SYNTHESIS AND ANTHELMINTIC EVALUATION OF [2,5']-BIS-HETEROCYCLES AS BENGAZOLE ANALOGS

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ABSTRACT

As part of our search for compounds as candidates for anticancer or antiparasitic drugs employing molecular simplification, we reported the preparation of uncommon [2,5'] bis-heterocycles employing efficient synthetic strategies. The synthesized compounds presents little modifications of the Bengazole heterocyclic fragment in order to preserve the biological properties to been employed in Bengazole derivatives analogs preparation. A limitation in the oxidation of 2-benzyl-oxazolines is described. We also presents the anthelmintic activities of these synthetized compounds.

Keywords: Bengazole, [2,5']bithiazole, [2,5']bis-heterocycle, Hantzsch, anthelmintic activity.

RESUMEN

Como parte de la búsqueda de compuestos candidatos a fármacos de uso contra el cáncer o parásitos empleando como estrategia la simplificación molecular, presentamos la preparación de los sistemas poco comunes [2,5'] bis-heterocíclicos, empleando estrategias sintéticas eficientes. Los compuestos sintetizados presentan en sus estructuras modificaciones simples del fragmento bis-heterocíclico de los Bengazoles con el fin de preservar las propiedades biológicas para ser empleados en la síntesis de análogos a derivados de Bengazoles. Se describe la limitante de oxidar oxazolinas que presenten sustituyentes de tipo bencílico en posición 2 del heterociclo. También presentamos los resultados obtenidos del ensayo de actividad antihelmíntica sobre los compuestos sintetizados.

Palabras claves: Bengazole, [2,5']bitiazol, [2,5']bis-heterociclo, Hantzsch, actividad antihelmintica.

INTRODUCTION

Natural products play an important role in drug development particularly in anticancer, antibiotics and antiparasitic drugs (Newman and Cragg, 2012). Its structural diversity is a source of inspiration for drug discovery and the preparation of analogs as simplified, synthetically more accessible and stable models are broadly described in the literature (Molinski *et al.*, 2009; Njardarson *et al.*, 2004).

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Bis-1,3-azole scaffolds linked by different chain length and connectivity points between the rings, are present in numerous natural products with interesting biological activities (Davyt and Serra, 2010; Jin, 2006; Yeh, 2004). Representative examples include Cystothiazole A, with a [2,4'] bithiazole system (Ojika *et al.*, 1998); Bengazoles containing an uncommon [2,5'] bioxazole (Adamczeski *et al.*, 1988; Rodriguez *et al.*, 1993; Rudi *et al.*, 1994); Leucamide A a cyclic heptapeptide with a [2,4'] oxazole-thiazole system (Kehraus *et al.*, 2002); Largazole a depsipeptide containing a [2,4'] thiazoline-thiazole system (Taori *et al.*, 2008); and cyclic peptides containing 1,3-azoles as Venturamide A (Linington *et al.*, 2007).

The most common moiety is the [2,4'] bis-1,3-azole, consistent with the biogenesis of these heterocycles which are derived from Ser, Thr or Cys peptide by cyclodehydration and oxidation process (Riego *et al.*, 2005). There are many synthetic methods reported for the preparation of (2,4-disubstituted) oxazoles (Taylor and Wipf, 2003). On the other hand, there are few methods to prepare (2,5-disubstituted) oxazoles. The most efficient methodology was developed by Schöllkopf (Schöllkopf and Gerhart, 1968; Schöllkopf and Schöder, 1971), starting from isocyanides, and then the use of TosMIC (tosylmethylisocyanide) reagent was pioneered by van Leusen (van Lausen *et al.*, 1972).

Bengazoles are a representative family of natural products containing the uncommon [2,5'] bioxazole, so several academic groups have been involved in the synthesis of some members. Molinski (Mulder *et al.*, 1999) and Shioiri's group (Chittari *et al.*, 2003) reported the synthesis of bis-1,3-oxazole of Bengazole A and Deacylbengazole respectively with an optimized modification of Vedejs methods (Vedejs and Monahan, 1996) from 5-oxazolecarboxaldehyde using Schöllkopf methodology. Ley and coworkers (Bull *et al.*, 2007) had explored differents routes to completed the total synthesis of Bengazole A and B and the best result was obtained employing TosMIC and ethyl glyoxylate.

