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QUARTERLY

N_4-Amino-acid derivatives of 6-azacytidine: Structure–activity relationship*

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Several N_4-derivatives of 6-azacytidine were synthesized using of Vorbrüggen's condensation method. Their antiviral activity with respect to the adenovirus serotypes 2 and 5 in Hep-2 cells culture was studied and primary specific activity was determined. Correlation between chemical structure of new 6-azacytidine derivatives and their biological properties is discussed.

6-Azacytidine (6-azaCyd) is a structural cytidine analogue showing anticancer, antimycoplasmal and antiviral effects [1–7].

Structural parameters, such as the nature of the heterocyclic base, carbohydrate moiety and absolute configuration, are all recognized as important for biological activity. It was interesting to compare effects of modifications of heterocyclic base and sugar moiety. This paper deals with the structure–activity relationship of 6-azaCyd, 6-azacytosine (6-azaCyt) and their N_4-substituted derivatives, in order to establish their inhibitory effect on adenovirus reproduction. In this connection we performed the modification in C_4-position by various amino acids whose side chains had different structures and glycoside modifications.

MATERIALS AND METHODS

The following reagents were used: 6-azauracil from "Biolar" (Latvia), 6-azacytosine from "Calbiochem", 4-thio- and 4-methyl-

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Abbreviations: Adh, human adenovirus; 6-azaCyd, 6-azacytidine; 6-azaCyt, 6-azacytosine; CTI, chemotherapeutic index; HMDS, hexamethyldisilazane; MIC, minimal inhibitory concentration; MTD, maximal tolerated dose; TCS, trimethylchlorosilane; TMS, tetramethylsilane.

thio-6-azauracils, synthesized according to previously described procedures [8, 9], and 1-*O*-acetyl-2′,3′,5′-tri-*O*-benzoyl-ribofuranose prepared by the procedure of guanosine acidolysis.

Hexamethyldisilazane (HMDS), trimethylchlorosilane (TCS) and tin tetrachloride ($SnCl_4$) were from "Reachim" (Russia); solvents (acetonitrile, methylene chloride, 1,4-dioxane) were purified and dried under reflux over phosphorus pentoxide; calcium hydride was distilled and stored over 3 Å molecular sieves.

Flash chromatography was carried out on silica gel 60 (40–63 μm, Merck) and analytical thin-layer chromatography on precoated plates of silica gel F_{254} (Merck) in the following solvent systems: A — 1-butanol/acetic acid/water (5:2:3, by vol.), B — chloroform/methanol (9:1, v/v). The spots were visualized with UV light (254 nm). Melting points were determined on a Kofler apparatus and are uncorrected. ^{1}H-NMR spectra were recorded with a Varian VXR-300 instrument. The UV absorption spectra were obtained on "Specord M-40" spectrometer (Germany). All compounds showed correct elementary analysis data (± 0.3%).

Determination of antiadenovirus activity of N_4-substituted analogues of 6-azaCyd

In this study we used reference strains of human adenovirus (Adh) types 2, 5 and Hep-2 cell culture. Antiadenovirus activity was determined by a method developed in our laboratory, based on calculation of infected cells carrying DNA-containing inclusion bodies.

Cells were grown in tubes with strips of cover glasses in a medium consisting of equal volumes of medium-199 and Eagle medium, supplemented with 10% (v/v) heat-inactivated bovine serum. After 48 h cells were infected with Adh-2 or Adh-5, further, after 60 min adsorption of virus at room temperature cells were washed with Hanks' solution and preincubated in maintenance medium (Eagle medium without serum) carrying new test compounds of varying concentrations. Forty eigth hours following infection the cells were fixed with 96% alcohol, washed with Hanks' solution, stained with 0.01% acridine orange solution and counted under ML-2 (LOMO) luminescent microscope. Control cells were infected but not treated with test substances. Infected cells were identified by the presence of specific intranuclear inclusions. On each of 3 slides 500 cells were counted and the percentage of infected cells between them determined. The compound's ability to inhibit virus reproduction was assessed based on reduction of percentage of infected cells in treated *vs.* untreated cultures.

The minimal inhibitory concentration (MIC) of each compound was its dose decreasing the percentage of infected cells by 50%. The maximal tolerated dose (MTD) was determined by a cytomorphological method. The chemotherapeutic index (CTI) is the ratio of MTD to MIC.

