ADENOSINE N^1-OXIDE ANALOGUES AS INHIBITORS OF ORTHOPOX VIRUS REPLICATION

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Dedicated to Professor Antonín Holý on the occasion of his 70th birthday in recognition of his outstanding contributions to the area of organic and medicinal chemistry.

Several new types of adenosine N^1-oxide (ANO) derivatives including N^1-alkoxy and N^6-alkyl as well as the analogues with a trihydroxycyclopentane ring in place of the ribose residue were synthesized and their antiviral properties were evaluated in Vero and LLC-MK2 cell cultures infected with vaccinia, mousepox, monkeypox, cowpox, and different isolates of smallpox viruses. The antiviral activity of ANO and its derivatives significantly depended on the virus type and cell cultures. Mousepox and monkeypox viruses were the most sensitive to these compounds, while vaccinia and cowpox viruses were inhibited at the concentrations 1–1.5 orders of magnitude higher. The toxicity of the synthesized compounds was much lower than that of ANO. Modifications of the ANO N^6-position did not offer any advantages over the parent compound. The synthesized N^1-oxide derivatives of noraristeromycin retained the activity comparable with noraristeromycin and displayed a decreased toxicity. No direct correlation between antiviral activity and stability of the compounds was found.

Keywords: Nucleosides; Purines; *N*-Oxides; Carbocyclic nucleosides; Noraristeromycin; Antivirals; Pox viruses.

The prophylaxis and treatment of the disease induced by smallpox virus are among the most topical problems of modern medicine and science. Since the vaccination was discontinued more than 25 years ago, only few individuals are now protected against this infection. The concern about the use of smallpox virus as a weapon for bioterrorism or biowarfare has greatly increased the interest in compounds capable of inhibiting the orthopox virus replication. The data on the antipox virus activity of compounds of various

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structures were recently summarized in reviews by De Clercq[1], Baker et al.[2], and Smee et al.[3] Of the tested compounds, adenosine N^1-oxide (**1**) and some of its N^1-benzyloxy derivatives were shown to exhibit a significant activity against vaccinia virus in a cell culture assay and in a mouse model of vaccinia-virus-induced tailpox lesion[4–6]. Kane and Shuman[7] showed that adenosine N^1-oxide inhibits vaccinia virus replication *in vitro* by selectively blocking the translation of viral mRNA, although the mechanism was undetermined. We describe in this work the synthesis of four new types of ANO analogues, namely, N^1-alkoxy (**2a**–**2e**) and N^6-alkyl (**3a**–**3e**) of ANO as well as ANO carbocyclic analogues **4** and **5a**, **5b** (Chart 1) and their antipox virus properties evaluated in cell cultures infected with vaccinia, monkeypox, cowpox, and smallpox viruses.

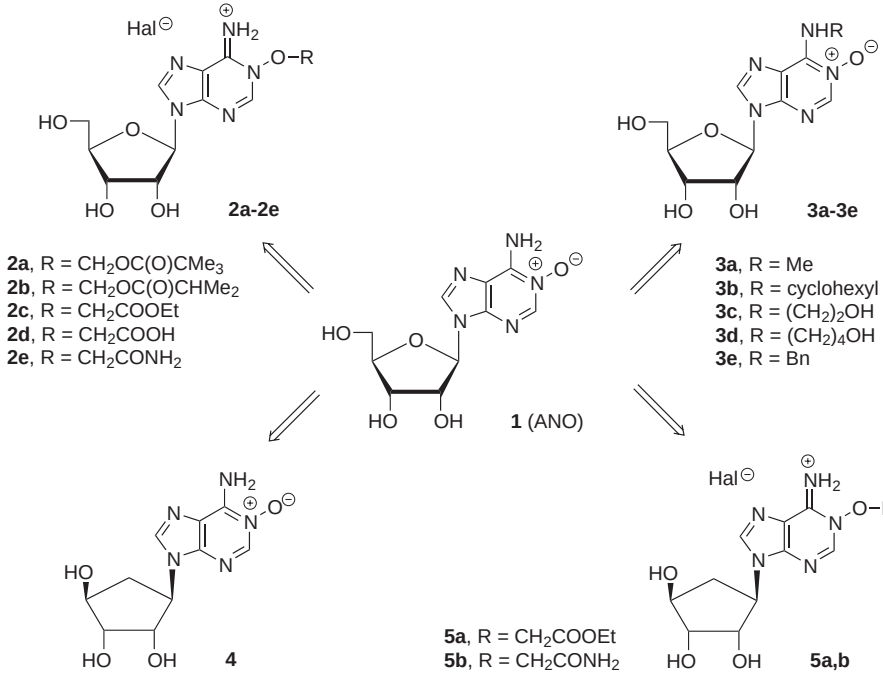
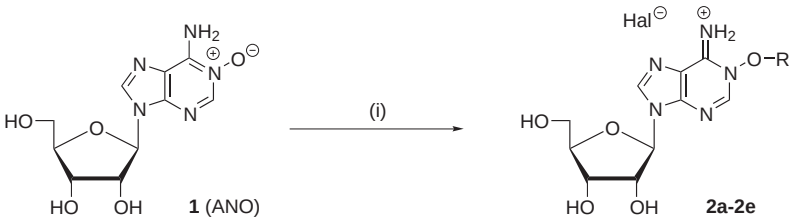


CHART 1

RESULTS AND DISCUSSION

Esters **2a** and **2b** were obtained by alkylation of ANO (**1**) with (pivaloyloxy)methyl chloride or (butyryloxy)methyl chloride, respectively, in DMF similarly to the method described in a previously published procedure[5]. As compound **2b** was partially degraded in the process of reversed-phase chro-

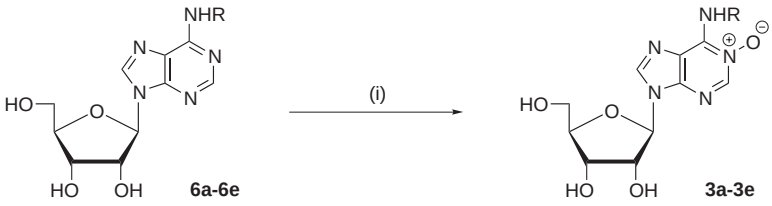
matography to give ANO, we purified it by dissolution in ethanol and subsequent precipitation with chloroform. Esters **2c**–**2e** were synthesized in a similar manner using the corresponding derivatives of haloacetic acids (Scheme 1). Esters **2** were isolated as halides (chlorides in the case of **2a**, **2b**, bromide **2c** and iodide **2e**). Compound **2d** was isolated in the form of inner salt. It is noteworthy that amide **2e** was unstable during isolation; the product of degradation was adenosine.



