SYNTHESIS AND IN VITRO ANTHelmINTIC ACTIVITY AGAINST NIPPOSTRONGYLUS BRASILIENSIS OF NEW 2-AMINO-4-HYDROXY-5-VALEROLACTAM DERIVATIVES

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Summary - The synthesis of a series of 2-amino-4-hydroxy-5-valerolactam derivatives is described (compounds 4 to 10). These compounds showed a high anthelmintic in vitro activity against the Nippostrongylus brasiliensis model.

INTRODUCTION

Chemotherapy represents an important tool in the treatment of parasitic infections due to helminthes in both human and veterinary practice. However, the common use of anthelmintics in farm animals has resulted in the appearance of drug resistant strains of worms (mainly nematodes), while the currently used anthelmintic agents have proved to be poorly effective in the treatment of some systemic parasitic diseases in man. This loss of effectiveness and raising sanitary and economic questions have therefore brought to search for new broad-spectrum anthelmintic drugs.

A series of 2-amino-4-hydroxy-5-valerolactam and derivatives have been synthesized. They are structurally related to a group of secondary metabolites of marine origin, which contain a myristic ester of heterocyclic amino acids as basic structure, and exhibit an interesting in vivo and in vitro anthelmintic activity.

For the compounds obtained [4 to 10] the anthelmintic in vitro activity has been studied against Nippostrongylus brasiliensis model. With the purpose of approaching to the pharmacopote properties responsible for the activity, a series of derivatives (compounds 6-10), with different substituents on the NH2 group of C-2 and on the -OH group of C-4 were obtained.

CHEMISTRY

Compounds 4-10 were prepared from racemic 2-amino-4-hydroxy-3-oxo-5-valerolactam 3-carboxylic acid [10] according to the sequence shown in Scheme 1.

The bromolactonization of 1 with NBS/THF/H2O afforded a mixture of g-butyrolactones 2a-b in a 5:1 ratio. This ratio (determined with 400 MHz H NMR in CDCl3) was evaluated from NH proton (5.65 ppm cis-isomer, 7.53 ppm trans-isomer), and 9H (4.89 ppm trans-isomer, 4.60 ppm cis-isomer) integrations.

The major isomer cis-2a was easily purified by crystallization (EtOH; n-hexane), and subsequently converted to cis-amino-lactone 3 by direct nucleophilic displacement of the bromine with azide ion. When the trans-3-butyrolactone 2b (minor isomer) was treated with NaN3 under the same conditions, the trans-amino derivative was obtained. Spectroscopic data of this compound are identical to previously reported values. This is an additional proof of the relative stereochemistry assignment to compound 2a.

When 3 was submitted to catalytic hydrogenation, reduction of azide function with spontaneous lactone-lactam interconversion occurred; examples of reduction of azide function followed by a spontaneous esteramide interconversion, can be found in the literature. The trans-3-5-valerolactam 4a was obtained as the major reaction product. Under these reaction conditions the cis-isomer 4b was formed as minor product. The mixture was clearly separated by silica gel flash chromatography, to provide 4a and 4b in 75 and 12% isolated yields respectively.

Introduction of the myristoyl moiety onto the hydroxyl group of 4a, to afford 5, was performed using myristic acid and a mixture of DCC/HOBt/DMAP as condensing agents.

Relative stereo configuration for 4a was deduced by NMR experiments and confirmed by X-ray crystallographic analysis of its myristoyl ester 5 (Figure 1).

The acetylation of compound 4a employing usual conditions (Ac2O/Pyr), afforded quantitative yield of the acetyl derivative 6.
Scheme 1

Compound 7 was obtained from 5 by treatment with TFA. Compounds 8 and 9 were obtained from 7 by acetylation and alkaline hydrolysis respectively.

Treatment of 5 with MeI in the presence of Ag₂O afforded the N-methyl derivative 10.

Chemical Experimental

General Methods

Melting points were determined on a Gallenkamp capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin Elmer 1600 spectrophotometer. NMR spectra were recorded on various machines (Varian FT 300, 500, 600, 800, or a Varian XL 90). Chemical shifts are related to TMS as internal standard. Multiplicities of 1H NMR for compound 10 were assigned using a standard DEPT experiment, while those for compound 4a were arbitrary. Proton and carbon-13 NMR assignments for compound 5 are based on literature data for the corresponding compounds.

