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SYNTHESIS AND ANTILEUKAEMIA ACTIVITY OF N-(2,4-DIMETHYLPHENYL)HYDRAZINE CARBOTHIOAMIDE AND ITS AZOMETHINE DERIVATIVES

A .Gulea[1], A. Sargun[1], A. Barba[2], A. Jalba[1], D. Poirier[3], P. Petrenko[4], Yu. Chumakov[4]

[1]*Department of Inorganic and Physical Chemistry, Moldova State University, Chisinau, Moldova*

[2]*Institute of Chemistry, Academy of Sciences of Moldova, Chisinau, Moldova*

[3]*Oncology and Molecular Endocrinology Research Centre, CHUL Research Centre and Université Laval, Québec City, Canada*

[4]*Institute of Applied Physics, Academy of Sciences of Moldova, Chisinau, Moldova*

Rezumat

Prezenta lucrare conţine date despre sinteza, caracterizarea şi evaluarea *in vitro* a activităţii biologice a N-(2,4-dimetilfenil)hidrazinocarbotioamidei şi a cinci derivaţi azometinici ai acesteia, obţinuţi prin condensarea N-(2,4-dimetilfenil)hidrazincarbotioamidei cu diferiţi compuşi carbonilici. Compoziţia şi structura cristalină a compuşilor sintetizaţi a fost determinată cu ajutorul spectroscopiei ^{1}H, ^{13}C RMN şi a difracţiei cu raze X a monocristalelor. Toţi compuşii obţinuţi au fost testaţi ca inhibitori ai proliferării celulelor de leucemie umană HL-60. A fost stabilit că N-(2,4-dimetilfenil)-2-(tiofen-3-ilmetilen)hidrazinocarbotioamida şi N-(2,4-dimetilfenil)-2-(2-hidroxibenziliden) hidrazinocarbotioamida sunt cei mai activi agenţi antiproliferativi obţinuţi în acest studiu.

Cuvinte-cheie: N-(2,4-dimetilfenil)hidrazinocarbotioamida – azometine – anti-proliferativ – leucemie

Introduction

Leukaemia is a type of cancer of the blood or bone marrow characterised by an abnormal increase of immature white blood cells called "blasts". Like other cancers, it results from mutations in the DNA. Certain mutations can trigger leukaemia by activating oncogenes or deactivating tumor suppressor genes, and thereby disrupting the regulation of cell death, differentiation or division.

Since 1960s, an anthracycline antibiotic called *Doxorubicin* has been widely used in cancer chemotherapy. It is closely related to the natural product daunomycin, and like all anthracyclines, it works by intercalating DNA. However, *Doxorubicin* has a series of side-effects that strongly jeopardize patients' lives. For instance, when the cumulative dose of *Doxorubicin* reaches 550 mg/m², the risks of developing cardiac side effects, including congestive heart failure, dilated cardiomyopathy, and death, dramatically increase. *Doxorubicin* cardiotoxicity is characterised by a dose-dependent decline in mitochondrial oxidative phosphorylation. Reactive oxygen species, generated by the interaction of *Doxorubicin* with iron, can afterwards damage the myocytes, causing myofibrillar loss and cytoplasmic vacuolisation [1, 2].

For these reasons, more efforts are now focused on developing novel antitumor medicines with improved clinical effectiveness, broadened spectrum of activity, and with reduced general toxicity [3]. As DNA can interact with many bio-molecules and synthetic compounds, it is the main target for therapeutic treatment of cancer. Therefore, we have started a project directed towards the synthesis of different classes of compounds that can act as molecular inhibitors of cancer cells proliferation [4-6]. Particularly interesting are thiosemicarbazones, which have demonstrated potent cytotoxic activities against a series of murine and human tumor cells in culture [7]. In continuation of this approach, the present paper describes the chemical synthesis, characterisation and *in vitro* biological evaluation of N-(2,4-dimethylphenyl)hydrazinecarbothioamide and its azomethine derivatives. The composition and the structure of the synthesised substances have been determined by ¹H and ¹³C NMR spectroscopy and X-ray diffraction. All substances were tested as inhibitors of human leukaemia (HL-60) cells growth.