2	R	reagent (i)
a	$CH_2OC(O)CMe_3$	$ClCH_2OC(O)CMe_3$
b	$CH_2OC(O)CHMe_2$	$ClCH_2OC(O)CHMe_2$
c	CH_2COOEt	$BrCH_2COOEt$
d	CH_2COOH	ICH_2COOH
e	CH_2CONH_2	ICH_2CONH_2

SCHEME 1

6-Alkyl N^1-oxides **3a**–**3e** were obtained by oxidation of the corresponding N^6-alkylpurines **6a**–**6e** with 3-chloroperbenzoic acid in aqueous methanol (Scheme 2). Nucleosides **6b**–**6d** were prepared in high yields by treating 6-chloropurine with the corresponding amines in aqueous dioxane. Compounds **6a**, **6e** were purchased from Fluka. The yields of target **3a**–**3e** ranged from 45 to 65%.



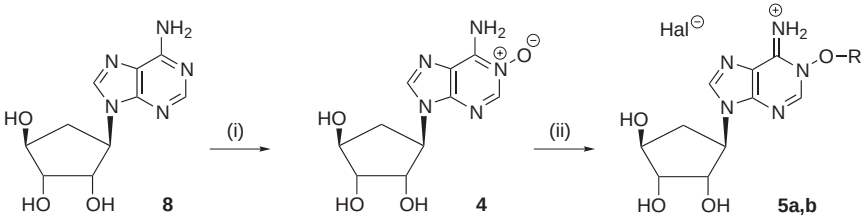
3	R
a	Me
b	cyclohexyl
c	$(CH_2)_2OH$
d	$(CH_2)_4OH$
e	Bn

(i) 3-chloroperbenzoic acid, H_2O/MeOH

SCHEME 2

A similar oxidation of N^6-disubstituted purines (6-morpholinopurine riboside and N^6,N^6-dimethyladenosine) gave inosine as a major product and N^1-hydroxyinosine (**7**) as a minor component. The structure of **7** was confirmed by physicochemical methods (UV, NMR and elemental analysis) as well as by oxidation of inosine with 3-$ClC_6H_4CO_3H$ and by deamination of ANO with sodium nitrite in acid conditions similarly to ref.[8]

Oxidation of a cyclopentene adenosine analogue (±)-5′-noraristeromycin (**8**) smoothly afforded N^1-oxide **4** (Scheme 3). As expected, alkylation of N^1-oxide **4** with pivaloyloxymethyl chloride yielded the corresponding N^1-ester. However, unlike ester **2a**, its carbocyclic analogue was very unstable during chromatography, and we failed to isolate it in pure state. Although the reaction of N^1-oxide **4** with bromoacetic ester or (iodomethyl)-acetamide proceeded effectively, the overall yield of esters **5a**, **5b** was rather low because of purification losses.



3	R
a	CH_2COOEt
b	CH_2CONH_2

(i) 3-chloroperbenzoic acid, H_2O/MeOH
(ii) $BrCH_2COOEt$ for **5a** or ICH_2CONH_2 for **5b**

SCHEME 3

The synthesized compounds were tested in Vero and LLC-MK2 cell cultures infected with vaccinia, mousepox virus, monkeypox virus, cowpox virus, and different isolates of smallpox virus. The results are summarized in Tables I–III. The representative data from a single experiment for compounds **2a** and **5a** are shown in Figs 1 and 2. As one can see from the tables, the antiviral activity of parent ANO and its derivatives varied significantly with the virus type and cell culture. Mousepox virus and monkeypox virus were most susceptible to ANO (IC_{50} 0.001–0.05 μg/ml), whereas the inhibition of vaccinia and cowpox virus replication required a 100–150-fold increase in the ANO concentrations (IC_{50} 0.4–0.7 μg/ml). The data for vaccinia virus agree well with ANO inhibitory concentrations (>0.32 μg/ml) reported earlier by Kwong et al.[5]

In the assay with vaccinia virus, compounds **2a**, **2c** were about 35–80 times more active than ANO. The activities of compounds **2a**–**2c** were at

least one order of magnitude higher against monkeypox virus compared with ANO in Vero cell culture. Except **2c**, modifications of this type did not have any advantages over ANO against cowpox virus. The other compounds of this type showed similar or lower activity when compared with ANO. The toxicity assays showed essential advantages of the synthesized compounds, as their toxicities were 10–20-fold lower in non-infected Vero cells compared with the parent compound.

Compounds of type **3** present another type of ANO modifications. It was shown earlier that proper modifications of the N^6-position of purine-based agents led to highly active inhibitors of vaccinia and cowpox viruses[9,10]. However, the potency of compounds **3**, in which the N^6-amino group is

TABLE I
Antipox virus activity of the synthesized compounds in assay with Vero cells

Compd	CD_{50} μg/ml	IC_{50}, μg/ml			
		vaccinia	mousepox	monkeypox	cowpox
1	10	0.7	0.001	0.05	0.4
2a	100	0.01	0.007	0.006	14.4
2b	70	1.1	0.001	0.003	4.0
2c	>200	0.02	0.013	0.003	0.012
2d	>200	0.13	0.1	0.04	0.3
2e	30	0.4	0.4	0.3	1.3
3a	>200	0.7	0.6	0.28	0.8
3b	>100	23.2	35	24.7	>100
3c	>200	1.8	1.9	1.7	>100
3d	>200	1.5	0.6	1.9	49.0
3e	>200	2.2	2.4	2.2	6.3
4	100	0.75	0.6	0.57	21.0
5a	>200	0.1	0.2	0.03	1.6
5b	100	0.4	0.4	0.16	0.3
7	>200	16	6.4	23	>100
8	10	0.05	0.01	0.14	0.4

IC_{50}, the concentration that causes a 50% reduction in virus replication; CD_{50}, the concentration that causes a 50% inhibition of cell growth. Averages of the data from three independent experiments. Experimental error 40–60%.

substituted with an alkyl group, was lower than that of ANO towards all the tested viruses, although they were non-toxic up to the concentration of 200 μmol/l.

