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Anti-HIV Activity of some Mannich Bases of Isatin Derivatives

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A number of Mannnich bases of Isatin were synthesised and screened for anti-HIV activity. The Schiff's base derived from 5-Methylisatin and trimethoprim was converted into Mannich bases. The N-dimethyl (Pan I) and morpholino (Pan M) were the most active compounds in the series and gave maximum protection against HIV-1 (III B) strain.

IN the search for more selective and effective agents for human immuno-deficiancy virus (HIV), which is the causative agent of the acquired immuno deficiancy syndrome (AIDS)[1,2], various cyclic urea derivatives have been designed to inhibit HIV-1 replication[3]. Further diazepine class of compound nevirapine is a potent HIV-1 inhibitor, acting at the level of the Reverse transcriptase (RT)[4]. In certain investigations of HIV-Protease inhibitors, morpholino group forms a part of the active pharmacophore of L-689502[5]. Isatin and its derivatives have been traditionally used as antivirtal drugs[6-8] like methisazone. The trimethoprim molecule itself has been widely used for control of infection caused by bacteria. In the present study it was envisaged that a drug molecule containing the above mentioned pharmacophores could be in a advantageous position and was expected to exhibit antiviral activity especially against HIV. Therefore, Schiff's base of isatin and its derivatives with trimethoprim was synthesised and these Schiff's bases were converted into various Mannich bases taking secondary amines and formaldehyde (Scheme I). These compounds have not been described earlier in the literature. All compounds (Table 1) gave satisfactory elemental analysis. IR and NMR spectra consistant with the assigned structure. All the synthesised compounds were tested for Anti-HIV activity against replication of HIV-1 (III B) and HIV-2 (ROD) in MT-4 cells.

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MATERIALS AND METHODS

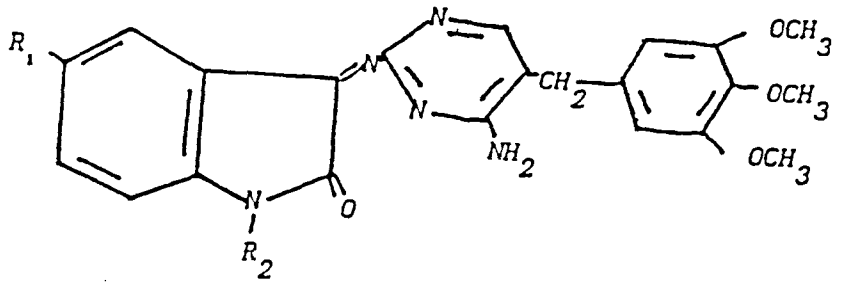
Melting points were taken in open capillary tubes on a Thomas Hoover melting point apparatus and are uncorrected. Infrared spectra were recorded on Jasco Infrared spectrometer in KBr. NMR spectra were recorded at 90 MHZ on a Jeol FX 90Q FT-NMR spectrometre using tetramethyl silane as the internal reference.

Pan A. synthesis of 3[4-amino, 5'-(3", 4", 5"-trimethoxybenzyl) pyrimidinyl] imino isatin.

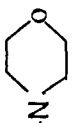
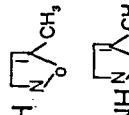
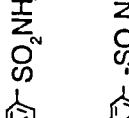
Equimolar quantities (0.06 moles) of isatin (8.82 g) and trimethoprim (17.4 g) were dissolved in 75 ml of warm alcohol containing 1 ml of glacial acetic acid. The reaction mixture was refluxed for 4 hours and set aside. The resulting solid was washed with dilute alcohol, dried and recrystallised from ethanol: chloroform mixture. Yield-24.5 g (96%); m.p.-180-185°; IR (KBr) – 1660 (C=N), 3050 (C-H), 1580 (C=C), 1620 (C=O), 3300 (NH); NMR ($CDCl_3$) δ ppm - (4, 5, 6, 7H) 7.0, (3H) 6.8, (6'H) 6.2, (4'NH_2) 5.8, 5 (CH_2) 4.2, (3", 5", OCH_3) 3.4, (4"OCH_3) 3.3 Anal ($C_{22}H_{21}N_5O_4$) C, H, N.