Materials and methods

General. All reagents and chemicals have been obtained from commercially available sources, have been of analytical- or reagent-grade purity and have been used without further purification. ¹H and ¹³C nuclear magnetic resonance (NMR) spectra have been recorded at room temperature with a Bruker DRX 400 spectrometer. All chemical shifts (¹H, ¹³C) are given in ppm versus $SiMe_4$ using DMSO-d$_6$ as solvent. X-ray diffraction has been performed with a Bruker X8 diffractometer.

Antileukaemia bioassay. Human promyelocytic leukaemia cells HL-60 (ATCC, Rockville, MD, USA) were routinely grown in 90% suspension RPMI-1640 (Sigma Saint Louis, USA) containing *L*-glutamine (2 nM), antibiotics (100 IU penicillin/mL, 100 μg streptomycin/mL) and supplemented with 10% (v/v) foetal bovine serum (FBS), in a 5% CO_2 humidified atmosphere at 37°C. Cells were currently maintained twice a week by diluting the cells in RPMI-1640 medium containing 10% FBS. The cell proliferation assay was performed by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Cell Titre 96 Aqueous, Promega, USA), which allowed us to measure the number of viable cells. In a

nutshell, triplicate cultures of 10,000 cells in a total of 100 µg medium in 96-well microtitre plates (Becton Dickinson and Company, Lincoln Park, NJ, USA) were incubated at 37°C, 5% CO_2. The synthesized compounds were dissolved in ethanol to prepare the stock solution of $1 \cdot 10^{-2}$ M. Both, the compounds and *Doxorubicin* (Novopharm, Toronto, Canada), as a positive control, were diluted at multiple concentrations with culture media, added to each well and incubated for 3 days. Following each treatment, MTS (20 µL) was added to each well and the mixture has been incubated for 4 hours. MTS is converted to water-soluble colored formazan dehydrogenase enzymes present in metabolically active cells. Subsequently, the plates were read at 490 nm using a microplate reader (molecular Devices, Sunnyvale, CA).

The synthesis of N-(2,4-dimethylphenyl)hydrazinecarbothioamide and its azomethine derivatives has been performed according to the published methods (Scheme 1), with some particular modifications:
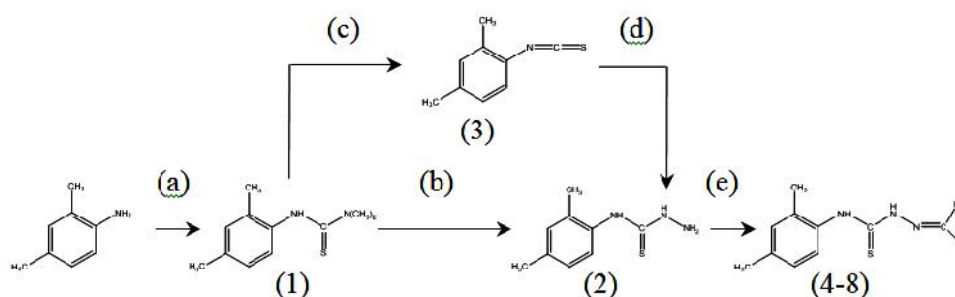
R = (4) pyridine-3-yl; (5) pyridine-4-yl; (6) thiophene-3-yl; (7) quinoline-2-yl; and (8) 2-hydroxyphenyl.

Scheme 1. (a) tetramethylthiuram disulphide (TMTD), benzene, 83°C, Yield 93%; (b) hydrazine hydrate, benzene, 85°C, Yield 90%; (c) HCl-H_2O (1:1), toluene, 112°C, Yield 82%; (d) hydrazine hydrate, diethyl ether, ethanol, 30°C, Yield 84%; (e) 3-formylpyridine (4), 4-formylpyridine (5), 3-formylthiophene (6), 2-formylquinoline (7), salicylaldehyde (8), ethanol, acetic acid, 75°C ,Yield 85-90%.

