SYNTHESIS AND BIOLOGICAL EVALUATION OF 1*H*-PYRAZOLO[3,4-*b*]PYRIDINE-5-CARBOXYLIC ACIDS AGAINST VACCINIA VIRUS

Alexandre R. Azevedo[a], Vitor F. Ferreira[a], Heloisa de Mello[a], Luiz R. Leão-Ferreira[b], Alfredo V. Jabor[b], Izabel C.P.P. Frugulhetti[b], Helena S. Pereira[b], Nissin Moussatche[c], Alice M. Rolim Bernardino*[a],

a*. Departamento de Quimica Orgânica, Instituto de Química, Universidade Federal Fluminense, Outeiro São João Batista, Niterói, R J. , Valonguinho, Niterói, RJ, 24020-150, Brasil; e-mail : alice@rmn.uff.br
b. Departamento de Biologia Celular e Molecular, Instituto de Biologia, Universidade Federal Fluminense, Outeiro São João Batista, Niterói, R J. , Valonguinho, Niterói, RJ, 24020- 150, Brasil.
c. Departamento de Biologia Molecular de Vírus, Instituto de Biofisica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, CCS, bloco I, Ilha do Fundão, Rio de Janeiro, RJ.

ABSTRACT: Several new 3-phenyl and 3-alkyl-1*H*-pyrazolo[3,4-*b*]pyridine derivatives (**3a-e**) were prepared and evaluated against Vaccinia virus on BSC-40 cells. The derivatives **3a, 3b** and **3d** showed an inhibitory activity above 90% at 30 μM, 40 μM and 50 μM concentrations.

INTRODUCTION

Among various important parent ring systems of the pyrazolo-pyridine[1,2,3], substituted 1*H*-pyrazolo[3,4-*b*]pyridine is known to have several biological[4] activities such as anxiyolitic[5], anticonvulsant[6], antinflammatory, analgesic, hypoglycemic, antipyretic and vasodialators[7,8] . Antiviral activity[9] has also been investigated for several compounds of this system.

The number of antiviral agents available for the treatment of viral infections has increased dramatically during the last decade. There is currently three classes of antiviral agents used tin the chemotherapy of HIV infection: nucleoside, non-nucleoside reverse transcriptase inhibitors and protease inhibitors. In non-nucleoside reverse transcriptase inhibitors many heterocyclic compounds played prominent activity against several types of retroviruses.

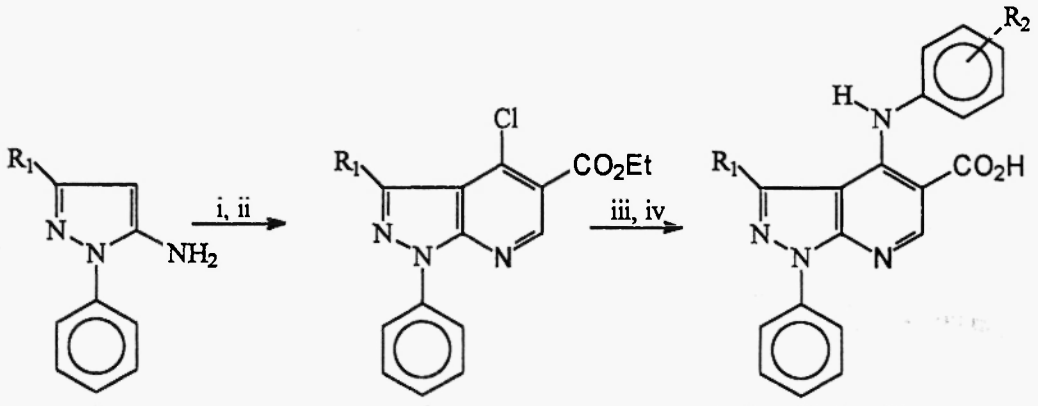
Several synthetic substances are currently approved for the treatment of virus infection, such as, acyclovir, gancyclovir, ribavarin, azidothimidine, ddI and ddC. Compounds such as TIBOR are non-nucleoside substances with good perspectives for clinical trial. Other non-nucleoside compounds such as Efavirinz was approved by FDA in 1998 and has become an important component of combination treatment for AIDS treatment. These compounds can be very potent inhibitors of RT, with low toxicity and favorable pharmakokinetic properties.

As an ongoing program devoted to produce new heterocyclic compounds with potential biological activities[10,11], several derivatives of the 1*H*-pyrazolo[3,4-*b*]pyridine were synthesized. The structural formulas of these compounds (3a-e) are shown in Scheme 1. Since the antiviral activity of 4-anilino-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxylic acid have already been described[9], we used vaccinia virus infected cells as a model for investigating the biological effects of these compounds on virus replication.