Fig. 1 - ORTEP drawing of the X-ray structure of compound 5a. Displacement ellipsoids are drawn at 50% probability level. The absolute configuration is not implied.
CHEMICAL SYNTHESIS

cis-4,10-dimethyl-7,13-dioxo-16,16,18,18-tetramethyl-
undecahydropyridine-2-carboxaldehyde, 3

A solution of 2a (2.0 g, 6.79 mmol) in dry DMF (30 mL), NaN₃ (3.50 g, 26.33 mmol) was added at room temperature, and the mixture was stirred at 60 °C under nitrogen for 4 h. The solution was poured into ice-water and extracted with EtOAc. The organic layer was washed with water, dried over Na₂SO₄, and evaporated in vacuo. The residue was chromatographed on silica gel using CH₂Cl₂:MeOH (10:1) as eluent.

trans-2-2-tetrahydroanilino[2,3-d][1,3]dioxol-4,10-dione, 3a

A solution of 1a (1.2 g, 6.79 mmol) in MeOH (20 mL), containing 0.19 g of 18°P Pd/C was hydrogenated (1.1 atm) at room temperature over 4 h. The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated under reduced pressure. The residue was separated by flash chromatography (silica gel, CH₂Cl₂:MeOH 95:5) to afford a product.

trans-2-tert-tert-butylcarbonylcinnamoyl[2,3-d][1,3]dioxol-4,10-dione, 4a and 4b

A solution of 3a (1.2 g, 6.79 mmol) in MeOH (20 mL) containing 0.19 g of 18°P Pd/C was hydrogenated (1.1 atm) at room temperature over 4 h. The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated under reduced pressure.

trans-2-tert-tert-butylcarbonylcinnamoyl[2,3-d][1,3]dioxol-4,10-dione, 4a and 4b

A solution of 3a (1.2 g, 6.79 mmol) in MeOH (20 mL) containing 0.19 g of 18°P Pd/C was hydrogenated (1.1 atm) at room temperature over 4 h. The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated under reduced pressure. The residue was separated by flash chromatography (silica gel, CH₂Cl₂:MeOH 95:5) to afford a product.

trans-2-Acetylcinnamyl-2-tetrahydroanilino[2,3-d][1,3]dioxol-4,10-dione, 5

A solution of 1a (1.2 g, 6.79 mmol) in MeOH (20 mL) containing 0.19 g of 18°P Pd/C was hydrogenated (1.1 atm) at room temperature over 4 h.
TABLE I - In vitro anthelmintic activity against *N. brasiliensis* of standards drugs and compounds 4a-10

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>PM</th>
<th>EC50 μg/ml</th>
<th>EC50 nM</th>
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<tbody>
<tr>
<td>Albendazole</td>
<td>1553</td>
<td>0.39</td>
<td>34.108</td>
</tr>
<tr>
<td>Fenbendazole</td>
<td>299.1</td>
<td>0.10</td>
<td>12.109</td>
</tr>
<tr>
<td>Levamisole</td>
<td>294.3</td>
<td>0.094</td>
<td>11.109</td>
</tr>
<tr>
<td>Pyrantel</td>
<td>112.6</td>
<td>&gt;100</td>
<td>23.107</td>
</tr>
<tr>
<td>4a</td>
<td>229.3</td>
<td>48.106</td>
<td>48.108</td>
</tr>
<tr>
<td>5</td>
<td>640.1</td>
<td>35.104</td>
<td>35.104</td>
</tr>
<tr>
<td>6</td>
<td>214.1</td>
<td>35.104</td>
<td>35.104</td>
</tr>
<tr>
<td>7</td>
<td>143.5</td>
<td>48.106</td>
<td>48.108</td>
</tr>
<tr>
<td>8</td>
<td>392.2</td>
<td>35.104</td>
<td>35.104</td>
</tr>
<tr>
<td>9</td>
<td>150.1</td>
<td>51.108</td>
<td>39.107</td>
</tr>
<tr>
<td>10</td>
<td>485.0</td>
<td>2.104</td>
<td>5.9710</td>
</tr>
</tbody>
</table>

**Conidence Limit**: 1% LC90.

The model was calibrated using Albendazole (ABZ), Fenbendazole (FBZ), Levamisole (LVZ) as standards with known anthelmintic activity, and the EC50 of every drug determined (Table I).

The EC50 of compounds 4a and 5 were 2.10×10⁻⁷ and 8.8×10⁻⁸ mM respectively, corresponding to an EC50 more than one thousandfold lower than the EC50 of the standards (ABZ, FBZ, LVZ) used to calibrate the model.