3-(2,4-dimethylphenyl)-1,1-dimethylthiourea (1): 2.42 g (20 mmol) of 2,4-dimethylaniline was added to a hot (75°C) solution of 2.4 g (10 mmol) TMTD in 20 mL benzene. The resulting solution was refluxed at 83°C for 4h. Then, the solution was allowed to reach ambient temperature and cooled to 7°C overnight. The crude product was filtered, dried and recrystallized from concentrated HCl. Yield, 3.86 g (93%), m.p. 155°C. ^{1}H-NMR (DMSO-d_6) δ: 2.13 (s, 3H, *o*-CH_3); 2.28 (s, 3H, *p*-CH_3); 3.27 (s, 6H, N(CH_3)$_2$); 6.92, 6.97, 7.02 (m, 3H, phenyl); 8.73 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6) δ: 18.33 (*o*-CH_3); 21.06 (*p*-CH_3); 41.16 (N(CH_3)$_2$); 126.88, 129.32, 131.02, 135.73, 135.89, 137.78 (phenyl); 182.12 (C=S).

N-(2,4-dimethylphenyl)hydrazinecarbothioamide (2), 1st method: 0.40 g (8 mmol) of hydrazine hydrate was added to a hot (75°C) solution of 2.08 g (10 mmol) of **1** in 18 mL benzene. The resulting solution was refluxed at 83°C for 1h. Then, the solution was allowed to reach ambient temperature and cooled to 7°C overnight. The crude product was filtered, dried and recrystallized from ethanol. Yield, 1.76 g (90%), m.p. 153°C. ^{1}H-NMR (DMSO-d_6) δ: 2.16 (s, 3H, *o*-CH_3); 2.26 (s, 3H, *p*-CH_3); 4.75 (s, 2H, NH_2); 6.96, 7.02, 7.34 (m, 3H, phenyl); 9.00 (s, 1H, NH–Ph); 9.31 (s, 1H, C(S)

NH). ^{13}C-NMR (DMSO-d$_6$) δ: 18.14 (*o*-CH$_3$); 21.01 (*p*-CH$_3$); 126.65, 127.58, 130.97, 133.73, 135.08, 135.80 (phenyl); 180.96 (C=S).

N-(2,4-dimethylphenyl)hydrazinecarbothioamide (2), 2nd method: A warm solution (30-36°C) of 1.63 g (10 mmol) of **3** in 3 mL diethyl ether was added dropwise to a solution of 0.75 g (15 mmol) hydrazine hydrate in 3 mL ethanol and the resulting mixture was stirred until the solid product **2** began to precipitate. Then, the solution was allowed to reach ambient temperature. The crude product was filtered, dried and recrystallized from ethanol. Yield, 1.64 g (84%), m.p. 153°C. ^{1}H-NMR (DMSO-d$_6$) δ: 2.16 (s, 3H, *o*-CH$_3$); 2.26 (s, 3H, *p*-CH$_3$); 4.75 (s, 2H, NH$_2$); 6.96, 7.02, 7.34 (m, 3H, phenyl); 9.00 (s, 1H, C$_{Ph}$–NH); 9.31 (s, 1H, C(S)NH). ^{13}C-NMR (DMSO-d$_6$) δ: 18.14 (*o*-CH$_3$); 21.01 (*p*-CH$_3$); 126.65, 127.58, 130.97, 133.73, 135.08, 135.80 (phenyl); 180.96 (C=S).

1-isothiocyanato-2,4-dimethylbenzene (3): A mixture of 4.16 g (20 mmol) of **1** in 40 mL toluene and 40 mL HCl-H$_2$O (1:1) was refluxed at 112°C for 2h. The toluene layer containing the product **3** was separated from water and the solvent was removed by vacuum distillation. Isothiocyanate **3** was dissolved in n-hexane and purified on a column of silica gel (eluting with n-hexane). n-hexane was removed by vacuum distillation. Yield, 2.67 g (82%), m.p. 28-29°C. ^{1}H-NMR (DMSO-d$_6$) δ: 2.27 (s, 3H, *o*-CH$_3$); 2.28 (s, 3H, *p*-CH$_3$); 7.05, 7.14, 7.23 (m, 3H, phenyl). ^{13}C-NMR (DMSO-d$_6$) δ: 18.20 (*o*-CH$_3$); 21.14 (*p*-CH$_3$); 126.12, 126.95, 128.16, 131.77, 133.85, 134.97 (phenyl); 138.22 (C=S).

