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Laboratory note

Synthesis and antitumour activity of 4-hydroxy-2-pyridone derivatives

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Abstract – 4-hydroxy-2-pyridone derivatives **2** were prepared by reaction of 3-amino-3-dialkylaminopropenoates with bis(2,4,6-trichlorophenyl)malonate. These compounds were further reacted with a set of aldehydes to give bis(pyridyl)methanes **3** and **4**. The newly synthesized compounds **2**, **3** and **4** were evaluated in vitro as antitumour agents against 60 human tumour cell lines. Some derivatives exhibit tumour growth inhibition activity. In particular, derivative **4g**, the most active of the series, possesses significant activity on all cell lines at concentrations ranging from 1×10^{-6} to 1×10^{-5} M. © 2000 Éditions scientifiques et médicales Elsevier SAS

4-hydroxy-2-pyridone derivatives / bis(pyridyl)methanes / antitumour activity

1. Introduction

The ongoing efforts of research on treatment of malignancy are focused on the discovery of novel products that enable us to modulate the pharmacological activity of drugs used currently and to decrease the toxicity of cancer chemotherapy. On the other hand, novel approaches to cancer chemotherapy are represented by inhibitors of angiogenesis and inhibitors of signal transduction pathways upon which cancer cells selectively depend. At the same time random screening continues to be one of the main routes to discover new leads with antineoplastic activity and the National Cancer Institute (NCI), Bethesda, USA, has played a pivotal role in this field. NCI screening program [1] started with the selection of defined chemical structures. This selection was based on the uniqueness of the chemical structures. Obviously, new chemical classes of agents that have not had extensive clinical evaluation are prime targets.

Among the wide variety of heterocycles that have been explored for developing pharmaceutically important molecules, pyridin-2(1*H*)-ones have received attention as antimicrobial and antiviral agents [2–5]. On this basis and in pursuing the interest in the study on novel compounds bearing potentially anticancer and antiviral activity, our research group has became interested in 4-hydroxy-2-pyridone derivatives and a list of these was submitted to NCI. We report here the synthesis, the physical-chemical properties of the new derivatives and the results of primary antitumour screening of compounds selected by NCI.

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2. Chemistry

The 4-hydroxy-6-oxopyridines **2** and their bis(pyridyl)-methane derivatives **3** and **4** were prepared by the sequence of reactions shown in *figure 1*. The key starting materials, ethyl 3,3-diaminopropenoates **1**, were available from ethyl cyanoacetate as previously described [6]. The aminopropenoates **1** were converted to pyridones **2** by heating with bis(2,4,6-trichlorophenyl)malonate by slight adjustment of the Kappe procedure [7, 8]. We found that better yields were obtained when the reaction was performed in toluene solution at about 95 °C for 2 h. Utilizing more vigorous reaction conditions afforded a complex mixture of compounds. Finally, the preparation of bis(pyridyl)methanes **3** and **4** was achieved in good yields by treatment of two equivalents of pyridones **2** with the appropriate aldehydes in ethanol using piperidine as catalyst.

The reaction was initiated by condensation of compound **2** with aldehyde to give intermediate **A** followed by addition of another molecule of **2**. With the aim of

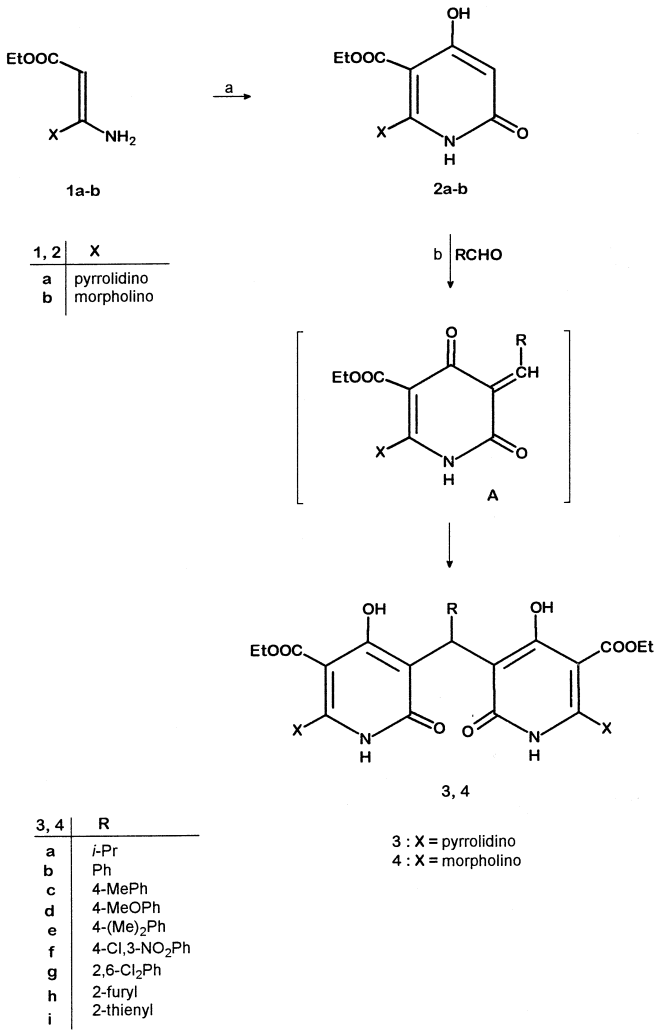

Figure 1. Conditions: a) bis(2,4,6-trichlorophenyl)malonate, toluene, 95 °C; b) ethanol, piperidine, reflux.