Some interesting results were obtained for noraristeromycin derivatives **4** and **5a**, **5b**. 5′-Noraristeromycin (**8**) is known to be active against a wide spectrum of viruses including the pox virus family[11–13] and is targeted at cellular *S*-adenosyl-L-homocysteine (SAH) hydrolase, which is crucial for RNA maturation[14]. SAH hydrolase inhibitors may serve as promising antiviral agents. Some known SAH hydrolase inhibitors were active *in vitro* against a broad spectrum of viruses including vaccinia virus but not against cowpox virus[6]. Herein, we showed that compounds **5a**, **5b** obtained by modifying the N^1-atom of noraristeromycin, exhibit the similar activity in Vero cell culture against all the tested viruses including cowpox virus com-

TABLE II
Antipox virus activity of the synthesized compounds in the assay in LLC-MK2 cells

Compd	CD_{50} μg/ml	IC_{50}, μg/ml			
		vaccinia	mousepox	monkeypox	cowpox
1	14	3.6	0.03	3.7	4.2
2a	32	6.0	0.1	0.6	9.7
2b	24	32	0.06	0.2	3.4
2c	94.5	10.5	0.624	3.6	23
2d	>200	105	0.2	17	24
2e	100	70	11	8.5	21
3a	23	20	0.5	3.0	5.7
3b	>100	>100	>100	>100	>100
3c	>200	>100	>100	>100	>100
3d	>200	>100	18.6	41	16
3e	>200	>100	>100	>100	>100
4	>100	85	9.0	>100	>100
5a	>200	>100	2.7	1.0	>100
5b	>200	0.4	0.2	0.1	>100
7	>200	>100	>100	16.52	22
8	>100	>100	0.5	0.8	18

For symbols, see Table I.

TABLE III
Activities and cytotoxicities of the selected compounds against smallpox virus strains in Vero cell culture

Compd	CD_{50} μg/ml	India 3a		6-58		Congo-9		Butler	
		IC_{50} μg/ml	IS	IC_{50} μg/ml	IS	IC_{50} μg/ml	IS	IC_{50} μg/ml	IS
1	~10	0.01	1110	0.004	2270	0.001	10000	0.007	1538
2a	~200	0.008	25000	0.008	25000	0.007	28570	0.005	40000
2b	70	0.004	17500	0.003	10000	0.003	10000	0.006	5000
4	>100	0.13	>770	0.47	>215	0.8	>120	1.3	>80
8	10	0.01	1000	0.044	227	0.044	227	0.04	250

For symbols, see Table I. IS = CD_{50}/IC_{50}

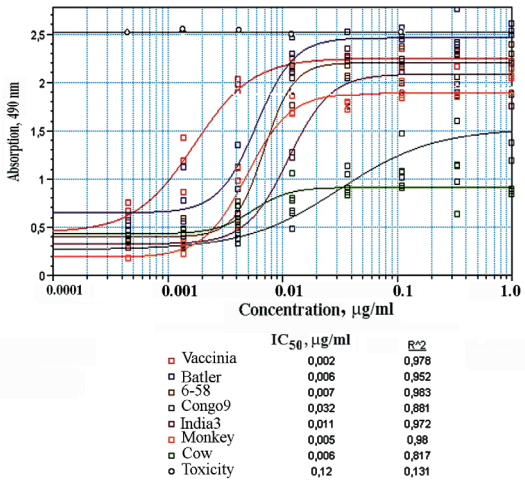


FIG. 1
Representative data from a single experiment on the efficacy of N^1-[(pivaloyloxy)methoxy]-adenosine chloride (**2a**) in Vero cells infected with various pox viruses. IC_{50} values (μg/ml) for each virus are shown below the graph. R^2 are point deviations to the curve

pared with parent **8** (Table I). It should be also noted that compounds **5a**, **5b** showed decreased toxicity towards non-infected Vero cell culture.

The synthesized compounds were also tested in LLC-MK2 cell culture (Table II). A comparison of Tables I and II demonstrates that drug efficacies and cellular toxicities varied considerably and depended both on the virus type and cell line. As a rule, the synthesized compounds showed a much lower efficacy in LLC-MK2 cell line compared with the results for Vero cells. Compounds **2a**, **2b**, which inhibited monkeypox virus in LLC-MK2 cells with the activity higher than the parent compound, were an exception. These data partially correlate with the earlier published data, where it was shown that 1-[(2,4-difluorobenzyl)oxy]adenosine perchlorate (DFBA) manifested a significant activity against poxvirus family in Vero cells, but not in LLC-MK2 cells[2].

The selected compounds were also tested in Vero cell culture against a number of smallpox virus strains (Table III). The efficacy of esters **2a**, **2b** in these experiments was higher than that of the reference ANO due to both lower toxicity and slightly higher activity. At the same time, carbocyclic derivative **4** showed a decreased activity compared with the reference 5′-noraristeromycin for the tested virus strains, although it was tenfold less toxic.