N-(2,4-dimethylphenyl)-2-(pyridine-3-ylmethylene)hydrazinecarbothioamide (4): 1.18 g (11 mmol) of 3-formylpyridine was added to a hot (65°C) solution of 1.95 g (10 mmol) of **2** in 4 mL ethanol. 2 to 4 drops of glacial acetic acid was added as a catalyst. The resulting mixture was refluxed at 75°C for 2h. Then, the solution was allowed to reach ambient temperature and cooled to 0°C overnight. The crude product was filtered, dried and recrystallized from ethanol-DMF solvent mixture. Yield, 2.56 g (90%), m.p. 236°C. ^{1}H-NMR (DMSO-d$_6$) δ: 2.19 (s, 3H, *o*-CH$_3$); 2.30 (s, 3H, *p*-CH$_3$); 7.03, 7.09, 7.13 (m, 3H, phenyl); 7.45 (s, 1H, azomethine); 8.16, 8.39, 8.57, 9.00 (m, 4H, pyridyl); 10.03 (s, 1H, C$_{Ph}$–NH); 11.93 (s, 1H, NH–N=). ^{13}C-NMR (DMSO-d$_6$) δ: 18.21 (*o*-CH$_3$); 21.11 (*p*-CH$_3$); 119.63, 126.30, 130.97, 132.73, 139.18, 140.80 (phenyl); 143.3 (azomethine); 123.9, 130.4, 133.7, 149.0, 151.9 (pyridyl), 177.70 (C=S).

N-(2,4-dimethylphenyl)-2-(pyridine-4-ylmethylene)hydrazinecarbothioamide (5): 1.18 g (11 mmol) of 4-formylpyridine was added to a hot (65°C) solution of 1.95 g (10 mmol) of **2** in 4 mL ethanol. 2 to 4 drops of glacial acetic acid was added as a catalyst. The resulting mixture was refluxed at 75°C for 2h. Then, the solution was allowed to reach ambient temperature and cooled to 0°C overnight. The crude product was filtered, dried and recrystallized from ethanol-DMF solvent mixture. Yield, 2.50 g (88%), m.p. 244-245°C. ^{1}H-NMR (DMSO-d$_6$) δ: 2.19 (s, 3H, *o*-CH$_3$); 2.31 (s, 3H, *p*-CH$_3$); 7.04, 7.10, 7.13 (m, 3H, phenyl); 8.10 (s, 1H, azomethine); 7.88, 8.61 (m, 4H, pyridyl); 10.10 (s, 1H, C$_{Ph}$–NH); 12.04 (s, 1H, NH–N=). ^{13}C-NMR (DMSO-d$_6$) δ: 18.19 (*o*-CH$_3$); 21.12 (*p*-CH$_3$); 119.61, 126.14, 131.20, 132.90, 139.22, 143.82 (phenyl); 145.3 (azomethine); 120.45, 144.31, 149.17 (pyridyl), 177.93 (C=S).

N-(2,4-dimethylphenyl)-2-(thiophene-3ylmethylene)hydrazinecarbothioamide (6): 1.23 g (11 mmol) of 3-formylthiophene was added