isolating this intermediate the reaction was performed without catalyst utilizing equimolar amounts of the two reagents. However, this approach was unsuccessful, in fact compounds **3** and **4** were exclusively obtained in poor yields and unreacted aldehyde was recovered.

Structures of new compounds **2**, **3** and **4** were assigned by analytical and spectral data. For compounds **2**, the IR spectra are characterised by a C=O stretching absorption band shifted at 1 620–1 670 cm^{-1}, probably due to formation of an intramolecular hydrogen bond. The ^{1}H-NMR spectra of pyridones **2**, registered in DMSO-d_6, exhibit a deuterium oxide exchangeable broad singlet corresponding to OH and NH protons in the region 10.26–10.90 ppm and a signal at 5 ppm assigned to the H-5 proton. The presence of these signals indicates that compounds **2** exist exclusively as 4-hydroxy-2(1*H*)pyridone tautomers. For compounds **3** and **4** the ^{1}H-NMR spectra show a singlet in the range 5.5–5.8 ppm due to H-1 and a downfield resonating D_2O exchangeable broad signal integrating for four protons.

3. Pharmacology

Evaluation of anticancer activity on 15 of the 18 synthesized compounds **2**, **3** and **4** was performed at the NCI following the known in vitro disease-oriented antitumour screening program, which is based upon use of multiple panels of 60 human tumour cell lines [1, 9]. Each compound is tested at a minimum of five concentrations at 10-fold dilution against every cell line in the panel. A 48 h continuous drug exposure protocol is used and a sulforhodamine B (SRB) protein assay is used to estimate cell viability or growth [10]. The anticancer activity of each compound is deduced from dose–response curves and is presented in three different tables according to the data provided by NCI [11].

The response parameters GI_{50}, TGI and LC_{50} (*tables I–III*) refer to the drug concentration that produced 50% inhibition, total growth inhibition and 50% cytotoxicity, respectively, and are expressed in micromolar concentrations (μM). In the tables we report only the activity of those compounds which exhibited GI_{50}, TGI and LC_{50} less than 100 μM.

Table I. GI_{50} values (μM) of compounds **2**, **3** and **4** against different tumour cell lines.

Panel/cell line	Compound											
	2b	**3a**	**4a**	**3d**	**3e**	**4e**	**3f**	**4f**	**4g**	**3h**	**4h**	**3i**
Leukemia												
CCRF-CEM	–	–	–	–	–	54.2	23.6	4.76	1.59	–	–	78.2
HL-60 (TB)	–	n.t.	–	n.t.	n.t.	n.t.	n.t.	n.t.	1.96	–	–	n.t.
K-562	–	–	–	–	–	–	63.2	30.4	1.68	–	–	–
MOLT-4	–	–	–	–	–	–	46.5	25.2	1.87	–	–	–
RPMI-8226	–	–	–	–	–	46.6	42.8	26.7	1.98	84.3	–	54.6