As part of our search for compounds as candidates for anticancer or antiparasitic drugs employing molecular simplification (Scarone *et al.*, 2004; Sellanes *et al.*, 2006; Mahler *et al.*, 2006; Sellanes *et al.*, 2007; Incerti *et al.*, 2008; Peña *et al.*, 2011), we reported our results on the synthesis of [2,4'] and [2,5'] bis-heterocycles with an ethylene bridge between the rings as scaffolds for Bengazole analogs using Robison-Gabriel, van Lausen and cyclodehydration-oxidation reactions (Scarone *et al.*, 2009). (Figure 1)

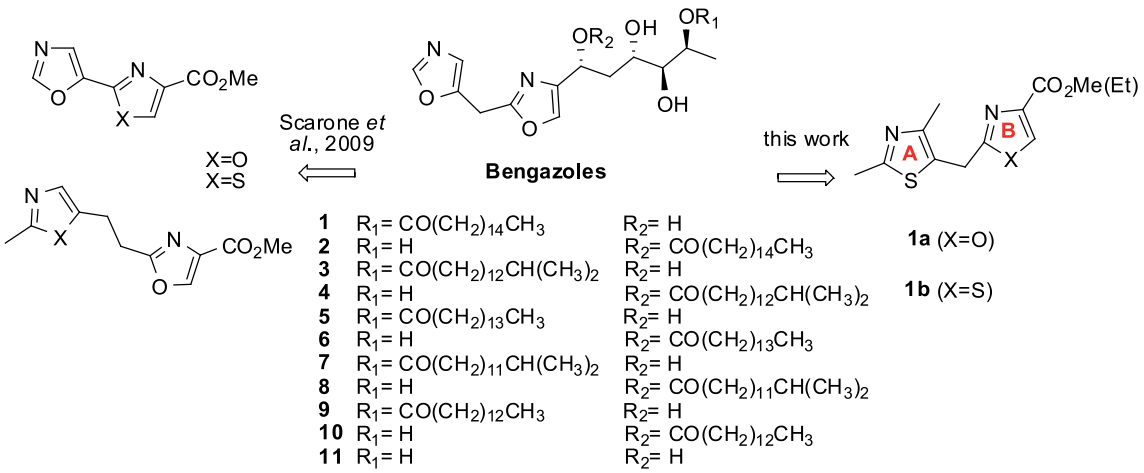


Figure 1: Bengazoles and proposed bis-heterocycles

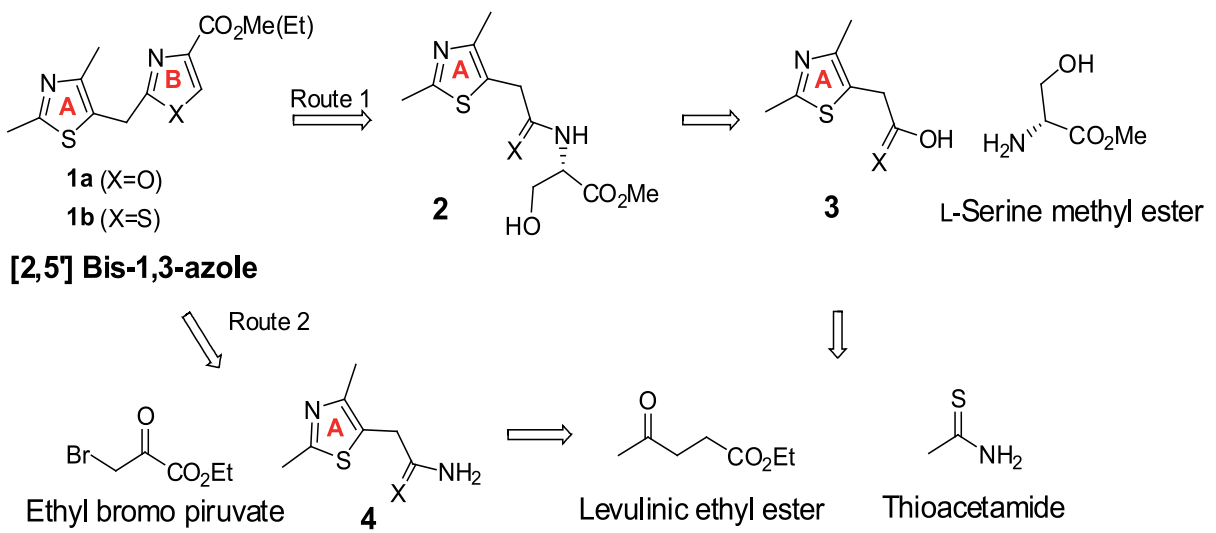
Bengazoles **1**- **10** were found to be very active against *Candida albicans*, with MIC values from 0.8 to 1.5 μg/mL, and the hydrophilic derivative bengazole **11** (no esterification with a fatty acid) was inactive in these assays (Fernández *et al.*, 1999). Bengazoles have been evaluated for their cytotoxicity in the NCI's 60 cell lines screen and Bengazole A has shown in vitro potency against two human tumor cell lines. In contrast, Bengazole **Z** (11, figure 1) was inactive. Furthermore, Bengazole A shows complete anthelmintic activity at a 50 μg/mL against *Nippostrongylus brasiliensis* (Adamczeski *et al.*, 1988; Rodriguez *et al.*, 1993).

