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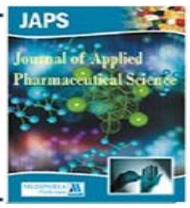

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Synthesis, characterization, anti-trypanosomal activity and toxicity against *Artemia salina* Leach of thiobenzamides and derivatives

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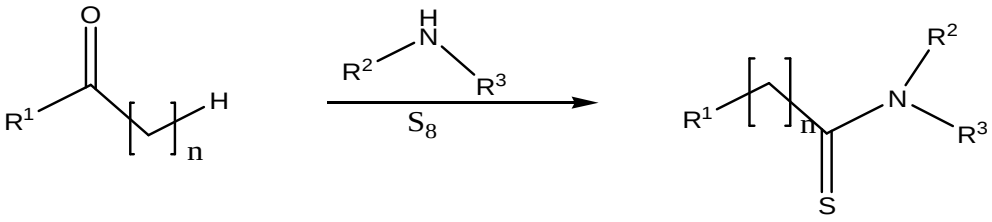
ABSTRACT

This work aims to synthesize, characterize of thioamides benzaldehyde and 4-(dimethylamino)benzaldehyde and assess their *in votro*trypanocidal activity and totoxicity. The Willgerodt-Kindler reaction preferred for the synthesis of thioamides morpholin-4-yl (phenyl) methanethione 1 and [4 - (dimethylamino) phenyl] - (morpholin-4-yl) methanethione 2, is catalyzed with montmorillonite K-10 and in a microwave oven. The structures of the thioamides were characterized and confirmed by IR spectrometry, nuclear magnetic resonance (^{1}H and ^{13}C NMR) and mass spectrometry (MS) Their trypanocidal activity was evaluated in the blood stream form of the strain of *Trypanosoma brucei brucei* 427 using the "Lilit, Alamar Blue" (Baltz *et al.*, 1985; Hirumi *et al*., 1994; Räz *et al*., 1997) and cytotoxicity on brine shrimp larvae (*Artemia salina* Leach) using the method of Michael *et al.* (1956) resumed by Vanhaecke *et al.* (1981) and Sleet and Brendel (1983). The compounds**1** (IC_{50}> 483.09 μM) and **2** (IC_{50}> 400 μM) have weak trypanocidal activities. However the larvae were sensitive to **2** (LD_{50} = 214 ± 9 μM) and therefore it could be used in cancer treatment.

Keywords: Willgerodt-Kindler, Montmorillonite K-10, morpholin-4-yl (phenyl)-methanethione, [4-(di-methylamino) phenyl] (morpholin-4-yl) methanethione, *Artemia salina* Leach, trypanocidal

INTRODUCTION

The thiobenzamides are interesting molecules in medicinal chemistry. Previous studies revealed their antifungal and antibacterial properties (Matysiak *et al.*, 2000). They are inhibitors of aldose reductase (Sestani *et al.*, 1984) and estrogen receptor antagonists (Stauffer *et al.*, 2000; Waisser *et al*., 2000). However, the bibliography is not case of their antitrypanosomal activity. The trypanosome is a protozoan parasite responsible for Chagas disease. One of its species, Trypanosoma brucei causes the human African trypanosomiasis and livestock respectively in humans and animals in Africa. The World Health Organisation (WHO), estimated over 250 homes active mostly in sub-Saharan Africa in some member states of the WHO African region and Sudan.



Scheme. 1: Willgerodt-Kindler's reaction.

