The Synthesis and Biological Activities Of Some New 2-(4-Methoxy-phenoxymethyl)benzoic Acid Thioureides

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This paper presents the synthesis, structure confirmation and biological (antimicrobial and cytotoxicity) activities of some new 2-(4-methoxy-fenoxymethyl)benzoic acid thioureides. The new thioureides were obtained in three stages. In the first stage we prepared the 2-(4-methoxy-fenoxymethyl)benzoic acid by reacting potassium para-methoxy-phenoxide with phtalide. The second stage consisted in the synthesis of the 2-(4-methoxy-fenoxymethyl) benzoic acid chloride and in the third phase, the acid chloride afore mentioned was refluxed with ammonium thiocyanate and the resulting 2-(4-methoxy-fenoxymethyl)benzoyl isothiocyanate was treated with primary aromatic amines to obtain the new compunds. The new synthetised compounds structures were confirmed by spectral ^{1}H-NMR, ^{13}C-NMR and elemental analysis. The in vitro antimicrobial activity was evaluated using qualitative screening of the susceptibility spectra of different microbial strains to these compounds by three adaptated diffusion methods: paper filter disk impregnation with the tested substances solutions, the disposal of tested solutions in agar wells and the spotting of tested solutions on microbial inoculums seeded medium. The quantitative assay of the antimicrobial activity was performed by nutrient broth microdilution method in order to establish the minimal inhibitory concentration (MIC). The new thioureides presented a significant antimicrobial activity with MICs ranging from 128 μg/ mL to 256 μg/mL. The tested compounds exhibited reduced cytotoxicity on different cell lines and influenced the eukariotic cell cycle.

Key words: thioureides, antimicrobial activity, cytotoxicity

The specialized literature mentions a series of thioureides tested for a large spectrum for biological activities, such as: vermicides, antineoplastics, diuretics, platelet aggregation inhibitors, anticonvulsants, H_2-antagonists, antidiabetics, pesticides, insecticides or herbicides and also antibacterial activity [1, 2].

In previous papers we presented the synthesis, the structural proofs and some research on the antimicrobial action of some thioureides of the 2-phenoxymethylbenzoic acid and the 2-(4-methyl-phenoxymethyl) benzoic acid [3, - 6].

The preliminary positive results determined us to continue this research which resulted in the obtaining of 2-(4-methoxy-phenoxymethyl)benzoic acid thioureides [7, 8]. As we mentioned in previous papers, the general synthesis method used was the addition of some primary aromatic amines to the 2-(4-methoxy-phenoxy-methyl)benzoyl isothiocyanate. The chemical structures were confirmed by ^{1}H-NMR and ^{13}C-NMR spectral analysis and elemental analysis.

The *in vitro* antimicrobial activity was evaluated using qualitive screening of the susceptibility spectra of different microbial strains to these compounds using adaptated diffusion methods: paper filter disk impregnation with the tested substances solutions, the disposal of tested solutions in agar wells and the spotting of tested solutions on microbial inoculums seeded medium.

The quantitative assay of the antimicrobial activity was performed by nutrient broth microdilution method in order to establish the minimal inhibitory concentration. Cytotoxicity and cellular cycle influence were also tested.

Experimental part

The new 2-(4-methoxy-phenoxymethyl)benzoic acid thioureides synthesis was completed in three stages. The synthesis of the compounds **1**, **4**, ***6a-k***. and their spectral ^{1}H-NMR and ^{13}C-NMR analyses were published in previous papers [7-9].

In the first step 2-(4-methoxy-phenoxymethyl)benzoic acid (**1**) was obtained by treating phtalide (**2**) with potassium *para*-methoxyphenoxide in xylene. First the potassium salt of 2-(4-methoxy-phenoxymethyl)benzoic acid (**3**) is obtained and, by having a good solubility in a 10% sodium hydroxide aqueous solution, can be separated from xylene. The acid is removed from the salt by treatment with a hydrochloric acid solution. The potassium *para*-methoxyphenoxide was synthesized from *para*-methoxyphenol and solid potassium hydroxide in a xylene reaction medium, the resulting water being removed by azeotropic distillation.