In the present work, we report our investigations to the synthesis of [2,5']-bis-heterocycles of type **1** with a methylene between the rings. (Scheme 1) The bioisosterism between thiazole and oxazole rings and the use of a straightforward synthetic route employing comercially available reagents as starting materials were considered. Due to our interest in antiparasitic drugs, we present an *in vitro* preliminary sceening of the effect on the L_4 larvae of *Nippostrongylus brasiliensis* of these bis-heterocycles and intermediates.

MATERIAL AND METHODS

IR spectra were recorded on a Shimadzu FTIR 8101A spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance DPX- 400. Chemical shifts are related to TMS as an internal standard. Mass spectra (EIMS) were obtained on a GCMS Shimadzu QP-2010 plus. Flash column chromatography was carried out with Silica gel 60 (J.T. Baker, 40 μm average particle diameter). All reactions and chromatographic separations were monitored by TLC, conducted on 0.25 mm Silica gel plastic sheets (Macherey/Nagel, Polygram_ SIL G/UV 254). TLC plates were analyzed under 254 nm UV light, iodine vapor, p-hydroxybenzaldehyde spray or ninhydrine spray. Yields are reported for chromatographically and spectroscopically (^{1}H and ^{13}C NMR) pure compounds.

Ethyl 2-(2,4-dimethylthiazol-5-yl) acetate (8): Ethyl levulinate (7.0 mmoles) in diethyl ether (2 ml) was cooled to 0°C and bromine (0.18 ml, 3.5 mmoles) was added dropwise with stirring. The reaction mixture was stirred at room temperature overnight, washed with water (4x 5ml) and dried



Scheme 1: Retrosynthetic analysis

with Na_2SO_4. Evaporation *in vacuo* of the diethyl ether gave α-bromoketones (**5** and **6**) in 1:1 mixture by ^{1}HNMR spectroscopy. The α-bromoketones were dissolved in dry EtOH (4 ml) and thioacetamide (7.0 mmoles) was added. The mixture was refluxed for 2 h, after cooling to room temperature the EtOH was evaporated under reduced pressure. The residue was dissolved in EtOAc (10 ml) and extracted with water (10 ml). The organic layer was dried with Na_2SO_4, filtered and concentrated *in vacuo*. Flash chromatography (AcOEt: *n*-hexane, 1:4) afforded **8** in 50 % yield. R_f= 0.42. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 1.27 (t, *J* = 7.1 Hz, 3H), 2.32 (s, 3H), 2.63 (s, 3H), 3.69 (s, 2H), 4.18 (q, *J* = 7.1 Hz, 2H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 14.2, 14.9, 19.1, 32.1, 61.4, 121.8, 149.3, 163.7, 170.2.

Methyl 2-(2,4-dimethylthiazol-5-yl) acetamido)-3-hydroxypropanoate (10): An aqueous KOH (10%) (3 ml) solution was added to the ester **8** (3 mmoles) solution in THF (3 ml). The reaction mixture was stirred at room temperature during 2 h and THF was evaporated under reduced pressure. HCl 1M was added until pH 4 and the solution was extracted with EtOAc. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to afford the acid **9**. To a stirred solution of L-serine methyl ester hydrochloride (1.4 mmol) in dry CH_2Cl_2 (10 mL) cooled at 0 °C under N_2, Et_3N (1.4 mmol) was added. The reaction mixture was stirred during 30 minutes. Then acid **9** (1.4 mmol), DCC (1.54 mmol) and HOBt (1.54 mmol) were added and the reaction mixture was stirred at room temperature during 24 hours. The precipitated DCU was filtered, water (10 mL) was added and the mixture was extracted with AcOEt (4x15 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under vacuo. Flash chromatography (AcOEt: MeOH, 4:0.5) afforded β-hydroxyamide **10** in 54% yield. R_f= 0.40. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 2.36 (s, 3H), 2.66 (s, 3H), 3.72 (s, 2H), 3.80 (s, 3H), 3.97 (ddd, *J* = 3.4, 3.7, 11.1 Hz, 2H), 4.67-4.70 (m, 1H), 6.51 (d, *J* = 6.1 Hz, 1H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 14.9, 19.1, 33.8, 52.9, 54.8, 63.1, 122.0, 150.0, 164.3, 169.6, 170.6. **EIMS** (70 eV), *m/z* (%) 272 (M^+, 14), 153 (24), 127 (100), 86 (31), 61 (61). **IR** ν_{max}/cm^{-1} (liquid film): 1074, 1207, 1369, 1544, 1647, 1743, 2945, 2953, 3288.