Panel/cell line	Compound											
	2b	**3a**	**4a**	**3d**	**3e**	**4e**	**3f**	**4f**	**4g**	**3h**	**4h**	**3i**
SR	–	37.5	–	88.9	–	–	40.2	36.1	2.83	–	–	–
Non-small cell lung												
A549/ATCC	–	–	–	–	–	–	–	–	17.5	–		–
EKVX	–	–	–	44.7	–	–	33.6	–	18.6	–	–	–
HOP-62	–	94.3	60.3	46.6	–	–	27.4	34.9	15.0	50.6	–	–
HOP-92	–	28.7	80.6	12.3	–	–	18.5	23.9	1.15	–	81.0	–
NCI-H226	–	–	–	–	–	–	63.6	–	10.4	–	–	–
NCI-H23	–	71.1	–	82.0	95.6	–	22.0	25.8	6.86	–	–	–
NCI-H322M	–	–	–	81.5	–	–	–	–	18.1	–	–	–
NCI-H460	n.t.	–	–	–	–	–	–	–	20.3	–	–	–
NCI-H522	–	43.5	–	–	–	–	24.5	28.0	1.83	84.8	–	–
Colon cancer												
COLO 205	–	–	–	–	–	–	–	–	3.55	–	–	–
HCC-2998	–	52.8	–	–	–	–	–	–	18.2	–	–	–
HCT-116	–	–	–	–	–	–	34.1	20.3	1.68	–	–	–
HCT-15	–	–	–	–	–	–	–	–	3.40	–	–	–
HT29	–	–	–	n.t.	–	–	n.t.	20.9	3.56	–	–	–
KM12	–	–	–	–	–	–	–	–	14.5	–	–	–
SW-620	–	–	–	–	–	–	33.2	23.5	1.71	–	–	–
CNS cancer												
SF-268	–	72.4	–	49.5	–	–	23.0	44.1	6.09	85.4	–	–
SF-295	–	–	58.4	25.7	–	–	37.6	–	16.5	40.3	–	–
SF-539	–	–	–	–	–	–	19.2	26.5	1.57	–	–	–
SNB-19	–	70.6	83.6	–	–	–	15.5	26.5	3.84	38.7	–	–
SNB-75	n.t.	25.3	22.4	21.3	48.5	–	9.78	41.8	3.42	23.5	–	–
U251	–	37.9	–	48.9	–	–	14.9	19.6	4.72	73.7	–	-
Melanoma												
LOX IMVI	–	–	–	–	–	59.0	19.3	19.5	2.37	37.4	–	–
MALME-3M	–	–	–	–	–	–	38.9	41.4	2.00	–	–	–
M14	–	–	–	–	–	–	20.8	20.7	1.74	–	–	–
SK-MEL-2	–	n.t.	–	5.00	n.t.	–	21.3	n.t.	4.75	–	–	–
SK-MEL-28	–	–	–	–	–	–	35.3	42.6	1.69	–	–	–
SK-MEL-5	–	–	–	–	–	–	37.8	24.3	4.07	82.5	–	–
UACC-257	–	–	–	–	–	–	41.5	70.0	5.13	–	–	–
UACC-62	–	–	–	–	–	–	26.8	40.1	4.88	–	–	–
Ovarian cancer												
IGROV1	–	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	4.09	n.t.	n.t.	n.t.
OVCAR-3	–	–	–	–	–	–	29.9	17.2	2.48	–	–	–
OVCAR-4	–	–	–	–	–	–	–	42.8	6.76	–	–	–
OVCAR-5	–	33.2	73.8	52.7	–	–	30.2	–	13.1	68.0	–	–
OVCAR-8	–	–	–	86.1	–	–	15.5	18.4	6.31	92.9	–	–
SK-OV-3	–	–	86.5	–	–	–	85.6	–	7.80	–	–	–
Renal cancer												
786-0	–	61.8	60.1	–	–	–	27.2	28.1	1.81	29.3	–	–
A498	–	–	–	–	–	–	33.1	–	7.57	–	–	–
ACHN	–	–	–	56.0	–	–	34.2	55.1	2.97	95.5	–	–
CAKI-1	–	47.9	–	28.5	–	–	24.4	82.5	1.84	–	–	–
RXF 393	–	43.0	–	57.3	–	–	31.7	–	2.06	54.4	–	–
SN12C	–	–	–	–	–	–	36.2	20.5	1.58	–	–	–
TK-10	–	42.4	–	42.7	94.3	–	19.2	39.9	3.38	–	–	–
UO-31	–	–	–	–	–	–	24.6	34.0	1.88	–	–	–
Prostate cancer												
PC-3	–	33.5	–	–	–	–	28.0	14.2	14.1	–	–	–
DU-145	–	–	–	–	–	–	60.8	22.9	9.96	–	–	–

Panel/cell line	Compound											
	2b	**3a**	**4a**	**3d**	**3e**	**4e**	**3f**	**4f**	**4g**	**3h**	**4h**	**3i**
Breast cancer												
MCF7/ADR-RES	–	–	–	–	–	–	28.6	22.0	2.07	–	–	–
NCI/ADR-RES		–	–	–	–	–	28.7	39.0	16.6	–	–	–
MDA-MB-231/ATCC	–	21.8	–	20.8	82.3	–	22.0	15.2	11.2	–	–	–
HS 578T	–	42.0	47.9	–	–	–	6.54	–	3.47	27.9	–	–
MDA-MB-435	–	–	–	–	–	–	29.5	30.6	2.53	–	–	–
MDA-N	–	–	–	–	–	–	24.5	26.1	1.94	–	–	–
BT-549	–	32.3	–	76.0	30.4	–	16.3	24.5	4.95	–	–	–
T-47D	63.0	–	–	–	–	–	28.1	19.7	3.56	19.4	–	–

n.t.: means not tested
(–) values > 100 μM

Table II. TGI values (μM) of compounds **3** and **4** against different tumour cell lines.