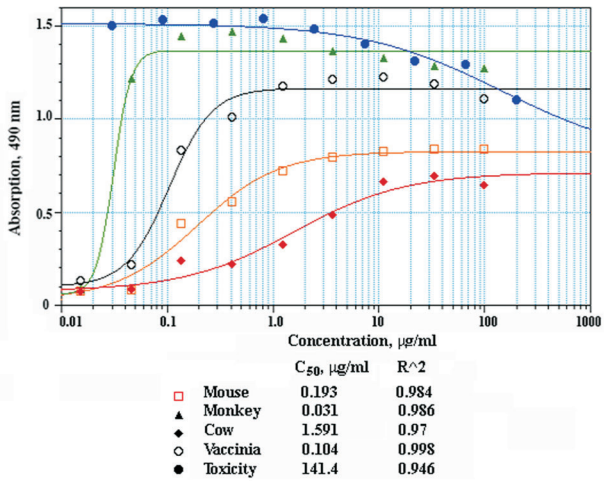


FIG. 2

Representative data from a single experiment on the efficacy of N^1-[(ethoxycarbonyl)-methoxy]-9-[(1β,2α,3α,4β)-2,3,4-trihydroxycyclopentyl]adenine bromide (**5a**) in Vero cells infected with various pox viruses. For other symbols, see legend to Fig. 1

We assumed that the synthesized compounds can be depot forms of ANO (**2a**–**2e**) or noraristeromycin (**5a**, **5b**), which underwent intracellular hydrolysis to release parent ANO or noraristeromycin, respectively. The HPLC analysis of product composition after chemical or enzymatic hydrolysis of **2a** and **5a**, **5b** confirmed that the major hydrolysis products were ANO and noraristeromysin. The half-life ($t_{0.5}$) of ester **2a** in the phosphate buffer (pH 7.0, 37 °C) was about 2.5 h, whereas for **2c** it fell to 20 min (TLC and ^{1}H NMR monitoring). In 100% human blood serum, $t_{0.5}$ of **2a** was only 10 min, with ANO as a major hydrolysis product. Carbocyclic ester **5a** was stable in PBS, but its half-life in human blood serum did not exceed 15 min, and the major hydrolysis products were *N*-oxide **4** and noraristeromycin. These data allow a suggestion that these compounds are depot forms of ANO and noraristeromycin, respectively. A higher activity of **2a** might be accounted for by a better cellular uptake due to its higher hydrophobicity and intracellular degradation to ANO, which manifests significant antipox virus activity[5]. At the same time, antiviral activity of compound **2c** against different pox viruses in Vero cell culture is rather high, although it is hydrolyzed to give a mixture of products, in which a portion of ANO is considerably lower compared with **2a**. A comparison of antiviral activity and stability of the compounds in different media did not enable a direct activity–stability correlation. The data obtained imply that the mechanism of action of different N^1-oxide derivatives may vary according to their structures. One cannot exclude that some ANO derivatives are phosphorylated to give the corresponding triphosphates that might be incorporated into viral RNA and block its translation similar to the mechanism described earlier for ANO[7]. It is also noteworthy that most of the synthesized compounds are markedly less toxic than the parent compound.

EXPERIMENTAL

N^6-Methyladenosine (**6a**), N^6-benzyladenosine (**6e**), inosine and 3-chloroperbenzoic acid were from Fluka; pyridine and DMF were from Aldrich. Column chromatography was performed on Silica gel 60 (40–63 μm); reversed-phase chromatography was carried out on LiChroprep RP-8 and LiChroprep RP-18 (25–40 μm) (Merck); HPLC was performed on a Gilson chromatograph (France) supplied with an LKB 2220 integrator. Nucleosil 100 C-18 (5 μm) column (4 × 150 mm) and two eluting systems were used: (a) Buffer A: 5 mM NaH_2PO_4 buffer, pH 5.4; buffer B: 70% acetonitrile; gradient: 0–5 min, 0% B; 5–7 min, 0–10% B; 7–20 min, 10–20% B; 20–35 min, 20–70%; 35–40 min, 70–100% B; the flow rate was 0.5 ml/min. (b) Buffer A: H_2O; buffer B: 100% ethanol; gradient: 0–5 min, 0% B; 5–30 min, 0–30% B; 30–40 min, 30–32% B; 40–45 min, 32–100% B; the flow rate was 0.5 ml/min.

NMR spectra were recorded on an AMX III-400 spectrometer (Bruker) with the working frequency 400 MHz for ^{1}H NMR (Me_4Si as an internal standard for organic solvents and sodium 3-(trimethylsilyl)propane-1-sulfonate for D_2O) and 101 MHz for ^{13}C NMR. Chemical shifts (δ-scale) are given in ppm, coupling constants (*J*) in Hz. UV spectra (λ in nm) were recorded on a Shimadzu UV-1201 spectrophotometer (Japan) in methanol or H_2O at pH 7.0. Elemental analysis was performed on a Thermo Finigan (CHNS) analyzer, model EA1112 (Italy).

N^1-[(Pivaloyloxy)methoxy]adenosine Chloride (**2a**)

(Pivaloyloxy)methyl chloride (200 μl, 1.39 mmol) was added to a suspension of ANO (130 mg, 0.46 mmol) in DMF (2 ml), and the mixture was stirred at 20 °C for 18 h. The solution was quenched with water (10 ml), evaporated in vacuum, diluted with water (2 ml) and applied onto a column LiChroprep RP-18 eluted with water. The target fractions were evaporated and lyophilized to give 155 mg (78%). UV (MeOH): λ_{max} 258. ^{1}H NMR (DMSO-d_6): 10.56 s and 9.94 s, 2 H (NH_2); 8.97 s, 1 H (H-8); 8.82 s, 1 H (H-2); 5.95 m, 3 H (H-1′ and OCH_2O); 5.62 br s, 1 H (2′-OH); 5.34 br s, 1 H (3′-OH); 5.09 br s, 1 H (5′-OH); 4.49 m, 1 H (H-2′); 4.15 m, 1 H (H-3′); 3.99 m, 1 H (H-4′); 3.63 m, 2 H (H-5′); 1.19 s, 9 H ($(CH_3)_3$). ^{13}C NMR (D_2O): 180.1 (C=O); 149.7 (C-6); 146.1 (C-4); 144.8 (C-8 and C-2); 120.0 (C-5); 93.5 (OCH_2O); 89.4 (C-1′); 86.0 (C-4′); 74.9 (C-2′); 70.6 (C-3′); 61.6 (C-5′); 39.2 (CMe_3); 26.5 (Me_3). For $C_{16}H_{24}ClN_5O_7 \cdot 0.5H_2O$ (442.9) calculated: 43.42% C, 5.69% H, 15.82% N; found: 43.53% C, 5.81% H, 15.75% N.