to a hot (65°C) solution of 1.95 g (10 mmol) of **2** in 4 mL ethanol. 2 to 4 drops of glacial acetic acid was added as a catalyst. The resulting mixture was refluxed at 75°C for 2h. Then, the solution was allowed to reach ambient temperature and cooled to 0°C overnight. The crude product was filtered, dried and recrystallized from ethanol-DMF solvent mixture. Yield, 2.46 g (85%), m.p. 220°C. ^{1}H-NMR (DMSO-d$_6$) δ: 2.19 (s, 3H, *o*-CH$_3$); 2.30 (s, 3H, *p*-CH$_3$); 7.02, 7.08, 7.15 (m, 3H, phenyl); 8.17 (s, 1H, azomethine); 7.59, 7.84, 7.96 (m, 3H, thiophenyl); 9.81 (s, 1H, C$_{Ph}$–NH); 11.69 (s, 1H, NH–N=). ^{13}C-NMR (DMSO-d$_6$) δ: 18.23 (*o*-CH$_3$); 21.11 (*p*-CH$_3$); 126.89, 131.07, 135.54, 135.97, 136.20, 138.07 (phenyl); 126.24 (azomethine); 127.54, 128.49, 128.96 (thiophenyl), 177.29 (C=S).

N-(2,4-dimethylphenyl)-2-(quinoline-2-ylmethylene) hydrazinecarbothioamide (7): 1.73 g (11 mmol) of 2-formylquinoline was added to a hot (65°C) solution of 1.95 g (10 mmol) of **2** in 4 mL ethanol. 2 to 4 drops of glacial acetic acid was added as a catalyst. The resulting mixture was refluxed at 75°C for 2h. Then, the solution was allowed to reach ambient temperature and cooled to 0°C overnight. The crude product was filtered, dried and recrystallized from ethanol-DMF solvent mixture. Yield, 2.87 g (86%), m.p. 208°C. ^{1}H-NMR (DMSO-d$_6$) δ: 2.22 (s, 3H, *o*-CH$_3$); 2.31 (s, 3H, *p*-CH$_3$); 7.05, 7.11, 7.14 (m, 3H, phenyl); 8.34 (s, 1H, azomethine); 7.63, 7.78, 7.98, 8.04, 8.37, 8.63 (m, 6H, quinolyl); 10.22 (s, 1H, C$_{Ph}$–NH); 12.15 (s, 1H, NH–N=). ^{13}C-NMR (DMSO-d$_6$) δ: 18.24 (*o*-CH$_3$); 21.12 (*p*-CH$_3$); 118.87, 127.04, 130.38, 135.85, 136.54, 136.68 (phenyl); 143.08 (azomethine); 119.6, 127.0, 128.3, 128.9, 129.9, 130.9, 136.5, 148.5, 149.3 (quinolyl), 177.89 (C=S)

N-(2,4-dimethylphenyl)-2-(2-hydroxybenzylidene)hydrazinecarbothioamide (8): 1.23 g (11 mmol) of salicylaldehyde was added to a hot (65°C) solution of 1.95 g (10 mmol) of **2** in 4 mL ethanol. 2 to 4 drops of glacial acetic acid was added as a catalyst. The resulting mixture was refluxed at 75°C for 2h. Then, the solution was allowed to reach ambient temperature and cooled to 0°C overnight. The crude product was filtered, dried and recrystallised from ethanol-DMF solvent mixture. Yield, 2.66 g (89%), m.p. 187-188°C. ^{1}H-NMR (DMSO-d$_6$) δ: 2.18 (s, 3H, *o*-CH$_3$); 2.29 (s, 3H, *p*-CH$_3$); 7.01, 7.07, 7.15 (m, 3H, phenyl); 8.47 (s, 1H, azomethine); 6.82, 6.88, 7.23, 8.07 (m, 4H, hydroxyphenyl); 9.82 (s, 1H, OH); 9.95 (s, 1H, C$_{Ph}$–NH); 11.69 (s, 1H, NH–N=). ^{13}C-NMR (DMSO-d$_6$) δ: 18.18 (*o*-CH$_3$); 21.06 (*p*-CH$_3$); 119.69, 126.84, 131.09, 133.62, 135.36, 136.00 (phenyl); 140.42 (azomethine); 117.82, 118.58, 121.43, 127.52, 136.18, 156.99 (hydroxyphenyl), 177.31 (C=S).

