Synthesis of Some Cytidine Derivatives and Their Antibacterial Effects

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Summary: Reaction of cytidine-component of RNA-with alkylating agent (such as methyl iodide) and subsequent halogenation led to the synthesis of novel compounds which are expected to be molecular medicines, These have been identified and characterised by paper chromatography, UV and ^{1}H-NMR spectroscopy. Amongst them the 2′-O-methyl-5-bromo-cytidine (2′-O-m-5-Br-cytd) has shown significant inhibitory effects of some common pathogenic bacteria. The overall results are reported in this paper.

Introduction

The derivatives of nucleic acid bases and nucleosides have been synthesized by various workers in which extensive variations in purine, pyrimidine and pentose moieties have been made [1]. These derivatives as reviewed by the author [2] have been reported to be useful in therapy of cancer, infection against viruses e.g. herpes; retro-virus, varicella zoster virus etc. The citation for the design and syntheses of such derivatives also called analogs may be found in the review of Clercq and Torrence [1]. Amongst them the halogenated analogs of deoxynucleosides have been reported to inhibit the multiplication of DNA viruses and some retroviruses, too [3]. As for example 5-iodo-2′-deoxyuridine (Idoxuridine, IDU) is the first antiviral nucleoside synthesized by Prusoff in 1959 [4]. It is a prototype of pyrimidine nucleoside antiviral agent. It is used against herpes infection in man to prevent blindness. Bromination on deoxy series of nucleosides and nucleotides has been reported to be inhibitors of certain viruses e.g. 5-Bromo-2′-deoxycytidine, bromodeoxy cytidine (BrdCytd) has been studied against vaccinia viruses in the HeLa, chick embryo and rabbit kidney cells and herpes simplex virus (HSV) [1]. The same compound i.e. BrdCytd also proved to be a potent nematicide against *Meloidegine javanica,* [5]. We report in this paper the alkylation of cytidine with methyl iodide followed by subsequent bromination, using bromine. Bromination in cytidine of ribonucleoside in which the 2′- and 3′-OH functions have been ceased by methyl groups is first ever attempt in the area of pharmacodynamics of

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nucleoside analogs. The products i.e. methyl bromocytidine compounds (m-Br-Cytd) were studied against pathogenic microorganisms.

Results and Discussion

With reference to the known nucleoside i.e. cytidine it is possible to assign the structure of cytidine derivatives by a combination of UV and ^{1}H-NMR spectroscopy. Any perturbation of UV spectrum from that of unsubstituted cytidine is suggestive of a substitution at the base moiety [6,7]. The shift of anomeric proton of ribose to a lower field as compared to that of unsubstituted cytidine is indicative of ribose substitution in ^{1}H-NMR spectroscopy. The 2′-O-substitution being at a lower field as compared to 3′-O-substitution [8].

From the reaction of cytidine with methyl iodide the product corresponding to Rf 0.22in solvent 'A was obtained in 7 % yield as an amorphous powder. There was no shift in UV spectrum. The ^{1}H-NMR spectrum showed the anomeric proton at δ 5.72 which is shifted upfield as compared to the unreacted one indicating alkylation at 3′-OH group of ribose; further the singlet integrating for three methyl protons was observed at δ 3.4. This compound was therefore assigned as 3′-O- methyl cytidine (**2**). When this compound was brominated showed Rf value of 0.69 in solvent system A and was obtained in 40 % yield. Its UV spectrum was profoundly perturbed in both acidic and alkaline media when compared with unbrominated 3′-O-methylated cytidine. Perturbation of λ_{max} to the extent of 300 nm in 0.01 N HCI is reported to be diagnostic of the formation of 5-brominated cytidine [9]. It was conveniently assigned as 3′-O-m-5-Br.cytd.(**3a**). The antibacterial activity of this compound is shown in Table-1.

The product corresponding to Rf 0.25 in solvent A was obtained in 29 % yield as amorphous powder. Its UV spectrum was similar to unreacted cytidine (**1**) indicating the absence of alkylation in the base moiety. The anomeric proton of ribose was located at δ 6.03 and was shifted downfield as compared to that of unreacted cytidine a singlet integrating for three protons located at δ 3.52, substantiating the assignment as 2′-O-methyl cytidine (**3**) When this compound was brominated showed the highest Rf value in solvent A as 0.71. It was obtained in 70% yield. Its UV spectrum was also profoundly perturbed in both acidic and alkaline media with the same magnitude as that of the compound (**2a**), when compared with unbrominated (**3**). This compound was therefore assigned as 2′-O-methyl-5-bromo-cytidine (2'-O-m-5Br-Cytd) (**3a**). The antibacterial activity of this compound is shown in Table 1.