Moreover, the estimates made in 2006 give a variation from 50,000 to 70,000 of people infected (WHO, 2006). After incessant efforts to reduce this disease, the number of cases reported in 2009 fell below 10,000 for the first time in 50 years. However, the number of actual cases is currently estimated at 30000 people (WHO, 2010). Animal trypanosomiasis due to *Trypanosoma brucei brucei* (Kuboki *et al.,* 2003; Darsaud *et al.,* 2004) and affecting domestic animals is a major obstacle to economic development in affected rural areas. Drugs needed to treat these parasitic diseases are inaccessible to all populations and also present in some cases significant risk of toxicity (Aguiarre *et al.*, 2004). Several synthesis methods have been developed to give the pharmacological properties of thioamides and require a validated reaction of Willgerodt-Kindler (WK) (Nihed, 2009). This reaction is a known method for the synthesis of thioamides (Amupitan *et al*., 1983). It consists in condensation of the carbonyl compound with an amine in the presence of sulphur (Kawai *et al.*, 1999). This reaction was performed for the first time by Willgerodt in 1887 by transforming a terminal amide carbonyl compound into a salt $(NH_4)_2S$ (Brown, 1975; Willgerodt, 1887). In 1923, Karl Kindler suggested a modification of this reaction by using sulphur and an amine such as morpholine on the carbonyl compound thus leading to the thioamide (Kindler, 1923; DeTar *et al.*, 1946). (Scheme 1).

This reaction has been the subject of several studies on the optimal conditions.

Carlson et al, 1986 and 1987 showed the influence of solvents, the substrate structure and the structure of the amine reagent to know about the optimum conditions.

Recently, Gbaguidi *et al.* (2010) showed that an acid catalyst such as montmorillonite K-10 also participates in optimal conditions during its use for the synthesis of 1-morpholino-2-(naphthalen-1-yl) éthanethione. The microwave technology used in chemistry since the 1970s (Sarjani *et al.,* 2010) is applied to the reaction of WK. This technology promotes a decrease in the formation of H_2S and a reduction in the reaction time (Poupaert *et al*., 2004). The aim of this work is to synthesize thiobenzamides by Willgerodt-Kindler reaction with microwave technology, to confirm their structures and to assess their antitrypanosomial activity and their toxicity against *Artemia salina Leach.*

MATERIALS AND METHODS

Chemistry

General Technical

The synthesis were made with radiation in a microwave oven "type Brandt MO 18T (940W, 2450MHz). TLC analyses were performed using silica gel pre coated plates. The evaporation of volatile solutions using a rotary evaporator (Laborota Heidolph type 4000-efficient Melting points of the products were taken on a fusionometer of the type electrothermal 1A 9000 and are uncorrected. The IR spectra were recorded with a spectrophotometer Perkin-Elmer FTIR 286. The NMR spectra were performed and recorded with a Bruker type 400 MHz for proton and 100 MHz ^1H to ^{13}C in $CDCl_3$ (chloroform). Mass spectra were performed using a LCQ advantage mass spectrometer (Thermo Fisher Scientific) with a source at atmospheric pressure chemical ionization (APCI) in positive mode.

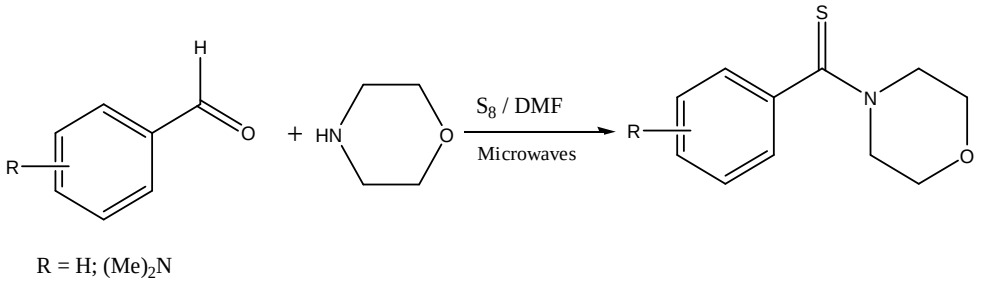
Reagents and catalyst

benzaldehyde, 4-(dimethylamino)benzaldehyde, sulphur, morpholine, montmorillonite K-10, dimethylformamide (DMF), ethyl acetate, ethanol, n-hexane, magnesium sulphate were obtained from Prolabo, Acros Organics and Sigma-Aldrich. Montmorillonite K-10 used for the acid catalysis of the reaction is a commercial clay obtained by acidification of natural montmorillonite smectite group belonging to the family of phyllosilicates of formula (Na, Ca) 0.3 (Al, Mg) $2Si_4O_{10}$ (OH) 2 • nH_2O (Alas *et al.* , 1994; do Rego *et al.*, 2010).