In the second stage of the synthesis the 2-(4-methoxy-phenoxymethyl)benzoyl chloride (**4**) was obtained by reacting, for three hours, the acid (**1**) with thionyl chloride, in 1,2-dichlorethane made anhydric with calcium chloride. After the removal of the excess reactant and reaction medium, the raw acid chloride was used in the next stage.

In the third stage 2-(4-methoxy-phenoxymethyl)benzoyl chloride was reacted with ammonium thiocyanate, dried at 100^{o}C, and 2-(4-methoxy-phenoxymethyl)benzoyl isothiocyanate (**5**) was obtained. The reaction time was one hour and the reaction medium was acetone dried on potassium carbonate. The obtained isothiocyanate was not separated and the new thioureides (**6a- k.**) resulted after adding some primary aromatic amines in the reaction

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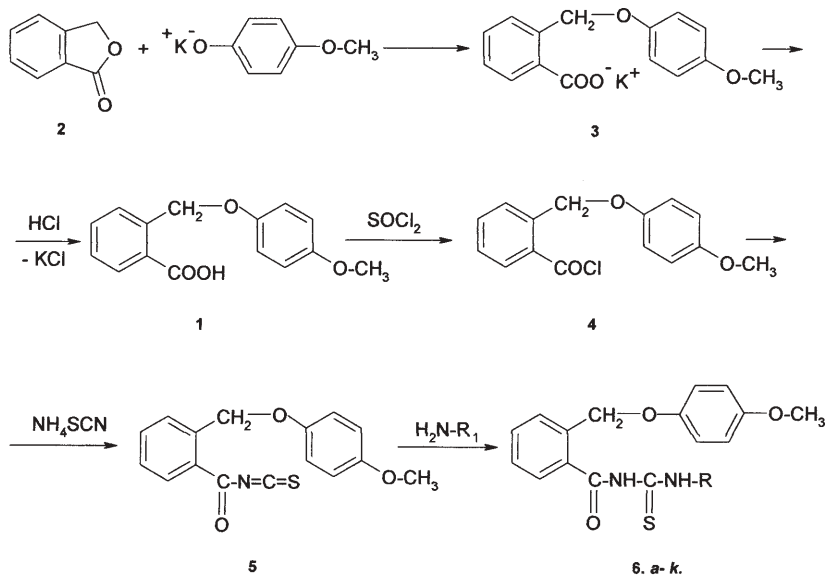


R= -C_6H_5 (6.a.), -$C_6H_4(CH_3)(4)$(6.b.), -$C_6H_4(OCH_3)(4)$(6.c.), -$C_6H_4(NO_2)(4)$(6.d.),

-$C_6H_4(Cl)(4)$(6.e.), -$C_6H_3(Cl_2)(2,4)$(6.f.), -$C_6H_3(Cl_2)(2,5)$(6.g.), -$C_6H_3(Cl_2)(2,6)$(6.h.),

-$C_6H_4(Br)(4)$(6.i.), - $C_6H_3(Br_2)(2,6)$(6.j.), -$C_6H_5(I)(4)$(6.k.)

Scheme 1. The synthesis pathway for the new thioureides

medium, while the reflux continued for another hour. The mentioned reactions are presented in the scheme 1.

Antimicrobial activity assay

The antimicrobial properties of the new thiouiredes were tested against bacterial and fungal strains recently isolated from clinical specimens belonging to the following genera and species: *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Candida albicans* and *Bacillus* subtilis, cultivated on liquid/solidified Mueller Hinton (for bacterial strains) and YPG (Yeast Peptone Glucose) medium (for yeast strains). The microbial inoculums were prepared in sterile saline from 15-18 h microbial cultures (4-5 isolated colonies) developed on solid medium for adjusted by nephelometry (using a McFarland standard) to a standard density of $1,5 \cdot 10^8$ CFU/mL (corrersponding to 0.5 MacFarland).

