Synthesis of Furonaphth[1,3]oxazine and Furo[1,3]oxazinoquinoline Derivatives as Precursors for an *o*-Quinonemethide Structure and Potential Antitumor Agents

Leila BENAMEUR,[a] Zouhair BOUAZIZ,[a] Pascal NEBOIS,[a] Marie-Hélène BARTOLI,[b] Michèle BOITARD,[b] and Houda FILLION*,[a]

Laboratoire de Chimie Organique, Institut des Sciences Pharmaceutiques et Biologiques, Université Claude Bernard,[a] 8 Avenue Rockefeller, F-69373 Lyon Cedex 08, France, Laboratoire de Biotechnique et de Pharmacie Clinique GREPO, UFR de Pharmacie de Grenoble, Université Joseph Fourier,[b] Domaine de La Merci, 38706 La Tronche Cedex, France. Received July 28, 1995; accepted October 31, 1995

The synthesis of dihydro furonaphth[1,3]oxazine derivatives 3 was performed through a Mannich-type condensation between 2-cyano-5-hydroxy-3-methylnaphtho[1,2-*b*]furan 2a, 1.5 eq of a primary amine and 3 eq of formaldehyde. Similarly, 2-cyano-5-hydroxy-3-methylfuro[2,3-*f*]quinoline 2b gave the dihydro furo[1,3]oxazinoquinoline compounds 4. Heating a mixture of the naphthofuran 2a, *tert*-butylamine and formaldehyde at toluene reflux led to the furonaphthoxazine 3e, which decomposes to afford an *o*-quinonemethide intermediate 5. The latter was trapped with 1-morpholinopropene to give a dihydro furonaphthopyran derivative 6. All compounds 2, 3, 4 and 6 were assayed for *in vitro* cytotoxic activity toward L 1210, MDA-MB 231 and PC3 tumor cells. Among them, furonaphth[1,3]oxazines 3b, 3c, and furo[1,3]oxazinoquinolines 4c, 4d showed significant activity against L 1210 cells, while furoquinoline 2b was the most cytotoxic compound towards all three cell lines.

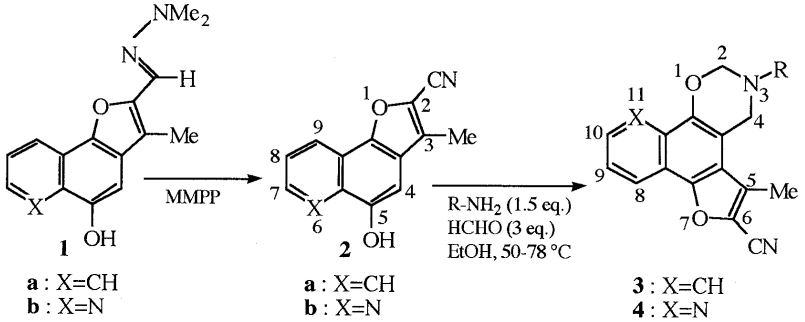
Key words dihydro-1,3-oxazine; furonaphth[1,3]oxazine; furo[1,3]oxazinoquinoline; *o*-quinonemethide; cytotoxicity

As part of our investigations on the biological properties of fused 3-methylfuran derivatives, we developed an efficient synthesis of 3-methyl-5-hydroxynaphthofurans and 3-methyl-5-hydroxyfuroquinolines through an addition-cyclization process of 2-ethoxybut-2-enal *N*,*N*-dimethylhydrazone with naphthoquinones or quinolinediones.[1] Evaluation of the *in vitro* cytotoxic activity of these compounds was performed on murine lymphocytic leukemia cells (L 1210), human mammary adenocarcinoma cells (MDA-MB 231) and human prostate cells (PC3).[2] Several naphthofurans and furoquinolines showed significant IC_{50} values towards L 1210 cells while cytotoxicity against MDA-MB 231 and PC3 tumor cells was only retained in the furoquinoline series. On the other hand, some *N*-substituted dihydro[1,3]oxazines condensed with aromatic rings were reported to possess cytotoxic or antifungal activities.[3-6] In order to check whether aminomethylation of the 5-hydroxynaphthofuran and 5-hydroxyfuroquinoline skeletons would influence the inhibition of tumor cell proliferation, we planned to synthesize and test a series of furonaphth[1,3]oxazine and furo[1,3]oxazinoquinoline derivatives.