Method of synthesis

In a mixture of aldehyde (5 mmol) and morpholine (7.5 mmol) stirred, 15 ml of DMF (solvent) were added. In the mixture under stirred, is added 0.35 g of the catalyst K-10 and 8 mmol of sulfur. Stirring was continued until obtaining of a brown coloration and the reaction mixture was subjected to 10 sequences of microwave pulses for about 10 to15 minutes. Pulses were spaced of one minute 20 seconds. After cooling to room temperature, the mixture was then poured into an ethyl acetate solution for precipitation of the sulfur that was then removed with the K-10 by filtration. The filtrate was first treated with 100 mL of hydrochloric acid (0.1 M) to protonate the amine in excess, then with 100 mL of a saturated solution of NH_4Cl and finally washed with 2 × 100 mL of distilled water. The organic phase was concentrated with a rotary evaporator after drying over $MgSO_4$. The crystals formed were recrystallized from ethyl alcohol 95 ° and purification was achieved on silica gel column to ensure the removal of sulfur and secondary products. The mobile phase used is a mixture of hexane and ethyl acetate (6/4; v / v).

All synthesized products were dried before the determination of melting points, spectroscopic analyzes and biological tests.



R = H; $(Me)_2N$

Scheme 2 : Formation of thioamides by WK reaction.

PHARMACOLOGY

Anti-trypanosomal activity (LILIT, Alamar Blue™)

Anti-trypanosomal activity (Lilit ALAMAR Blue™) The test is performed on the blood stream form of the strain of Trypanosoma brucei brucei 427 using the "Lilit, Alamar Blue" (Baltz *et al*., 1985; Hirumi *et al.*, 1994; Räz *et al*., 1997). Stock solutions of morpholin-4-yl (phenyl) méthanethione 1 and [4 - (dimethylamino) phenyl] - (morpholin-4-yl) méthanethione 2 were carried out at an initial concentration of 10 mg / mL in DMSO. Trypanosomes are cultured in medium containing 10% fetal calf serum and factor supporting the blood stream form trypanosome. Suspensions of trypanosomes have been adjusted to 5104 tryps / mL. A 50 L of various dilutions of the stock solution was added 50 L of suspension of trypanosomes in different wells. The plates were then incubated at 37 ° C for 72 hours in an atmosphere of 5% CO_2. Then added to each well 10 µl of the dye "Alamar Blue™" again and then incubated for 4 hours. The dye " Alamar Blue™ " is a reagent for detection of enzyme activity; colored wells are those in which the concentration of the synthetic product is insufficient to inhibit proliferation of trypanosomes. The plates are read in comparison with control wells on a drive fluorescence at a wave length of 530 nm excitation and emission wave length of 590 nm.

Toxicity Against Artemia Salina Leach

This test was performed on larvae of brine shrimp (Artemia salina Leach) by method of Michael *et al.* (1956) resumed by Vanhaecke *et al.* (1981) and by Sleet and Brendel (1983). Thus, encysted *Artemia salina* eggs are incubated in seawater at pH 7-8(48h). Then, series of solutions of test substances at varying concentrations and progressive were prepared in DMSO (dimethyl sufoxide)/seawater. A defined number of larvae introduced into each solution. All solution sand control solutions containing no active substance were left stirring for 24hours.Counting under a microscope the number of death larvae in each solution used to evaluate the toxicity of the solution. In the case where there was death in the control medium, the data was corrected by Abbott's formula:

%death= [(test- control**)** /control**)]** x100 (Abbott, 1925)

Data (dose-response) are transformed by logarithm and the half-lethal concentration LC_{50} is determined by linear regression (Hafner *et al.*, 1977). Tests were carried out in triplicate. All data were expressed as means±standard deviation of triplicate measurements.