Panel/cell line	Compound						
	3a	**4a**	**3d**	**3f**	**4f**	**4g**	**3h**
Leukemia							
CCRF-CEM	–	–	–	79.3	40.4	3.50	–
HL-60 (TB)	n.t.	–	n.t.	n.t.	n.t.	4.37	–
K-562	–	–	–	–	–	3.51	–
MOLT-4	–	–	–	–	57.8	5.23	–
RPMI-8226	–	–	–	–	–	4.87	–
SR	–	–	–	–	–	8.22	–
Non-small cell lung							
A549/ATCC	–	–	–	–	–	31.2	–
EKVX	–	–	–	–	–	32.6	–
HOP-62	–	–	–	–	–	29.8	–
HOP-92	–	–	58.6	58.3	69.4	4.51	–
NCI-H226	–	–	–	–	–	22.5	–
NCI-H23	–	–	–	48.9	98.8	20.7	–
NCI-H322M	–	–	–	–	–	32.0	–
NCI-H460	–	–	–	–	–	41.2	–
NCI-H522	–	–	–	74.1	55.2	3.72	–
Colon cancer							
COLO 205	–	–	–	–	–	14.1	–
HCC-2998	–	–	–	–	–	32.3	–
HCT-116	–	–	–	–	63.6	3.21	–
HCT-15	–	–	–	–	–	12.3	–
HT29	–	–	n.t..	n.t.	42.1	12.9	–
KM12	–	–	–	–	–	27.6	–
SW-620	–	–	–	–	58.8	3.08	–
CNS cancer							
SF-268	–	–	–	55.2	–	19.0	–
SF-295	–	–	–	–	–	30.4	–
SF-539	–	–	–	41.6	59.7	3.98	–
SNB-19	–	–	–	49.9	79.4	16.2	–
SNB-75	96.5	69.7	69.0	34.4	–	9.48	60.6
U251	–	–	–	36.9	41.9	15.6	–
Melanoma							
LOX IMVI	–	–	–	49.4	48.1	6.29	–
MALME-3M	–	–	–	84.6	–	5.95	–
M14	–	–	–	42.7	58.5	3.18	–

Panel/cell line	Compound						
	3a	**4a**	**3d**	**3f**	**4f**	**4g**	**3h**
SK-MEL-2	–	–	53.9	45.6	n.t.	16.2	–
SK-MEL-28	–	–	–	–	–	3.37	–
SK-MEL-5	–	–	–	–	74.5	15.6	–
UACC-257	–	–	–	–	–	17.7	–
UACC-62	–	–	–	86.0	–	16.6	–
Ovarian cancer							
IGROV1	–	n.t.	n.t.	n.t.	n.t.	15.0	n.t.
OVCAR-3	–	–	–	–	35.5	8.07	–
OVCAR-4	–	–	–	–	–	19.6	–
OVCAR-5	–	–	–	–	–	25.7	–
OVCAR-8	–	–	–	33.5	37.0	18.8	–
SK-OV-3	–	–	–	–	–	20.8	–
Renal cancer							
786-0	–	–	–	–	–	3.20	–
A498	–	–	–	–	–	19.7	–
ACHN	–	–	–	–	–	8.32	–
CAKI-1	–	–	71.1	60.6	–	4.27	–
RXF 393	–	–	–	99.6	–	4.36	–
SN12C	–	–	–	–	51.0	4.54	–
TK-10	–	–	–	59.2	–	11.3	–
UO-31	–	–	–	66.6	95.5	3.73	–
Prostate cancer							
PC-3	–	–	–	83.9	50.1	27.6	–
DU-145	–	–	–	–	55.8	21.5	–
Breast cancer							
MCF7/ADR-RES	–	–	–	–	69.6	3.86	–
NCI/ADR-RES	–	–	–	73.4	–	31.5	–
MDA-MB-231/ ATCC	64.3	–	88.1	50.5	41.9	23.4	–
HS 578T	–	–	–	–	–	15.9	–
MDA-MB-435	–	–	–	–	–	7.49	–
MDA-N	–	–	–	79.6	70.1	3.73	–
BT-549	–	–	–	51.8	75.1	15.7	–
T-47D	–	–	–	–	48.8	9.27	–

n.t. means not tested
(–) values > 100 μM

Table III. LC_{50} values (μM) of compounds **3f**, **4f** and **4g** against different tumour cell lines.