Results and discussion

Antileukaemia activity. Six of the synthesized compounds were tested as inhibitors of HL-60 cells proliferation. These human promyelocytic leukaemia cells were incubated for three days in the presence of synthetic compounds and the number of viable cells was measured using the MTS assay (Scheme 2).

The results are expressed as the percentage of cell growth inhibition at three concentrations (Table 1). As it can be concluded from the data above, N-(2,4-dimethylphenyl) hydrazinecarbothioamide **2** has a pretty low antiproliferative activity. Thereby, at the concentration of 0.1 μM **2** shows its highest activity, almost equal to that of *Doxorubicin* at the same concentration. Thiosemicarbazones **4** and **5** do not inhibit cell proliferation at any concentration.

The highest antiproliferative activity shows **6** in 10 μM solution and **8** in 10 μM and 1 μM solutions.

Table 1. Antiproliferative activity of compounds 2-8 against human myeloid leukaemia (HL-60) cancer cells

Compound	Inhibition of cell proliferation (%)		
	10 μM	**1 μM**	**0.1 μM**
2	8.5	10.4	16
4	0	0	0
5	0	0.4	0
6	90.3	0	0
7	0.2	6.6	0
8	89.9	96.5	9.7
Doxorubicin	99	98	15

Therefore, it can be inferred that the antiproliferative activity of the compounds **4**-**8** is influenced by the nature of R and it grows in the following order: pyridine-3-yl $\leq$ pyridine-4-yl < quinoline-2-yl < thiophene-3-yl < 2-hydroxyphenyl. When comparing the activity of the thiosemicarbazide **2** to the activities of thiosemicarbazones **4**-**8,** it becomes conspicuous that the replacing of hydrogen atoms from NH_2 group with arilmethylene group (aril = carbocyclic or heterocyclic aromatic rest) leads either to decrease or significant increase of the antiproliferative activity. When the aromatic rest from arilmethylene group is pyridine-3-yl, pyridine-4-yl or quinoline-2-yl the antiproliferative activity is brought down to zero. On the contrary, when the aril rest is a thiophene-3-yl or a 2-hydroxyphenyl, the activities rise up to values comparable to those of the currently used in medical practice *Doxorubicin*. Hence, thiosemicarbazones **6** and **8** are of interest for further studies as potential alternatives to traditional antileukaemia medicines.
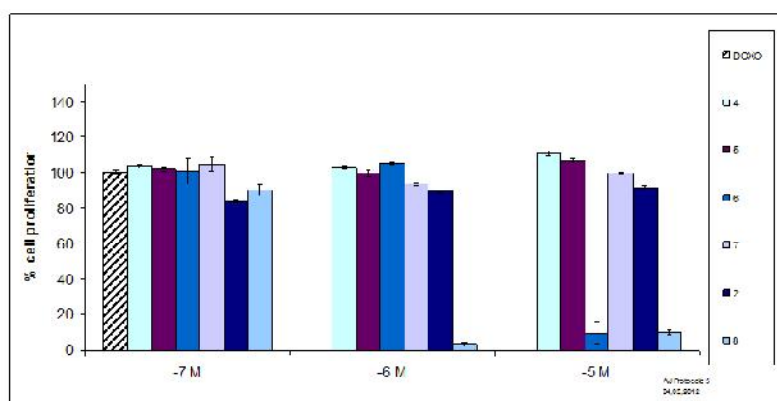
Scheme 2. The effect on HL-60 cancer cells proliferation

X-ray diffraction. N-(2,4-dimethylphenyl) hydrazinecarbothioamide crystallises in a P1 21/n 1 (14) monoclinic space group. The parameters of elementary cell are: a = 11.5215(5) Å, b = 7.5680(5) Å, c = 11.7534(7) Å; β = 99.24(1)°; V = 1011.54(89) Å^3.

As the length of the C–S bond is 1.696 Å, value closer to the theoretical C–S

double bond distance (1.615 Å), it could be concluded that in the solid state this thiosemicarbazide is more likely to adopt the thio-ketone form (Figure 1).

