, 2 H); 2.27 (d,  $J = 9.4$ , 1 H); 3.85 (s, OH); 3.96–4.03 (m, 2 H); 4.21–4.27 (m, 2 H); 5.42–5.46 (m, 1 H); 6.16 (dd,  $J = 9.4$ , 16.0, 1 H); 6.31 (d,  $J = 2.6$ , 1 H); 6.35 (d,  $J = 16.0$ , 1 H); 7.36 (d,  $J = 2.6$ , 1 H).  $^{13}\text{C}$ -NMR (50 MHz,  $\text{CDCl}_3$ ): 22.4; 22.4; 26.2; 27.1; 30.7; 31.6; 54.0; 55.1; 62.0; 103.0; 122.3; 123.5; 131.4; 133.7; 134.4; 151.9. Anal. calc. for  $\text{C}_{16}\text{H}_{24}\text{N}_2\text{O}$ : C 73.81, H 9.29, N 10.76; found: C 74.18, H 9.12, N 10.63.

*N,N*-Dimethyl-5-[2-(2,6,6-trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazole-1-carbothioamide (**4c**). From thiosemicarbazide at 25° (*Method a*). After evaporation to dryness, the residue was chromatographed on  $\text{SiO}_2$  eluting with toluene, red oil. Yield 55%. For IR, and  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data, see [17].

4-Amino-5-[5-[2-(2,6,6-trimethylcyclohex-1-en-1-yl)ethenyl]pyrazol-1-yl]-4H-[1,2,4]triazole-3-thiol (**4d**). From *Purpald*® at reflux (*Method a*). After evaporation to dryness, the residue was chromatographed on  $\text{SiO}_2$  eluting first with toluene and then with toluene/AcOEt 9:1. The resulting yellow solid was filtered, washed with  $\text{H}_2\text{O}$ , dried, and crystallized from cyclohexane. Yield 80%. M.p. 138–139°. For IR, and  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data, see [17].

1-Phenyl-5-[2-(2,6,6-trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazole (**4e**) and 1-Phenyl-3-[2-(2,6,6-trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazole (**5e**). From  $\text{PhNHNH}_2 \cdot \text{HCl}$  at 25° (*Method b*). After evaporation to dryness, the residue was chromatographed on  $\text{SiO}_2$  eluting with toluene. The first eluate gave **5e** in 10% yield as thick oil. The second eluted fraction gave **4e** in 80% yield as a thick oil.

*Data for 4e.* For IR, and  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data, see [17].

*Data for 5e.* IR (film): 2958, 2915, 1600, 1518, 1457, 1384.  $^1\text{H}$ -NMR (200 MHz,  $\text{CDCl}_3$ ): 0.88 (s, 3 H); 0.94 (s, 3 H); 1.17–1.23 (m, 1 H); 1.43–1.53 (m, 1 H); 1.63 (s, 3 H); 2.01–2.05 (m, 2 H); 2.29 (d,  $J = 9.1$ , 1 H); 5.41–5.48 (m, 1 H); 6.14 (dd,  $J = 9.1$ , 15.8, 1 H); 6.50 (d,  $J = 15.8$ , 1 H); 6.53 (d,  $J = 2.6$ , 1 H); 7.22–7.28 (m, 1 H); 7.39–7.45 (m, 2 H); 7.64–7.68 (m, 2 H); 7.83 (d,  $J = 2.6$ , 1 H).  $^{13}\text{C}$ -NMR (50 MHz,  $\text{CDCl}_3$ ): 22.3; 22.3; 26.2; 27.0; 30.7; 31.7; 53.9; 103.6; 118.0; 120.5; 122.4; 125.3; 126.8; 128.6; 133.1; 133.6; 139.3; 151.7. Anal. calc. for  $\text{C}_{20}\text{H}_{24}\text{N}_2$ : C 82.15, H 8.27, N 9.58; found: C 81.89, H 8.33, N 9.45.

1-(4-Chlorophenyl)-5-[2-(2,6,6-trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazole (**4f**) and 1-(2-Chlorophenyl)-3-[2-(2,6,6-trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazole (**5f**). From 2-chlorophenylhydrazine hydrochloride at reflux (*Method b*). After evaporation to dryness, the residue was chromatographed on  $\text{SiO}_2$  eluting with toluene. The first eluate gave **5f** in 10% yield as a red thick oil. The second eluted fraction gave **4f** in 87% yield as a red thick oil.

*Data of 4f.* For IR,  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data, see [17].

