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Synthesis and Anti-HIV Studies of Some Substituted Pyrimidinediones, Ethoxy Pyrano [3,2 -C] Quinolines and Hydrazino Pyrano [3,2-C] Quinolines

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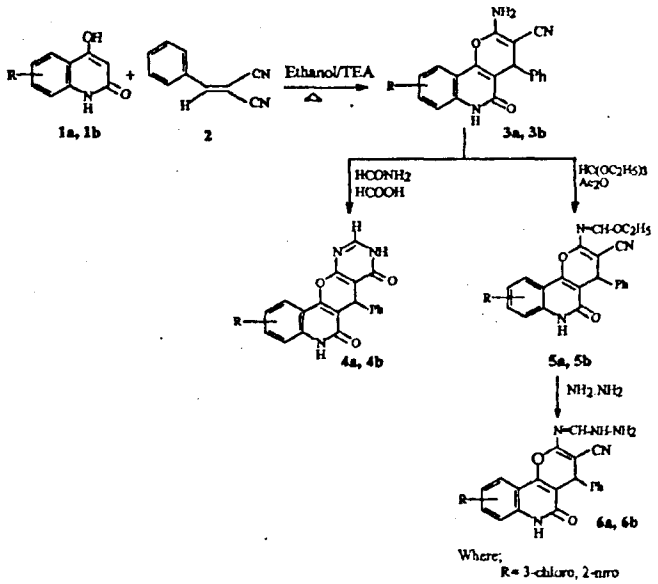
Recent reports[1] describe a novel class of 4-hydroxycoumarins and its derivatives as Non-nucleoside ReverseTransscriptase Inhibitiors (NNRTIs) in the Human Immunodeficiency Virus (HIV).The chemistry of 4-hydroxycoumarins and 4-hydroxy carbostyryls (4-hydroxy 2-quinolones) was much studied for evaluating tautomerism and pharmacology as well[2-4]. Our current interest is to synthesize pyrano [3, 2-c) quinolines and their transformation into a variety of fused heterocycles substituted at 3,4 - positions, which may prove to be interesting antiviral agents.

In the present synthetic work, 4-hydroxy carbostyryls, which are fused with pyrimidines and pyran systems, were synthesized and screened for antiHIV activity against both HIV-1 and HIV-2. Thus substituted 2-amino-5-oxo-6-hydro-4H-pyrano[3,2-c]quinoline-3-carbonitriles were prepared by the reaction of 4-hydroxy-2-quinolones with cinnamonitriles. These compounds were further cyclised as pyrimidinediones, ethoxy vinyl pyrano [3,2-c] and hydrazino pyrano [3,2-c] quinolines. All these compounds were screened for AntiHIV activity. The compounds were characterized on the basis of IR, PMR, Mass spectral and elemental analysis.

MATERIALS AND METHODS

Melting points were determined in open glass capillariers in a liquid paraffin-bath and are uncorrected. Purity of compounds was checked by TLC. IR spectra were recorded on a Nicolet-megna IR 550 series -II spectrophotometer and PMR spectra in $CDCl_3$+DMSO-D_6 in a BRUKER AC (300 MHz) FT-NMR spectrometer usingTMS as internal standard (chemical shifts) in δppm.

7-chloro-4-hydroxy-2-quinolone (Ia) was prepared according to reported methods[5,6]. Other compounds that were prepared are as follows.



REACTION SCHEME FOR TRICYCLIC AND TETRACYCLIC FUSED SYSTEMS BUILT ON 4-HYDROXY CARBOSTYRYLS

2-amino-8-chloro-5-oxo-4-phenyl-6-hydro-4H-pyrano[3,2-c]quinoline-3-carbonitrile(3a):

A mixture of 7-chloro-4-hydroxy-2-quinolone Ia (0.01 mol) and cinnamonitrile 2 (0.01 mol) absolute ethanol (30 ml) and catalytic amount of triethyl amine (0.1 ml) was

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taken in a round bottom flask and refluxed in a water bath for 45 min. The reaction mixture was cooled and the product was collected by filtration and recrystallized from N,N dimethyl formamide (DMF). Melting point 80°, IR (KBr) cm^{-1}, 1672 (C=O str.); 2203 (C=N str.); 3474 (N-H str.); 679 (C-Cl); PMR ($CDCl_3$+DMSO-d_6) δppm: 4.90 (s, IH, 4H-pyran), 6.22-8.17 (m, 10H, 8 aromatic, 2H, NH_2); [Found: C, 65.11; H, 3.39; N, 12.00; 0, 9.10; Cl, 10.12; Required: C, 65.18; H, 3.43; N, 12.01; 0, 9.14; Cl, 10.14].