Vaccinia virus (VV), the prototype member of the Poxviridae family, was used in the first global eradication program of a human disease, smallpox[12,13]. Although, VV is not a pathogenic virus, this Orthopoxvirus has been extensively used as a suitable model for evaluating biological activity of several antiviral compounds on pathogenic poxviruses[14].

The VV replicative cycle can be described in terms of sequential steps beginning with the binding and entry of virus particle. In the cytoplasm of the host cell, early gene expression precedes virus DNA replication, which in turn shuts the early gene expression off and stimulate the intermediate and late gene expression[15]. The virus replicative cycle, which occurs entirely in the cytoplasm of the host-cell, culminates with virus particle morphogenesis and release by fusion of the intracellular enveloped-virus (IEV) with the plasma membrane of the host-cell. The WR strain of VV remains associated on the cell surface of the host-cell and is the virus form that mediates cell-to-cell spread[16]. The cytoplasmic replication cycle of VV is mainly carried out by the viral counterparts of cellular enzymes involved in the replication and transcription of virus DNA[18]. Infection of monolayers of cultured-cells induces several morphological and metabolic modifications of the host-cells[18,17].

For preparing compounds **3a-e** was used a known synthetic sequence (Scheme 1), which usually involve formation of the pyrazolo-piridine system, typically via reaction the amino-pyrazole (**1**) with diethyl ethoxymethylenemalonate followed by chlorocyclization to produce 3-phenyl or 3-methyl-4-chloro-1*H*-pyrazolo[3,4-*b*]pyridine (**2**), which at a late stage of the sequence is transformed into the compounds **3a-e** by reaction with the appropriated amines[19]. This general, easily and extensible technology for the construction of this heterocycles system was very suitable for preparing this structural family of substituted pyrazole.



1 **2**

i) Ethoxymethylenemalonate, EtOH, reflux, 2h;
ii) POCl3; reflux 5h;
iii) Aniline, 130 oC, 2h;
iv) EtOH, 20% NaOH, reflux.

3a, R_1 = Ph, R_2 = H
3b, R_1 = Ph, R_2 = *p*-OMe
3c, R_1 = Ph, R_2 = *o*-OMe
3d, R_1 = Me, R_2 = H
3e, R_1 = Me, R_2 = *p*-OMe

Scheme 1. Synthetic sequence for preparing compounds **3a-e**

EXPERIMENTAL

A mixture of **1**[20] (0.46 mole) and diethyl ethoxymethylenemalonate (0.46 mole) in 200 mL of ethanol was refluxed for 2 hours under nitrogen. The resulting mixture was concentrated under reduced pressure to give a solid material, which was recrystallized from anhydrous ethanol. This material was mixed with phosphorus oxychloride and refluxed for 5 hours 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 **2**, which was reacted with a slightly excess with the appropriated aniline and heated at 130^{0}C for 2 hours producing a precipitate which was then washed with water and sodium hydroxide solution (1N). The solid was filtered off, washed with water and recrystallized from ethanol and, then 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 compounds **3a-e** as a solid that was filtered off and recrystallized from ethanol.

Biological Assay

Cells and Virus

An epithelial-derived cell line from monkey kidney cells, BSC-40, was grown in monolayer cultures in Dulbecco's modified Eagle's medium (Gibco laboratories) containing 2% heated-inactivated fetal bovine serum (purchased from Fazenda Pigue), 8% calf serum (purchased from Centro Pan-Americano de Febre Aftosa), 2.25% sodium bicarbonate, 500U/ml penicilin, 100μg/ml streptomycin, 50μg/ml gentamycin, 2,5μg/ml anphotericin B. Cell cultures were incubated at 37^{0}C in humidified air containing 5% CO_2.

Vaccinia virus (WR strain) was obtained from American Type Culture Collection and was routinely propagated and titrated in Confluent BSC-40 cells, as described by Dâmaso & Mousatché(1998)[21].

Virus Plaque Assay

Sub-confluent BSC-40 cells, grown in 6-well plates (10^6cells/well), were infected with 250 PFU of VV per well. After one hour of virus adsorption at 37^{0}C in 200μl of DMEM, virus inoculum was replaced by culture medium, containing or not testing drugs, as indicated in the text. Infection proceeded for 36-48 hours at 37^{0}C in humidified air containing 5% CO_2, when culture medium was aspirated and cell monolayers were then fixed and stained by PBS containing 0.1% crystal violet, 3.7% formaldehyde, for 30 minutes. Plates were extensively washed with water and virus plaque number was determined. The results were expressed as % of inhibition of virus plaque number, by using the number of plaques obtained in the absence of drugs as reference[22].