Methyl 2-((2,4-dimethylthiazol-5-yl) methyl)oxazoline-4-carboxylate (11): A solution of β-hydroxyamide **10** (0.37 mmol) in dry CH_2Cl_2 (4 mL) at -78 °C under N_2, DAST (0.05 mL, 0.40 mmol) was added dropwise. After stirring for an hour, the reaction mixture was quenched with K_2CO_3 (0.17g, 1.23 mmol) at – 20 °C. After warming to room temperature, the mixture was further diluted with saturated aqueous solution of Na_2CO_3 and then extracted with CH_2Cl_2 (4x 20 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated in vacuo. Flash chromatography (EtOAc: MeOH, 4:0.5) afforded thiazole-oxazoline **11** in 96% yield. R_f= 0.44. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 2.36 (s, 3H), 2.64 (s, 3H), 3.75 (d, *J*= 6.2 Hz, 2H), 3.81 (s, 3H), 4.45 (dd, *J*= 8.8, 10.6 Hz, 1H), 4.54 (dd, *J*= 7.8, 8.8 Hz, 1H), 4.77 (dd, *J*= 7.8, 10.6 Hz, 1H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 14.9, 19.1, 25.6, 52.7, 68.0, 70.0, 122.3, 149.6, 163.7, 167.7, 171.4.

Methyl 2-((2,4-dimethylthiazol-5-yl) methyl)oxazole-4-carboxylate (1a): Oxazoline **11** (0.37 mmol) was dissolved in dry CH_2Cl_2 (2 mL). The reaction mixture was cooled at -20 °C and $BrCCl_3$ (1.4 mmol, 3.5 eq.) was slowly added. Then it was allowed to reach 0 °C and DBU (1.4 mmol, 3.5 eq.) was slowly dripped. The reaction mixture stirred at room temperature overnigth. Then was quenched with a saturated solution of $NaHCO_3$ and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. Flash chromatography (AcOEt) afforded thiazole-oxazole **1** in 32% yield. R_f= 0.56 (AcOEt). **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm) 2.39 (s, 3H), 2.63 (s, 3H),

3.93 (s, 3H), 4.24 (s, 2H), 8.18 (s, 1H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 14.9, 19.1, 25.5, 52.3, 122.4, 133.3, 144.3, 149.6, 161.5, 162.4, 163.9. **EIMS** (70 eV), *m/z* (%) 252 (M^+, 100), 220 (27), 192 (48), 126 (74), 112 (34), 85 (39). **IR** ν_{max}/cm^{-1} (liquid film): 1109, 1139, 1199, 1321, 1437, 1585, 1740, 2853, 2928.

2-(2,4-dimethylthiazol-5-yl)acetamide (12): To a solution of acid **9** (4.6 mmol) in dry THF (15 mL) was added DIPEA (5.6 mmol) under N_2 at 0 °C. The reaction mixture was stirred at 0 °C for 30 minutes before 2,2,2-trichloroethyl chloroformate (5.6 mmol) was added rapidly and continued stirring for 30 minutes. Then aqueous NH_3 solution (1.7 mL) in THF (1.7 mL) was added. The resulting reaction mixture was allowed to reach room temperature and stirred for additionally 16 h. The reaction mixture was concentrated *in vacuo* and the resulting residue was partitioned between EtOAc (40 mL) and H_2O (40 mL). The aqueous layer was extracted with EtOAc (2 x 30 mL) and the combined organic layers were washed with saturated aqueous $NaHCO_3$ solution (30 mL), water (30 mL), brine (30 mL); dried with Na_2SO_4 and concentrated *in vacuo*. Flash chromatography (EtOAc: *n*-hexane, 3:1) afforded amide **12** in 53%. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 2.36 (s, 3H), 2.67 (s, 3H), 3.67 (s, 2H), 5.55 (bs, 2H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 14.9, 19.1, 33.4, 122.6, 150.0, 164.3, 171.4.

