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Design, synthesis, in vitro evaluation and preliminary SAR studies of *N*-(2-(heteroaryloxy)propyl)phenothiazines against *Rhipicephalus microplus* cattle tick

Mauricio Maldonado-Domínguez [a], David Alavez-Rosas [a], Humberto Aguilar [a], José Manuel Méndez-Stivalet [a], Margarita Romero-Ávila [a], Blas Flores-Pérez [a], Froylán Ibarra [b], Yazmín Alcalá-Canto [b], Yolanda Vera-Montenegro [b], Héctor García-Ortega [a,*]

[a] Facultad de Química, Departamento de Química Orgánica, Universidad Nacional Autónoma de México, 04510 México, D.F., Mexico
[b] Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, 04510 México, D.F., Mexico

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ABSTRACT

A family of 15 N-substituted phenothiazines was designed, synthesized and their acaricidal activity against *Rhipicephalus microplus* was determined in vitro. The synthetic methodology is simple and can be employed in multigram scale. The rationale for the structure-based design of these compounds is the potential for azines and phenothiazine to engage in π–π interactions; these fragments, joined together by a short, flexible alkoxide linker, structurally resemble phenothiazine-based cholinesterase inhibitors, while their weak basicity implies a neutral active form, rather than a cationic one, thus facilitating penetration of the cuticle of ticks. One compound displayed excellent acaricidal activity (LD_{50} = 0.58 μg/mL). Preliminary SAR analysis suggests that the activity is influenced by the presence of a weakly basic nitrogen atom, as well as the substitution pattern within the heterocycles.

Ticks represent a plague affecting both domestic and wild fauna. As obligate hematophages, these arthropods practice parasitism in animals and humans. Among almost 800 registered species, the tick *Rhipicephalus microplus* (formerly *Boophilus microplus*) is the most important ectoparasite in cattle.[1] It is a very important species producing significant economic losses in tropical and subtropical regions around the world,[2] as well as being a vector for the transmission of diseases such as rickettsiosis, babesiosis and *Lyme disease*.[3] In many countries, stockbreeding in pastures constitutes an important economic activity and tick infestations are a limiting factor in productivity.[4]

For the chemical control of tick infestations, typically organophosphorates,[5] arsenic derivatives,[6] amidines,[7] avermectines,[8] carbamates and pyrethroids[9] are the tools of the trade, but resistance[10–12] to these families of ixodicides has led to the search for new, structurally divergent compounds with different mechanisms of action, as well as alternative approaches such as biological control[13] and vaccines.[14]

Phenothiazines are a family of heterotricyclic compounds with two *o*-phenylene rings bridged by nitrogen and sulfur atoms. Since their introduction as pesticides in 1935, these molecules have attracted attention due to their wide spectrum of biological activity not only as an aid in pest management, but also as antipsychotic[15] and antioxidant with positive impact on symptoms of Parkinson's disease.[16] The biological targets spanned by these molecules include, but are not limited to, calmodulin,[17] MALT1 protease[18] and cholinesterases.[19] Among these enzymes, butyrylcholinesterase (BuChE) has long been thought of as an auxiliary agent,[20] but evidence suggests this protein plays an important role in maintaining adequate levels of acetylcholine (ACh) within the nervous system, thus corregulating cholinergic function along with AChE;[21] this has been exploited in the tailored design of molecules to treat symptoms of dementia due to Alzheimer's disease.[19] Since cholinesterase inhibitors usually act upon both acetyl and butyrylcholinesterase, it is difficult to determine if biological activity arises from inhibition of AChE, BuChE or a combination of both. Therefore, the design, synthesis and evaluation of selective inhibitors for each cholinesterase are important to comprehend their potential pharmacological applications.

* Corresponding author. Tel./fax: +52 55 5622 3722.
E-mail address: hector.garcia@unam.mx (H. García-Ortega).

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Properly functionalized phenothiazines have been found to selectively inhibit BuChE;[22] due to its relatively large catalytic pocket, this enzyme is able to accommodate the bulky tricyclic moiety.[23] The active site of BuChE is rich in aromatic residues; from a de-novo-design point of view, this can be exploited with ligands capable of involvement in π–π interactions.[24] Some phenothiazine derivatives are known to make use of this property, with a proposed binding mode implying a tyrosine and a phenylalanine residue contained in the E-helix in human BuChE.[25] N-functionalization with flexible substituents has proved to be an effective method of conferring BuChE inhibitory activity on phenothiazine-containing compounds. That strategy was employed throughout this work since it provides a facile and rational benchmark for the design, synthesis and biological screening in the search for lead molecules.