Panel/cell line	Compound		
	3f	**4f**	**4g**
Leukemia			
CCRF-CEM	–	–	7.73
HL-60 (TB)	n.t.	n.t.	9.73
K-562	–	–	7.31
MOLT-4	–	–	–
RPMI-8226	–	–	–
SR	–	–	–
Non-small cell lung			
A549/ATCC	–	–	55.9
EKVX	–	–	57.1
HOP-62	–	–	55.6
HOP-92	–	–	22.6
NCI-H226	–	–	48.8
NCI-H23	–	–	50.1
NCI-H322M	–	–	56.5
NCI-H460	–	–	83.4
NCI-H522	–	–	7.58
Colon cancer			
COLO 205	–	–	37.5
HCC-2998	–	–	57.2
HCT-116	–	–	6.13
HCT-15	–	–	57.9
HT29	n.t.	84.8	36.0
KM12	–	–	52.6
SW-620	–	–	5.55
CNS cancer			
SF-268	–	–	44.3
SF-295	–	–	56.1
SF-539	90.2	–	10.2
SNB-19	–	–	40.2
SNB-75	–	–	30.7
U251	91.7	89.5	40.0
Melanoma			
LOX IMVI	–	–	26.3
MALME-3M	–	–	22.1
M14	87.4	–	5.83
SK-MEL-2	–	n.t.	40.2
SK-MEL-28	–	–	6.69
SK-MEL-5	–	–	39.5
UACC-257	–	–	44.3
UACC-62	–	–	40.7
Ovarian cancer			
IGROV1	n.t.	n.t.	51.8
OVCAR-3	–	73.3	28.2
OVCAR-4	–	–	44.3
OVCAR-5	–	–	50.7
OVCAR-8	72.1	74.5	44.9
SK-OV-3	–	–	46.0
Renal cancer			
786-0	–	–	5.66
A498	–	–	44.4
ACHN	–	–	30.6
CAKI-1	–	–	9.91
RXF 393	–	–	9.24
SN12C	–	–	15.8
TK-10	–	–	37.3
UO-31	–	–	7.39
Prostate cancer			
PC-3	–	–	54.3
DU-145	–	–	46.4
Breast cancer			
MCF7/ADR-RES	–	–	7.19
NCI/ADR-RES	–	–	59.7
MDA-MB-231/ATCC	–	–	48.9
HS 578T	–	–	63.9
MDA-MB-435	–	–	26.5
MDA-N	–	–	7.15
BT-549	–	–	39.6
T-47D	–	–	50.9

n.t means not tested
(–) values > 100 μM

4. Results and discussion

From the biological data reported in *tables I–III*, it can be observed that several compounds show an interesting activity level often associated with high or moderately different cellular sensitivity and/or with a significantly different subpanel sensitivity. In particular compound **4g** was the most active in the series with a significant inhibitory activity on all the 60 cell lines at 1.57–20.3 μM concentrations and cytotoxic activity on 57 cell lines at the 5.56–57.9 μM concentrations. Compound **4f** exhibited similar inhibitory activity although over a smaller range (43 over 60), but show cytotoxicity on four cell lines only. Compounds **3a**, **3d** and **3f** show significant sensitivity, with high values of inhibition, on 11 subpanel cell lines (SR, HOP-62, HOP-92, NCI-H23, SF-268, SNB-75, OVOCAR-5, CAKI-1, TK-10, MDA-MB-231/ATCC, BT-549). The limited number of compounds tested and the lack of many reference compounds does not allow us to establish structure–activity relationships. However, it seems that although the examined compounds have closely related structures, the results obtained from the NCI screen showed clear differences in activity. The 4-hydroxypyridones **2** were devoid of activity. At the present stage we may infer that, in bis(pyridyl) methanes **3** and **4**, replacement of isopropyl moiety on the methyne bridge with a phenyl ring resulted in improved cytostatic activity. As regards to the influence of the substituents on the phenyl ring we can observe that the presence of electron-withdrawing substituents favourably

affects the antitumour activity. Furthermore, the replacement of the phenyl ring with a furane or thiophene leads to a drop off in activity.

Due to its good activity and because, in addition, it lies outside the category of adequately studied classes of antitumour agents, compound **4g** has been selected by NCI Biological Evaluation Committee for further testing in an in vivo Hollow Fibre Assay [12].

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a Kofler hot stage and are uncorrected. IR spectra were recorded on Nujol mulls between salt plates in a Perkin-Elmer 398 spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian Unity 300 spectrometer. It was not possible to register the ^{1}H-NMR spectra of some compounds since they are insoluble in all common deuterated solvents. Elemental analyses were carried out with a Carlo Erba Model 1106 Elemental Analyzer. Ethyl 3-amino-3-pyrrolidino-2-propenoate and ethyl 3-amino-3-morpholino-2-propenoate were synthesized as previously described [6].

5.1.1. 4-Hydroxy-2-pyrrolidino-1,6-dihydro-6-oxopyridine-3-carboxylic acid ethyl ester **2a**

A mixture of ethyl 3-amino-3-pyrrolidino-2-propenoate (3.68 g, 0.02 mol) and bis (2,4,6-trichlorophenyl) malonate (9.26 g, 0.02 mol) in toluene (20 mL) was heated in water bath for 2 h. After concentration in vacuo, the residue was treated with diethyl ether, filtered off and recrystallized. Yield 98%. M.p. 185–187 °C (from 2-propanol). Calcd. For $C_{12}H_{16}N_2O_4$ (252.27); % C 57.13; % H 6.39; % N 11.11. Found: % C 57.09; % H 6.40; % N 11.08. IR (Nujol): 3 100, 1 660, 1 620, 1 560 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 1.19 (t, J = 6.8 Hz, 3H, CH_3), 1.77, 3.31 (m, 8H, pyrrolidinyl), 4.14 (q, J = 6.8 Hz, 2H, CH_2), 5.15 (s, 1H, H-5), 10.26 (br s, 2H, NH and OH).