N^1-[(Isobutyryloxy)methoxy]adenosine Chloride (**2b**)

(Isobutyryloxy)methyl chloride (200 μl, 1.50 mmol) was added to a suspension of ANO (130 mg, 0.46 mmol) in DMF (2 ml), and the mixture was stirred at 20 °C for 18 h. The solution was diluted with CCl_4 (5 ml) and cooled to 0 °C. After 18 h, the oil-like residue was separated and dissolved in ethanol (1 ml). The solution was quenched with chloroform (10 ml), and the mixture was stirred at 0 °C for 18 h. The precipitate was filtered off, dried in vacuum, dissolved in water (5 ml), and lyophilized to give 38 mg (20%) of the product. UV (MeOH): λ_{max} 258. ^{1}H NMR (DMSO-d_6): 10.54 s and 9.96 s, 2 H (NH_2); 9.04 s, 1 H (H-8); 8.83 s, 1 H (H-2); 5.95 d, 1 H, *J* = 5.3 (H-1′); 5.93 s, 2 H (OCH_2O); 5.65 br s, 1 H (2′-OH); 5.38 br s, 1 H (3′-OH); 5.12 br s, 1 H (5′-OH); 4.50 m, 1 H (H-2′); 4.16 m, 1 H (H-3′); 4.00 m, 1 H (H-4′); 3.63 m, 2 H (H-5′); 2.72 m, 1 H (CH (i-Pr)); 1.19 d, 6 H, *J* = 6.9 (Me_2). For $C_{15}H_{22}ClN_5O_7 \cdot 0.25H_2O$ (424.3) calculated: 42.47% C, 5.34% H, 16.51% N; found: 42.56% C, 5.41% H, 16.74% N.

N^1-[(Ethoxycarbonyl)methoxy]adenosine Bromide (**2c**)

(Ethoxycarbonyl)methyl bromide (165 μl, 1 mmol) was added to a suspension of ANO (300 mg, 0.9 mmol) in DMF (5 ml), and the mixture was stirred at 20 °C for 18 h. The solution was diluted with CCl_4 (5 ml) and cooled to 0 °C. After 18 h, the oil-like residue was separated and dissolved in ethanol (1 ml). The solution was quenched with chloroform (10 ml), and the mixture was stirred at 0 °C for 18 h. The precipitate was filtered off, dried in vacuum, dissolved in water (5 ml), and lyophilized to give 78 mg (21%) of ester **2c**. UV (H_2O): λ_{max} 258 (ε 14500). ^{1}H NMR (DMSO-d_6): 10.48 s and 9.79 s, 2 H (NH_2); 9.09 s, 1 H (H-2); 8.81 s, 1 H (H-8); 5.94 d, 1 H, *J* = 5.6 (H-1′); 5.62 m, 1 H (2′-OH); 5.35 m, 1 H

(3′-OH); 5.12 s, 2 H (OCH_2); 5.11 m, 1 H (5′-OH); 4.46 m, 1 H (H-2′); 4.25 q, 2 H, *J* = 7.2 ($OC\mathbf{H}_2CH_3$); 4.55 m, 1 H (H-3′); 3.99 m, 1 H (H-4′); 3.66–3.58 m, 2 H (H-5′); 1.25 t, 3 H, *J* = 7.2 ($OCH_2C\mathbf{H}_3$). For $C_{14}H_{20}BrN_5O_7 \cdot 0.5H_2O$ (459.3) calculated: 36.63% C, 4.61% H, 15.26% N; found: 36.86% C, 4.72% H, 15.05% N.

N[1]-(Carboxymethoxy)adenosine (**2d**)

Iodoacetic acid (70 mg, 0.45 mmol) was added to a suspension of ANO (100 mg, 0.3 mmol) in DMF (3 ml), the mixture was stirred at 20 °C for 18 h and evaporated. The residue was dissolved in water applied onto a LiChroprep RP-18 column and eluted with water to give 31% (30.6 mg) of product **2d**. UV (H_2O): λ_{max} 258 (ε 14500). ^{1}H NMR (D_2O): 8.97 s, 1 H (H-2); 8.51 s, 1 H (H-8); 6.08 d, 1 H, *J* = 5 (H-1′); 4.92 s, 2 H (OCH_2); 4.66 m, 1 H (H-2′); 4.40 m, 1 H (H-3′); 4.22 m, 1 H (H-4′); 3.66–3.88 m, 2 H (H-5′). For $C_{12}H_{15}N_5O_7 \cdot 1.5H_2O$ (368.3) calculated: 39.16% C, 4.92% H, 19.02% N; found: 39.23% C, 5.04% H, 18.85% N.

N[1]-(Carbamoylmethoxy)adenosine Iodide (**2e**)

Carbamoymethyl iodide (80 mg, 0.5 mmol) was added to a suspension of ANO (130 mg, 0.4 mmol) in DMF (2 ml), and the mixture was stirred at 20 °C for 18 h and evaporated. The residue was dissolved in water, applied onto a LiChroprep RP-18 column and eluted with 4% MeOH to give 44% (66.2 mg) of the product. UV (H_2O): λ_{max} 258 (ε 14500). ^{1}H NMR (CD_3OD): 9.10 s, 1 H (H-2); 8.71 s, 1 H (H-8); 6.10 d, 1 H, *J* = 5 (H-1′); 5.15 s, 2 H (OCH_2); 4.62 m, 1 H (H-2′); 4.36 m, 1 H (H-3′); 4.16 m, 1 H (H-4′); 3.77–3.81 m, 2 H (H-5′). For $C_{12}H_{17}IN_6O_6 \cdot 0.75H_2O$ (481.7) calculated: 29.92% C, 3.87% H, 17.45% N; found: 29.73% C, 3.83% H, 17.59% N.