*Data of 5f.* IR (film): 2910, 2854, 1890, 1593, 1495, 1382, 1364.  $^1\text{H}$ -NMR (200 MHz,  $\text{CDCl}_3$ ): 0.89 (s, 3 H); 0.92 (s, 3 H); 1.22–1.26 (m, 1 H); 1.40–1.46 (m, 1 H); 1.63 (s, 3 H); 2.01–2.05 (m, 2 H); 2.30 (d,  $J = 8.6$ , 1 H); 5.42–5.47 (m, 1 H); 6.20 (dd,  $J = 8.6$ , 15.8, 1 H); 6.40 (d,  $J = 15.8$ , 1 H); 6.54 (d,  $J = 2.4$ , 1 H); 7.24–7.34 (m, 3 H); 7.36–7.40 (m, 1 H); 7.82 (d,  $J = 2.4$ , 1 H).  $^{13}\text{C}$ -NMR (50 MHz,  $\text{CDCl}_3$ ): 22.2; 22.3; 26.2; 27.3; 30.6; 31.7; 54.0; 104.1; 122.0; 122.5; 127.9; 129.5; 130.5; 130.8; 131.3; 131.4; 133.7; 136.7; 138.0; 154.0. Anal. calc. for  $\text{C}_{20}\text{H}_{23}\text{ClN}_2$ : C 73.49, H 7.09, Cl 10.85, N 8.57; found: C 73.44, H 7.12, Cl 10.90, N 8.68.

1-(4-Chlorophenyl)-5-[2-(2,6,6-trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazole (**4g**) and 1-(4-Chlorophenyl)-3-[2-(2,6,6-trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazole (**5g**). From 4-chlorophenylhydrazine hydrochloride at 25° (*Method b*). According to the *GP* the first eluate gave **5g** as yellow thick oil, yield 10%. The second eluate gave **4g** as red oil. Yield 78%.

*Data of 4g.* For IR,  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data, see [17].

*Data of 5g.* IR (film): 2920, 2857, 1522, 1498, 1383, 1363.  $^1\text{H}$ -NMR (200 MHz,  $\text{CDCl}_3$ ): 0.88 (s, 3 H); 0.94 (s, 3 H); 1.23–1.27 (m, 1 H); 1.41–1.45 (m, 1 H); 1.63 (s, 3 H); 2.00–2.03 (m, 2 H); 2.40 (d,  $J = 8.6$ , 1 H); 5.45–5.51 (m, 1 H); 6.12 (dd,  $J = 8.6$ , 16.1, 1 H); 6.48 (d,  $J = 16.1$ , 1 H); 6.52 (d,  $J = 2.6$ , 1 H); 7.37 (d,  $J = 8.8$ , 2 H); 7.60 (d,  $J = 8.8$ , 2 H); 7.78 (d,  $J = 2.6$ , 1 H).  $^{13}\text{C}$ -NMR (50 MHz,  $\text{CDCl}_3$ ): 22.2; 22.3; 26.1; 27.1; 30.6; 31.7; 53.9; 104.1; 118.4; 119.5; 120.6; 122.1; 126.7; 131.7; 133.1; 134.2; 138.6; 151.8. Anal. calc. for  $\text{C}_{20}\text{H}_{23}\text{ClN}_2$ : C 73.49, H 7.09, Cl 10.85, N 8.57; found: C 73.45, H 7.17, Cl 10.86, N 8.63.

1-(4-Bromophenyl)-5-[2-(2,6,6-trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazole (**4h**) and 1-(4-Bromophenyl)-3-[2-(2,6,6-trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazole (**5h**). From 4-bromophenylhy-

drazine hydrochloride at 25° (*Method b*). According to the *GP*, the first eluate gave **5h** as yellow solid, 8% yield. M.p. 68–70° from cyclohexane. The second eluate gave **4h** as thick red oil. Yield 88%.

*Data of 4h.* For IR, and <sup>1</sup>H- and <sup>13</sup>C-NMR data, see [17].