Synthesis

The hydrazone function of **1a** or **1b** was converted to a cyano group by the use of magnesium monoperoxyphthalate hexahydrate (MMPP) according to a known procedure.[7] Thus, compounds **2a** and **2b**[8] were obtained in excellent yields (94 and 90% respectively). Then, the dihydro[1,3]oxazine derivatives **3** and **4** were prepared through a Mannich-type condensation by heating an ethanol solution of the corresponding phenol **2** with a primary amine (1.5 eq) and three equivalents of a 37% aqueous solution of formaldehyde (Chart 1 and Table 1).

Mannich bases of phenols and dihydro[1,3]oxazines are known to be thermally unstable and to decompose to *o*-quinonemethide and the respective amine or imine.[9-11] Heating to reflux a toluene solution of the naphthofuran **2a** and a mixture of *tert*-butylamine (1 eq) and formaldehyde (2 eq) in the same solvent gave the furonaphthoxazine **3e**, which decomposed quickly to an *o*-quinonemethide **5** through a retro Diels–Alder reaction. Due to its instability, **5** was not isolated, but was trapped with *trans*-1-morpholinopropene. Thus, the furonaphthopyran **6** was obtained as a single stereoisomer in 71% yield through a one-pot procedure. Formation of compound **6**



1 **a**: X=CH **b**: X=N **2** **a**: X=CH **b**: X=N **3**: X=CH **4**: X=N

Chart 1

* To whom correspondence should be addressed.

Table 1. Synthesis of Furonaphth[1,3]oxazines **3** and Furo[1,3]oxazinoquinolines **4**

3	X	R	Time (h)	Yield (%)	4	X	R	Time (h)	Yield (%)
3a	CH	$CH_2C_6H_5$	1	80	**4a**	N	$CH_2C_6H_5$	6	78
3b	CH	$CH_2C_6H_4OCH_3$	0.5	90	**4b**	N	$CH_2C_6H_4OCH_3$	6	79
3c	CH	CH_2–$CH=CH_2$	0.5	80	**4c**	N	CH_2–$CH=CH_2$	2	75
3d	CH	$CH_2CH_2N(CH_3)_2$	0.5	65	**4d**	N	$CH_2CH_2N(CH_3)_2$	0.5	87
3e	CH	$C(CH_3)_3$	0.75	60					

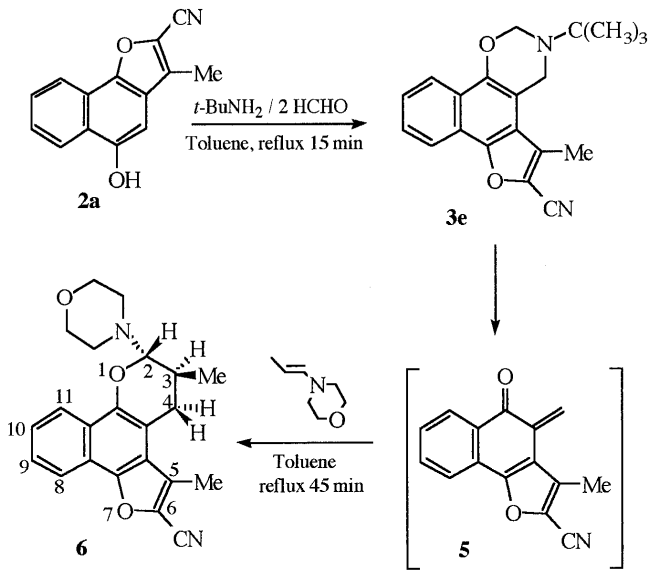


Chart 2

resulted from a regiospecific and stereospecific [4+2] cycloaddition with an inverse electron demand (Chart 2). Its relative *trans* configuration was established from its ^{1}H-NMR spectrum at 300 MHz. Indeed, H-2 gave a doublet at 4.44 ppm with a 9.4 Hz *J*-value which is in accord with literature data for *J-trans* coupling in analogous dihydropyran derivatives.[12]