Methyl 2-((2,4-dimethylthiazol-5-yl) methyl)thiazole-4-carboxylate (1b): Lawesson's reagent (2.24 mmol) was added to a solution of amide **12** (1.4 mmol) in dry THF (15 mL) and the reaction mixture was stirred under N_2 atmosphere at room temperature for 24 h. The reaction was quenched by addition of saturated aqueous $NaHCO_3$ and was stirred for 1 h before been extracted with EtOAc (3 x 15 mL). The organic layers were washed with brine (20 mL); dried with Na_2SO_4 and the solvent evaporated under reduced pressure. Flash chromatography (EtOAc) afforded thioamide **13** in 89% yield. A solution of ethyl bromopiruvate (2.1 mmol), thioacetamide **13** (1.1 mmol) and pyridine (3.2 mmoles) in dry EtOH (4 mL) under N_2, was refluxed during 6 hours. Then, the reaction mixture was concentrated under vacuo and HCl aq. (sol. 5%) was added until pH 4. The aqueous layer was extracted with Et_2O (4 x 20 mL), and the combined organic layers were dried over Na_2SO_4, filtered and concentrated under vacuo. Flash chromatography (AcOEt: *n*-hexane, 3:1) afforded bithiazole **1** in 41% yield. R_f= 0.52. **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.43 (t, J= 7.1 Hz, 3H), 2.39 (s, 3H), 2.67 (s, 3H), 4.45 (q, J= 7.1 Hz, 2H), 4.47 (s, 2H), 8.10 (s, 1H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 14.4, 15.0, 19.2, 30.4, 61.6, 125.4, 127.9, 147.1, 149.8, 161.6, 164.4, 170.6. **EIMS** (70 eV), *m/z* (%) 282 (M^+, 81), 236 (17), 208 (100), 167 (6), 126 (25), 85 (21). **IR** ν_{max}/cm^{-1} (liquid film): 1093, 1205, 1240, 1319, 1477, 1719, 2924, 2980.

Anthemintic Assay: Animal protocol was in conformity with Uruguayan Law No. 18611 (http:/www.presidencia.gub.uy/web/leyes/2009/EC1395.pdf) and harmonized with The Canadian Guidelines on Animal Care. The experimental protocol of the study was reviewed and approved by IACUC of Facultad de Química - Universidad de la República, Uruguay, under approval number 06-05-09 (http:/www.chea.udelar.edu.uy).

Parasite specimens of *N. brasiliensis* L4 were obtained from rat small intestines as per the procedure described previously for the *in vitro* model of anthelmintic activity developed by our team (Gordon *et al.*, 1997). Briefly, Wistar rats were infected subcutaneously with 5000 L3 larvae of *N. brasiliensis* and euthanized by cervical dislocation at 72 h post-infection. L4 parasites were recovered from intestines, washed, and kept in a 24-well tissue culture plate (disposable plates of 24 wells x 2 mL, pfs SIGMA) containing 1.8 mL of culture medium and 50 L4s suspended in 0.2 mL of medium per well. Samples in each well