*Data of 5h.* IR (KBr): 2918, 2856, 1592, 1523, 1496, 1382. <sup>1</sup>H-NMR (200 MHz, CDCl<sub>3</sub>): 0.88 (s, 3 H), 0.94 (s, 3 H); 1.17–1.23 (m, 1 H); 1.42–1.46 (m, 1 H); 1.63 (s, 3 H); 2.02–2.05 (m, 2 H); 2.26 (d, *J* = 8.5, 1 H); 5.43–5.49 (m, 1 H); 6.14 (dd, *J* = 8.5, 15.8, 1 H); 6.48 (d, *J* = 15.8, 1 H); 6.54 (d, *J* = 2.6, 1 H); 7.54 (s, 4 H); 7.80 (d, *J* = 2.6, 1 H). <sup>13</sup>C-NMR (50 MHz, CDCl<sub>3</sub>): 22.3; 22.4; 26.2; 27.1; 30.7; 31.8; 53.9; 104.1; 118.5; 119.4; 120.6; 122.1; 126.8; 131.6; 132.9; 134.3; 138.2; 152.0. Anal. calc. for C<sub>20</sub>H<sub>23</sub>BrN<sub>2</sub>: C 64.69, H, 6.24, Br 21.52, N 7.54; found: C 64.55, H 6.06, Br 21.43, N 7.45.

*4-[5-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]benzoic Acid (4i).* From 4-hydrazinobenzoic acid (*Method a*) at 25°. The resulting white solid was crystallized from AcOEt. Yield 93%. M.p. 209–210°. IR (KBr): 2917, 2508, 1704, 1606, 1512, 1420, 1384, 1260. <sup>1</sup>H-NMR: 0.85 (s, 3 H); 0.88 (s, 3 H); 1.18–1.22 (m, 1 H); 1.38–1.42 (m, 1 H); 1.58 (s, 3 H); 1.99–2.03 (m, 2 H); 2.25 (d, *J* = 7.4, 1 H); 5.40–5.44 (m, 1 H); 6.08–6.22 (m, 2 H); 6.72 (d, *J* = 1.6, 1 H); 7.57 (d, 2 H); 7.67 (d, *J* = 1.6, 1 H); 8.1 (d, 2 H). <sup>13</sup>C-NMR: 23.3; 23.5; 27.4; 28.2; 31.6; 32.9; 54.7; 105.9; 115.1; 119.1; 122.1; 125.2; 130.4; 130.7; 131.2; 133.7; 137.02; 141.6; 143.4; 167.3. Anal. calc. for C<sub>21</sub>H<sub>24</sub>N<sub>2</sub>O<sub>2</sub>: C 74.97, H 7.19, N 8.33; found: C 74.74, H 7.09, N 8.40.

*Ethyl 4-[5-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]benzoate (4l) and Ethyl 4-[3-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]benzoate (5l).* From ethyl 4-hydrazinobenzoate at 25° (*Method a*). After evaporation to dryness, the residue was chromatographed on SiO<sub>2</sub> eluting with toluene. The first eluate gave **5l** as yellow oil. Yield 7%. The second eluted fraction gave **4l** as yellow oil. Yield 92%.

*Data of 4l.* IR (film): 2958, 2921, 1715, 1607, 1515, 1463, 1366, 1247. <sup>1</sup>H-NMR: 0.91 (s, 3 H); 0.95 (s, 3 H); 1.26–1.28 (m, 1 H); 1.40–1.48 (m, 4 H); 1.63 (s, 3 H); 1.99–2.03 (m, 2 H); 2.22 (d, *J* = 8.4, 1 H); 4.40 (q, *J* = 7.2, 2 H); 5.40–5.44 (m, 1 H); 6.10 (dd, *J* = 8.4, 15.4, 1 H); 6.25 (d, *J* = 15.4, 1 H); 6.50 (d, *J* = 2.2, 1 H); 7.62 (d, *J* = 8.4, 2 H); 7.64 (d, *J* = 2.2, 1 H); 8.18 (d, *J* = 8.4, 2 H). <sup>13</sup>C-NMR: 14.4; 23.1; 23.1; 26.9; 27.9; 31.5; 32.8; 54.9; 61.4; 105.0; 118.8; 122.0; 124.5; 129.3; 130.6; 133.1; 137.0; 140.1; 140.9; 143.3; 165.1. Anal. calc. for C<sub>23</sub>H<sub>28</sub>N<sub>2</sub>O<sub>2</sub>: C 75.79, H 7.74, N 7.69; found: C 75.82, H 7.70, N 7.64.