8-Chloro-6-penyl-5,9-dihydro quinolino[3',4'-5,6-]-pyrano [2,3-d]-pyrimidin-6, 8-dione (4a):

A mixture of **3a** (0.01 mol) was refluxed in formic acid (5 ml)/formamide (15 ml) for 24 h. Then it was cooled and poured into crushed ice. The solid obtained and collected by filtration and washed with 100 ml of water. It was recrystallized from ethanol. Melting point 149°, IR (KBr) cm^{-1}, 1670 (C=O str.); 2208 (C=N str.); 3470 (N-H str.); 686 (C-Cl); PMR ($CDCl_3$+DMSO-d_6) δppm: 4.81 (s, IH, 4H-pyran), 7.17-8.25 (m, 10H, 1-CH and 9 aromatic), 11.29 (s, IH, NH); [Found: C, 63.49; H, 3.14; N, 11.10; O, 12.68, Cl, 9.34; Required: C, 63.52; H, 3.17; N, 11.11; O, 12.70; Cl, 9.39].

2-[(Ethoxymethylenamino)-1-aza-α-ethoxyvinyl]-8-chloro-5-oxo-4-phenyl-6-hydro-4H-pyrano [3,2-c] quinoline-3-carbonitrile (5a):

A mixture of **4a** (0.01 mol) and ethylorthoformate (3 ml) in aceticanhydride (15 ml) was refluxed for 2 h. After cooling, the product was filtered off and washed with chilled ethnol. The product was recrystallized from ethanol. Melting point 295°, IR (KBr) cm^{-1}; 1658 (C=O str.), 2223 (C=N str.); 3500-3030 (broad, N-H str.); 1218 (C-O-C str); 678 (C-Cl); PMR ($CDCl_3$+DMSO-d_6) δppm: 1.15 (3H, CH_3); 4.31 (2H, CH_2); 4.67 (s, IH, 4H-pyran), 7.22-8.45 (m, 10H, I-CH and 9 aromatic), 11.80 (s, IH, NH); [Found: C, 65.02; H, 3.91; N, 10.29; O, 11.80; Cl, 8.70; Required: C, 65.05; H, 3.94; N, 10.34; O, 11.82; Cl, 8.74].

2-[(Ethoxymethylenamino)-1-aza--2(hydrazino-methyleneaminovinyl)-8-chloro-5-oxo-4-phenyl-6-hydro-4H-pyrano [3,2-c] quinoline-3-carbonitrile (6a):

A mixture of **5a** (0.01 mol) and 90% hydrazine hydrate (0.01 mol) in absolute ehtanol was stirred at room temperature for 30 min. The product was filtered and washed with chilled ethanol. And it was recrystallized from ethanol. Melting point 118°, IR (KBr) cm^{-1}, 1660 (C=O str.); 2200 (C=N str.); 3380 and 3300 (NH_2, and NH); 1243 (C-O-C str); 667 (C-Cl); PMR ($CDCl_3$+DMSO-d_6) δppm: 4.89 (s, IH, 4H-pyran), 5.35 (s, 2H, NH_2); 7.19-8.53 (m, 10H, 1-CH and 9H aromatic), 11.73 (s, IH, NH); [Found: C, 64.28; H, 3.53; N, 10.69; O, 12.20; Cl, 9.02; Required: C, 64.31; H, 3.57; N, 10.71; O, 12.25; Cl, 9.06].

Other compounds 3a, 3b, 4a, 4b, 5a, 5b, 6a, 6b were also synthesized and their physical and analytical characteristics are shown in Table 1.