5.1.2. 4-Hydroxy-2-morpholino-1,6-dihydro-6-oxopyridine-3-carboxylic acid ethyl ester **2b**

Compound **2b** was prepared following the same procedure reported for **2a**, starting from ethyl 3-amino-3-morpholino-2-propenoate. Yield 96%. M.p. 196–198 °C (from acetonitrile). Calcd. for $C_{12}H_{16}N_2O_5$ (286.26). % C 57.72; % H 6.01;% N 10.44. Found: % C 57.64; % H 6.02; % N 10.40. IR (Nujol): 1 670, 1 640, 1 575 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 1.22 (t, J = 7.1 Hz, 3H, CH_3), 3.15, 3.56 (m, 8H, morpholinyl), 4.18 (q, J = 7.1 Hz, 2H, CH_2), 5.41 (s, 1H, H-5), 10.63, 10.90 (br s, 2H, NH and OH).

5.2. Synthesis of derivatives **3** *and* **4**

A mixture of 4-hydroxypyridone **2** (0.0025 mol) and aldehyde (0.00125 mol) in ethanol (20 mL) was added a few drops of piperidine and heated at reflux for 4–17 h. The formed precipitate was filtered off and washed with diethyl ether.

5.2.1. 1,1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-pyrrolidino-1,6-dihydro-6-oxopyridin-5-yl)]-2-methylpropane **3a**

Prepared in 73% yield starting from compound **2a** and isobutyraldehyde, refluxing for 6 h. M.p. 260 °C dec. Calcd. for $C_{28}H_{38}N_4O_8$ (558.63). % C 60.20; % H 6.86; % N 10.03. Found: % C 60.27; % H 6.85; % N 10.07. IR (Nujol): 3 240, 3 100, 2 620, 1 705, 1 685 cm^{-1}.

5.2.2. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-pyrrolidino-1,6-dihydro-6-oxopyridin-5-yl)methyl]benzene **3b**

Prepared in quantitative yield starting from compound **2a** and benzaldehyde, refluxing for 4 h. M.p. 263 °C dec. Calcd. for $C_{31}H_{36}N_4O_8$ (592.65). % C 62.83; % H 6.12; % N 9.45. Found: % C 62.90; % H 6.13; % N 9.48. IR (Nujol): 3 220, 3 100, 2 600, 1 710, 1 570 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.18 (m, 6H, CH_3), 1.84, 3.25 (m, 16H, pyrrolidinyl), 4.12 (m, 4H, CH_2), 5.63 (s, 1H, CH), 7.13 (m, 5H, Ar), 10.47, 12.40 (br s, 4H, D_2O exchangeable).

5.2.3. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-pyrrolidino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-methoxybenzene **3d**

Prepared in 84% yield starting from compound **2a** and 4-methoxybenzaldehyde, refluxing for 4 h. M.p. 213–215 °C. Calcd. for $C_{32}H_{38}N_4O_9$ (622.68). % C 61.73; % H 6.15; % N 9.00. Found: % C 61.68; % H 6.14; % N 9.05. IR (Nujol): 3 180, 3 050, 2 640, 1 730, 1 620 cm^{-1}.

5.2.4. 4-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-pyrrolidino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-N, N-dimethylbenzenamine **3e**

Prepared in 66% yield starting from compound **2a** and 4-(dimethylamino)benzaldehyde, refluxing for 4 h. M.p. 262 °C dec. Calcd. for $C_{33}H_{41}N_5O_8$ (635.72). % C 62.35; % H 6.50; % N 11.02. Found: % C 62.40; % H 6.51; % N 11.06. IR (Nujol): 3 280, 3 080, 2 600, 1 700, 1 580 cm^{-1}.

5.2.5. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-pyrrolidino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-chloro-3-nitrobenzene **3f**

Prepared in 75% yield starting from compound **2a** and 4-chloro-3-nitrobenzaldehyde, refluxing for 4 h. M.p.

233 °C dec. Calcd. for $C_{31}H_{34}ClN_5O_{10}$ (672.09). % C 55.40; % H 5.10; % N 10.42. Found: % C 55.46; % H 5.08; % N 10.46. IR (Nujol): 3 330, 1 730, 1 710, 1 590 cm^{-1}.

5.2.6. 1-[Bis(3-(ethoxycarbonyl)-2-pyrrolidino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene **3g**

Prepared in 52% yield starting from compound **2a** and 2,6-dichlorobenzaldehyde, refluxing for 8 h. M.p. 290 °C dec. Calcd. for $C_{30}H_{34}Cl_2N_4O_8$ (649.53). % C 55.48; % H 5.28; % N 8.63. Found: % C 55.43; % H 5.30; % N 8.69. IR (Nujol): 3 400, 3 080, 2 730, 2 640, 1 650, 1 630 cm^{-1}.