Pharmacology

All of compounds **2**, **3**, **4** and **6** were assayed for *in vitro* cytotoxic activity against the three tumor cell lines mentioned above. The IC_{50} values are reported in Table 2.

The IC_{50} values given in Table 2 show that the furonaphth[1,3]oxazine derivatives are more cytotoxic towards L 1210 cells than the starting naphthofuran **2a**. In the furo[1,3]oxazinoquinolines, the best IC_{50} values against L 1210 cells are observed when R is an allyl (**4c**) or a dimethylaminoethyl group (**4d**). But, these two compounds remain less active than the parent furoquinoline **2b**. Finally, the furonaphthopyran **6** is inactive. A comparison of the IC_{50} values of **2a** and **2b** indicated that the furoquinoline structure is essential for significant cytotoxicity against the three tumor cell lines used. Moreover, aminomethylation of **2a** slightly reduces the cytotoxicity of compounds **3b** and **3c** to L 1210 cells, whereas the cytotoxicity of **4** is weaker than that of **1b**.

Table 2. Effect of Compounds **2**, **3**, **4** and **6** on the Growth of L 1210, MDA-MB 231 and PC3 Cells (IC_{50}, M)

Compound	L 1210	MDA-MB 231	PC3
2a	18.071×10^{-6}	17.009×10^{-6}	30.941×10^{-6}
2b	0.459×10^{-6}	0.950×10^{-6}	2.007×10^{-6}
3a	10.002×10^{-6}	$>28\times10^{-6}$	$>28\times10^{-6}$
3b	2.995×10^{-6}	$>26\times10^{-6}$	$>26\times10^{-6}$
3c	2.256×10^{-6}	16.612×10^{-6}	14.127×10^{-6}
3d	4.958×10^{-6}	7.512×10^{-6}	10.481×10^{-6}
4a	6.0×10^{-6}	10.137×10^{-6}	10.932×10^{-6}
4b	3.013×10^{-6}	12.286×10^{-6}	10.260×10^{-6}
4c	1.499×10^{-6}	7.073×10^{-6}	4.596×10^{-6}
4d	2.261×10^{-6}	4.754×10^{-6}	16.319×10^{-6}
6	$>28\times10^{-6}$	$>28\times10^{-6}$	$>28\times10^{-6}$
Doxorubicin	0.035×10^{-6}	0.082×10^{-6}	0.515×10^{-6}

Experimental

Melting points were measured on a Büchi apparatus (capillary tube). The infrared (IR) spectra were obtained on a Perkin-Elmer 1310 spectrophotometer. The proton nuclear magnetic resonance spectra were recorded at 300 MHz on a Bruker AM 300 apparatus. Chemical shifts are reported in ppm (δ) from tetramethylsilane (TMS) as an internal reference. Elemental analysis was done at the Centre de Microanalyse du CNRS at Solaize, France.

All the amines were freshly distilled before use. Compounds **1a**,[1] **1b**[1] and **2b**[8] were prepared according to the cited procedures. The naphthofuran derivative **2a** was obtained as reported.[8] 1-Morpholinopropene was also prepared as reported.[13]