Table 1: Some Physical data of compounds 3a-e.

Substances	Yield (%)	m.p. (°C)	Crystallization	I.v. bands (cm^{-1})[a]
3a	80	234-235	EtOH	3220 (N-H), 1675 (C=O)
3b	99	235-236	EtOH	3220 (N-H), 1670 (C=O)
3c	80	218-219	EtOH	3220 (N-H), 1670 (C=O)
3d	98	207-8	EtOH	3220 (N-H), 1670 (C=O)
3e	88	231-32	EtOH	3220 (N-H), 1670 (C=O) 1500, 1330 (NO_2)

[a] Infrared spectra were recorded on a Perkin-Elmer 1420 spectrophotometer.

Table 2: ^1H NMR[a] and ^{13}C NMR[a] data of 3a-e obtained by the following experiments 1D (^1H, PND e DEPT, $\theta=90^0$ e 135^0) e 2D (^1H x ^1H- COSY, ^1H x ^{13}C-COSY, nJ_{CH} n=1,2 ou 3.

	3a (DMSO - d_6)		3b (DMSO - d_6)		3c (DMSO - d_6)		3d (DMSO - d_6)		3e (DMSO - d_6)	
CH	δ_c (ppm)	δ_H (J, Hz)	δ_c (ppm)	δ_H (J, Hz)	δ_c (ppm)	δ_H (J, Hz)	δ_c (ppm)	δ_H (J, Hz)	δ_c (ppm)	δ_H (J, Hz)
3	147,12	-	147,32	-	147,04	-	147,12	-	147,32	-
3a	104,70	-	104,04	-	104,33	-	104,70	-	104,04	-
4	149,67	-	150,88	-	150,34	-	149,67	-	150,88	-
5	106,34	-	105,36	-	106,18	-	106,34	-	105,36	-
6	153,16	9,16(s)	153,33	9,16(s)	153,19	9,04(s)	153,16	9,16(s)	153,33	9,16(s)
7a	153,87	-	154,04	-	153,90	-	153,87	-	154,04	-
CO_2H	169,97	10,71(s)	170,28	10,72(s)	168,83	10,34(s)	169,97	10,71(s)	170,28	10,72(s)
NH	-	10,71(s)	-	10,72(s)	-	10,34(s)	-	10,71(s)		10,72(s)
OCH_3	-	-	55.58	3,97(s)	55,36	3,80(s)	-	-	55.38	3,80(s)

[a] ^1H and ^{13}C NMR spectra were recorded with a Varian Unity Plus 300 spectrometer operating at 300 and 75 MHz respectively, with TMS as the internal standard.

Table 3: Effects of 1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxylic acids derivatives 3a-e on against Vaccinia vírus on BSC-40 cell.

	Cl	0.1µM	0.5µM	1µM	5µM	10µM	20µM	30µM	40µM	50µM
3a	100%	6.87%	7.08%	10.20	7.70%	4.37%	3.95%	94.60%	78.30%	-
3b	100%	1.25%	-	6.25%	10.80%	-	5.20%	8.54%	98.50%	-
3c	100%	8.54%	9.58%	-	8.54%	4.79%	3.54%	5.62%	55.70%	-
3d	100%	-	-	-	4.80%	31.65%	46.66%	61.66%	78.33%	95.00%
3e	100%	-	-	-	16.65%	8.32%	24.99%	41.66%	61.66%	78.33%

RESULTS AND DISCUSSION

The compounds 3**a-c** reduced virus plaque number in a dose-dependent manner (table 3). The highest inhibition level for the compound 3**a** was found at concentrations as high as 30 μM. At the concentration of 40μM, the compound **3b** reached 98.5%. It is important to emphasize that compounds 3**a-c** were not cytotoxic at the concentration of 40μM as indicated by the trypan blue dye exclusion method[23].

CONCLUSIONS

In summary, we have described a mild and efficient synthesis of five new 1*H*-pyrazolo[3,4-*b*]pyridine derivatives (**3a-e**). The derivatives **3a, 3b** and **3d** showed an inhibitory activity above 90% at 30 μM, 40 μM and 50 μM concentrations. These results may provide some important information for future design of antiviral drugs. The fact that these compounds exerted minimal effects on cellular DNA synthesis trend for low cellular toxicity may indicate that should be tested in other type of virus.

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