*Data of 5l.* IR (film): 2959, 2926, 1715, 1608, 1524, 1367, 1276. <sup>1</sup>H-NMR (200 MHz, CDCl<sub>3</sub>): 0.88 (s, 3 H); 0.94 (s, 3 H); 1.22–1.26 (m, 1 H); 1.42 (t, *J* = 7.2, 3 H); 1.49–1.53 (m, 1 H); 1.63 (s, 1 H); 2.01–2.05 (m, 2 H); 2.30 (d, *J* = 7.8, 1 H); 4.38 (q, *J* = 7.2, 2 H); 5.44–5.48 (m, 1 H); 6.16 (dd, *J* = 7.8, 16.0, 1 H); 6.50 (d, *J* = 16.0, 1 H); 6.57 (d, *J* = 2.6, 1 H); 7.74 (d, *J* = 9.0, 2 H); 7.90 (d, *J* = 2.6, 1 H); 8.10 (d, *J* = 9.0, 2 H). <sup>13</sup>C-NMR (50 MHz, CDCl<sub>3</sub>): 13.6; 22.3; 22.4; 26.2; 27.0; 30.6; 31.8; 53.9; 60.3; 104.7; 116.9; 120.7; 122.1; 126.9; 130.3; 132.9; 134.6; 142.4; 152.5; 165.2. Anal. calc. for C<sub>23</sub>H<sub>28</sub>N<sub>2</sub>O<sub>2</sub>: C 75.79, H 7.74, N 7.69; found: C 75.80, H 7.71, N 7.63.

*4-([5-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]methyl)phenol (4m) and 4-([3-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]methyl)phenol (5m).* From 3-hydroxybenzylhydrazine dihydrochloride at 25° (*Method b*). The residue was chromatographed on SiO<sub>2</sub> eluting with toluene. The first eluate gave **5m** as a yellow oil. Yield 10%. The second eluate gave **4m** as already pure yellow crystals in 87% Yield.

*Data of 4m.* M.p. 128–130° from AcOEt. IR (KBr): 3149, 2959, 2865, 1602, 1458, 1282. <sup>1</sup>H-NMR (200 MHz, CDCl<sub>3</sub>): 0.78 (s, 3 H); 0.87 (s, 3 H); 1.15–1.19 (m, 1 H); 1.33–1.37 (m, 1 H); 1.48 (s, 3 H); 1.98–2.02 (m, 2 H); 2.17 (d, *J* = 8.97, 1 H); 5.24 (s, 2 H); 5.41–5.45 (m, 1 H); 5.98 (dd, *J* = 9.0, 15.9, 1 H); 6.15 (d, *J* = 15.9, 1 H); 6.31 (d, *J* = 2.0, 1 H); 6.44 (s, 1 H); 6.61 (dd, *J* = 2.2, 7.8, 1 H); 6.67 (dd, *J* = 2.2, 7.8, 1 H); 7.13 (t, *J* = 7.8, 1 H); 7.35 (d, *J* = 2.0, 1 H). <sup>13</sup>C-NMR (50 MHz, CDCl<sub>3</sub>): 22.0; 22.3; 26.0; 27.1; 30.5; 31.7; 52.2; 53.9; 102.4; 112.8; 114.5; 116.7; 116.9; 121.1; 128.9; 132.3; 136.4; 137.3; 137.5; 140.8; 156.8. Anal. calc. for C<sub>21</sub>H<sub>26</sub>N<sub>2</sub>O: C 78.22, H 8.13, N 8.69; found: C 78.20, H 8.10, N 8.47.

*Data of 5m.* IR (film): 3260, 2958, 2923, 1602, 1459, 1280. <sup>1</sup>H-NMR: 0.84 (s, 3 H); 0.91 (s, 3 H); 1.17–1.23 (m, 1 H); 1.39–1.45 (m, 1 H); 1.58 (s, 3 H); 1.98–2.03 (m, 2 H); 2.20 (d, *J* = 8.9, 1 H); 5.17 (s, 2 H); 5.23–5.27 (m, 1 H); 6.03 (dd, *J* = 8.9, 16.1, 1 H); 6.31 (d, *J* = 2.4, 1 H); 6.35 (d, *J* = 16.1, 1 H); 6.42 (s, 1 H); 6.70 (dd, *J* = 1.8, 7.1, 2 H); 7.12 (t, *J* = 7.1, 1 H); 7.28 (d, *J* = 2.4, 1 H). <sup>13</sup>C-NMR: 23.0; 23.0; 26.9; 27.8; 31.5; 32.5; 54.6; 55.4; 102.9; 114.2; 115.6; 118.7; 121.3; 122.6; 129.8; 130.9; 133.8; 133.9; 137.7; 151.0; 157.2. Anal. calc. for C<sub>21</sub>H<sub>26</sub>N<sub>2</sub>O: C 78.22, H 8.13, N 8.69; found: C 78.23, H 8.09, N 8.64.