The testing of the antimicrobial and antifungal activity of the new thioureides was investigated by qualitative screening of the susceptibility spectrum of different microbial strains to the tested compounds solubilised in DMSO (1mg/mL) using adapted variants of the diffusion method [10, 11]. In the 1st variant, 5 µL of the compound solution were equally distributed on the paper filter disks placed on Petri dishes previously seeded "in layer" with the tested bacterial strain inoculums. In the 2nd variant, 5 µL of the tested compounds solutions were placed in the agar wells cut in the solidified culture medium seeded with the microbial inoculum [10, 11]. In the 3rd variant of the qualitative antimicrobial activity assay, 5 µL of the compounds solutions were spotted on Petri dishes seeded with bacterial/yeast inoculum. In all three variants, the Petri dishes were left at room temperature to ensure the equal diffusion of the compound in the medium or to allow the drop of the solution to be adsorbed in the medium and afterwards the dishes were incubated at 37°C for 24 h [10, 11]. The solvent used was also tested by all three methods to evaluate a potential antimicrobial activity.

For the quantitative assay of the antimicrobial activity of the new compounds by the microdilution method in liquid medium distributed in 96-well plates, binary serial dilutions of the tested compounds solutions were perfomed (there were obtained concentrations from 1024 µg/mL to 32 µg/mL) in a 200 µL culture medium final volume, afterwards each well was seeded with a 50 µL microbial suspension of 0,5 MacFarland density. In each test a microbial culture control (a series of wells containing exclusively culture medium with microbial suspension) and a sterility control (a series of wells containing exclusively culture medium) were performed. The plates were incubated for 24 h at 37°C [10, 12].

Cytotoxicity assay

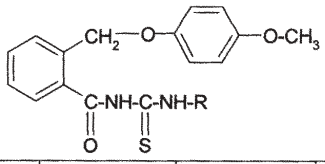
For the cytotoxicity assay two methods were used: the tetrazolium salts reduction method and the neutral red reduction test [13, 14].

The analysed compounds cytotoxicity was studied on HeLa (human cervical carcinoma) (ECACC # 93021013) cells and TV cells (human laryngeal carcinoma). The cells were cultivated in 96-well plates in DMEM medium (Dulbecco's Modified Essential Medium Sigma) supplemented with 10% fetal calf serum (Sigma) at 37°C, 5% CO_2, in a humid atmosphere. The eukaryotic cells were treated with the same concentrations of the tested compounds solution like those in the antimicrobial activity tests. After 24 h, XTT is added which, after 4 h of incubation, generates an orange colour. The method is based on the active cells metabolic capacity to reduce the tetrazolium salts to formazan compounds with orange color, which can be colorimetrically measured at 490 nm.

In the *neutral red reduction test*, eukaryotic cells with a density of $5 \cdot 10^4$ cell/mL were cultivated in 96-well plates incubated in a CO_2 atmosphere at 37°C and treated with variate concentrations of the tested compounds solutions. After 24 h the culture medium was changed with Hank's medium added with 50 µg/mL neutral red and then incubated 3 h at 37°C. The neutral red in excess was