2-Cyano-5-hydroxy-3-methylnaphtho[1,2-*b*]furan (2a) A solution of the hydrazone **1a** (0.223 g, 1 mmol) in 3 ml of methanol was added under stirring to magnesium monoperoxyphthalate hexahydrate (1.224 g, 2.5 mmol) in the same solvent (8 ml) cooled to 0 °C. At the end of the addition (5 min), stirring was continued for 5 min at 0 °C. Then, dichloromethane (25 ml) and water (25 ml) were added. The organic layer was washed twice with a saturated aqueous solution (25 ml) of sodium chloride. The solution was dried over magnesium sulfate and evaporated under vacuum. The pink solid obtained was recrystallized from ethanol to yield compound **2a** (94%). mp 167—170 °C. IR (KBr): 3400, 2220 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 10.41 (1H, s, OH), 8.25 (1H, dd, J=7.7, 1.1 Hz, H-6 or H-9), 8.18 (1H, dd, J=7.7, 1.1 Hz, H-6 or H-9), 7.70 (1H, dt, J=7, 1.3 Hz, H-7 or H-8), 7.63 (1H, dt, J=7, 1.3 Hz, H-7 or H-8), 6.93 (1H, s, H-4), 2.50 (3H, s, CH_3). *Anal.* Calcd for $C_{14}H_9NO_2$: C, 75.32; H, 4.06; N, 6.27. Found: C, 75.02; H, 4.14; N, 6.36.

General Procedure for the Synthesis of 3-Substituted-6-cyano-5-methyl-3,4-dihydro-2*H*-furo[3′,2′ : 3,4]naphth[2,1-*e*][1,3]oxazines (3) The corresponding primary amine (1.5 mmol) was added to a 37% aqueous solution of formaldehyde (3 mmol) in ethanol (15 ml) previously cooled in an ice bath. The resulting mixture was stirred at 0 °C for 30 min. Then, compound **2a** (1 mmol) was added and the reaction mixture was heated at 50 °C for a variable time, the evolution of the reaction being followed by TLC. Precipitates of the corresponding dihydrofuronaphthoxazines **3** were formed. They were collected and recrystallized from ethanol.

3-Benzyl-6-cyano-5-methyl-3,4-dihydro-2*H*-furo[3′,2′ : 3,4]naphth[2,1-*e*][1,3]oxazine (3a) Compound **3a** was obtained as a white solid (80% yield). mp 190 °C. IR (KBr): 2220 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 8.23 (2H, m, H-8 and H-11), 7.62 (2H, m, H-9 and H-10), 7.32 (5H, m, H aromat.), 5.07 (2H, s, H-2), 4.35 (2H, s, H-4), 4.0 (2H, s, N–CH_2–C_6H_5), 2.42 (3H, s, CH_3-5). *Anal.* Calcd for $C_{23}H_{18}N_2O_2$, 0.33 H_2O: C, 76.65; H, 5.22; N, 7.77. Found: C, 76.56; H, 5.09; N, 8.01.

6-Cyano-3-*p*-methoxybenzyl-5-methyl-3,4-dihydro-2*H*-furo[3′,2′ : 3,4]-naphth[2,1-*e*][1,3]oxazine (3b) Compound **3b** was obtained as a white solid (90% yield). mp 158 °C. IR (KBr): 2220 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 8.24 (2H, dd, J=6.2, 2.2 Hz, H-8 and H-11), 7.64 (2H, m, H-9 and H-10), 7.33 (2H, d, J=8.6 Hz, H aromat.), 6.92 (2H, d, J=8.6 Hz, H aromat.), 5.07 (2H, s, H-2), 4.40 (2H, s, H-4), 3.99 (2H, s, N–C<u>H</u>$_2$–C_6H_5–OCH_3), 3.84 (3H, s, OCH_3), 2.44 (3H, s, CH_3-5). *Anal.* Calcd for $C_{24}H_{20}N_2O_3$: C, 74.98; H, 5.24; N, 7.29. Found: C, 75.0; H, 5.27; N, 7.37.