2-[5-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]pyridine (**4n**) and 2-[3-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]pyridine (**5n**). From 2-hydrazinopyridine dihydrochloride at 50° (*Method b*). The residue was chromatographed on SiO<sub>2</sub> eluting with toluene. The first eluate gave **5n** as a yellow oil. Yield 11%. The second eluate gave **4n** as yellow oil. Yield 72%.

*Data of 4n.* IR (film): 2900, 2915, 2864, 1590, 1470, 1436, 1378. <sup>1</sup>H-NMR (200 MHz, CDCl<sub>3</sub>): 0.91 (s, 3 H); 0.96 (s, 3 H); 1.18–1.22 (m, 1 H); 1.42–1.47 (m, 1 H); 1.68 (s, 3 H); 1.99–2.03 (m, 2 H); 2.18 (d, *J* = 9.1, 1 H); 5.25–5.27 (m, 1 H); 6.07 (dd, *J* = 9.1, 15.8, 1 H); 6.51 (d, *J* = 1.8, 1 H); 7.12 (d, *J* = 15.8, 1 H); 7.20–7.28 (m, 1 H); 7.62 (d, *J* = 1.8, 1 H); 7.82–7.86 (m, 2 H); 8.44–8.46 (m, 1 H). <sup>13</sup>C-NMR (50 MHz, CDCl<sub>3</sub>): 23.2; 23.2; 27.1; 27.9; 31.6; 32.7; 54.7; 105.4; 117.1; 121.0; 121.5; 121.5; 134.7; 135.5; 138.5; 140.9; 142.6; 147.9; 151.9. Anal. calc. for C<sub>19</sub>H<sub>23</sub>N<sub>3</sub>: C 77.78, H 7.90, N 14.32; found: C 77.74, H 7.50, N 14.17.

*Data of 5n.* IR (film): 2900, 2914, 2862, 1592, 1470, 1436, 1383. <sup>1</sup>H-NMR (200 MHz, CDCl<sub>3</sub>): 0.88 (s, 3 H); 0.94 (s, 3 H); 1.18–1.22 (m, 1 H); 1.49–1.55 (m, 1 H); 1.62 (s, 3 H); 2.00–2.05 (m, 2 H); 2.33 (d, *J* = 9.3, 1 H); 5.44–5.48 (m, 1 H); 6.17 (dd, *J* = 9.3, 16.3, 1 H); 6.50 (d, *J* = 16.3, 1 H); 6.53 (d, *J* = 2.8, 1 H); 7.08–7.16 (m, 1 H); 7.72–7.82 (m, 1 H); 7.94 (d, *J* = 6.9, 1 H); 8.30–8.40 (m, 1 H); 8.46 (d, *J* = 2.8, 1 H). <sup>13</sup>C-NMR (50 MHz, CDCl<sub>3</sub>): 22.3; 22.4; 26.2; 27.0; 30.8; 31.8; 53.98; 104.0; 111.4; 120.2; 120.6; 122.3; 127.1; 132.9; 134.6; 137.9; 147.1; 150.6; 152.8. Anal. calc. for C<sub>19</sub>H<sub>23</sub>N<sub>3</sub>: C 77.78, H 7.90, N 14.32; found: C 77.72, H 7.57, N 14.20.

4-[5-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]benzenesulfonic Acid (**4o**) and 4-[3-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]benzenesulfonic Acid (**5o**). From 4-hydrazinobenzenesulfonic acid hemihydrate at reflux (*Method a*). The residue was chromatographed on SiO<sub>2</sub> eluting first with toluene/AcOEt 1:1 and then with EtOH. The first eluate gave **5o** as an already pure yellow solid. Yield 16%. The second eluate gave **4o** as a yellow oil. Yield 49%.

*Data of 4o.* IR (film): 3445, 2959, 2917, 1599, 1502, 1387, 1182. <sup>1</sup>H-NMR (200 MHz, CDCl<sub>3</sub>): 0.80 (s, 3 H); 0.84 (s, 3 H); 1.17–1.21 (m, 1 H); 1.37–1.42 (m, 1 H); 1.51 (s, 3 H); 1.94–1.98 (m, 2 H); 2.19 (d, *J* = 8.9, 1 H); 5.38–5.42 (m, 1 H); 6.05–6.13 (m, 2 H); 6.49 (d, *J* = 1.8, 1 H); 7.45 (d, *J* = 8.4, 2 H); 7.68 (d, *J* = 1.8, 1 H); 7.89 (d, *J* = 8.4, 2 H); 8.70 (s, 1 H, D<sub>2</sub>O exchangeable). <sup>13</sup>C-NMR (50 MHz, CDCl<sub>3</sub>): 22.2; 22.2; 26.1; 27.0; 30.7; 31.8; 54.1; 103.6; 117.7; 121.1; 124.1; 124.1; 126.2; 126.2; 132.2; 136.3; 139.7; 140.2; 140.8; 142.7. Anal. calc. for C<sub>20</sub>H<sub>24</sub>N<sub>2</sub>O<sub>3</sub>S: C 64.49, H 6.49, N 7.52, S 8.61; found: C 64.69, H 6.40, N 7.64, S 8.50.

