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Study of benzoin 4-phenyl-3-thiosemicarbazone and benzil bis(4-phenyl-3-thiosemicarbazone) : Synthesis and their antiparasitic activity on trypanosome

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Abstract: Thiosemicarbazones represent a large family of molecules with extremely diverse pharmacological properties. Their interest in medicinal chemistry has stimulated the development of new methods of preparing these molecules. In this work, benzoin and benzil were used with 4-phenyl-3-thiosemicarbazide to synthesize benzoin 4-phenyl-3-thiosmicarbazone ***(Bzn-4PhT)*** with AAG and benzyl bis(4-phenyl-3-thiosemicarbazone) ***(Bzl-b4PhT)*** with NaOH, according to a new approach in the synthesis. Steric aspect and reaction conditions played an important role in the synthesis and yields of 75% and 67% respectively. The products presented physical properties compatible with reasonable pharmacokinetics and drug availability. The molecules were characterized by proton and carbon 13 NMR spectrometric analyzes (^{1}H & ^{13}C). The antiparasitic activity of the products was evaluated on *Trypanosoma brucei brucei* and their larvae toxicity on *Aremia salina* Leach It emerges after the test that the compounds have inhibited a moderate trypanocidal activity on the parasite and a toxic activity on the larvae with their inhibitory (and lethal) half-concentrations IC_{50} = 15.43 and 26.40 µM an LC_{50} = 165 and 257 µM respectively).

Products turned out selective on the parasite with their selectivity index greater than to unit (SI = 9 and 10). Compounds could be able to have applications in the treatment of parasitic diseases.

Keywords: synthesis, benzoin, benzil, 4-phenyl-3-thiosemicarbazones, trypanocidal activity, selectivity

INTRODUCTION

African trypanosomiases are still a serious health and economic problem that requires not only the application of the knowledge and resources currently available, but also their improvement through multidisciplinary research [1,2]. Animal trypanosomiasis is a major constraint for the livestock industry in developing countries [3]. In East Africa, animal trypanosomiasis is caused by numerous protozoan parasites transmitted by tsetse flies, including *Trypanosoma vivax*, *T. congolense* and subspecies of *T. brucei* sl (*T. brucei* and *T. b* zoonotic *rhodesiense* infectious for humans) that can co-circulate in domestic and wild animals [4]. The challenge of drug resistance, emerging and re-emerging diseases is a serious concern to the field of phytomedicine, pharmacognosy and pharmaceutical microbiology and chemistry [5]. The extensive use of antibacterial drugs and their resistance against bacterial infections is positively correlated with the use of antibacterial agents in clinical practice. That is why it is very much essential to find out safe, more effective and inexpensive new chemical compounds or plants extracts as antibacterial agents. Bacterial resistance to antibacterial drugs has led to severe health and economic problems [5].

Thiosemicarbazones are of considerable interest due to their pharmacological application, on the one hand, and their versatility as ligands to generate a wide variety of coordination modes, on the other hand [6]. In addition to their various chemical and structural characteristics, the interest of these compounds is also due to their broad spectrum of biological activity [7-9]. Recently, due to these spectrum biological activity, interest in these compounds has been considerably increased in the pharmaceutical sector at present [10-13], particularly in the fields of antimicrobials, anticonvulsants, anti-malarial, antiviral [8-12], and especially anti-tumor [13-21]. They are widely used as intermediates in the synthesis of heterocycles of molecules of great biological importance [20]. Numerous works reported in the scientific literature mention interesting aspects of the chemistry of these compounds, such as the methods to synthesize them [13], their stereochemistry [22], their spectral characteristics and the diversity of crystal structures [23-24] they generate. They represent validated drug leads that kill several species of protozoan parasites through the inhibition of cysteine proteases as well as other novel targets [25].

In the light of this important data which have been achieved with the literature survey considering that the thiosemicarbazones are biologically active compounds, the aim of this work is to synthesis the thiosemicarbazone derivatives expected to show positive activity.