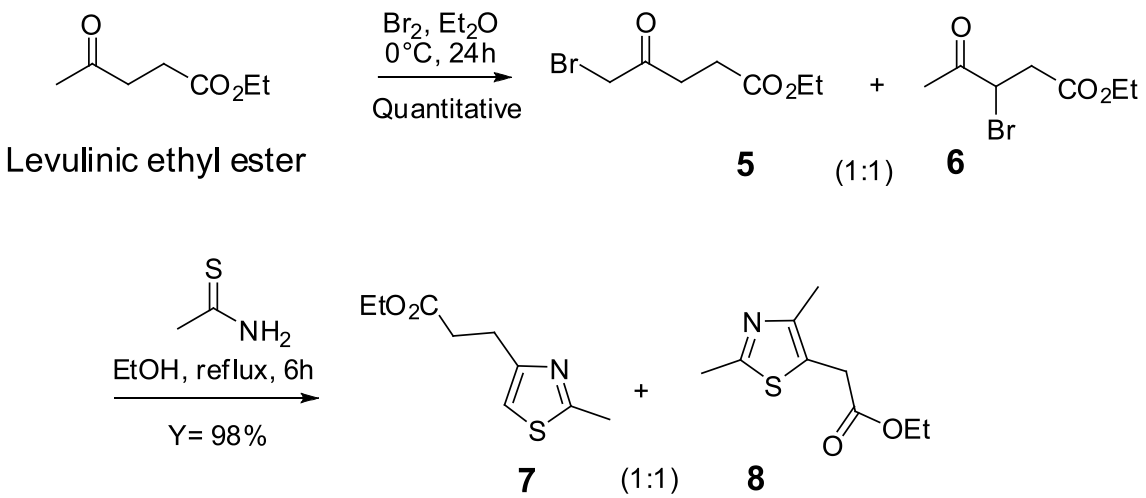
were dissolved and diluted to the desired final concentration by the addition of 10 µL of DMSO. Controls with and without the addition of DMSO were also included. Plates were incubated at 37°C and the number of dead parasites read under an inverted microscope (Nikon TS 100) on day five. Readings were corrected against DMSO controls (corrections amounting to less than 15%). Results were analyzed by ANOVA and Tukey´s post-run test ($p < 0.05$). The mean effective concentration (EC_{50}) —corresponding to 50% of dead parasites— was calculated for each product by the probit method at a confidence level of 95% by means of Prism GraphPad 5.00 software (2008, San Diego, USA), and later confirmed experimentally.

RESULTS AND DISCUSSION

Synthesis of ring A: Thiazole ring is a very important scaffold in medicinal chemistry, so several methods were developed for the synthesis of this ring by Hantzsch, Cook-Heilbron, Gabriel among others (Zagade and Senthilkumar, 2011). However, the most widely used and relied method for the preparation of 2,4-disubtituted thiazoles is Hantzsch's synthesis.

Thiazoles have been used previously in our group as building blocks to the synthesis of natural products analogs (Peña *et al.*, 2011; Peña *et al.*, 2012). Now, we decided to explore the Hantzsch's reaction for the synthesis of 2,5-disubstitued thiazoles. Overend protocol (Overend *et al.*, 1950) was employed to prepare both α-bromoketones (**5** and **6**) which were obtained as a 1:1 mixture. These compounds were used, without further purification, in Hantzsch's reactions with thioacetamide. Thiazoles **7** and **8** were obtained in excellent yield (1:1 relationship). (Scheme 2) Then, thiazoles were purified by flash chromatography and we continue our synthetic route employing thiazole **8**.

Synthesis of ring B: The ethyl ester hy-



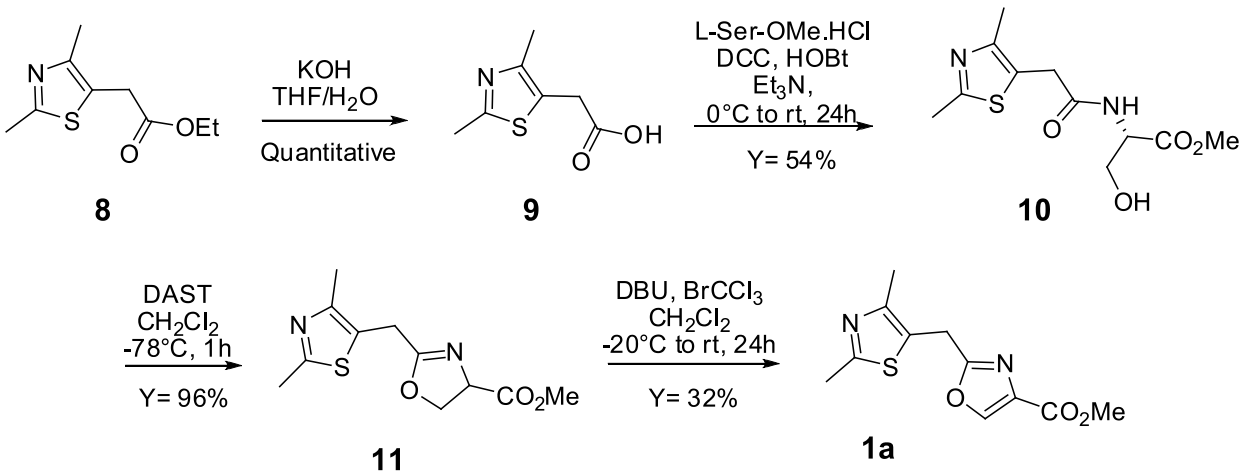
Scheme 2: Synthesis of ring A

drolisis of thiazole **8** afforded the carboxilic acid **9** as reagent to amide bond formation with the L-serine methyl ester hydrochloride employing *N,N*-dicyclohexylcarbodiimide (DCC) and *N*-hydroxybenzotriazole (HOBt) as coupling reagents. β-hydroxyamide **10** was obtained in 54% yield. (Scheme **3**) It is important to highlight that if the triethylamine (Et_3N): L-serine methyl ester hydrochloride relationship (1:1) is increased the reaction results in a mixture of inseparable products.