Literature reports m.l.c for these compounds (ranging between 0.05 μg/ml for ABZ and >100 μg/ml for PZQ), but we cannot compare data because a different criteria to calibrate the model was used[2][7].

All the derivatives studied showed interesting anthelmintic activity which is reported in Table I.

**PHARMACOLOGICAL EXPERIMENTAL**

1. **CULTURE AND MAINTENANCE OF *N. BRASILIENSIS*** (**N. BRASILIENSIS**)

1.1. Infective larvae (LL)

Two pairs of *N. brasiliensis* eggs were incubated for 500 L3 of *N. brasiliensis* by subcutaneous injection of 0.1-0.2 ml of one a week.

Five days post-infection the animals were isolated in metabolic cages and the feces of both rats were together collected every day during 4 days.

Feces were placed on slides of filter paper and maintained in Petri dishes. The temperature was maintained at 27 °C and 80% relative humidity for a week. The larvae were recovered and counted by Baermann apparatus filled with distilled water at 37 °C on L1, L3, and 5 days post-infection, the recovery rates were 1150% at 10 days, 195% at 14 days, and 5400% larva successfully.

The maintenance of the strain was done in vivo by weekly infections.

1.2. Fourth stage larvae (L4)

Three male rats of 60 days old were infected with 5000 infective third L3 of *N. brasiliensis* by subcutaneous injection.
2. EVALUATION OF ANTHOCYANIN ACTIVITY

2.1. Calibration of the model using E6 lavender

Three standards with known anthocyanin activity were used to calibrate the model. They were AB2, PB2 and E2W.

The corresponding calibration curves were performed using a double wave dilution method with six wells for each concentration assayed.

Controls loaded with and without vehicle used identically to that used to prepare the samples were always considered in each study.

The percentage of death was determined on day 5 and corrected by the controls in each only vehicle was used.

The results of corresponding ESW for each standard drug used are shown in Table 1.

A dose was used in one of the absence of first culture diluent, the following experimental protocol (Table 1).

Appropriate dilutions of DM5 were prepared for each compound in order to obtain the desired concentration after the addition of 10 μl into each well.

The control groups were put in the same conditions and 10 μl of DM5 were added.

2.2. Interpretation of the results

The activity of the compounds was assessed by comparing the mean percentage of death of the vehicle-exposed control on day 5.

The results of the assay were analyzed using the Student's t-test for each compound.

The corresponding linear regression curves were calculated for each standard drug used and the ESW for each compound was determined and experimentally confirmed.

2.4. Statistical analysis

The accuracy and precision of the model were evaluated using ANOVA (F-test) in large differences.

The variance analysis was performed using the Student's t-test for each compound.

The coefficient of linear variation was estimated as 1%

There were no significant differences between the variances of the 4 assays (F-test), the mean percentage of death did not show significant differences (Student's t-test).

The ESW for each compound was calculated using the Student's t-test.

The percentage of death was corrected by the controls, with a coefficient of variation of 1% over the total amount of death.

RESULTS AND DISCUSSION

Taking into account the preliminary results of the in vivo anthocyanin activity using the N. brasiliensis model we can highlight the following observations:

1) All the compounds 4-10 showed high anthocyanin activity.
2) The compound with no substitution on the OH group of C-2 and the OH group of C-4 (9) showed the greatest activity (E50 = 3.9x10^-4 mM) of the series of derivatives studied.
3) For the series of derivatives studied, substitutions in the OH group of C-2 affect more the loss of activity than substitutions in the OH group of C-4 (5, 7, 4a, 4b).
4) N-methylation of the arylidene nitrogen decreases the activity (10).
5) Activities of compounds 4a, 6, and 5 seemed to demonstrate that esterifications of OH group of C-4 does not produce significant changes in the activity when OH group of C-2 is substituted.
6) OH group of C-4 is not substituted (7, 9) esterification in OH group of C-4 decreases the activity.

Taking into account the decrease of activity between compounds 8 and 9, we can hypothesize that the type of substituent on the OH group of C-2 affects the activity.

We can conclude that the compounds studied have shown a high anthocyanin in vitro activity against N. brasiliensis, that has not yet been reported in literature, while studies of the same model in vivo as well as preliminary in vivo test are being carried out. We are developing in vivo and in vitro models using casesteroles in order to know the spectrum of action of these compounds. Results will be communicated shortly.

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