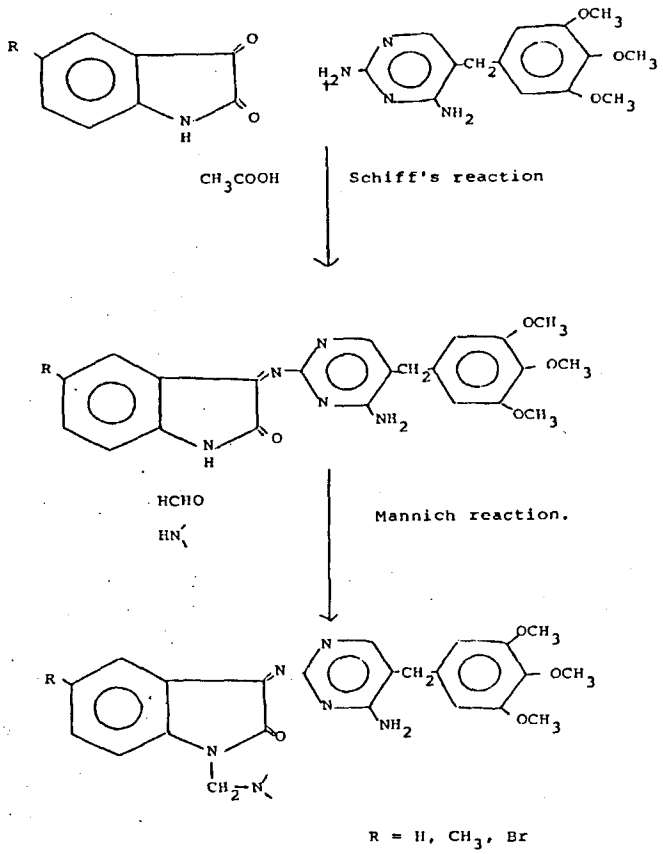
Pan I. Synthesis of I-(N, N-dimethylamino) methyl-5-methyl-3-[4'amino-(3", 4", 5"-trimethoxybenzyl) pyrimidinyl] imino isatin.

Table-1



Code	R_1	R_2	m.p. °C	Molecular Formula*	IR cm^{-1}
Pan. A	H	H	185	$C_{22}H_{21}N_5O_4$	1660, 3450, 3300, 1580
Pan. B	CH_3	H	232	$C_{23}H_{23}N_5O_4$	1670, 1590, 1620, 3405
Pan. C	Br	H	143	$C_{22}H_{20}N_5O_4Br$	1640, 3300, 1120, 3340
Pan. D	H	$-CH_2N(CH_3)_2$	205	$C_{25}H_{28}N_6O_4$	2970, 2890, 2850, 1660
Pan. E	H	$-CH_2N(C_2H_5)_2$	140	$C_{27}H_{32}N_6O_4$	2950, 2880, 2840, 1660
Pan. F	H	$--CH_2$-N(CH$_2$)$_4$	145	$C_{28}H_{32}N_6O_4$	3000, 2800, 2850, 1236
Pan. G	H	$-CH_2$-N(CH$_2$)$_4$	125	$C_{27}H_{30}N_6O_4$	1650, 2850, 2960
Pan. H	H	$-CH_2$-N(CH$_2$)$_2$O(CH$_2$)$_2$	125	$C_{27}H_{30}N_6O_5$	1230, 1210, 1660, 2870
Pan. I	CH_3	$-CH_2-N(CH_3)_2$	102	$C_{26}H_{30}N_6O_4$	2970, 2890, 2850
Pan. J	CH_3	$-CH_2-N(C_2H_5)_2$	105	$C_{28}H_{34}N_6O_4$	2850, 1660, 2910, 1236