9-[(1β,2α,3α,4β)-2,3,4-Trihydroxycyclopentyl]adenine-1-oxide (**4**)

A suspension of (±)-5′-norariste romycin (**8**) (50 mg, 0.2 mmol), DMF (5 ml), MeOH (5 ml) and H_2O (5 ml) was heated under stirring until complete dissolution. 3-Chloroperbenzoic acid (250 mg, 1 mmol) was added to the solution, and the mixture was stirred at 20 °C for 18 h. The solution was evaporated up to the volume of 2 ml, diluted with water (20 ml), evaporated to the volume 2 ml, and diluted with water (20 ml). The filtrate was evaporated and chromatographed on a LiChroprep RP-18 column eluted with water. The yield of the product was 84% (44.7 mg). UV (H_2O): λ_{max} 262 (ε 14500). ^{1}H NMR (DMSO-d_6): 8.59 s, 1 H (H-8); 8.35 s, 2 H (H-2); 5.17 m, 5.03 m and 4.91 m, 3 H (3 × OH); 4.69 m and 4.48 m, 2 H (H-2′ and H-3′); 3.90 m and 3.76 m, 2 H (H-1′ and H-4′); 2.63 m and 1.82 m, 2 H (H-5′). For $C_{11}H_{15}N_5O_4 \cdot 0.5H_2O$ (290.3) calculated: 45.56% C, 5.56% H, 24.15% N; found: 45.63% C, 5.68% H, 24.22% N.

N[1]-[(Ethoxycarbonyl)methoxy]-9-[(1β,2α,3α,4β)-2,3,4-trihydroxycyclopentyl]adenine Bromide (**5a**)

(Ethoxycarbonyl)methyl bromide (15 μl, 0.13 mmol) was added to a suspension of **4** (32 mg, 0.12 mmol) in DMF (1.5 ml), and the mixture was stirred at 20 °C for 18 h, evaporated in vacuum of oil pump without heating, and diluted with ethyl acetate. The resulting precipitate was washed with ethyl acetate, dissolved in water, and lyophilized to give 76% of compound **5a**. UV (H_2O): λ_{max} 262 (ε 14500). ^{1}H NMR (DMSO-d_6): 10.40 br s and 9.71 br s, 2 H

(NH_2); 9.05 s and 8.64 s, 2 H (H-2 and H-8); 5.25 m, 1 H (OH); 5.11 s, 2 H (O-CH_2-COO); 5.09 m and 5.06 m, 2 H (2 × OH); 4.77 m and 4.49 m, 2 H (H-2′ and H-3′); 4.21 q, 2 H, *J* = 7.2 (C**H**$_2$$CH_3$); 3.93 m and 3.77 m, 2 H (H-1′ and H-4′); 2.63 m and 1.76 m, 2 H (H-5′); 1.24 t, 3 H (CH_2C**H**$_3$). For $C_{15}H_{22}BrN_5O_6 \cdot 0.75H_2O$ (461.8) calculated: 39.03% C, 5.13% H, 15.17% N; found: 39.28% C, 5.21% H, 14.96% N.

N^1-(Carbamoylmethoxy)-9-[(1β,2α,3α,4β)-2,3,4-trihydroxycyclopentyl]adenine Iodide (**5b**)

Carbamoylmethyl iodide (25 mg, 0.13 mmol) was added to a suspension of **4** (32 mg, 0.12 mmol) in DMF (2 ml), and the mixture was stirred at 20 °C for 18 h, evaporated, dissolved in water, and chromatographed on a LiChroprep RP-18 column eluted with water. Yield 57%. UV (H_2O): λ_{max} 262 (ε 14500). ^{1}H NMR (DMSO-d_6): 9.85 br s, 2 H (NH_2); 9.03 s and 8.45 s, 2 H (H-2 and H-8); 7.65 s and 7.09 s, 2 H ($CONH_2$); 5.22 m, 5.09 m and 5.02 m, 3 H (3 × OH); 4.65 s, 2 H (O-CH_2-COO); 4.48 m, 3.92 m, 3.83 m and 3.76 m, 4 H (H-1′, H-2′, H-3′ and H-4′); 2.62 m and 1.74 m, 2 H (H-5′). For $C_{13}H_{19}IN_6O_5 \cdot 0.75H_2O$ (479.8) calculated: 32.55% C, 4.30% H, 17.52% N; found: 32.71% C, 4.36% H, 17.41% N.

Synthesis of *N*^{6}-Substituted Adenosines **6**. General Procedure

Alkylamine (1.0 mmol) and water (0.1 ml) were added to a suspension of 6-chloropurine riboside (57.5 g, 0.2 mmol) in dioxane (3 ml) and the suspension was stirred at room temperature. After 3 h, the clear solution was evaporated, and the residue was diluted with methanol–ether (1:1 for **6b**, 1:5 for **6c**, and 1:3 for **6d**). After 18 h at +4 °C, the supernatant was decanted, evaporated, and repeatedly treated with the methanol–ether solution. Combined precipitates were purified by reverse-phase chromatography and lyophilized.

N^6,*N*^{6}-Dimethyladenosine and 6-morpholinopurine riboside were prepared in a similar manner from 6-chloropurine riboside and dimethylamine or morpholine, respectively.

N^6*-Cyclohexyladenosine chloride* (**6b**): Yield ca. 90%. UV (MeOH): λ_{max} 269 (ε 15400). ^{1}H NMR (CD_3OD): 8.23 s and 8.14 s, 2 H (H-2 and H-8); 5.98 d, 1 H, *J* = 5.8 (H-1′); 4.70 t, 1 H, *J* = 5.5 (H-2′); 4.26 dd, 1 H, *J* = 5.3 and 3.9 (H-3′); 4.21 q, 1 H, *J* = 3.5 (H-4′); 3.80 dd, 1 H, *J* = 3.1 and 12.6 (H-5′a); 3.72 dd, 1 H, *J* = 3.8 and 12.6 (H-5′b); 1.96 br s, 1 H (cyclohexyl); 1.65–1.49 m, 4 H (cyclohexyl); 1.42–1.19 m, 6 H (cyclohexyl). For $C_{16}H_{24}ClN_5O_4 \cdot 0.25H_2O$ (300.4) calculated: 49.25% C, 6.32% H, 17.95% N; found: 49.16% C, 6.21% H, 18.04% N.