5.2.7. 2-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-pyrrolidino-1,6-dihydro-6-oxopyridin-5-yl)methyl]furane **3h**

Prepared in quantitative yield starting from compound **2a** and furane-2-carboxyaldehyde, refluxing for 4 h. M.p. 252 °C dec. Calcd. for $C_{29}H_{34}N_4O_9$ (582.61). % C 59.79; % H 5.88; % N 9.62. Found: % C 59.84; % H 5.90; % N 9.57. IR (Nujol): 2 650, 1 715, 1 590 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.19 (t, J = 6.8 Hz, 6H, CH_3), 1.82, 3.26 (m, 16H, pyrrolidinyl), 4.13 (q, J = 6.8 Hz, 4H, CH_2), 5.54 (s, 1H, CH), 5.90, 6.29, 7.38 (s, 3H, furyl), 10.44, 12.43 (br s, 4H, D_2O exchangeable).

5.2.8. 2-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-pyrrolidino-1,6-dihydro-6-oxopyridin-5-yl)methyl]thiophene **3i**

Prepared in quantitative yield starting from compound **2a** and thiophene-2-carboxyaldehyde, refluxing for 4 h. M.p. 240 °C dec. Calcd. for $C_{29}H_{34}N_4O_8S$ (598.67). % C 58.18; % H 5.72; % N 9.36. Found: % C 58.12; % H 5.73; % N 9.40. IR (Nujol): 3 080, 1 700, 1 580 cm^{-1}.

5.2.9. 1,1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)]-2-methylpropane **4a**

Prepared in 40% yield starting from compound **2b** and isobutyraldehyde, refluxing for 17 h. M.p. 240 °C dec. Calcd. for $C_{28}H_{38}N_4O_{10}$ (590.63). % C 56.94; % H 6.48; % N 9.49. Found: % C 57.00; % H 6.47; % N 9.52. IR (Nujol): 3 200, 3 080, 2 600, 1 720, 1 680, 1 615 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.73 (d, J = 5.9 Hz, 6H, CH_3), 1.18 (t, J = 6.8 Hz, 6H, CH_3), 2.98, 3.09, 3.53 (m, 17H, morpholinyl and CH), 3.71 (m, 1H, H-1), 4.14 (q, J = 6.8 Hz, 4H, CH_2), 11.52, 12.98 (br s, 4H, D_2O exchangeable).

5.2.10. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]benzene **4b**

Prepared in quantitative yield starting from compound **2b** and benzaldehyde, refluxing for 4 h. M.p. 248 °C dec. Calcd. for $C_{31}H_{36}N_4O_{10}$ (624.65). % C 59.61; % H 5.81; % N 8.97. Found: % C 59.54; % H 5.82; % N 9.01. IR (Nujol): 3 100, 1 715, 1 590 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.21 (t, J = 6.8 Hz, 6H, CH_3), 3.13, 3.59 (m, 16H, morpholinyl), 4.16 (q, J = 6.8 Hz, 4H, CH_2), 5.82 (s, 1H, CH), 7.01–7.22 (m, 5H, Ar), 11.50, 12.27 (br s, 4H, D_2O exchangeable).

5.2.11. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-methylbenzene **4c**

Prepared in quantitative yield starting from compound **2b** and 4-methylbenzaldehyde, refluxing for 4 h. M.p. 247 °C dec. Calcd. for $C_{32}H_{38}N_4O_{10}$ (637.67). % C 60.18; % H 6.00; % N 8.77. Found: % C 60.25; % H 6.01; % N 8.73. IR (Nujol): 3 100, 1 590 cm^{-1}.

5.2.12. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-methoxybenzene **4d**

Prepared in 86% yield starting from compound **2b** and 4-methoxybenzaldehyde, refluxing for 4 h. M.p. 238 °C dec. Calcd. for $C_{32}H_{38}N_4O_{11}$ (654.67). % C 58.71; % H 5.85; % N 8.56. Found: % C 58.78; % H 5.84; % N 8.59. IR (Nujol): 3 140, 2 560, 1 720, 1 600 cm^{-1}.

5.2.13. 4-[Bis(3-(ethoxycarbonyl)-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-N, N-dimethylbenzenamine **4e**

Prepared in 52% yield starting from compound **2b** and 4-(dimethylamino)benzaldehyde, refluxing for 4 h. M.p. 246 °C dec. Calcd. for $C_{33}H_{41}N_5O_{10}$ (667.72). % C 59.36; % H 6.19; % N 10.49. Found: % C 59.30; % H 6.18; % N 10.53. IR (Nujol): 3 220, 3 120, 2 500, 1 715, 1 580 cm^{-1}.

5.2.14. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-chloro-3-nitrobenzene **4f**

Prepared in 75% yield starting from compound **2b** and 4-chloro-3-nitrobenzaldehyde, refluxing for 4 h. M.p. 245 °C dec. Calcd. For $C_{31}H_{34}ClN_5O_{12}$ (704.09). % C 52.88; % H 4.87; % N 9.95. Found: % C 52.93; % H 4.88; % N 9.90. IR (Nujol): 3 250, 3 160, 2 540, 1 700, 1 630, 1 590 cm^{-1}.