Here, we described the synthesize of N(4)-phenyl-3-thiosemicarbazones of benzoin and benzil and then evaluated their parasitic activity against *Trypanosoma brucei brucei* and their toxicity on the *Artemia salina* Learch.

EXPERIMENTAL

Reagents : All reagents were obtained from chemical societies: Sigma-Aldrich, Acros Organic, Janssen Chimica, Prolabo and Riedel-de Haen. Substrates, reagents, catalysts and solvents were used directly for syntheses without any further purification. There are : benzoin, benzil,

phenylisothiocyanate, hydrazine hydrate, glacial acetic acid (AAG), sodium hydroxid (NaOH), technical ethanol (EtOH, 96°).

Equipment : All synthesized products were characterized by Nuclear Magnetic Resonance spectra using Bruker Avance 400. UltraSheild with dimethylsufoxide (DMSO)-d_6 or chloroform $CDCl_3$. The frequencies for ^1H and ^{13}C are 400.130 and 100.612 MHz respectively. Chemical shifts are given in parts per million (ppm) relative to tetramethylsilane as internal standard. Multiplicity was designated as singlet (s) and multiplet (m).

Methods : Many synthesis protocols were used in this work.

Synthesis of molecules

Preparation of benzil from benzoin (using synthesis method of Ashnagar et al. 2009) [26]

Benzoin (50 g, 0.235 mol) was placed in a 1000 mL Erlenmeyer flask and concentrated nitric acid (250 mL) was added into it in a fumecupboard. The mixture was heated on a hot plate with occasional shaking until all the red coloured nitrogen oxide gas was evolved (about 2 hours). The mixture was transferred to another 2000 mL Erlenmeyer flask which contained 1000 mL distilled water and stirred vigorously until the oil solidified as a yellow crystalline material. It was filtered over a Buchner funnel and washed with a liberal quantity of cold water until all the excess HNO_3 was removed. The solid material was recrystallized from 95% ethanol which resulted yellow needle crystalline material (44 g, 0.21 mol, 89%).

4-phenyl-3-thiosemicarbazide (valided in our laboratory)

In a 100 mL round-bottomed flask, place 3.5 g of hydrazine hydrate in 40 mL of ethanol. Prepare a solution of phenylisothiocyanate (7.5 g) in 30 mL of ethanol and transfer it to a dropping funnel. Place the "flask and ampoule" system in an ice bath and keep it stirred while adding the phenylisothiocyanate solution dropwise. Leave to stir for a further hour, filter off the precipitate obtained and recrystallize it from technical ethanol.

Benzoin 4-phenyl-3-thiosemicarbazone [27] (**Bzn-4PhT**)

An equimolar mixture (0.01 mol) of 4-phenyl-3-thiosemicarbazide dissolved in 10 mL of ethanol (EtOH 96°) was added slowly to a solution (0.01 mol) of benzoin dissolved in 30 mL of EtOH in presence of glacial acetic acid (GAA, 1.5 mL). The mixture was heated at reflux (80°C) for 4 h with stirring. After cooling, the precipitate was filtered, washed with cold distilled water until neutrality, dried and then recrystallized in technical ethanol and dried.

Benzil bis(4-phenyl-3-thiosemicarbazone) (valided in our laboratory) **(Bzl-b4PhT)**

A solution (0.01 mol) of benzil dissolved in 30 mL of EtOH was added slowly to a solution (0.01 mol) of 4-phenyl-3-thiosemicarbazide disoolved in 10 mL of EtOH and 2 mL of sodium hydroxide (NaOH 40%) during 30-45 min with stirring. The mixture was heated at reflux (80°C) for 4 hours with stirring. After cooling, the precipitate was filtered, washed with cold distilled water until neutrality, dried and then recrystallized in technical ethanol and dried.

