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Article

Synthesis of 4-Anilino-1*H*-Pyrazolo [3,4-*b*] Pyridine Derivatives and their *in vitro* Antiviral Activities

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Novos derivados do sistema 1*H*-pirazolo[3,4-*b*]piridina (**9a-e**) foram preparados e avaliados quanto à atividade polimerásica das enzimas transcriptase reversa (RT) do vírus HIV-1 e das DNA-polymerases humanas α e ε. Os derivados **9c** e **9e** inibiram a atividade da transcriptase reversa em concentrações micromolares. Entretanto, as mesmas substâncias não foram capazes de inibir a atividade polimerásica das enzimas DNA-polimerase humanas α e ε.

Several new 1*H*-pyrazolo[3,4-*b*]pyridine derivatives (**9a-e**) were prepared and evaluated on the catalytic activity of recombinant reverse transcriptase (RT) of HIV-1 and on the human DNA polymerases α and ε. Some of them inhibited the RT activity at micromolar concentrations, whereas they were not able to inhibit the human placental DNA.

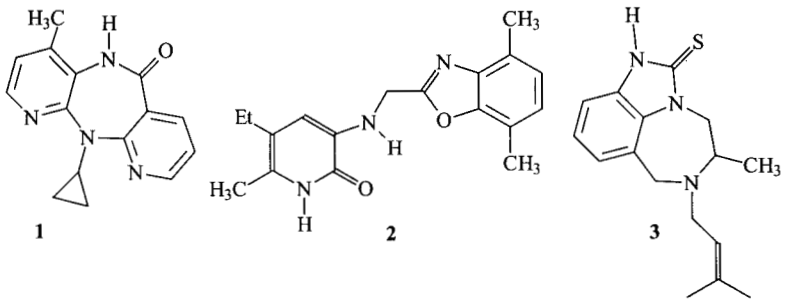
Keywords: *pyrazolopyridine, antiviral, reverse transcriptase*

Introduction

A new candidate substance for a drug should cure, or at least improve, the patient's clinical conditions without causing side effects. Several nucleoside[1,2] and non-nucleoside[3,4] substances that inhibit HIV reverse transcriptase enzyme have been discovered. Reverse transcriptase (RT) are key enzymes in the life cycle of retrovirus, since they are responsible for the transcription of the viral RNA into the provirus DNA. This process of reverse transcription is absolutely required for viral replication. No cellular homologue of viral reverse transcriptases has been discovered, although many normal cells carry endogenous retrovirus and retrotransposons that encode this enzyme[5].

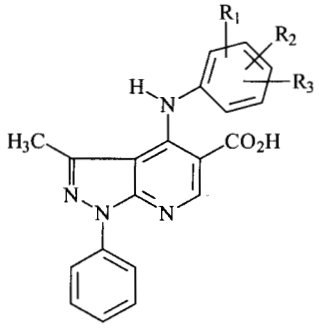
The viral reverse transcriptase is, for this reason, a potential target for drug therapies designed to interfere with the life cycle of retroviruses, most notably the human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS). Much effort is being made to find new chemotherapeutic agents that might result in more potent as well as less toxic drugs than 3'-azido-3'- dideoxythymidine (AZT) for the treatment of AIDS. In general terms, the inhibition of viral DNA synthesis poses the problem that such drugs may also interfere with cellular DNA synthesis and this is reflected on the side effects of AZT involving actively proliferatins tissues, such as the bone marrow[6]. Both suramin and HPA-23, for example, are highly toxic in patients[7], and part of this toxicity may be due to the concomitant inhibition of cellular DNA polymerases[8].

Compounds such as **1** (BI-RG-587), **2** (L697639) and **3** (TIBO R-82150) are among the non-nucleoside substances with good perspectives for clinical trial. This kind of antiviral substances interacts directly with the enzyme in a very specific sense and they do not need to be phosphorilated to get into the cells. This specificity might be of great importance for inhibiting the virus without interfering with functions shared with the host[9].



Structures 1-3.