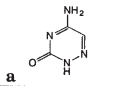
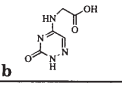
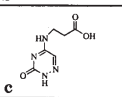
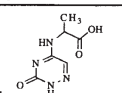
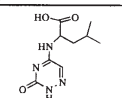
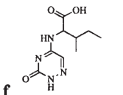
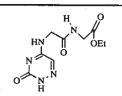
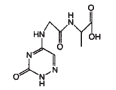
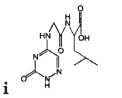
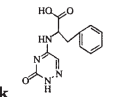
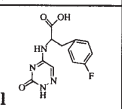
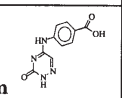
RESULTS AND DISCUSSION

Chemistry

To introduce amino-acid residues into the azapyrimidine molecule we used the reaction of nucleophylic substitution of 4-methylmercapto group in the heterocycle by an amino component [10]. N_4-Substituted 6-azacytosines were obtained by reaction of 1 equiv. of 4-methylmercapto-6-azauracil with 1.5 equiv. of amino acid and 1.5 equiv. NaOH in aqueous medium at 22°C with satisfactory (55–85%) yields. Unfortunately, new 6-azaCyt derivatives dissolved very poorly in water. This property complicated the study of their biological activity. Spectroscopic properties of 6-azaCyt N_4-derivatives are presented in Table 1.

Further investigation involved glycosylation of 6-aza-bases and modification of new nucleosides. Stereospecific formation of the

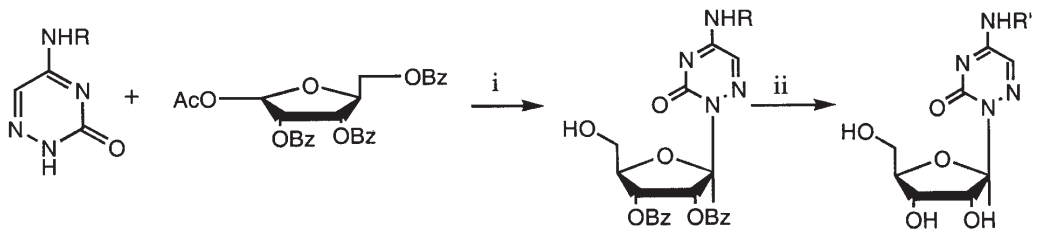
Table 1. Chemical shifts of selective protons in Me_2SO-d_6 (δ in p.p.m. from TMS)

Compounds	$\lambda_{max}^{H_2O}$	N_1 - H	N_4 - H	C_5 - H	NH - amide
 a	258	11.390	7.734, 7.685	7.347	—
 b	276	11.971	8.557	7.552	———
 c	277	11.716	8.612	7.475	———
 d	275	11.969	8.584	7.514	———
 e	279	11.986	8.541	7.474	———
 f	280	11.962	8.408	7.590	———
 g	274	11.950	8.640	7.553	8.531
 h	275	11.943	8.450	7.539	8.432
 i	276	11.905	8.510	7.545	8.606
 k	279	11.996	8.631	7.494	———
 l	278	11.990	8.609	7.491	———
 m	329	12.360	10.560	7.640	———

glycoside bond with β-configuration was the key step of 6-azanucleoside synthesis, using a simplified, one step stereo-selective approach described by Vorbrüggen [11]. The condensation of ethyl esters of N_4-substituted 6-azacytosines (1) with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (acetonitrile, 60–80°C, 6–8 h) led to formation of the corresponding acylnucleosides 2 [10] (Scheme 1).

$SnCl_4$ – a catalyst providing a stereo-selective formation of β-nucleosides was used in quantity 2.0–2.4 equiv. *vs.* ribose. The subsequent unblocking of the sugar moiety led to final compounds 3. Some of their characteristics are described in Table 2.

By transformation of the carbohydrate moiety of compound **3a** we obtained 2′-deoxy-6-azaCyd (2′d6-azaCyd), 2′,3′-didehydro-2′,3′-dideoxy-6-azacytidine (4d6-azaCyd) [12] and N_1-tetrahydrofuranyl-6-azacytosine (N_1-fur-6-azaCyt), synthesized as previously described [13].



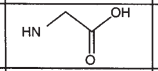
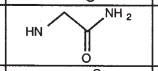
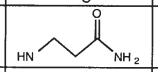
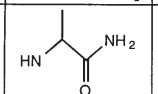
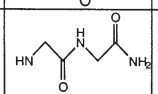
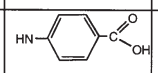
1a: R=H
Ethyl
ester 1 b-d, g, m 2 a-d, g, m 3
a, b-g, m
comp.
i: 1-O-acetyl-2,3,5-tri-O-benzoyl-ribofuranose (1 eq.), TMSCl (1.6 eq.), HMDS (0.8 eq);
ii: NH_3 in MeOH or 0.1 N NaOMe / MeOH.