Most biologically relevant phenothiazine derivatives display N-functionalization, typically an alkyl group with a basic nitrogen atom (Fig. 1), although acyl[26] or alkoxycarbonyl[25] chains have also proven to imprint cholinesterase inhibitory activity; this suggests that a complementary substituent is needed for phenothiazines to effectively inhibit BuChE. Considering this, we hypothesized that pyridine and pyrazine rings would serve such purpose. Firstly, these aromatic systems are capable of involvement in π–π interactions with the electron-rich phenylalanine, tyrosine and tryptophan residues within the active-site cavity.[27] Secondly, azines are weakly basic and are not protonated at neutral pH. This is desirable because acaricides are usually incorporated into the parasite through direct absorption and neutral, lipophilic molecules are expected to be more easily absorbed through the cuticle of ticks.[28] Also, these substituents can be installed via S_NAr reaction of a suitable, commercially available haloazines with an appropriate nucleophile. Pyridines and pyrazines find application in market areas where bioactivity is important, as in medicinal drugs[29] and in agricultural products such as herbicides, insecticides, fungicides, and plant growth regulators.[30–32]

In this Letter, we describe the design of a family of *N*-(2-(heteroaryloxy)propyl)phenothiazines (Fig. 2), their structural characterization, in vitro acaricidal evaluation, and the identification of a lead compound for further acaricidal optimization and biological evaluation.

Propylene oxide was selected as a potential flexible unit to link the phenothiazine moiety with nitrogen containing aromatic systems (Fig. 2). It is readily available, is expected to be easily attached to the tricylic system by nucleophilic cleavage, and the oxyanion produced should, without isolation, react with a suitable aromatic haloazine species. This one pot process was successfully applied on a multigram scale as described below in Scheme 1.

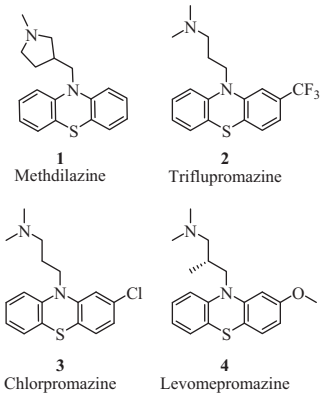


Figure 1. Some biologically relevant N-substituted phenothiazines. Cholinesterase inhibition is a common feature in these compounds.

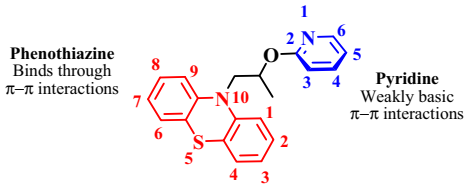
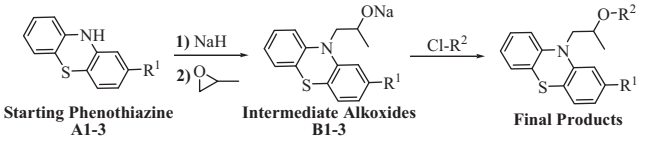


Figure 2. General structure of the synthesized compounds and numeration for each heterocycle. The alkoxy bridge plays also an important role by acting as a flexible chain.

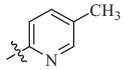
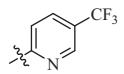
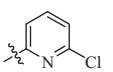
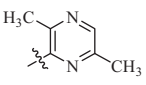


Scheme 1. General synthetic procedure employed throughout this work which can be performed without isolation of the intermediate alkoxides **B1**–**B3**.

Deprotonation of phenothiazine with NaH followed by the addition of propylene oxide readily affords the expected sodium alkoxide which, without isolation, is reacted with a haloazine system. In this manner, a total of 15 molecules of the structural type desired were prepared for in vitro testing against *R. microplus*. All compounds display predicted molecular volumes larger than 300 Å^3, excluding AChE inhibition as a possible mechanism of action (Table 1,all compounds were fully characterized by standard spectroscopic techniques).[23]

To determine the in vitro activity of the compounds under study (previously purified), the modified larval packet test was used.[35] N-functionalized phenothiazine was dissolved in a mixture of trichloroethylene and olive oil (2:1), the resulting solution was absorbed onto filter papers folded into packets using bulldog clips. After solvent evaporation, 100 *R. microplus* larvae were placed into each treated filter paper packet, which was then sealed with additional bulldog clips and placed in an incubator at 27 °C and 80% relative humidity for 24 h. After this time had elapsed, mortality was determined. Three replicates and a control (filter paper with trichloroethylene and olive oil, kept in a different incubator) were performed for each molecule. Only larvae that had the ability to walk were considered alive. The results are

Table 1
Structure, yield and computed Log*P* for the family of compounds synthesized

Compound	R^1	R^2	Yield (%)	*c*Log*P*[a]	*V*[b] (Å^3)
C1	–H		90	5.44	344
C2	–Cl		89	6.05	368
C3	$-SCH_3$		65	5.94	389
D1	–H		21	5.76	373
D2	–Cl		75	6.37	389
D3	$-SCH_3$		50	6.26	409
E1	–H		92	6.2	390
E2	–Cl		92	6.82	406
E3			70	6.7	427
F1	–H		95	6.14	369
F2	–Cl		92	6.75	384
F3	$-SCH_3$		55	6.64	403
G1	–H		57	5.2	388
G2	–Cl		80	5.82	403
G3	$-SCH_3$		60	5.7	427

[a] Values were predicted using the OSIRIS Property Explorer.[33]
[b] Volumes computed at the AM1 level using Spartan '08.[34]