5.2.15. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene **4g**

Prepared in 60% yield starting from compound **2b** and 2,6-dichlorobenzaldehyde, refluxing for 6 h. M.p. 260 °C dec. Calcd. for $C_{30}H_{34}Cl_2N_4O_{10}$ (681.53). % C 52.87; % H 5.03; % N 8.22. Found: % C 52.95; % H 5.02; % N 8.25. IR (Nujol): 3 060, 1 710, 1 620, 1 570 cm^{-1}.

5.2.16. 2-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]furane **4h**

Prepared in quantitative yield starting from compound **2b** and furane-2-carboxyaldehyde, refluxing for 4 h. M.p. 242 °C dec. Calcd. for $C_{29}H_{34}N_4O_{11}$ (614.61). % C 56.67; % H 5.58; % N 9.12. Found: % C 56.73; % H 5.60; % N 9.17. IR (Nujol): 3 080, 1 710, 1 590 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.21 (t, J = 6.8 Hz, 6H, CH_3), 3.13, 3.57 (m, 16H, morpholinyl), 4.17 (q, J = 6.8 Hz, 4H, CH_2), 5.74 (s, 1H, CH), 5.89, 6.29, 7.40 (s, 3H, furyl), 11.50, 12.22 (br s, 4H, D_2O exchangeable).

5.2.17. 2-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]thiophene **4i**

Prepared in 96% yield starting from compound **2b** and thiophene-2-carboxyaldehyde, refluxing for 4 h. M.p. 250 °C dec. Calcd. for $C_{29}H_{34}N_4O_{10}S$ (630.67). % C 55.23; % H 5.43; % N 8.88. Found: % C 55.17; % H 5.41; % N 8.93. IR (Nujol): 3 070, 1 710, 1 630, 1 590 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.21 (t, J = 6.8 Hz, 6H, CH_3), 3.13, 3.58 (m, 16H, morpholinyl), 4.18 (q, J = 6.8 Hz, 4H, CH_2), 5.99 (s, 1H, CH), 6.57, 6.83, 7.22 (s, 3H, thienyl), 11.53, 12.44 (br s, 4H, D_2O exchangeable).

5.3. Pharmacology

The compounds were tested by NCI in an in vitro assay as a primary cancer screen. A total of 60 human tumour cell lines, derived from nine cancer types (leukaemia, lung, colon, brain, melanoma, ovarian, renal, prostate and breast) formed the basis of this test. The tumour cells were cultured in RPMI1640 medium supplemented with 5% foetal calf serum and 2 mM L-glutamine. The tumour cells are inoculated over a series of standard 96-well microtitre plates in 100 µL of medium [1, 9]. Density of inoculum depends on the type of tumour cell and from its growth characteristics [13]. These cells are then pre-incubated on the microtitre plate for 24 h before adding the compounds. These were tested in DMSO solution at five different concentrations (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M). After an incubation of the chemical agent for 48 h with the tumour cell lines a sulforhodamine B (SRB) protein assay was used to estimate cell viability or growth. The cytotoxic effects are evaluated and the assay results and dose response parameters were calculated as previously described [11].

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References

[1] Grever M.R., Schepartz S.A., Chabner B.A., Semin. Oncol. 19 (1992) 622–638.

[2] Dannhardt G., Meindl W., Schober B.D., Kappe T., Eur. J. Med. Chem. 26 (1991) 599–604.

[3] Saari W.S., Hoffman J.M., Wai J.S., Fisher T.E., Rooney C.S., Smith A.M. et al., J. Med. Chem. 34 (1991) 2925–2928.

[4] Dollé V., Fan E., Nguyen C.H., Aubertin A., Kirn A., Andreola M.L. et al., J. Med. Chem. 38 (1995) 4679–4686.

[5] William D.R., Bremmer M.L., Brown D.L., Antuono J.D., J. Org. Chem. 50 (1985) 2807–2809.

[6] Cocco M.T., Congiu C., Maccioni A., Plumitallo A., Schivo M.L., Palmieri G., Farmaco Ed. Sci. 43 (1988) 103.

[7] Kappe T., Ajili S., Stadlbauer W., J. Heterocycl. Chem. 25 (1988) 463–468.

[8] Kappe T., in: Pauette L.A. (Ed.), Encyclopaedia of Reagents for Organic Synthesis Vol. 1, John Wiley & Sons, Chichester, 1995, pp. 557–559.

[9] Boyd M.R., Paul K.D., Drug Dev. Res. 34 (1995) 91.

[10] Skehan P., Storeng R., Scudiero D. et al., J. Natl. Cancer Inst. 82 (1990) 1107.

[11] Boyd M.R., Princ. Pract. Oncol. 3 (1989) 1.

[12] Plowman J., Dykes D.J., Hollingshead M., Simpson-Herren L., Alley M.C., Teicher B., Anticancer Drug development guide: preclinical Screening, Clinical trials, and Approval, Humana Press Inc., Totowa, NJ, 1997, pp. 101–125.

[13] Monks A., Scudiero D., Skehan P., Shoemaker R., Paull K., Vistica D. et al., J. Natl. Cancer Inst. 83 (1991) 757.