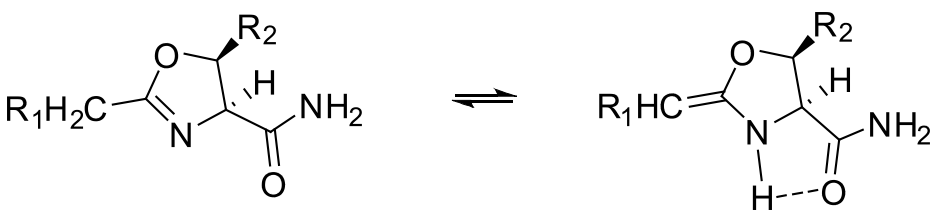
Next step involved the cyclodehydation of **10** using DAST to afford oxazoline **11** in excellent yield. Then, we oxidazed oxazoline **11** using William's protocol (Williams *et al.*, 1997) to obtain oxazole **1a** in low 32% yield.

A bibliographic revision was carried out in order to explain the low yied for oxazoline oxidation. Ley's group reported 47% as the best yield for oxazoline oxidation in the total synthesis of Bengazoles A and B. Xi's group (Xi *et al.*, 2005) reported yield between 31-46% for the oxidation reactions of 2-benzyl-oxazolines. On the other hand, Cossu and co-workers (Cossu *et al.*, 1994) reported an unusual reactivity of 4-carboxyamido-2-benzyl-oxazolines in the aim to obtain the 4-nitrile derivatives and assumed the presence of an equilibrum between two species with the presence of a hydrogen bond, probably due to the presence of exocyclic protons in position 2 of the heterocycle. (Scheme 4)

In the case of oxazoline **11** (I, scheme



Scheme 3: Synthesis of ring **B** to obtain **1a**

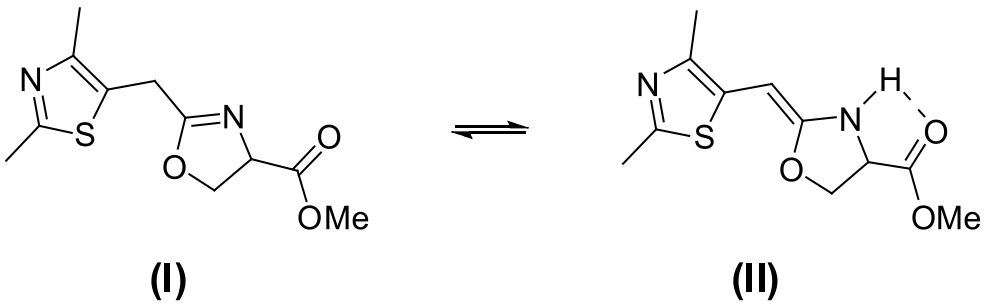


Scheme 4: Equilibrium species of 4-carboxyamido-2-benzyl-oxazolines.