Table 1

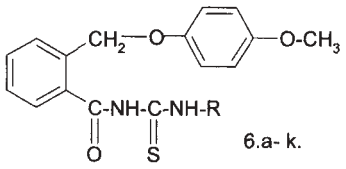
DATA ON THE NEW THIOUREIDES 6a-k



Nr. crt.	R	C%		H%		N%		S%		Melting point (°C)	Yield (%)
		t.	e.	t.	e.	t.	e.	t.	e.		
6a.	$-C_6H_5$	70.13	69.97	5.31	5.35	7.44	7.39	8.50	8.56	109.9-111.2	62.5
6b.	$-C_6H_4CH_3$ (4)	70.68	70.77	5.63	5.51	7.17	7.21	8.19	8.03	138.4-140.1	63.6
6c.	$-C_6H_4OCH_3$ (4)	67.90	67.74	5.41	5.36	6.89	6.88	7.87	7.85	127.2-128.7	59.5
6d.	$-C_6H_4NO_2$ (4)	62.64	62.46	4.51	4.39	9.97	10.06	7.59	7.59	148.6-150.3	57.4
6e.	$-C_6H_4Cl$ (4)	61.84	61.65	4.45	4.38	6.56	6.43	7.50	7.43	124.5-126.3	64.7
6f.	$-C_6H_3Cl_2$ (2,4)	57.22	57.09	3.90	3.78	6.07	6.16	6.94	6.89	140.7-143.3	63.4
6g.	$-C_6H_3Cl_2$ (2,5)	57.22	57.14	3.90	3.91	6.07	6.18	6.94	6.97	123.4-124.9	64
6h.	$-C_6H_3Cl_2$ (2,6)	57.22	57.31	3.90	3.87	6.07	6.12	6.94	6.85	183.2-185.9	51.5
6i.	$-C_6H_4Br$ (4)	56.01	56.28	4.03	4.12	5.94	5.87	6.79	6.77	109.7-112.4	67.9
6j.	$-C_6H_3Br_2$ (2,6)	47.98	47.77	3.27	3.34	5.09	4.98	5.82	5.69	183.5-187.1	44.6
6k.	$-C_6H_4I$ (4)	50.93	50.59	3.67	3.59	5.40	5.31	6.17	6.26	120.8-122.3	59.5

Table 2

THE RESULTS OF THE ANTIMICROBIAL ACTIVITY OF THE NEW THIOUREIDES



6.a- k.

No.	R	*Escherichia coli*	*Salmonella enteritidis*	*Pseudomonas aeruginosa*	*Listeria monocytogenes*	*Staphylococcus aureus*	*Bacillus subtilis*	*Candida albicans*
6a.	$-C_6H_5$	> 1024	> 1024	512	> 1024	512	> 1024	**256**
6b.	$-C_6H_4CH_3$ (4)	**256**	**256**	**128**	**256**	> 1024	**256**	**128**
6c.	$-C_6H_4OCH_3$ (4)	> 1024	> 1024	**256**	> 1024	> 1024	> 1024	> 1024
6d.	$-C_6H_4NO_2$ (4)	**256**	512	**256**	**256**	**256**	**128**	**128**
6e.	$-C_6H_4Cl$ (4)	> 1024	> 1024	**256**	**256**	**256**	**128**	**256**
6f.	$-C_6H_3Cl_2$ (2,4)	**256**	**256**	**128**	**256**	**256**	**128**	**128**
6g.	$-C_6H_3Cl_2$ (2,5)	> 1024	**256**	**128**	> 1024	> 1024	> 1024	**128**
6h.	$-C_6H_3Cl_2$ (2,6)	512	**256**	**256**	> 1024	**256**	**256**	**256**
6i.	$-C_6H_4Br$ (4)	> 1024	**256**	**256**	**256**	**256**	**256**	**256**
6j.	$-C_6H_3Br_2$ (2,6)	> 1024	**256**	**128**	**256**	**256**	**256**	**256**
6k.	$-C_6H_4I$ (4)	**256**	**256**	> 1024	**256**	> 1024	> 1024	> 1024

removed with a saline phosphate buffer and the incorporated dye was extracted with an ethanol: acetic acid: distilled water mix (49:1:50) and measured by 540 nm absorbancy measuring.