The product corresponding to Rf 0.40 in solvent A was obtained in 12% yield and showed the shift of λ_{max} to lower wave length in both acidic and basic pH. This shift corresponded in magnitude exactly to that of N^3 substituted cytidine [10,11]. The proton NMR spectrum exhibited a singlet at δ 3.20 integrating for three protons and the location of methyl signal and the shift in UV spectrum were enough to assign the compound as N^3-methyl cytidine (**4**).

When this compound was brominated showed Rf value of 0.65 in solvent A and was obtained in 30% yield. Its UV spectrum was also perturbed. The λ_{max} was shifted to 292 nm. acidic and 280 nm. in alkaline media. This shift was found to be characteristic for 5-bromo substitution, when compared with unsubstituted (**4**). It was assigned as N^3-m-5-Br-cytd.(**4a**). The antibacterial activity of this compound is shown in Table-1.

Table-1 Antibacterial Activity of Bromo-Alkylated Cytidine derivatives

Name of Microorganisms	Name of compounds Cytidine (1) (Control)	3′-0-m-5-Br Cytd. (2a)	2′-0-me-5-Br Cytd. (3a)	N^3-m-5-Br Cytd (4a)	Fortum Ceflaridine 30 µg	Sparaxin (Sparfloxacin) 5 ug	Rocephin (Ceftrioxane) 30 µg
Salmonella typhi	- ve	(1.0 mm)	(29 mm)	-ve	(20 mm)	(18 mm)	(30 mm)
Staphylococcus aureus	-ve	(1.0 mm)	(30 mm)	-ve	(9 mm)	(20 mm)	(14 mm)
Escherichia coli	-ve	(1.0 mm)	(23 mm)	-ve	(8mm)	-ve	(22 mm)
Proteus morganii	-ve	+ve negligible	(22 mm)	-ve	(9 mm)	(31 mm)	(45 mm)
Pseudomonas aeruginosa	-ve	+ve negligible	(21 mm)	-ve	-	-	-

The antibacterial activity was determined by measuring diameter of the zones in mm showing inhibition and growth inhibition was calculated with reference to control. Compounds (2a), (3a) and (4a) were also compared with known antibiotics such as Fortum, Sparaxin and Rocephin. Compound (3a) (2′-O-m-5-Br-Cyt) showed significant activity against *Salmonella typhi, Staphlococcus aureus, Escherichia coli, Proteus morganii* and *Pseudomonas aeruginosa.* The inhbition zones formed by this compound were significantly larger than that of known antibiotics (Table-1). This indicated that compound 2′-O-m-5-Br-cytidine (3a) is more potent than Fortum, Sparaxn and Rocephin.

Experimental

Standard chemicals such as cytidine, methyl iodide and solvents were purchased from B.D.H. England. Bromine ampules [F.W. 79.90] from Analar. Paper chromatographies were carried out on Whatman No.1 and No. 3. UV spectra were run in λ on a 4C Perking Elmer spectrophotometer. ^{1}H NMR spectra were taken on BRUKER-300 NMR spectrometer 5mg / 0,5 ml solution in D_2O. Solvents being A (Isopropanol: Ammonium hydroxide: Water 7:1:2 v/v), solvent B: (Ethanol: IM Ammonium acetate pH 7.4, 7:3 v/v) and solvent C: (*n*-Butanol :Water, 86:14v/v) were used for analytical as well as preparative paper chromatography. Ion exchange chromatography over Dowex 1x2 in the formate form was carried out in a glass column, fractions of 4 ml each were collected using 0. 1M formic acid.

Different pathogenic organisms such as *Salmonella typhi, Staphlococcus aureus, Eschericia coli, Pseudomonas aerogenosa* and *Proteus morganii* were obtained from the Department of Microbiology, University of Karachi. The organisms were checked for their purity and activity and were maintained on nutrient agar (Merck, Darmstadt, Germany) slants by sub-culturing every month. Test samples were sterilized on cellular acetate filter media with polypropylene housing 100 units:13 mm diameter 0.2 .μm pore size.