Scheme 1

Spectroscopy

The data obtained from NMR spectra confirmed the glycoside bond formation in compounds **3**. Substitution at the NH_2 group of 6-azaCyd apparently caused a significant splitting of amino proton signal of compounds **3** at downfield (Table 2). That explained increased acid properties of nucleosides **3** ($\Delta\delta$**3b–d** were 0.6–0.7 p.p.m). It was interesting to notice that introduction of an aryl residue into the amino group (compound **3m**) results in the maximal splitting of proton – 2.79 p.p.m.

Table 2. Some characteristics of synthesized nucleosides

Compound	R'	M.p., °C	Chemical shift of selective protons (δ, ppm from TMS)				UV-spectra in H_2O λ_{max}(log ε)
			of aglycon			of ribose	
			N_4-H	C_5-H	amidic proton 1 & 2	Anomeric proton $C_{1'}$-H (j, H_2)	
3a	NH_2	225-227	8.018 7.901	7.508	—	5.977 (3.9)	264 (3.90)
3b		82-84	8.664	7.638	—	5.980 (3.9)	269 (3.92)
3b'		108-110	8.684	7.670	7.553 7.171	5.979 (3.9)	271 (3.96)
3c		124-126	8.611	7.535	7.417 6.929	5.976 (3.9)	275 (3.95)
3d		115-117	8.651	7.567	7.446 7.344	5.975 (3.9)	272 (4.02)
3g		191-193	8.744	7.678	7.242 7.044 8.330	5.973 (3.9)	272 (3.85)
3m		275-276	10.80	7.766	—	6.024	320 (4.23)

We believe the latter to be due to N_4-amino proton participation in an intramolecular hydrogen bond [14, 15]. This may be the cause of decreasing biological activity of some N_4-derivatives, as compared with 6-azaCyd (**3c, 3g, 3m** in Table 2).

Biology

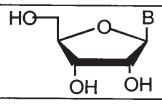
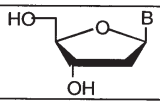
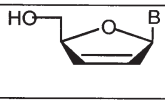
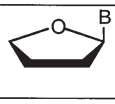
Biological activities were examined *in vitro* by following inhibition of reproduction of Adh types 2 and 5 in Hep-2 cell culture. It allowed to detect not only a compound's primary specific activity, but also to reveal some features of structure–activity relationship. Of interest was the influence of amino-acid side chains structure and carbohydrate moiety structure on biological activity of the new compounds. Selection of amino acids was made according to their primary structure and physiological activity. The modified bases (Table 1) showed no antiadenoviral activity. The structure–activity relationship of nucleosides is very well illustrated by the data presented in Tables 3 and 4.

The biological activity of N_4-amino acid-substituted derivatives of new ribonucleosides was dependent on the size and structure of the amino-acid substituents (Table 3). Those amino-acid substituents having a natural structure, i.e. with the carboxyl and amino groups in the closest proximity, allowed to retain 6-azaCyd antiviral activity (**3a** = **3b′** > **3b** > **3d**), while those having an unnatural struc-

Table 3. Effect of 6-azacytidine and its N_4-substituted derivatives on the Adh2 reproduction in Hep-2 cell culture

Compound	MEC, μg/ml	MIC, μg/ml	CTI
3a	125	1	125
3b	1000	125	8
3b′	>1000	8	125
3c	>125	absent	–
3d	>1000	250	4
3g	>1000	absent	–
3m	>250	250	>1

Table 4. Influence of 6-azacytosine glycosides on the human adenovirus type 5 reproduction in Hep-2 cell culture (% of inhibition)

Compounds				
Concentration, μg/ml				
125	100	100	100	5
16	100	100	100	0
8	100	41	57	0
4	100	—	—	0
2	100	16	15	0
0.5	61	0	0	0

B – 6-azaCyt

ture, such as β-alanine (**3c**) or *p*-aminobenzoic acid (**3m**), caused lowering or removal of antiviral activity. It should be noticed that amide introduction into the amino acid carboxyl group of **3b** (resulting in **3b′**) caused a significant increase of activity and allowed to retain CTI of 6-azaCyd (Table 3).

The results also demonstrated the contribution of the sugar moiety to biological activity (Table 4).

The most profitable 6-azaCyd conformation is *syn*. It is stabilized by the following hydrogen bonds: O5′H...O2; C1′H...N6; O3′H... O2′H [16]. 6-AzaCyd sugar moiety modifications without aglycon changes did not lead to a loss of biological activity, provided the furanous ring structure and 5′-OH group remained intact. However, substitution of the carbohydrate fragment by a furane ring resulted in a complete loss of inhibitory effect (Table 4).

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