The cellular cycle influence was tested for one compound (i.e. 6g.) by flow cytometry. After the cell's exposure to 100 μg/mL compound solution, for 24-48 h, they were trypsinized and the cells suspension was fixed in ethanol 70% for at least 30 min. After centrifugation, the sediment was taken with 1 mL phostate buffer solution containing 50 μg/mL of RNAase and propidium iodide (PI). The dyed cells were analyzed two hours at the flow cytometer. The percentage of cells at different cellular cycle stages was determined with the ModFIT software [13, 14].

Results and discussions

The structure, elemental analysis, melting point and yield of the new thioureides are presented in the table 1. The melting points were recorded with an Electrothermal 9100 apparatus and are uncorrected. The elemental analysis was performed on a PerkinElmer 2400 Series II CHNS/O Elemental Analyzer and allowed us to certify the new compounds molecular structures.

For the qualitative methods of paper filter disks impregnated with the tested compounds solution and

Standard	Compound 6g.
G0/G1=82.99	G0/G1=65.36
S=7.47	S=3.60
G2/M=7.49	G2/M=21.61

Table 3
THE INFLUENCE ON THE CELLULAR CYCLE

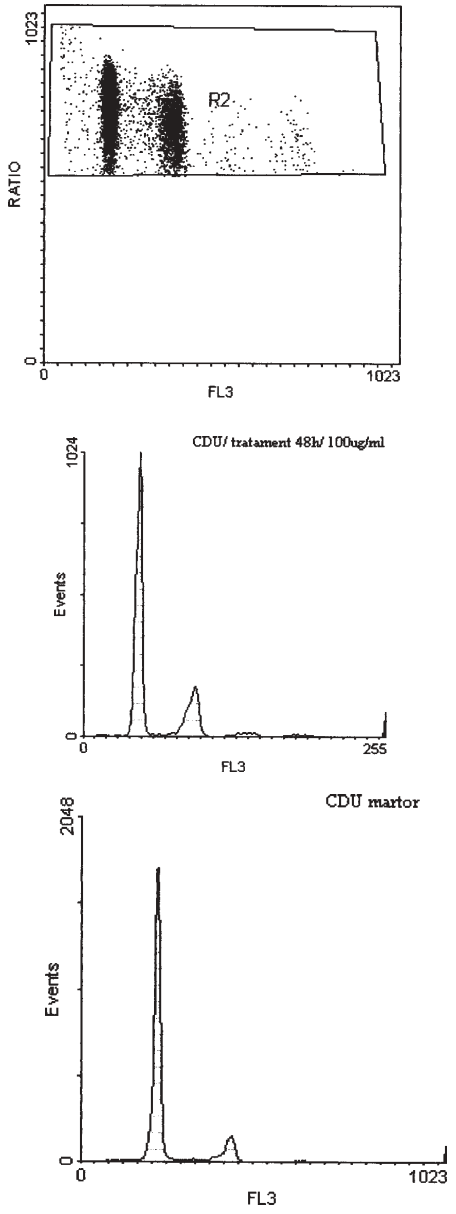


Fig. 1. The results of the flow cytometry assay of the influence of the compound no. 6.g. on cell cycle

disposal of the respective solutions in agar wells the reading of the results was done by measuring the microbial growth inhibition zones around the filter disks impregnated with the testing compounds and around the wells, respectively.

The most efficient qualitative method proved to be the spotting of the tested solutions on the seeded medium, the results being very good correlated with the results of the MIC quantitative assay.

Firstly, there were examined the standard culture plates to read and to analyze the qualitative method results, where the culture stripes had to be observed. If in a plate containing a compound the inoculated strain didn't grow, then it was considered that the respective compound has a *bactericidal effect*. If in the plate a bacterial growth could be observed, the culture density was compared with that of the standard culture plate. In the case of a bacterial growth less abundant than that of the standard culture, we appreciated that the substance exhibited a *bacteriostatic effect*.