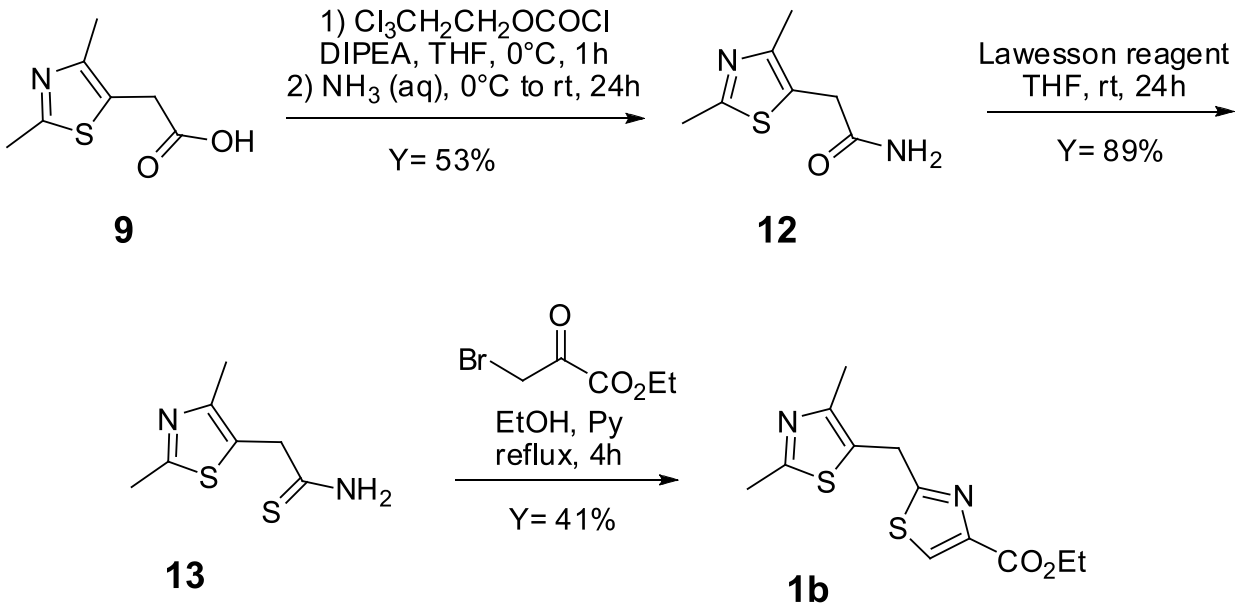
5), we propose an equilibrium with the oxazolidine II, stabilized by an hydrogen bond and consequently the desired oxazole synthesis proceed in poor yield.

For the synthesis of the bisthiazole of type **1**, we decided to employ the widely used Hantzsch's methodology. Amide **12** was prepared from acid **9** (Scheme 6), employing 2,2,2-trichloroethyl chloroformiate/aqueous ammonia in moderate yield. Further thionation of amide **12** with Lawesson's reagent, allowed us to obtain the thioamide **13** in good yield. Then, Hantzsch's reaction using ethyl bromo piruvate afforded bis-thiazole **1b**.

The anthelmintic effect on the parasitic stage (L_4) of *Nippostrongylus brasiliensis* was evaluated using Gordon protocol (Gordon *et al.*, 1997; Jenkins *et al.*, 1980). The results are summarized in Table 1 which includes the activities of some previously prepared [2, 5']bis-heterocycles.

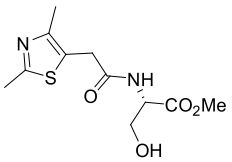
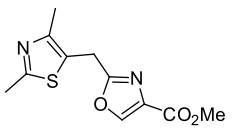
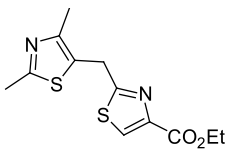


Scheme 5: Species in equilibria



Scheme 6: Synthesis of ring **B** to obtain **1b**

Table 1: Anthelmintic activities of synthetic derivatives

Entry	Compound	MW	LogP	LC_{50} (µM)
1	**Albendazole**	265	2.55	0.34 ± 0.02
2	**Bengazole A**	524	2.76	90*
3	 **10**	272	0.46	3.27 ± 0,06
4	 **1a**	252	2.42	69.2 ± 0.6
5	 **1b**	268	3.79	56.6 ± 0,3

*EC_{100} reported by Jenkins

Even thougth the overall level of activity was moderate, some remarks can be made. The open intermediate **10** (entry 3) show a 10-fold increase in activity compared with bis-heterocycles type **1**. The presence of a thiazole instead an oxazole slight increases the anthelmintic activity if we compared data of entries 4 and 5.

CONCLUSIONS

We have applied a straightforward synthetic method to obtain [2,5'] bi-1,3-azoles linked by a methylene bridge. The synthesis of [2,5'] bis-heterocycle **1a** was performed in just 5 steps with 16% overall yield and [2,5'] bis-heterocycle **1b** was obtained in 5 step with 21% overall yield.

We proposed an explanation to the low yield in the oxidation of 2-benzyl-oxazolines and thus could be a limitation of this reaction.

Our preliminary evaluation of the anthelmintic activity demonstrated a broad distribution of anthelmintic effects. Insights gained from these studies will serve for further preparations of new analogs of these natural products. These compounds will be usefull for a fragment-based lead discovery (Rees *et al.*, 2004) in order to improve the biological effects in a next-generation series.

ACKNOWLEDGEMENT

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