3-Allyl-6-cyano-5-methyl-3,4-dihydro-2*H*-furo[3′,2′ : 3,4]naphth[2,1-*e*][1,3]oxazine (3c) Compound **3c** was obtained as a white solid (80% yield). mp 164 °C. IR (KBr): 2220 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 8.22 (2H, m, H-8 and H-11), 7.63 (2H, m, H-9 and H-10), 6.03 (1H, m, CH_2=C<u>H</u>–CH_2), 5.31 (2H, m, C<u>H</u>$_2$=CH–CH_2), 5.09 (2H, s, H-2), 4.45 (2H, s, H-4), 3.54 (2H, d, J=6 Hz, CH_2=CH–C<u>H</u>$_2$), 2.53 (3H, s, CH_3-5). *Anal.* Calcd for $C_{19}H_{16}N_2O_2$, 0.1 H_2O: C, 74.54; H, 5.33; N, 9.15. Found: C, 74.39; H, 5.34; N, 9.26.

6-Cyano-3-(*N*,*N*-dimethyl)ethyl-5-methyl-3,4-dihydro-2*H*-furo[3′2′ : 3,4]naphth[2,1-*e*][1,3]oxazine (3d) Compound **3d** was obtained as a white solid (65% yield). mp 145 °C. IR (KBr): 2220 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 8.19 (2H, m, H-8 and H-11), 7.60 (2H, m, H-9 and H-10), 5.07 (2H, s, H-2), 4.46 (2H, s, H-4), 3.04 (2H, t, J=6.3 Hz, C<u>H</u>$_2$–$CH_2N(CH_3)_2$), 2.67 (2H, t, J=6.3 Hz, CH_2–C<u>H</u>$_2$N$(CH_3)_2$), 2.55 (3H, s, CH_3-5), 2.39 (6H, s, N$(CH_3)_2$). *Anal.* Calcd for $C_{20}H_{21}N_3O_2$, 0.1 H_2O: C, 71.24; H, 6.34; N, 12.46. Found: C, 71.34; H, 6.56; N, 12.49.

6-Cyano-5-methyl-3-*tert*-butyl-3,4-dihydro-2*H*-furo[3′2′ : 3,4]naphth-[2,1-*e*][1,3]oxazine (3e) Compound **3e** was obtained as a yellow solid (60% yield). mp 140 °C. IR (KBr): 2220 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 8.18 (2H, m, H-8 and H-11), 7.58 (2H, m, H-9 and H-10), 5.14 (2H, s, H-2), 4.48 (2H, s, H-4), 2.59 (3H, s, CH_3-5), 1.25 (9H, s, $(CH_3)_3$). *Anal.* Calcd for $C_{20}H_{20}N_2O_2$, 0.33 H_2O: C, 73.60; H, 6.38; N, 8.58. Found: C, 73.45; H, 6.32; N, 8.21.

General Procedure for the Synthesis of 3-Substituted-6-cyano-5-methyl-3,4-dihydro-2*H*-furo[2,3-*f*][1,3]oxazino[5,6-*h*]quinolines (4) Compounds **4** were prepared from **2b** according to the same procedure used for **3**. The reaction mixture was heated to reflux until the starting hydroxyfuroquinoline **2b** was completely consumed. The precipitate was collected and recrystallized from an appropriate solvent.

3-Benzyl-6-cyano-5-methyl-3,4-dihydro-2*H*-furo[2,3-*f*][1,3]oxazino-[5,6-*h*]quinoline (4a) Compound **4a** was obtained as a white solid. It was recrystallized from ethanol (78% yield). mp 212 °C. IR (KBr): 2220 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 9.10 (1H, d, J=4.4 Hz, H-10), 8.66 (1H, d, J=8.3 Hz, H-8), 7.66 (1H, dd, J=8.3, 4.4 Hz, H-9), 7.37 (5H, m, H aromat.), 5.24 (2H, s, H-2), 4.46 (2H, s, H-4), 4.10 (2H, s, N–C<u>H</u>$_2$–C_6H_5), 2.45 (3H, s, CH_3-5). *Anal.* Calcd for $C_{22}H_{18}N_3O_2$, 0.5 H_2O: C, 72.51; H, 4.98; N, 11.53. Found: C, 72.34; H, 4.83; N, 11.46.