RESULTS AND DISCUSSION

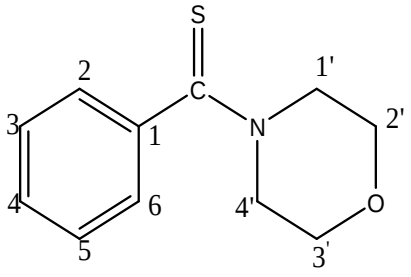
Chemistry

Willgerodt-Kindler (WK) reaction catalysed by Montmorillonite K-10 helped to synthetized morpholin-4-yl(phényl) methanethione (1)and [4-(dimethylamino)phenyl] (morpholin-4-yl) methanethione (2) with yields of 67% and 43% respectively. This work showed that the K-10 used as catalyst in the WK reaction for ketone compounds identified in the work of Gbaguidi *et al.* (2010) can also be used for derivatives of benzaldehyde. With this catalyst, benzaldehyde was the most reactive. It reacted quickly and leaded to better performance (> 60%). Acid catalysis with K-10 may influence the reactivity of the compounds having one amino group bound to the aromatic ring **2** (43%).

The difference between the calculated molar mass and molar mass obtained by mass spectrometry for each APCI thioamide was about of 0.05%. The spectrometric data of the synthesized molecules confirmed the structures proposed for the products. Indeed, the vibrational frequencies of thioamides in IR between 3030-3050 cm-1 and ^{1}H NMR chemical shifts ($\delta \approx 7$ ppm) revealed the presence of an aromatic ring. Furthermore the ^{13}C NMR analysis with a chemical shift of δ = 199.15 ppm for compound **1** and δ = 202.25 ppm for compound **2** characterized the thioamide group. These values are quite close to theoretical values obtained by chemdraw calculation.

Characterization of synthethised compounds

morpholin-4-yl(phenyl)methanethione

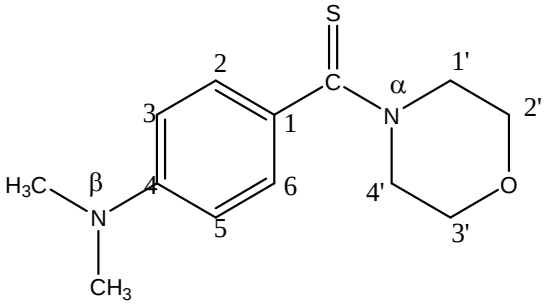


Rf (Hexane / Ethyl acetate 6/4 v/v) : 0,71 ; MP: 137-138°C ; MS : *207,06 m/z*

IR ν (KBr cm^{-1}) : 3025,16 ; 3009,17 ; 2971,68 (C aromatic) ; 1594,11 ; 1574,71 ; 1495,25 (thioamide). ; ***NMR ^{1}H(CDCl$_3$, δ ppm)*** : 7,3 (5H, m, aromatic) ; 4,4 (2H, t, C$^{1'}$H$_2$) ;

3,85 (2H, t, $C^{4'}H_2$) ; 3,6 (4H, t, $C^{2'}H_2$, $C^{3'}H_2$). ; **NMR ^{13}C ($CDCl_3$, δ ppm)**: 140,6 (C^1 aromatic) ; 126,99 (C^2, C^6 aromatic) ; 126,66 (C^3, C^5 aromatic) ; 123,99 (C^4 aromatic) ; 199,15(C=S) ; 47,16 ($C^{1'}$) ; 64,64 ($C^{2'}$) ; 64,86 ($C^{3'}$) ; 50,63 ($C^{4'}$).