AntiHIV activity:

The antiHIV screening was carried out at different concentration also. The exact methodology adopted for screening is given below. The MT-4 cells were grown in

TABLE 1: PHYSICAL AND ANALYTICAL DATA

Compound No.	R	Molecular Formula	Melting Point °C	% of yield	% of Nitrogen	
					Calculated	Found
3a	3-chloro	$C_{19}H_{12}N_3O_2Cl$	85	70	12.10	12.01
3b	2-nitro	$C_{19}H_{12}N_4O_4$	95	67	15.55	15.45
4a	3-chloro	$C_{20}H_{12}N_3O_3Cl$	149	77	11.12	11.12
4b	2-nitro	$C_{20}H_{12}N_4O_5$	111	59	11.39	14.43
5a	3-chloro	$C_{22}H_{16}N_3O_3Cl$	295	82	10.35	10.30
5b	2-nitro	$C_{22}H_{16}N_4O_5$	55	58	13.46	13.44
6a	3-chloro	$C_{21}H_{14}N_3O_3Cl$	118	70	17.87	17.88
6b	2-nitro	$C_{20}H_{14}N_4O_5$	245	67	20.85	20.29

RPMI 1640 DM (Dutch Modification) medium (Life Technologies, Merelbeke, Belgium), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 0.1% sodium bicarbonate, and 20 µg/ml gentamicin (equals complete medium). The cells were maintained at 37° in a humidified atmosphere of 5% CO_2 in air. Every 3-4 days and always 2 days before starting the experiment, cells were seeded at $3x10^5$ cells/ml. At regular time intervals, the MT-4 cells were analyzed for the presence of mycoplasama and consistently found to be mycoplasama-free. HIV (strain HTLV-III_B/LAI)[7] and HIV-2 (strain LAV-2_{ROD})[8] were obtained form the cultures supernatant of HIV -1 or HIV -2 infected MT -4 cell lines[9-10]. The virus titer of the supernatant was determined in MT-4 cells. The virus stocks were stores at - 70° until used. Flat bottom, 96 - well plastic microtitier trays (Nunc, Life Technologies, Merelbeke, Belgium) were filled with 100 µl of complete medium using a Titerttek^R Multidrop dispenser (ICN Biomedicals). Subsequently, stock solutions (10 X final test concentration) of compounds were added in 25 µl volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on HIV - and mock - infected cells. Serial five-fold dilutions were made directly microtiter trays using a Biomek 2000 robot (Beckman). Unreacted control HIV - and mock - infected cell samples were included for each compound. 50 µl of HIV at 100-300 $CCID_{50}$ or medium was added to either infected or mock-infected part of microtiter tray. Exponentially growing MT-4 cells were centrifuged for 5 min at 1000 rpms and the supernatants were discarded. The MT-4 cells were resuspended at $6x10^5$ cells/ml in a dispensing flask, which was connected with an autoclavabale dispensing cassette of a Titettek^R Multidrop dispenser. Under light magnetic stirring, 50 µl volumes were then transferred to the microtiter tray wells. The outer row wells were filled with 200 µl of medium. The cell cultures were incubated at 37° in humidified atmosphere of 5% CO_2 in air. The cells remained in contact with the test compounds during the whole incubation period. Five days after infection the viability of mock- and HIV-infected cells was examined spectrophotometrically by the MTT method.

MTT assay:

The MTT assay is based on the reduction of the yellow colored 3-(4,5-dimethylthaizol-2-yl)-2,5-diphenylterazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO) by mitochondrial dehydrogenase of metabolically active cells to a blue formazan, which can be measured spectrophotometrically. Therefore, to each well of the microtiter trays, 20 µl of a solution of MTT (7.5 mg/ml) in phosphate-buffered saline was added using the Titerttek^R Multidrop. The trays were further incubated at 37° in a CO_2 incubator for 1 h. A fixed volume of medium (150 µl) was then removed from each cup using a 96-well washer (Beun De Ronde) without disturbing the MT-4 cell cluster containing thew formazan crystals.