Alkylation of Cytidine

The reaction of alkylation was carried out by dissolving 500 mg of cytidine in a mixture of IN NaOH (5 ml) and I ,4-dioxane (5 ml) and allowed to stir it for 10-15 min. To this solution during stirring freshly distilled methyliodide (5 ml) was added and the stirring was continued at room temp. (25-30°C) for 4-5 hrs. The alkalinity of the reaction mixture was monitored constantly with the help of pH paper in order to maintain the reaction mixture in strongly alkaline condition throughout the course of reaction [10]. Paper chromatography using solvent A of the crude reaction mixture revealed the formation of three new compounds of higher mobilities having the Rf values of 0.22, 0.25 and 0.40 together with a small amount of unreacted cytidine at the base line comparable with the standard (unreacted) cytidine whose Rf was 0.15. These were visualised by using UV lamp, in dark room, equipped with wave lengths 254 nm and 366 nm. The reaction mixture was concentrated in vacuo to about 5 ml volume on a rotary evaporator at 37 °C. The products were separated by preparative paper chromatography in solvent A.

Preparative Paper Chromatography

The separation of the reaction mixture was carried out by loading 50 mg solids on each paper dissovled in 1-2 ml distilled water in the form of bands along with a spot of standard (unreacted) cytidine. Each paper was run in solvent A in a closed glass tank for 20-24 hrs. The paper was then dried in air and the bands were visualized under UV lamp at 254 nm. The bands faster than standard cytidine were marked and cut with the help of scissors into strips and eluted with water (20-25 ml) easily. It was then concentrated to minimum volume on a rotary evaporator at 40 °C. The concentrated samples were transferred to pre-weighed sample tubes and dried in desicator over $CaCl_2$. The drying completed in about 15 hours. The resulting compounds were amorphous.

3′-O-Methylcytidine (2)

The reaction of cytidine (1) with methyl iodide at room temperature resulted in the formation of three products of Rf 0.22, 0.25 and 0.40 in solvent A; 0.26, 0.36 and 0.60 in solvent B and 0.31, 0.48 and 0.64 in solvent C together with a small amount of unreacted cytidine (1) of Rf 0.15, 0.20 and 0.28 in solvents A, B and C respectively. On preparative paper chromatography the product of Rf 0.22 in solvent A was obtained as an amorphous powder (35 mg, 7 %) — UV (H_2O): pH 2.0 λ_{max} (ε) = 280 nm (10.4), λ_{min} 242 nm; pH 12.0 λ_{max} (ε) 270 nm, (10.1), λ_{min} 235 nm. ^{1}H NMR (D_2O): δ 5.72 (d, J=2.6 Hz, 1H), 3.47(s, 3H).

2'-O-Methylcytidine (3)

The product of Rf 0.25 in solvent A was obtained as an amorphous powder (145 mg, 29 %) - UV (H_2O): pH 2.0 λ_{max} (ε) 279 nm (12.1), λ_{min} 240 nm; pH 12.0 λ_{max} (ε) 270 nm (8.0), λ_{min} 235 nm. ^{1}H-NMR (D_2O): δ 6.03 (d,J=2.5 Hz, 1H), 3.52 (s, 3H).

N^3-Methylcytidine (4)

The product of Rf 0.40 in solvent A was obtained as amorphous solid (60 mg, 12 %)- UV (H_2O) pH 2.0 λ_{max} (ε) 276 nm (12.0), λ_{min} 240nm; pH 12.0 λ_{max} (ε) 267nm (8.2) λ_{min} 250 nm. ^{1}H-NMR (D_2O): δ 6.0 (d, J=3.0 Hz, IH), 3.20 (s, 3H).

Bromination of Methylated Cytidine

Bromination occurs in purine and pyrimidine nucleosides and nucleotides invariably at 8 and 5 positions respectively [12,13]. It was carried out on methylated cytidine by the same method given by Grunberg-Monago and Michelson for cytidine [14].