[4-(dimethylamino)phenyl](morpholin-4-yl) methanethione



Rf (Hexane / Ethyl acetate 6/4 v/v) :0,60 ; PF: 149-150°C ; MS : *250,12 m/z*

IR ν (KBr cm^{-1}) : 3000,38 ; 3020,40 (C aromatic) ; 2962,64 (methyl) ; 3448,83 (amine) ; 1547,15 ; 1520,63 ; 1487,27 (thioamide) ; **NMR ^1H ($CDCl_3$,δ ppm)**: 7,3 (2H, t, C^2H, C^6H aromatics) ; 6,7 (2H, t, C^3H, C^5H aromatics) ; 4 ,4 (2H, t, $C^{1'}H_2$) ; 3,8 (6H, t, $C^{4'}H_2$, $C^{2'}H_2$, $C^{3'}H_2$), 3 (6H, s, $2CH_3$) ; **NMR ^{13}C ($CDCl_3$, δ ppm)**: 129,78 (C^1 aromatic) ; 128,72 (C^2, C^6 aromatics) ; 111,1 (C^3, C^5 aromatics) ; 151,24 (C^4 aromatic) ; 202,25 (C=S) ; 52,89 ($C^{1'}$, $C^{4'}$) ; 66,78 ($C^{2'}$, $C^{3'}$) ; 40,27 ($2CH_3$)

Pharmacology

All thiomides were evaluated on the parasites *Trypanosoma brucei brucei.*Thioamides **1** and **2** synthesized showed no effect up to 400 µM.To our knowledge the literature has never reported the trypanocidal activity of thioamides, we accept as a reference molecule of thiosemicarbazones. Thioamide was a chemical function in which there is a sulfur. The trypanocidal activities of thiosemicarbazones have shown the importance of sulfur, which plays an essential role in the antimicrobial activity (Domagk *et al.,* 1946; Klayman *et al.,* 1984; Glinma *et al.,* 2011; Sakirigui *et al.,* 2011). According to Du *et al.* (2002) and Fujii *et al.* (2005), thiosemicarbazones with IC_{50} values below 10 µM were potential inhibitors of the trypanosome. Values between 10 and 100 µM, were considered as moderate inhibitory; above 100 µM anti-trypanosomal activity was low or zero (Du. *et al*. 2002, Fujii *et al.* 2005). Thioamides **1** and **2** synthesized showed no effect up to 400 µM and therefore had no significant trypanocidal activity.

Toxicity test against *Artemia salina* larvae shows that larvae were sensitive to morpholin-4-yl (phenyl) methanethione (**1**) (LD_{50} greater than 754 µM) and [4 - (dimethylamino) phenyl] (morpholin-4-yl) methanethione (**2**) to an LD_{50} = 214 ± 9 µM. Lapachol [2-hydroxy-3-(3-methylbut-2-enyl) naphthalene-1,4-dione] (LD_{50} = 281 µM) a reference compound was used as positive control. According to Santos-Pimenta *et al.* (2003) and Angelia *et al.* (2007) a compound having an LD_{50} less than 281 µM, was active on shrimp larvae. It is clear that the thioamide **2** (214 ± 9 µM) was active on shrimp larvae while the thioamide **1** (> 754 µM) was not.Since there is a correlation between the cytotoxicity of shrimp larvae and cell 9PS and 9KB nasopharyngeal carcinoma of a human hand (Pelka *et al.,* 2000), A-549 cells and lung carcinoma cells HT-29 colon carcinoma on the other hand (Carballo *et al.,* 2002), the thioamide **2** could be used in cancer treatment.

A structure - activity relationship of these thioamides showed that the substitution of a dimethylamino group at the para position on the aromatic ring induced toxicity of thioamide **1**. Bioactivity increased by fixing the dimethylamino group in the para position has already been noticed by Fatondji *et al.* (2011) during their work on the cinnamaldehyde thiosemicarbazone.

CONCLUSION

The application of the Willgerodt-Kindler reaction to benzaldehyde and a derivative thereof on 4 - (dimethylamino) benzaldehyde with microwaves in the acid catalysis with montmorillonite K-10 is valid. The search for biological activity of these thioamides on *Trypanosoma brucei brucei*, revealed a very low trypanocidal activity (IC_{50}> 100 µM). The toxicity of these thioamides was evaluated on larvae of *Artemia salina* Leach. The results showed that larvae are more sensitive to thioamide 2 (LD_{50} = 214 ± 9 µM) than thioamide 1 (LD_{50}> 754 µM) whose toxicity is relatively negligible. Therefore, the thioamide 2 could be used in cancer treatment. A structure-activity relationship of these thioamides showed that the substitution of a dimethylamino group at the para position on a thioamide aromatic ring increased his toxicity.

Conflict of interest statement

The authors declared no conflict of interest.

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