A superior bacterial growth in the presence of the tested compound then the standard culture could be observed. In this case the tested compound exhibited a stimulatory effect on the bacterial growth.

If the growth intensity is comparable for the tested plate and for the standard culture, then the substance doesn't influence notably the growth and the development of the tested bacterial stain.

The used solvent testing revealed that it hasn't antimicrobial activity, this being a practic advantage for the antimicrobial activity testing of the water insoluble compounds.

In the case of the quantitative assay of the antimicrobial activity of the tested compounds by the microdilution method in liquid medium the minimal inhibitory concentration was read by wells observation: in the first wells containing high concentrations of compounds the culture growth was not visible, the microbial cells being killed or inhibited by the tested compound. At lower concentrations of the tested compounds, the microbial culture becomes visible. The lowest concentration which inhibited the visible microbial growth was considered the MIC (μg/mL) value for the tested compound. In the next wells, including the standard culture growth control wells, the medium become muddy as a result of the microbial growth. In the sterility control wells series the medium had to remain clear. From the last well without any visible microbial growth and from the first one with a microbial growth, Gram stained smears were performed for the results confirmation.

In the table 2 are presented the results of the quantitative assay of the antimicrobial and antifungal activities of the new compounds, being known that a concentration of 32 μg/mL represents a very strong effect and a 256 μg/mL concentration represents a moderate effect. The tested compounds presented an antimicrobial activity at concentrations from 1024 to 128 μg/mL.

Many of the tested compounds presented broad spectrum antimicrobial activity, e.g. compounds **6d**. (N-[2-(4-methoxy-phenoxymethyl)-benzoyl]-N'-(4-nitrophenyl)-thiourea), **6f**. (N-[2-(4-methoxy-phenoxymethyl)-benzoyl]-N'-(2,4-dichlorophenyl)-thiourea), **6i**. (N-[2-(4-methoxyphenoxy-methyl)-benzoyl]-N'-(4-bromophenyl)-thiourea), **6j**. (N-[2-(4-methoxy-phenoxymethyl)-benzoyl]-N'-(2,6-dibromophenyl)-thiourea), being active at low concentrations both on Gram positive, Gram negative bacteria and fungus.

It's worthing to notice the good antimicrobial activity of most of the tested compounds against *Pseudomonas aeruginosa* and *Candida albicans*, which can represent new therapeutical options in the tratament of the *Pseudomonas* and fungal infections, which are difficult to treat and eradicate, because of the very high levels of natural and acquired resistance of these microorganisms. The activity on *Staphylococcus aureus* was moderate, having MIC values $\geq$ 256 μg/mL.

The cytotoxicity study results revealed that the tested compounds exhibited a reduced cytotoxicity on HeLa and TV lines.

The assessment of the cellular cycle influence for the compound **6g**. (N-[2-(4-methoxy-phenoxymethyl)-benzoyl]-

N'-(2,5-dichlorophenyl)-thiourea) by flow cytometry proved that the respective compound induced a rise of G2/M (meaning a blockade of G2/M) and a weak polyploidy after 48 h contact with the cell culture (table 3, fig.1).

Conclusions

This paper presents the synthesis, structure confirmation and some biological activities (including the antimicrobial, antifungal activity and the cytotoxicity) of some new 2-(4-methoxy-phenoxymethyl)-benzoic acid thioureides.

For the structure confirmation of the new compounds and theirs intermediaries the ^{1}H-NMR and ^{13}C-NMR spectra were studied and elemental analyses were performed.

This paper presents an original methodological approach for the assessment of the biological activities of newly synthesised chemical compounds. The *in vitro* qualitative and quantitative antimicrobial activity assay showed that the new thioureides exhibited significant antimicrobial activity with MICs ranging from 128 μg/mL to 256 μg/mL. The cytotoxicity study revealed a reduced cytotoxicity of the compounds on HeLa and TV cells, demonstrating the potential use of these substances in the selection of new antimicrobial agents.

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