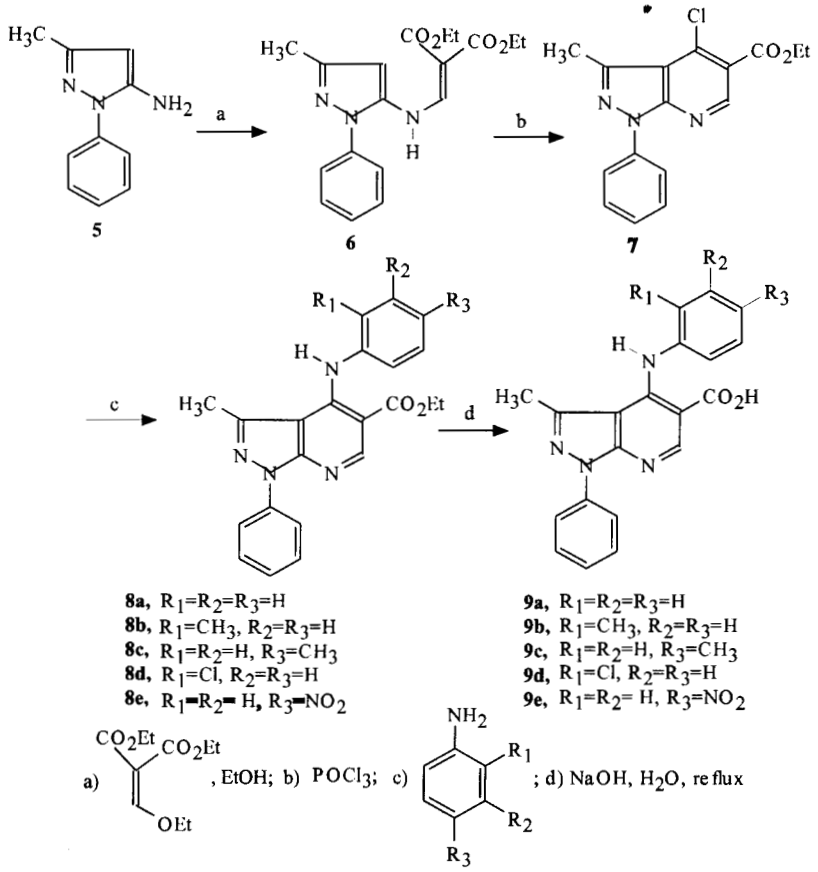
The considerable biological importance of 1*H*-pyrazolo[3,4-*b*]pyridine has stimulated much work on these susbtances[10-13]. As an ongoing program devoted to synthesize new antiviral compounds[14], we have decided to synthesize several derivatives of the 1*H*-pyrazolo[3,4-*b*] pyridine system with the general structure **4**.



Structure 4.

Results and Discussion

The reaction sequence for preparing the target substances **9a-e** is outlined in Scheme 1. The starting material diethyl [5-(3-methyl-1-phenylpyrazolyl]aminomethylenemalonate (**6**) was obtained from amino-pyrazole **5** and diethyl ethoxymethylenemalonate[15]. The chlorocycliza-



8a, $R_1=R_2=R_3=H$
8b, $R_1=CH_3$, $R_2=R_3=H$
8c, $R_1=R_2=H$, $R_3=CH_3$
8d, $R_1=Cl$, $R_2=R_3=H$
8e, $R_1=R_2=H$, $R_3=NO_2$

9a, $R_1=R_2=R_3=H$
9b, $R_1=CH_3$, $R_2=R_3=H$
9c, $R_1=R_2=H$, $R_3=CH_3$
9d, $R_1=Cl$, $R_2=R_3=H$
9e, $R_1=R_2=H$, $R_3=NO_2$

a) , EtOH; b) $POCl_3$; c) ; d) NaOH, H_2O, reflux

Scheme 1. Synthetic route used for preparing the substances **9a-e**.

tion method developed by Hoeln *et al.*[16] was applied and the corresponding 5-carbethoxy-4-chloro-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine (**7**) was obtained in 75% yield. Treatment of the latter compound with several amines led to the new products 4-anilino-5-carbethoxy-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridines (**8a-e**) in moderated yields. The desired carboxylic acids **9a-e** were obtained by saponification of the corresponding ester **8a-e** with aqueous 2 N sodium hydroxide in ethanol followed by neutralization of the former sodium salt. All the structures proposed for the compounds were confirmed by spectroscopic analysis and some of the data are presented in Table 1.