6-Cyano-3-*p*-methoxybenzyl-5-methyl-3,4-dihydro-2*H*-furo[2,3-*f*]-[1,3]oxazino[5,6-*h*]quinoline (4b) Compound **4b** was obtained as a white solid. It was recrystallized from ethanol (79% yield). mp 173 °C. IR (KBr): 2230 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 9.04 (1H, dd, J=4.3 and 1.7 Hz, H-10), 8.58 (1H, dd, J=8.3, 1.7 Hz, H-8), 7.59 (1H, dd, J=8.3, 4.3 Hz, H-9), 7.29 (2H, dd, J=8.7, 2.0 Hz, H aromat.), 6.89 (2H, m, H aromat.), 5.19 (2H, s, H-2), 4.39 (2H, s, H-4), 3.98 (2H, s, N–C<u>H</u>$_2$–C_6H_4–OCH_3), 3.82 (3H, s, OCH_3), 2.46 (3H, s, CH_3-5). *Anal.* Calcd for $C_{23}H_{19}N_3O_3$: C, 71.68; H, 4.96; N, 10.90. Found: C, 71.56; H, 4.90; N, 11.10.

3-Allyl-6-cyano-5-methyl-3,4-dihydro-2*H*-furo[2,3-*f*][1,3]oxazino[5,6-*h*]quinoline (4c) Compound **4c** was obtained as a beige solid. It was recrystallized from benzene (75% yield). mp 171 °C. IR (KBr): 2225 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 9.06 (1H, dd, J=4.5, 2 Hz, H-10), 8.61 (1H, dd, J=8.4, 2 Hz, H-8), 7.62 (1H, dd, J=8.4, 4.5 Hz, H-9), 6.05 (1H, m, CH_2=C<u>H</u>–CH_2), 5.30 (2H, dd, J=15, 2.4 Hz, C<u>H</u>$_2$=CH–CH_2), 5.20 (2H, s, H-2), 4.49 (2H, s, H-4), 3.56 (2H, d, J=6.4 Hz, CH_2=CH–C<u>H</u>$_2$), 2.56 (3H, s, CH_3-5). *Anal.* Calcd for $C_{18}H_{15}N_3O_2$, 0.1 H_2O: C, 70.39; H, 4.99; N, 13.68. Found: C, 70.36; H, 5.05; N, 13.48.

6-Cyano-3-(*N*,*N*-dimethyl)ethyl-5-methyl-3,4-dihydro-2*H*-furo[2,3-*f*][1,3]oxazino[5,6-*h*]quinoline (4d) Compound **4d** was obtained as a pink solid. It was recrystallized from ether (87% yield). mp 140 °C. IR (KBr): 2220 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 9.0 (1H, dd, J=4.4, 1.8 Hz, H-10), 8.56 (1H, dd, J=8.2, 1.8 Hz, H-8), 7.57 (1H, dd, J=8.2, 4.4 Hz, H-9), 5.17 (2H, s, H-2), 4.55 (2H, s, H-4), 3.09 (2H, t, J=6.3 Hz, CH_2–C<u>H</u>$_2$N$(CH_3)_2$), 2.68 (2H, t, J=6.3 Hz, CH_2–C<u>H</u>$_2$N$(CH_3)_2$), 2.59 (3H, s, CH_3-5), 2.38 (6H, s, N$(CH_3)_2$). *Anal.* Calcd for $C_{19}H_{20}N_4O_2$, 0.5 H_2O: C, 66.07; H, 6.13; N, 16.22. Found: C, 66.04; H, 6.13; N, 15.76.