N^6*-(2-Hydroxyethyl)adenosine chloride* (**6c**): Yield ca. 90%. UV (MeOH): λ_{max} 267 (ε 16200). ^{1}H NMR (CD_3OD/D_2O): 8.25 s and 8.23 s, 2 H (H-2 and H-8); 5.96 d, 1 H, *J* = 6.2 (H-1′); 4.74 t, 1 H, *J* = 5.1 (H-2′); 4.32 dd, 1 H, *J* = 5.4 and 2.5 (H-3′); 4.17 q, 1 H, *J* = 2.7 (H-4′); 3.88 dd, 1 H, *J* = 2.5 and 12.5 (H-5′a); 3.74 dd, 1 H, *J* = 2.8 and 12.5 (H-5′b); 3.77 m, 4 H (CH_2O and CH_2N (hydroxyethyl)). For $C_{12}H_{17}N_5O_5 \cdot 0.75HCl \cdot 0.5H_2O$ (347.6) calculated: 41.55% C, 5.44% H, 20.19% N; found: 41.76% C, 5.53% H, 20.44% N.

N^6*-(4-Hydroxybutyl)adenosine chloride* (**6d**): Yield ca. 90%. UV (MeOH): λ_{max} 269 (ε 16000). ^{1}H NMR (CD_3OD/D_2O): 8.26 s and 8.22 s, 2 H (H-2 and H-8); 5.98 d, 1 H, *J* = 6.5 (H-1′); 4.73 t, 1 H, *J* = 6.1 (H-2′); 4.33 dd, 1 H, *J* = 5.1 and 2.6 (H-3′); 4.22 q, 1 H, *J* = 2.5 (H-4′); 3.88 dd, 1 H, *J* = 2.8 and 12.8 (H-5′a); 3.74 dd, 1 H, *J* = 2.9 and 12.8 (H-5′b); 3.62 m, 4 H (CH_2O and CH_2N (hydroxybutyl)); 1.75 m and 1.67 m, 4 H (CH_2CH_2 (hydroxybutyl)). For $C_{14}H_{22}ClN_5O_5 \cdot 0.25H_2O$ (380.3) calculated: 44.23% C, 5.96% H, 18.42% N; found: 44.11% C, 5.88% H, 18.54% N.

Synthesis of N^6-Substituted Derivatives **3**. General Procedure

3-Chloroperbenzoic acid (100 mg, 40–63%, ~0.3 mmol) was added to a suspension of N^6-alkyladenosine (0.1 mmol) in aqueous methanol (1 ml). The reaction mixture was stirred at 37 °C for 18 h, then an excess of 3-chloroperbenzoic acid (50 mg, ~0.15 mmol) was added, and the reaction solution was stirred at 37 °C for another 24 h. The reaction mixture was evaporated and the residue was partitioned between water and ethyl acetate. The aqueous layer was evaporated and the residue was chromatographed on a reverse-phase LiChroprep RP-18 column in a gradient of acetonitrile in 0.05 M NH_4HCO_3. The target **3a**–**3e** were repeatedly purified by reverse-phase chromatography and lyophilized.

Oxidations of N^6,N^6-dimethyladenosine, 6-morpholinopurine riboside and inosine were carried out in a similar fashion. Isolation of hydroxyinosine **7** was performed similarly to the isolation of *N*-oxides **3**.

N^6-Methyladenosine N^1-oxide (**3a**): Yield 53%. UV (H_2O): λ_{max} 234 (ε 35000) and 269 (ε 9000). ^{1}H NMR (D_2O): 8.56 s and 8.44 s, 2 H (H-2 and H-8); 6.10 d, 1 H, *J* = 5.3 (H-1′); 4.74 t, 1 H, *J* = 5 (H-2′); 4.44 dd, 1 H, *J* = 4.4 and 5.0 (H-3′); 4.27 q, 1 H, *J* = 3.6 (H-4′); 3.91 dd, 1 H, *J* = 3.1 and 12.8 (H-5′a); 3.83 dd, 1 H, *J* = 4.1 and 12.8 (H-5′b); 3.54 s, 3 H (Me). For $C_{11}H_{15}N_5O_5 \cdot 0.5H_2O$ (306.3) calculated: 43.18% C, 5.26% H, 22.88% N; found: 43.36% C, 5.30% H, 22.69% N.

N^6-Cyclohexyladenosine N^1-oxide (**3b**): Yield 56%. UV (MeOH): λ_{max} 237 (ε 32000) and 273 (ε 10500). ^{1}H NMR (CD_3OD): 8.50 s and 8.37 s, 2 H (H-2 and H-8); 5.95 d, 1 H, *J* = 5.7 (H-1′); 4.70 t, 1 H, *J* = 5.3 (H-2′); 4.27 dd, 1 H, *J* = 5.4 and 3.9 (H-3′); 4.20 q, 1 H, *J* = 3.5 (H-4′); 3.79 dd, 1 H, *J* = 3.1 and 12.5 (H-5′a); 3.72 dd, 1 H, *J* = 3.9 and 12.5 (H-5′b); 1.97 m, 1 H (cyclohexyl); 1.70 m, 2 H (cyclohexyl); 1.57 m, 2 H (cyclohexyl); 1.41 m, 4 H (cyclohexyl); 1.20 m, 2 H (cyclohexyl). For $C_{16}H_{23}N_5O_5 \cdot 0.25H_2O$ (369.9) calculated: 51.97% C, 6.40% H, 18.94% N; found: 52.18% C, 6.46% H, 18.82% N.

N^6-(2-Hydroxyethyl)adenosine N^1-oxide (**3c**): Yield 45%. UV (H_2O): λ_{max} 234 (ε 31500) and 269 (ε 9800). ^{1}H NMR (D_2O): 8.44 s and 8.30 s, 2 H (H-2 and H-8); 5.96 d, 1 H, *J* = 6.2 (H-1′); 4.71 t, 1 H, *J* = 5.1 (H-2′); 4.32 t, 1 H, *J* = 4.4 (H-3′); 4.12 q, 1 H, *J* = 3.6 (H-4′); 4.06 t, 2 H, *J* = 5.5 (CH_2N (hydroxyethyl)); 3.76 dd, 1 H, *J* = 3.1 and 12.8 (H-5′a); 3.73 t, 2 H, *J* = 5.5 (CH_2O (hydroxyethyl)); 3.68 dd, 1 H, *J* = 3.9 and 12.8 (H-5′b). ^{13}C NMR (D_2O): 158.23 (C-6); 150.72 (C-4); 145.00 (C-2); 144.06 (C-8); 122.66 (C-5); 89.29 (C-1′); 86.30 (C-4′); 74.68 (C-2′); 71.31 (C-3′); 62.10 (C-5′); 60.61 (CH_2O (hydroxyethyl)); 40.11 (CH_2N (hydroxyethyl)). For $C_{12}H_{17}N_5O_6 \cdot 0.5H_2O$ (336.3) calculated: 42.89% C, 5.40% H, 20.84% N; found: 43.15% C, 5.51% H, 20.75% N.