*Data of 5o.* M.p. 234–237° from AcOEt. IR (KBr): 3447, 2960, 2912, 1601, 1521, 1384, 1195. <sup>1</sup>H-NMR (200 MHz, CDCl<sub>3</sub>): 0.81 (s, 3 H); 0.84 (s, 3 H); 1.12–1.16 (m, 1 H); 1.40–1.45 (m, 1 H); 1.56 (s, 3 H); 1.94–1.98 (m, 2 H); 2.26 (d, *J* = 8.7, 1 H); 5.39–5.43 (m, 1 H); 6.12 (dd, *J* = 7.4, 15.5, 1 H); 6.39 (d, *J* = 15.5, 1 H); 6.72 (d, *J* = 2.6, 1 H); 7.66–7.74 (m, 4 H); 8.32 (d, *J* = 2.6, 1 H); 8.72 (s, 1 H, D<sub>2</sub>O exchangeable). <sup>13</sup>C-NMR (50 MHz, CDCl<sub>3</sub>): 22.6; 22.6; 26.6; 27.4; 30.8; 31.9; 53.8; 105.0; 118.3; 121.2; 124.2; 124.2; 126.4; 126.4; 126.8; 132.9; 135.7; 139.4; 140.1; 151.5. Anal. calc. for C<sub>20</sub>H<sub>24</sub>N<sub>2</sub>O<sub>3</sub>S: C 64.49, H 6.49, N 7.52, S 8.61; found: C 64.60, H 6.43, N 7.66, S 8.51.

4-[5-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]benzonitrile (**4p**). From 4-cyanophenylhydrazine hydrochloride at 50° (*Method b*). After evaporation to dryness, the residue was washed and crystallized from cyclohexane. Yield 49%. White solid. M.p. 97–100°. IR (KBr): 3421, 2956, 2911, 2229, 1607, 1509, 1391. <sup>1</sup>H-NMR (200 MHz, CDCl<sub>3</sub>): 0.88 (s, 3 H); 0.92 (s, 3 H); 1.22–1.27 (m, 1 H); 1.39–1.43 (m, 1 H); 1.60 (s, 3 H); 1.99–2.04 (m, 2 H); 2.25 (d, *J* = 8.8, 1 H); 5.45–5.49 (m, 1 H); 6.02–6.16 (m, 2 H); 6.48 (d, *J* = 1.8, 1 H); 7.59–7.67 (m, 3 H); 7.76 (d, *J* = 8.8, 2 H). <sup>13</sup>C-NMR (50 MHz, CDCl<sub>3</sub>): 22.3; 22.3; 26.1; 27.1; 30.6; 31.9; 54.1; 55.5; 104.9; 110.2; 117.5; 117.5; 121.4; 124.2; 124.2; 131.9; 132.4; 132.4; 137.1; 140.5; 140.9; 141.0. Anal. calc. for C<sub>21</sub>H<sub>23</sub>N<sub>3</sub>: C 79.46, H 7.30, N 13.24; found: C 79.27, H 7.57, N 13.29.

4-[5-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]benzenesulfonamide (**4q**). From (4-sulfamoylphenyl)hydrazine hydrochloride at reflux (*Method b*). The resulting white solid was filtered and washed with EtOH. Yield 23%. M.p. 179–180°. IR (KBr): 3305, 2952, 2922, 1596, 1500, 1339, 1165. <sup>1</sup>H-NMR (200 MHz, DMSO): 0.77 (s, 3 H); 0.80 (s, 3 H); 1.10–1.14 (m, 1 H); 1.22–1.26 (m, 1 H); 1.49 (s, 3 H); 1.87–1.92 (m, 2 H); 2.21 (d, *J* = 8.8, 1 H); 5.34–5.38 (m, 1 H); 6.00–6.22 (m, 2 H); 6.65 (d, *J* = 2.0, 1 H); 7.44 (s, 2 H, D<sub>2</sub>O exchangeable); 7.54–7.60 (m, 3 H); 7.89 (d, *J* = 8.4, 2 H). <sup>13</sup>C-NMR (50 MHz, DMSO): 22.6; 22.6; 26.5; 27.4; 30.9; 32.1; 53.9; 55.5; 104.9; 118.0; 121.2; 124.6; 124.6; 126.7;

126.7; 132.7; 136.3; 140.6; 140.7; 141.4; 142.8. Anal. calc. for  $C_{20}H_{25}N_3O_2S$ : C 64.66, H 6.78, N 11.31, O 8.61, S 8.63; found: C 64.41, H 6.86, N 11.32, O 8.61, S 8.88.