Compounds were synthesized following the condensation reaction **(Figure 1)**

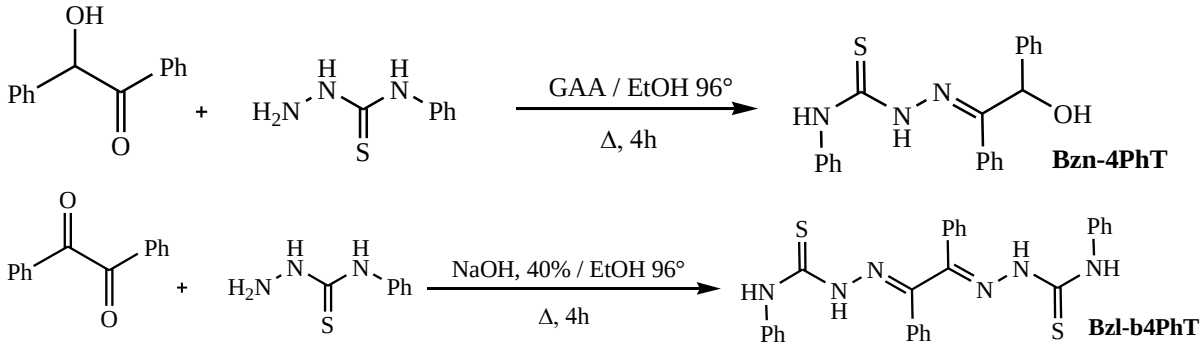


Figure 1 : Synthetic routes of 4-phenyl-3-thiosemicarbazones (scaffold).

All compounds after synthesis have been submitted to the *in vitro* anti-trypanosomal test on the bloodstream form of the strain 427 of *Trypanosoma brucei brucei* using lapachol as witness and the toxicity activity of products was screened on *Artemia salina* Leach following standard biological methods.

Pharmacology

Anti-trypanosomal test : The assessment is performed on the bloodstream form of the strain 427 of *Trypanosoma brucei brucei* by the «LILIT Alamar BlueTM» method [28]. The stock solutions of each thiosemicarbozone have been prepared from an initial concentration of 10 mg/mL in dimethylsufoxide (DMSO). The trypanosomes are grown in a medium containing 10% of heat inactivated fetal calf serum and bloodstream form supporting factor. The trypanosome suspensions were adjusted to 5×10^4 tryp/mL. In each well, 50 µL of different dilutions of the stock solution were added to 50 µL of suspension of trypanosomes. The plates were then incubated at 37°C for 72 hours in an atmosphere with 5% CO_2. 10 µL of dye "Alamar BlueTM" is added to each well and then incubated for 4 hours. The dye "Alamar BlueTM" is a reagent for detecting enzymatic activity. The wells in which the concentration of compound is insufficient to inhibit the proliferation of trypanosomes are stained. The half-inhibitory concentration is the concentration of unstained wells in which there is the lowest amount of thiosemicarbazones. The plate reading is made in comparison with control wells on a fluorescence plate reader using an excitation wavelength of 530 nm and an emission wavelength 590 nm. We carried out the test in triplicate for each compound. All data were expressed as means ± standard deviation of triplicate measurements.

Cytotoxicity screen: The cytotoxicity test was performed on larvae of brine shrimp (*Artemia salina* Leach) by the method of Sleet and Brendel (1983) [29]. *Artemia salina* eggs were incubated in seawater until hatching of young larvae (48 hours). Then, series of solutions of test compound at varying concentrations were prepared in DMSO/seawater. A defined number of larvae were introduced into each solution and incubated under rocking condition for 24 h. To evaluate the toxicity of the solution, counting of larvae viability was performed under microscope by determining the number of dead larvae in each solution. In the case where there was death in the control medium, the data was corrected by Abbott's formula:

% death = [(nd test - nd control)/ nd control)] x 100 [30] with nd = number of dead larvae.