N^6-(4-Hydroxybutyl)adenosine N^1-oxide (**3d**): Yield 62%. UV (H_2O): λ_{max} 235 (ε 31000) and 271 (ε 9600). ^{1}H NMR (CD_3OD/D_2O): 8.43 s and 8.30 s, 2 H (H-2 and H-8); 5.96 d, 1 H, *J* = 5.3 (H-1′); 4.70 t, 1 H, *J* = 5 (H-2′); 4.28 t, 1 H, *J* = 5.7 (H-3′); 4.12 q, 1 H, *J* = 4 (H-4′); 3.91 t, 2 H, *J* = 6.9 (CH_2N (hydroxybutyl); 3.77 dd, 1 H, *J* = 3.1 and 12.8 (H-5′a); 3.74 dd, 1 H, *J* = 4 and 12.8 (H-5′b); 3.49 t, 2 H, *J* = 6.7 (CH_2O (hydroxybutyl)); 1.65 m and 1.51 m, 4 H (CH_2CH_2 (hydroxybutyl)). ^{13}C NMR (CD_3OD/D_2O): 158.56 (C-6); 150.86 (C-4); 145.93 (C-2); 144.81 (C-8); 122.56 (C-5); 89.41 (C-1′); 86.48 (C-4′); 74.82 (C-2′); 71.44 (C-3′); 62.39 (C-5′); 58.75 (CH_2O (hydroxybutyl)); 39.14 (CH_2N (hydroxybutyl)); 26.07 and 29.33 (CH_2CH_2 (hydroxybutyl)). For $C_{14}H_{21}N_5O_6 \cdot 0.5H_2O$ (364.4) calculated: 46.18% C, 6.08% H, 19.24% N; found: 46.31% C, 6.13% H, 19.10% N.

N^6-Benzyladenosine N^1-oxide (**3e**): Yield 65%. UV (MeOH): λ_{max} 233 (ε 36500) and 271 (ε 8600). ^{1}H NMR (CD_3OD): 8.56 s and 8.35 s, 2 H (H-2 and H-8); 7.19–7.33 m, 5 H (Ph); 5.97 d, 1 H,

J = 6 (H-1′); 5.12 br s, 2 H (C**H**$_2$Ph); 4.64 t, 1 H, *J* = 5.3 (H-2′); 4.30 t, 1 H, *J* = 5 (H-3′); 4.14 q, 1 H, *J* = 4 (H-4′); 3.81 dd, 1 H, *J* = 3.2 and 12.8 (H-5′a); 3.72 dd, 1 H, *J* = 3.8 and 12.8 (H-5′b). For $C_{17}H_{19}N_5O_5 \cdot 0.25H_2O$ (377.9) calculated: 54.06% C, 5.20% H, 18.54% N; found: 53.97% C, 5.13% H, 18.76% N.

N^1-Hydroxyinosine (**7**): Yields < 5% (based on N^6,N^6-dimethyladenosine), 10% (based on 6-morpholinopurine riboside), 24% (oxidation of inosine), and ca. 60% (deamination of ANO [6]). UV (H_2O): λ_{max} 229 (ε 35000) and 257 (ε 8500). ^{1}H NMR (D_2O): 8.35 s and 8.15 s, 2 H (H-2 and H-8); 5.94 d, 1 H, *J* = 5.9 (H-1′); 4.69 t, 1 H, *J* = 5 (H-2′); 4.34 dd, 1 H, *J* = 4.4 and 5.2 (H-3′); 4.17 q, 1 H, *J* = 3.5 (H-4′); 3.81 dd, 1 H, *J* = 2.8 and 12.9 (H-5′a); 3.83 dd, 1 H, *J* = 4 and 12.9 (H-5′b). ^{13}C NMR (D_2O): 163.03 (C-6); 151.58 (C-4); 144.96 (C-2); 144.04 (C-8); 125.45 (C-5); 89.24 (C-1′); 86.26 (C-4′); 74.62 (C-2′); 71.07 (C-3′); 62.06 (C-5′). For $C_{10}H_{12}N_4O_6 \cdot 0.75NH_3 \cdot 0.25H_2O$ (301.5) calculated: 39.84% C, 4.93% H, 22.07% N; found: 39.75% C, 4.84% H, 22.21% N.

Antiviral Activity

All virus strains and cell cultures were obtained from the collection of State Research Center of Virology and Biotechnology “Vektor”, Koltsovo, Novosibirsk Region. The synthesized compounds were screened against cowpox (Grishak strain), monkeypox (Zair 599 strain), mousepox virus (K-1 strain), vaccinia virus (strain LIVP) used for vaccination of world population, and variola major (lethality 30–40%) of different geographical origin: India 3a (India), 6–58 (Pakistan), Congo-9 (Africa), and Butler variola minor (lethality 1–2%).

Antiviral activity of the compounds was tested similarly to the earlier described procedure[2]. Briefly, antiviral compounds were dissolved in DMSO or water to a concentration of 20 mg/ml followed by serial dilutions with RPMI media and added to 96-well microlitre plates containing Vero or LLC-MK2 cells. Final drug concentrations ranged from 100 to 0.0005 μg/ml. At each drug concentration, three wells were infected with 10^5 pfu/well of orthopox virus, while three wells were left uninfected for toxicity tests. The plates were incubated in a CO_2 incubator at 37 °C for 5 days, the medium-drug mixture was aspirated and cells were stained with Neutral Red as described earlier[2]. The absorbances were read at 490 nm on a Bio-tek plate reader. The drug concentrations that reduced cell viability by 50% (CD_{50}) and their antiviral efficacy (IC_{50}) were calculated using the software program (Molecular Devices, Menlo Park, U.S.A.).

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