Different concentrations of 4-anilino-1*H*-pyrazolo[3,4-*b*]pyridine derivatives were evaluated for their inhibitory activities against HIV-1 reverse transcriptase enzyme and human placental DNA polymerases α and ε, which are involved in human chromosomal DNA replication.

HIV-1 reverse transcriptase activity was performed as previously described[19] with some modifications. The standard reaction mixture contained, in a final of 50 µL, 50 mM Tris-HCL (pH 7.8), 50 mM KCl, 6 mM $MgCl_2$, 1 mM dithiotreitol, 1 mg/mL bovine serum albumin, 5 µM dTTP, 20 µCi/ mL of ^{3}H dTTP (47 Ci/mmol) and 0.26 OD/mL of poly(rA) p(dT) templete primer (from pharmacia). Incubations were stopped by adding ice cold 5 % TCA containing 20 mM sodium pyrophosphate. The precipitates were collected on Whatman GF/C filters and washed. Radioactivity was determinated by liquid scintillation.

The effect of these compounds on the DNA polymerase activity of HIV-1 reverse transcriptase with poly (rA)o(dT) templete/primer is shown in Table 2. In the presence of 50 µM of compounds **9c** and **9e**, HIV-1RT polymerase activity was inhibited by approximately 45% and 40%, respectively and substances **9a**, **9b** and **9d** proved to be ineffective.

Conclusion

An important consideration in evaluating any inhibitor of HIV-1 reverse transcriptase is examining its effect on human DNA polymerases α and ε, enzymes involved in cell replication. Inhibition of these enzymes could lead to cytoxicity, limiting the usefulness of the compound. Human placental DNA polymerase α was prepared as described by Toomey *et al.*[21], and had a major polypeptide of 180 KDa. DNA polymerases α and ε activities were measured as previously described[21,22]. Each assay contained 40 µg/mL BSA, 2% of glycerol, 100 µM of dATP, 1 mM Tris-HCl pH 7.8 and 0,26 OD of poly (dA)o(dT). Different from HIV-1 RT, none of the derivatives studied in concentrations of 0.5 mM and 5 mM, were potent inhibitors of DNA polymerase α and ε, as shown in Table 3.

These results may provide some important information in relation to the assessment of potential drug cytotoxicity for future design of antiviral drugs. The fact of these compounds exerted minimal effects on celular DNA polymers α and ε may predict a broader trend for low celular toxicity.

Table 2. Effects of 4-anilino-1*H*-pyrazolo[3,4-*b*]pyridine derivatives on HIV-1 reverse transcriptase activity.

	% Polymerase Inhibition RT at:			
Derivatives	10 µM	50 µM	100 µM	200 µM
9a	ND	ND	ND	0
9b	ND	ND	0	0
9c	0	45	50	80
9d	ND	0	0	0
9e	10	40	65	90

ND= not determined

Table 1. Some physical data[17-18] of compounds **8a-e** and **9a-e**.

	Yield (%)	m.p. (°C)	Crystallization	MS M^+ (m/z)	i.v. bands (cm^{-1})
8a	70	154-155	EtOH	372	3220 (N-H), 1675 (C=O)
8b	55	161-163	EtOH	386	3220 (N-H), 1670 (C=O)
8c	54	143-145	EtOH	386	3220 (N-H), 1670 (C=O)
8d	61	152-154	EtOH	406	3220 (N-H), 1670 (C=O)
8e	55	208-211	EtOH	417	3220 (N-H), 1670 (C=O) 1500, 1330 (NO_2)
9a	90	235-239	AcOH	344	3000(br,OH), 1650 (C=O)
9b	94	234-237	AcOH	358	3000(br,OH), 1670 (C=O)
9c	89	239-242	AcOH	358	3000(br,OH), 1670 (C=O)
9d	95	243-246	AcOH	378	3000(br,OH), 1680 (C=O)
9e	90	237-240	AcOH	389	3000 (br,OH), 1670 (C=O)

Table 3. Effects of 4-anilino-1*H*-pyrazolo[3,4-*b*]pyridine **(9a-e)** derivatives on human placental DNA polymerases α and ε.