Solublilization of the formazan crystals was achieved by adding 100 µl 10% (v/v) Triton X-100 in acidified isopropanol (2 ml concentrated HCl per 500 ml solvent) using a 96-well washer (Beun De Ronde). Complete dissolution of the formazan crystals should be obtained after the trays had been placed on a plate shaker for 10 - min. Finally, the absorbances were read in an eight-channel computer-controlled Titertek Microplate Reader and Stacker (Multiskan MCC, ICN Flow) at two wavelengths (540 and 690 nm). The absorbances measured at 690 nm were automatically subtracted form the absorbance at 540 nm, so as to eliminate the effects of non-specific absorption. Blanking was carried out directly on the micrortiter trays with the first column wells, which contained all reagents expect for the MT-4 cells, virus and compounds. All data were calculated using the 50% cytotoxic dose (CD_{50}), which was defined as the concentration of compound that reducer the absorbance (OD_{540}) of the mock-infected control sample by 50%. The percent protection achieved by the compound in HIV-infected cells was calculated by the following formula:

$$\frac{(OD_T)_{HIV} \cdot (ODc)_{HIV}}{(ODC)_{mock} \cdot (ODC)_{HIV}} \text{ expressed in \%}$$

where $(ODT)_{HIV}$ is the optical density measured with a given concentration of the test compound in HIV - infected cells; $(OD_C)_{HIV}$ is the optical density measured for the control untreated HIV - infected cells; $(OD_C)_{mock}$ is the optical density measured for the control untreated mock-infected cells. The dose achieving 50% protection according to the above formula was defined as the 50% effective dose (ED_{50}).

RESULTS AND DISCUSSION

All the compounds synthesized were studied for their antiHIV activities. Table 2 shows EC_{50}, EC_{90} data and also values of CC_{50} (cytotoxic concentraion). The anti HIV data suggests that in case of compounds 3a and 3b

TABLE 2: ANTIHIV ACTIVITY DATA

Compound No.	Strain	EC_{50}	EC_{90}	CC_{50}	Maximum Protection
	IIIB	>13	>13	12.9	7
3a	IIIB	>11	>11	11.1	3
	ROD	>10	>10	9.6	3
	IIIB	>12	>12	12.2	3
3b	IIIB	>11	>11	10.6	1
	ROD	>10	>10	10.4	3
	IIIB	>11	>11	11.0	2
4a	IIIB	>16	>16	15.9	3
	ROD	>12	>12	12.3	4
	IIIB	>53	>53	53.1	2
4b	IIIB	>46	>46	45.9	5
	ROD	>54	>54	54.4	6
	IIIB	>15	>15	14.7	7
5a	IIIB	>20	>20	20.4	0
	ROD	>13	>13	13.1	1
	IIIB	>25	>25	24.9	1
5b	IIIB	>13	>13	12.7	0
	ROD	>12	>12	12.3	0
	IIIB	>26	>26	26.3	3
6a	IIIB	>14	>14	14.1	2
	ROD	>34	>34	34.5	2
	IIIB	>102	>102	102.3	0
6b	IIIB	>125	>125	125.0	3
	ROD	>114	>114	113.6	0

EC = Effective concentration in µg/ml, CC = Cytotoxic concentration in µg/ml, III-B= HIV-1 strain, ROD= HIV-2 strain, Selective Index (SI)<1 in all the samples.

bearing electron withdrawing chloro or nitro group, equal potency is observed both against HIV -1 and HIV -2. Similarly 3a, when transformed into pyrimidine ring formation (4b) led to higher EC_{50} values against both the strains, while pyranoquinolines 5a and 5b showed potency at lower EC_{50} value. So is the case of another pyranoquinoline 6a. The compound 6b showed inhibition at higher concentration. These data suggest that further annelation leading to tetracyclic compounds may lead to either almost similar or better activity compared to the tricyclic precursors. However, modification in the structure by derivatization at amino group present at C_2 position did not result in better activity. The selectivity index (SI) is the ratio of CC_{50} to EC_{50} and it is necessary to increase SI value up to 10. For establishing structure-activity, further modification in present synthetic work is required as the selectivity index found to be <1 in the present study.

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