*Ethyl 2-[5-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]ethanoate (4r) and Ethyl 2-[3-[2-(2,6,6-Trimethylcyclohex-2-en-1-yl)ethenyl]-1H-pyrazol-1-yl]ethanoate (5r)*. From ethyl hydrazinoacetate hydrochloride at 50° (*Method b*). The residue was chromatographed on  $SiO_2$  eluting with toluene/AcOEt 9:1. The first eluate gave **5r** as an already pure white solid. Yield 18%. The second eluate gave **4r** as a yellow oil. Yield 72%.

*Data of 4r*. IR (film): 2959, 2915, 1758, 1464, 1375, 1206.  $^1H$ -NMR (200 MHz,  $CDCl_3$ ): 0.84 (s, 3 H); 0.91 (s, 3 H); 1.20–1.24 (m, 1 H); 1.25 (t,  $J = 7.3$ , 3 H); 1.41–1.46 (m, 1 H); 1.60 (s, 3 H); 2.00–2.06 (m, 2 H); 2.25 (d,  $J = 8.6$ , 1 H); 4.20 (q,  $J = 7.3$ , 2 H); 4.91 (s, 2 H); 5.42–5.46 (m, 1 H); 6.00 (dd,  $J = 8.6$ , 15.8, 1 H); 6.11 (d,  $J = 15.8$ , 1 H); 6.32 (d,  $J = 1.9$ , 1 H); 7.44 (d,  $J = 1.9$ , 1 H).  $^{13}C$ -NMR (50 MHz,  $CDCl_3$ ): 14.1; 22.9; 23.0; 26.9; 27.7; 31.4; 32.5; 50.9; 54.9; 61.8; 103.3; 117.2; 121.9; 132.9; 137.3; 139.4; 141.9; 167.7. Anal. calc. for  $C_{18}H_{26}N_2O_2$ : C 71.49, H 8.67, N 9.26; found: C 71.45, H 8.57, N 9.47.

*Data of 5r*. M.p. 45–46° from toluene. IR (KBr): 2954, 2914, 1741, 1464, 1373, 1235.  $^1H$ -NMR (200 MHz,  $CDCl_3$ ): 0.85 (s, 3 H); 0.91 (s, 3 H); 1.15–1.19 (m, 1 H); 1.25 (t,  $J = 7.5$ , 3 H); 1.41–1.46 (m, 1 H); 1.60 (s, 3 H); 1.98–2.03 (m, 2 H); 2.23 (d,  $J = 8.8$ , 1 H); 4.22 (q,  $J = 7.5$ , 2 H); 4.84 (s, 2 H); 5.40–5.44 (m, 1 H); 6.02 (dd,  $J = 8.8$ , 16.0, 1 H); 6.37 (d,  $J = 16.0$ , 1 H); 6.37 (d,  $J = 2.4$ , 1 H); 7.36 (d,  $J = 2.4$ , 1 H).  $^{13}C$ -NMR (50 MHz,  $CDCl_3$ ): 14.1; 23.1; 23.1; 26.9; 27.8; 31.5; 32.5; 52.9; 54.7; 61.8; 103.2; 121.2; 123.2; 131.5; 133.6; 133.9; 151.7; 167.9. Anal. calc. for  $C_{18}H_{26}N_2O_2$ : C 71.49, H 8.67, N 9.26; found: C 71.34, H 8.71, N 9.34.

*Cell Culture*. HL-60 Cells (promyelocytic cell line) were grown in RPMI 1640 (Gibco, Grand Island, NY, USA) containing 10% FCS (ICN), 100 U/ml penicillin (Gibco), 100 mg/ml streptomycin (Gibco), and 2 mM L-glutamine (Gibco) in 5%  $CO_2$  at 37°. To evaluate their viability, cells were stained with trypan blue and counted with a phase-contrast microscopy. Cells which showed trypan blue uptake were considered nonviable.