Bromination of (2)

To 25 mg of (2) in a mixture of 15 µl 0.2N HNO_3, and 50 µl 1,4-dioxane was added 12.5 µl bromine. The reaction mixture was kept in dark for overnight. After 18 hrs the reaction mixture was evaporated to dryness *in vacuo*. To the residue was added 10 ml ethanol and again evaporated. The nucleoside products were then taken in ether. Ether was also evaporated and the 5-bromo derivative of methylated cytidine (2a) was collected. The compound (2a) was separated and purified from unreacted (2) on Dowex 1x2 colunm in formate form. The elution solvent was 0.1M formic acid. Appropriate fractions were combined and dried *in vacuo* over $CaCl_2$ in cold. The 3'-O-methyl-5-bromocytidine (2a) showed Rf 0.69, 0.70 and 0.35 in solvent systems A, B and C respectively. UV (H_2O): pH 2.0 λ_{max} (ε) 298 nm (6.8), λ_{min} 251 nm; pH 12.0 λmax (ε) 286nm (6.1), λ_{min}251nm.

Bromination of (3)

To 100 mg (3) in a mixture of 60 µl 0.2 N HNO_3, 200 µl 1,4-dioxane was added 50 µl bromine and was kept in dark for overnight. After 18 hrs the reaction mixture was worked up and purified the product 2'-O-methyl-5-bromocytidine (3a) as for (2a). It showed Rf 0.71, 0.80 and 0.40 in solvent A, B and C respectively. UV (H_2O): λ_{max} (ε); pH 2.0 300nm (5.14), λ_{min} 253 nm, pH 12.0 λ_{max} (ε) 286 nm (4.25), λ_{min} 262 nm.

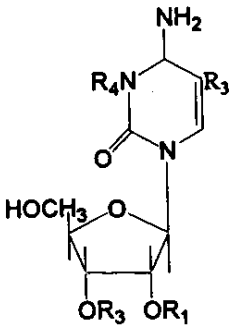

Figure No.

(1)	R_1 = H, R_2 = H, R_3 = H, R_4 = H	Cytidine
(2)	R_1 = H, R_2 = CH_3, R_3 = H, R_4 = H	3' – O – methyl – Cytidine
(3)	R_1 = CH_3, R_2 = H, R_3 = H, R_4 = H	2' – O – methyl – Cytidine
(4)	R_1 = H, R_2 = H, R_3 = H, R_4 = CH_3	N^3 – methyl Cytidine
(2a)	R_1 = H, R_2 = CH_3, R_3 = Br, R_4 = H	3' – O – methyl – 5 – bromocytidine
(3a)	R_1 = CH_3, R_2 = H, R_3 = Br, R_4 = H	2' – O – methyl – 5 – bromocytidine
(4a)	R_1 = H_2, R_2 = H, R_3 = Br, R_4 = CH_3	N^3 – methyl – 5 – bromocytidine

Bromination of (4)

To 50 mg (4) in a mixture of 30 µl 0.2N HNO_3, 100 µl 1,4-dioxane, was added 25 µl bromine. The reaction mixture was kept in dark for overnight. After 18 hrs the reaction mixture was worked up and purified the product N^3-methyl-5-bromocytidine (4a) like (2a). It showed Rf 0.69, 0.59 and 0.31 in solvent A, B and C respectively. UV (H_2O): λ_{max} (ε): pH 2.0, 292 nm (6.50), λ_{min} 253 nm, pH 12.0 λ_{max} (ε) 280 nm (6.13), λ_{min} 250 nm.

Pathogenecity

In order to ascertain the antibacterial activity of the novel compounds (2a, 3a, 4a) were tested against pathogenic microorganisms by Agar Well Diffusion method of Daba *et al.* [15]. Wells were precisely punched onto the surface of nutrient agar

plates using sterile metallic borer. The resulting agar buttons were removed and a drop of soft agar (1 % w/v) was placed to plug the button. Following which lawns of the test organisms duly grown in nutrient broth were made by using sterile swabs. The wells were filled with 100 µl of 0.1 mM solution of sterilized test samples of methyl-bromo-cytidine (m-Br-cytd) **(2a, 3a, 4a)** with methyl-cytidine **(2,3,4)** to act as control. The plates were incubated at 37 °C for 24 hrs. The zones of inhibitions formed were measured in mm and are show in Table 1.

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