Code	R_1	R_2	m.p. °C	Molecular Formula*	IR cm^{-1}
Pan. K	CH_3	$-CH_2-N$ 	109	$C_{29}H_{34}N_6O_4$	3000-2800, 2950, 2850
Pan. L	CH_3	$-CH_2-N$ 	96	$C_{28}H_{32}N_6O_4$	1660, 2850, 2960
Pan. M	CH_3	$-CH_2-N$ 	109	$C_{28}H_{32}N_6O_5$	1230, 1670, 2840
Pan. N	H	$-CH_2NH-$ 	121	$C_{33}H_{32}N_8O_7$	2850, 2860, 1360, 1160
Pan. O	CH_3	$-CH_2NH-$ 	195	$C_{34}H_{34}N_8O_7$	2840, 2950, 1340, 1660, 3240



Scheme I

To a slurry consisting of 5-methyl-3-[4'-amino-5'-(3", 4", 5"-trimethoxy benzyl) pyrimidinyl] imino isatin (0.04 moles), 50% ethanol and 37% formalin 1 ml was added to the dimethyl amine (0.04 moles) dropwise with cooling and shaking. The reaction mixture was allowed to stand at room temperature for one hour with occasional shaking. The solid which separated out was filtered and recrystallised from ether.

Yield (92%); m.p. - 230-232°; IR (KBr) -2850 (C-H str of CH_2) 1650 (C=N), 1120 (C-O-C); NMR($CDCl_3$) δ ppm - (1-2CH_3) 2.1, (1 CH_2) 1.8, (5CH_3) 1.1, (4'NH_2) 5.8,(2", 6"H)6.42 Anal ($C_{26}H_{30}N_6O_4$) C, H, N.

Anti-HIV Activity

Cell cultures

The MT-4 cells were grown in RPMI-1640 DM ("Dutch modification") medium (Flow Laboratories, Irvine,

Scotland), Supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and 20 µg/ml gentamicin (E. Merck, Darmstadt, F. R. G.). The cells were maintained at 37° in a humidified atmosphere of 5% CO_2 in air. Every 3-4 days, cells were spun down and seeded at $3x10^5$ cells/ml in new cell culture flasks. At regular time intervals, the MT-4 cells were analyzed for the presence of mycoplasma and consistently found to be mycoplasma-free.

Virus

HIV-1 (strain HTLV-III_B LAI)[9] and HIV-2 (strain LAV-2ROD)[10] were obtained from the culture supernatant of HIV-1 or HIV-2 infected MT-4 cell lines[11]. The virus titer of the supernatant was determined in MT-4 cells. The virus stocks were stored at -70° until used.

Anti-HIV assay

Flat bottom,96-well plastic microtiter plates (Falcon, Becton Dickinson, Mountain view, CA) were filled with 100 µl of complete medium using a Titertek Multidrop dispensor (Flow Laboratories). This eight-channel dispenser could fill a microtiter tray in less than 10s. Subsequently, stock solutions (10x 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 in the microtiter trays using a Biomek 1000 robot (Beckman). Untreated control HIV- and mock-infected cell samples were included for each compound.

50 µl of HIV at 100 $CCID_{50}$ medium was added to either infected or mock-infected part of a microtiter tray. Exponentially growing MT-4 cells were centrifuged for 5 min at 140xg and the supernatants were discarded.

The MT-4 cells were resuspended at $6x10^5$ cells/ml in a flask which was connected with an autoclavable dispensing cassette of a Titertek Multidrop dispenser. Under slight 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 a 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 were examined spectrophotometrically by the MTT method.

MTT assay

The MTT assay is based on the reduction of the yellow coloured 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium 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 plates 20 µl of a solution of MTT (7.5 mg/ml) in phosphate-buffered saline was added using the Titertek 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 M96 washer (ICN flow) without disturbing the MT-4 cell clusters containing the formazan crystals.