Synthesis of 6-Cyano-3,5-dimethyl-2-morpholino-3,4-dihydro-2*H*-furo-[3′2′ : 3,4]naphtho[1,2-*b*]pyran (6) *tert*-Butylamine (0.08 ml, 0.75 mmol) was added to a cooled, aqueous solution of 37% formaldehyde (0.14 ml, 1.5 mmol) in toluene (10 ml). The mixture was stirred at 0 °C for 30 min. A solution of compound **2a** (0.168 g, 0.75 mmol) in 5 ml of toluene was added and the reaction mixture was heated at reflux for 15 min. Then, a solution of 1-morpholinopropene (0.143 g, 1.125 mmol) in toluene (1 ml) was added and heating was continued for 45 min. A white precipitate was formed by cooling. It was recovered and recrystallized from toluene. Yield 71%. mp 270 °C. IR (KBr): 2220 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 8.19 (2H, m, H-8 and H-11), 7.58 (2H, m, H-9 and H-10), 4.44 (1H, d, J=9.4 Hz, H-2), 3.81 (4H, m, $2CH_2$), 3.21 (1H, dd, J=16.6, 5.6 Hz, H-4-a), 3.16 (2H, m, CH_2), 2.89 (2H, m, CH_2), 2.81 (1H, dd, J=16.6, 11 Hz, H-4-b), 2.56 (3H, s, CH_3-5), 2.32 (1H, m, H-3), 1.18 (3H, d, J=6.6 Hz, CH_3-3). *Anal.* Calcd for $C_{22}H_{22}N_2O_3$: C, 72.91; H, 7.73; N, 6.12. Found: C, 72.71; H, 7.72; N, 6.15.

***In Vitro* Cytotoxicity Assays** All compounds were dissolved in DMSO (Carlo–Erba, final concentration of DMSO = 0.2%) and were tested at various concentrations on three tumor cell systems. Assays included solvent and reference controls.

***In Vitro* Cytotoxic Activity towards L 1210 Cells** L 1210 murine leukemia cells were cultured in suspension in RPMI 1640 medium (Eurobio) with 10% heat-inactivated fetal calf serum (Boehringer, Mannheim), 2-mercaptoethanol (Sigma, 10 μM), L-glutamine (Boehringer Mannheim, 2 mM), and antibiotics at 37 °C in a humidified, 5% CO_2 air atmosphere. For the screening, the cell suspension (cells in an exponential growth phase) was adjusted to 10^4 viable cells per ml (cell viability was estimated by the Trypan blue exclusion test). Cells were distributed in wells of a microtest tissue culture plate (Falcon, 225 ml per well) before introducing the test compounds or the solvent (25 μl). Four days later, cells were counted with a Coulter Counter (Coultronics, France).

IC_{50} is defined as the concentration inhibiting by 50% the cell growth compared to the control after 96 h of culture, and was determined from the regression line of percentage cell growth inhibition against the logarithm of the dose.

***In Vitro* Cytotoxic Activity towards Human Cancer Cell Lines MDA-MB 231 and PC3** MDA-MB 231 cells were maintained in DMEM (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Eurobio), insulin 1 UI/ml, L-glutamine (2 mM) and antibiotics. PC3 cells were cultured in a solution composed of 65% F12 Nutrient Mixture Ham (Gibco), 25% DMEM and 10% heat-inactivated fetal calf serum, L-glutamine (2 mM) and antibiotics. All the cell suspensions were maintained at 37 °C in a humidified, 5% CO_2 air atmosphere. The cytotoxic activity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) colorimetric method based on that of Mosmann.[14] Cells (10^4 cells/ml) were seeded in 96-well tissue culture plates (135 μl per well). After 24 h of culture, wells received either 15 μl of medium with DMSO (controls) or 15 μl of medium containing a compound solubilized in this solvent. After 5 d of culture, each well received 15 ml of a sterile solution of MTT in phosphate-buffered saline (PBS) solution at 5 mg/ml and the plate was incubated for 2 h. Then, the culture medium was removed and 100 μl of DMSO was added to each well for quantitation of blue formazan by reading the absorbance at 540 nm on a Titertek Multiskan II (Flow Laboratories).

The IC_{50} values were calculated from the regression lines in plots of the percentage decrease in absorbance *versus* controls against the logarithm of the concentration.

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