	Polymerase Activity Inhibition at:			
	pol α		pol ε	
Derivatives	0.5 mM	5 mM	0.5 mM	5 mM
9a	0	23	0	3
9b	0	16	0	0
9c	0	3	0	0
9d	4	8	0	2
9e	0	9	0	0

Experimental

General procedures

Melting points were determined with a Thomas-Hoover apparatus and are uncorrected. The solvents used were of analytical grade and were purified and dried by using procedure described in the literature[23]. Column chromatography was performed on silica gel 60 (Merck). Infrared spectra were recorded with a Perkin-Elmer 783 spectrophotometer. NMR spectra were recorded with either a Varian VXR (300 MHz) or a Gemini (200 MHz) for solutions in $CDCl_3$. High (HRMS) and low resolution mass spectra (MS) were measured on a Autospec VG spectrometer.

Diethyl [5-(3-methyl-1-phenylpyrazolyl)]aminomethylenemalonate (6)

A mixture of **5**[16] (80.4 g, 0.46 mole) and diethyl ethoxymethylenemalonate (100.2 g, 0.46 mole) in 200 mL of ethanol was refluxed for 2 h under nitrogen. The resulting mixture was concentrated under reduced pressure to give a solid material which was recrystallized from anhydrous ethanol producing compound **6** (119 g) in 75% yield (m.p. 77-78 °C).

5-Carbethoxy-4-chloro-3-methyl-1-phenyl-1H-pyrazolo [3,4-b]pyridine (7)

A solution of **6** (19.0 g, 0.055 mole) in phosphorus oxychloride (46 mL) was refluxed for 5 h under nitrogen. The excess of the solvent was removed under reduced pressure and the resulting viscous material was poured onto crushed ice and the product was collected by filtration. The solid material was recrystallized from ethanol to yield **7** (10.30 g, 60%, m.p. 110 °C).

Typical procedure for preparation of compounds **8a-e**

A mixture of **7** (0,004 mole) and an aniline derivative (0,006 mole) was heated at 130 °C for 2 h producing a precipitate which was then washed with water and sodium hydroxide solution (1 N). The solid was filtered off, washed with water and recrystallized from ethanol to produce the ester **8a-e**. The structures were confirmed by elemental analysis[17] and some physical data are shown in Table 1.

Typical procedure for preparation of compounds **9a-e**

Derivatives **8a-e** (0,003 mole) were heated under reflux in a 20% ethanolic sodium hydroxide solution for one hour. The mixture was neutralized with HCl solution (1:3; v/v) producing a solid which was filtered off and recrystallized from glacial acetic acid. The structure was confirmed by ^{1}H-NMR analysis[18] and some physical data are reported in Table 1.

Acknowledgment

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17. Elemental analysis of compounds **8a-e** (%, calcd./found) for C, H and N; **8a**: C 70.95/71.10; H 5.41/5.58; N 15.04/15.29; **8b**: C 71.48/71.18; H 5.74/5.74; N 14.50/14.21; **8c**: C 71.48/71.19; H 5.74/5.86; N 14.50/14.20; **8d**: C 64.94/64.93; H 4.70/4.68; N 13.77/13.75; **8e**: C 63.29/63.38; H 4.59/4.67; N 16.78/16.63.
18. ^{1}H-NMR (300 Mhz, ppm, $CDCl_3$ + DMSO-d_6) **9a**, 1.72 (3H), 7.06-8.28 (10H), 9.01 (1H), 10.52-10.66 (2H); **9b**, 1.60 (3H), 7.04-8.30 (9H), 9.01 (1H), 10.42-10.56 (2H); **9c**, 1.72 (3H), 7.00-8.30 (9H), 9.02 (1H), 10.50-10.68 (2H); **9d**, 1.74 (3H), 7.14-8.28 (9H), 9.04 (1H), 10.56-10.68 (2H); **9e**, 1.74 (3H), 7.01-8.28 (9H), 9.04 (1H), 10.54-10.66 (2H).
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