*Morphological Evaluation of Apoptosis and Necrosis*. HL-60 Cells were exposed to different concentrations of each compound. After a 48-h period of culture, the effects of each compound on apoptosis were determined morphologically by fluorescent microscopy after labelling with acridine orange (Sigma) and ethidium iodide (EI). Cells ( $3 \times 10^5$ ) were centrifuged and resuspended in the dye mixture; 10  $\mu$ l of the cell suspension was placed on a microscope slide and examined with a fluorescence microscope. Living cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (EB; red fluorescence) stain. Apoptotic cells were identified by perinuclear condensation of chromatin stained with acridine orange and by the formation of apoptotic bodies (percentage of apoptotic cells). Necrotic cells were identified by uniform labelling of the cells with EB.

*Analysis of Cell Proliferation (MTT Assay)*. Cell proliferation was measured using mitochondrial respiration. It was assessed by a colorimetric test that detects the conversion of 3-(4,5-dimethyl-2H-thiazol-2-yl)-2,5-diphenyltetrazolium hydrobromide (MTT) into formazan product by enzymes of the respiratory chain. Briefly, 50,000 cells/well were seeded in 96-well plates in 100  $\mu$ l of medium alone and exposed to appropriate doses of each compound for 48 h. At the end of the incubation time, MTT soln. was added to the cell cultures at the final concentration of 5 mg/ml, and cells were incubated for an additional 4 h. Thereafter, cells were lysed in DMSO. The conversion of MTT to formazan, corresponding to mitochondrial respiratory activity, was monitored by automated microplate reader at 590 nm. The number of cells proliferating is calculated using different concentrations of cells cultured in 96-well plate under the same experimental conditions (calibration curve).

*Caspase Studies*. Caspase-3 inhibitor (DEVD-CHO, Ac-Asp-Glu-Val-Asp-aldehyde from Bachem, 20 mM) was added to cells 1 h before cell exposure to more active compounds among those tested. Experiments were carried out using the same experimental conditions of apoptosis assays.

*Cell-Cycle Analysis*. The technique of Nicoletti *et al.* [23] was used, with minor changes, to evaluate cell cycle and apoptosis. Briefly, after washing,  $10^6$  cells were permeabilized by adding 200  $\mu$ l of PBS plus 0.1% NP40 to cell pellet. Samples were gently shaken, and, after 30 s, 200  $\mu$ l of PBS plus 50  $\mu$ g/ml propidium iodide (PI) and 100  $\mu$ g/ml RNase (both from Sigma-Aldrich, St. Louis, MO) were added. Samples were incubated in the dark at r.t. for 10 min and then analyzed on a flow cytometer.

(FACScalibur, BD Biosciences, San Jose, CA). Ten thousand events were analyzed per sample. The amount of cells in the different phases of the cell cycle and the amount of cells in apoptosis was evaluated by measuring right and left shift from normal diploid DNA content, resp.

**Immunocytochemistry.** Bax and Bcl-2 protein expression was investigated by immunocytochemistry as described by Dibbert [24] with slight modification. Briefly, HL60 cells, collected after incubation with or without the more active compounds among those tested, were cytocentrifuged (*Cytospin*<sup>®</sup>). After rehydration in PBS, spots were submerged in peroxidase-quenching soln. for 10 min to neutralize endogenous peroxidase activity. Then, the slides were incubated with anti-human Bax polyclonal antibody (1 mg/ml, Santa-Cruz, CA) or with anti-human Bcl-2 mAb (1 mg/ml, Santa-Cruz, CA) diluted 1:200 in PBS. The secondary biotinylated IgG antibody (*Kit Histostain SP*, Zymed Laboratories, San Francisco, CA) was used. After washing and subsequent incubation with biotin-streptavidin-peroxidase (*Kit Histostain SP*), slides were incubated at r.t. for 5 min with the peroxidase-substrate soln. (*Kit Histostain SP*), rinsed with PBS, and counter-stained with ematoxylin. Then, cytopins were mounted in *Eukitt* (Merk), examined by light microscopy (*Leica*, Cambridge, UK), and evaluated by image analysis (*Leica*). Isotype-matched mAbs (*R&D Systems*) of irrelevant specificity were tested as negative control.

**Image Analysis.** Image analysis was performed by the *Leica Q500 MC* image analysis system (*Leica*). For each sample, 100 cells were randomly analyzed, and the optical density of the signal was quantified by computer analysis. The video image was digitalized for image analysis at 256 grey levels. Imported data were analyzed quantitatively by *Q500 MC Software-Qwin* (*Leica*). The operator randomly selected single cells using the cursor. Then, the positive area was analyzed automatically. The same optical threshold and filter combination were used throughout all of these experiments.

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