Data (dose-response) were transformed by logarithm and the half lethal concentration LC_{50} was determined by linear regression [31]. Tests were carried out in triplicates. All data were expressed as mean ± standard deviation of triplicate measurements.

RESULTS AND DISCUSSION

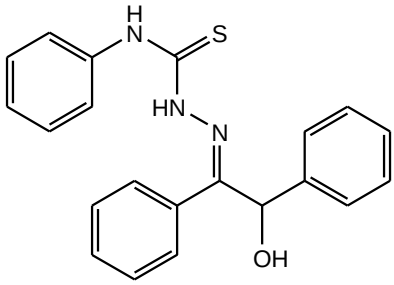
Chemistry : Before synthesizing the compounds, we carried out a theoretical study based on the pharmacokinetic properties rules [32-33] and results are summarized in the **table 1.**

Table 1: Theoretical pharmacokinetic and drug availability study

Compounds	Molecular weight ($g.mol^{-1}$)	*C logP*	Number of H-bond donors	Number of H-bond acceptors	Number of criteria met
Rules	**< 500**	**< 5**	**≤ 5**	**< 10**	**at least 3**
Bzn-4PhT	361.46	4.4346	3	5	all
Bzl-b4PhT	508.66	8.848	4	8	2

After synthesis, all molecules were characterized with spectrometrical analysis

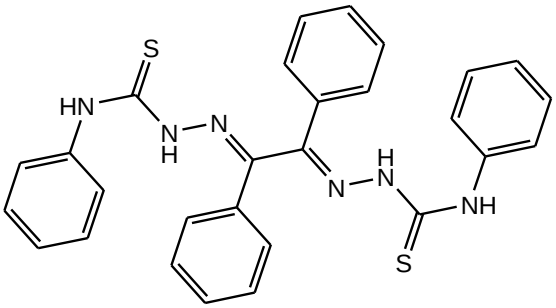
Characterization of products: We had focused this study of the thiosemicarbazones synthesized



Benzoin 4-phenyl-3-thiosemicarbazone **(Bzn-4PhT)**

^{1}H NMR ($CDCl_3$, 400 MHz, δ in ppm) : 10.21 (s, 1H, N**H**-Ph), 9.37 (s, 1H, =N-N**H**-C), 4.79 (s, 1H, OH), 5.91 (s, 1H, CH), 7.36 - 7.78 (m, 15H-aromatic).

^{13}CNMR ($CDCl_3$, 100 MHz, δ in ppm) : 177.25 (C=S), 145.17 (C=N), 149.89 (C-OH), 140.91, 138.85, 133.42, 133.80, 129.23, 129.14, 128.76, 128.65, 127.73, 127.27, 126.35, 124.87 (C-aromatic).



Benzil bis(4-phenyl-3-thiosemicarbazone) **(Bzl-b4PhT)**

^{1}H NMR ($CDCl_3$, 400 MHz, δ in ppm): 9.25 (s, 2H, N**H**-Ph), 9.67 (s, 2H, =N-N**H**-C), 7.48 - 7.98 (m, 20H-aromatic).

^{13}CNMR ($CDCl_3$, 100 MHz, δ in ppm): 175.64 (C=S), 144.67 (C=N), 137.18, 132.84, 130.76, 129.98, 129.79, 129.70, 128.89, 128.76, 128.30, 126.86, 124.19, 123.16 (C-aromatic).

Compound **Bzn-4PhT** respect (all) criteria met and then would be able to have biological activities while **Bzl-b4PhT** has just two valid criteria but very lipophilic. Molecules have physical properties compatible with reasonable pharmacokinetics and drug availability. The scaffold (Figure 1) has some advantageous properties: low molecular weight, reasonable *C*logP, good hydrogen bond donating and accepting capabilities (Table 1), easy and economical synthetic routes [32-33].