Moreover, as the [4]NH–C(S) bond has a *s-trans* conformation, the thiosemicarbazide rest NHC(S) NHNH$_2$ gets out of the plane of 2,4-dimethylphenyl ring and causes steric hindrances in the crystal packing (Figure 2).
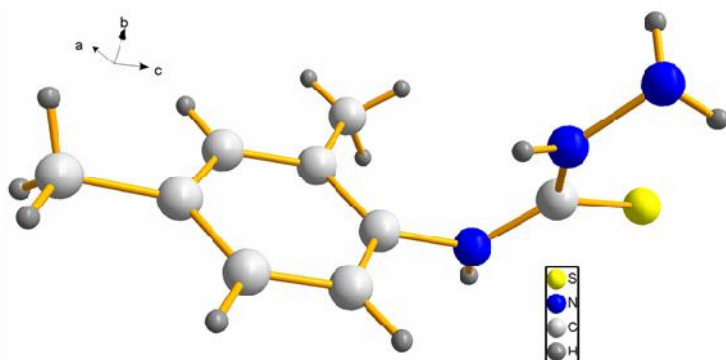

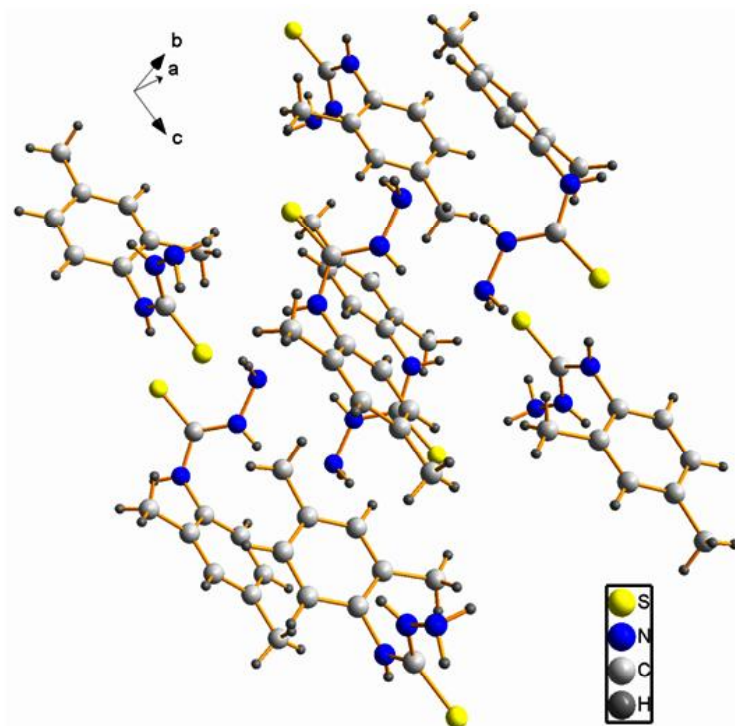**Figure 1. Molecular structure of N-(2,4-dimethylphenyl)hydrazine carbothioamide**

Figure 2. Crystal packing of N-(2,4-dimethylphenyl)hydrazinecarbothioamide

Conclusions

N-(2,4-dimethylphenyl) hydrazinecarbothioamide and its five azomethine derivatives have been synthesized starting from commercially available 2,4-dimethylaniline. The composition and the structure of the synthesized compounds have been defined by means of ^{1}H, ^{13}C NMR and X-ray diffraction. Antileukaemia bioassays have shown that

antiproliferative activity of the synthesized compounds is manifested mainly within the concentrations 10 μM and 1 μM and increases in the following series: $4 \leq 5 < 7 < 2 < 6 < 8$. Therefore, the most active compounds **6** and **8** should be further studied as potential alternatives to traditional antileukaemia medicines. Also, we have inferred from this study that in order to obtain highly antiproliferative active azomethines from N-(2,4-dimethylphenyl) hydrazinecarbothioamide, it should be condensed with aromatic carbocyclic or heterocyclic aldehydes or ketones, which contain donor atoms (such as O or N) in the *orto* position to the carbonyl group (e.g. salicylaldehyde, etc.).

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