Solubilization 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 the M96 washer. Complete dissolution of the formazan crystals could be obtained after the trays had been placed on a plate shaker for 10 min. Finally, the absorbances were read in a eight-channel computer-controlled photometer (Multiskan MCC, ICN Flow) at two wavelengths (540 and 690 nm). The absorbance measured at 690 nm was automatically subtracted from the absorbance at 540 nm, so as to eliminate the effects of non-specific absorption. Blanking was carried out directly on the microtiter plates with the first column wells which contained all reagents except the MT-4 cells.

All data represent the average values for a minimum of three wells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of compound that reduced the absorbance (OD_{540}) of the Mock-infected control sample by 50%. The percent protection achieved by the compounds in HIV-infected cells was calculated by the following formula:

$$\frac{(OD_T)\ HIV - (OD_C)\ HIV}{(OD_C)\ mock - (OD_C)\ mock} \quad \text{expressed in \%}$$

whereby (OD_T) HIV is the optical density measured with a given concentration of the test compound in HIV infected cells; (OD_C) mock is the optical density measured for the control untreated mock infected cells; all OD values determined at 540 nm. The dose achieving 50%

Table-2 : Anti-HIV activity

Code	Strain	EC_{50}	EC_{90}	CC_{50}	Max. Protection
Pan. A	IIIB	>67	>67	67.4	0
	ROD	>78	>78	77.6	7
Pan. B	IIIB	>48	>48	47.8	4
	ROD	>62	>62	62	3
Pan. C	IIIB	>8	>8	7.7	3
	ROD	>8	>8	8.5	3
Pan. D	IIIB	>47	>47	47.3	0
	ROD	>48	>48	48.5	1
Pan. E	IIIB	>52	>52	51.9	7
	ROD	>59	>59	58.7	9
Pan. F	IIIB	>11	>11	11.2	2
	ROD	>12	>12	11.6	2
Pan. G	IIIB	>12	>12	11.5	0
	ROD	>10	>10	10.4	0
Pan. H	IIIB	>11	>11	10.6	0
	ROD	>11	>11	10.6	2
Pan. I	IIIB	>58	>58	57.9	46
		19.3	>50	49.8	59
		7.6	13	69.9	138
	ROD	>49	>49	49.3	1
Pan. J	IIIB	>56	>56	56.2	2
	ROD	>45	>45	45	4
Pan. K	IIIB	>59	>59	59.2	0
	ROD	>53	>53	52.7	0
Pan. L	IIIB	>54	>54	54	44
	ROD	>52	>52	51.6	3
Pan. M	IIIB	23	>55	55.3	53
		12.3	>59	59.5	74
	ROD	>51	>51	51.3	0
Pan. N	IIIB	>12	>12	11.8	0
	ROD	>11	>11	11.3	1
Pan O	IIIB	>12	>12	11.2	3
	ROD	>12	>12	12.3	6

protection according to the above formula was defined as the 50% effective concentration (EC_{50}).

RESULTS AND DISCUSSION

The synthesised compounds have been screened for anti-HIV activity and cytotoxicity (Table 2) against HIV-1 (III_B) and HIV-2 (ROD) replicating in acutely infected MT-4 cells. From the results, the selectivity indices (SI), ratio of 50% cytotoxicity concentration (CC_{50}) to 50% effective concentration (EC_{50}) were rather low [approximately 3.5 to 6] against HIV-1 (III_B) strain. Compound Pan. I has exhibited on average EC_{50} that was more than 58/13.5 and for compound Pan. M average EC_{50} was 17.7 and these two compounds were active. All other compounds were inactive against the replication of HIV-1 (III_B) and HIV-2 (ROD) at subtoxic concentration in MT-4 cells.

It seems the Mannich bases with dimethyl amino group and morpholino group are active. The pyrollidino group is also showing good activity. In the isatin molecule according to present study, substitution of methyl group produces active compound as compared to unsubstitution. Further molecular modification in the isatin molecule can lead to active compounds.

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