Analysis of spectrometrical data: in ^{13}C NMR spectra, peaks of the thiocarbonyle C=S resoned at 177 and 175 ppm respectively in the molecules. The imine function C=N appeared at 145 and 144 ppm in the structure of products, which confirms the obtaining of the reaction products. In the substrates, we had the carbonyl group (C=O) resonating at 198.9 ppm in benzoin and at 194.5 ppm in benzil which have disappeared from the spectrum of products. In ^{1}H NMR specta analysis, evry characteristic proton have appeared, especially the H-N in molecules' structure. These results confirm the works of dos Reis et al., (2011), Hernandz et al. (2013) [34-35]. The analysis of these spectral data confirms generaly the structure of each molecule synthesized.

Pharmacology

The results of biological activities of products were obtained and expressed in IC_{50} and LC_{50} respectively. Selectivity of actives products are also determined. The different concentrations were expressed in micromolar for their comparison with the previous studies (Table 2).

Table 2: Trypanocidal, toxicity and selectivity of synthesized compounds

Products	**IC_{50} (µM)**	Trypanocidal activity	**LC_{50} (µM)**	Toxicity on larvae	**SI = LC_{50}/IC_{50}**	Selectivity
Bzn-4PhT	**15.75** ± 2.75	moderate	**165.19** ± 1.87	toxic	10.48	selective
Bzl-b4PhT	**26.40** ± 1.63	moderate	**257.32** ± 0.96	toxic	9.75	selective

Products showed interesting activity on the parasite. They exhibited a moderate trypanocidal activity with their IC_{50} low than 30 µM. These results are consistent with the scale of antitrypanosomal activity established in the previous works [25,36,37]. According their studies, thiosemicarbazones are trypanocidal when their IC_{50} values are lower than 10 µM, and are regarded as moderate anti-trypanosomal agents if these values are between 10 and 100 µM, and have little or no activity when their IC_{50} are higher than 100 µM. Product **Bzl-b4PhT** are the most lipophilic molecule and although it did not meet all the criteria of Lipinski's rules, it showed interesting activity against the parasite. This is the importance of the lipophilicity which is a main physico-chemical determinant influencing the bioavailability, permeability and frequently the toxicity of drugs [32]. A substance is all the more lipophilic as log P is positive. The calculation of the log P (C logP) involves the additivity rules of the hydrophobic constants of Rekker [38]. The higher the logP (*C* logP) the lower IC_{50} and the more active substance [36,37]. On the toxicity screening on *Artemia salina,* all products exerced a toxic activity on the larvae studied using the cytotoxicity of the lapachol (LC_{50} = 281 µM) as reference [39,40]. Shrimp larvae were selected in this study as biological model. Indeed, there is a correlation between the toxicity of the compounds on shrimp larvae and their cytotoxicity on 9KB and 9PS cells (human

carcinoma nasopharygien) [41], and on A-549 cells of lung carcinoma and HT-29 cells of colon carcinoma [42]. With their LC_{50} and IC_{50} values, we have determined the selectivity index of each compound (table 2). All products displayed greater selectivity with their SI = 8 and 10, so superior to 1, respectively. These results are in perfect agreement with the work of Tiuman et al., (2005) [43] in which if the SI value obtained is greater than unity, the tested compound is considered to be selective on the parasite and if SI value is less than unity, the test compound is more cytotoxic than anti-parasitic. We can, from this work, say that the benzoin 4-phenyl-3-thiosemicarbazone and benzil bis(4-phenyl-3-thiosemicarbazone) studied showed an interesting activity on the parasite *T. b. brucei*.

CONCLUSION

Two derivative of thiosemicarbazones were studied in this work. A new protocol of their synthesis whit NaOH was explored in the synthesis of the benzil bis(4-phenyl-3-thiosemicarbazone). All products exhibited a moderate trypanocidal activity on *Trypanosoma b. brucei*. They were selective on the parasite and could be used in the traitment of parasitic desease.

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