Bioorganic & Medicinal Chemistry 17 (2009) 4961–4967

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Reaction of the antitumor antibiotic olivomycin I with aryl diazonium salts. Synthesis, cytotoxic and antiretroviral potency of 5-aryldiazenyl-6-*O*-deglycosyl derivatives of olivomycin I

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ARTICLE INFO

Article history:
Received 22 April 2009
Revised 25 May 2009
Accepted 31 May 2009
Available online 6 June 2009

Keywords:
Olivomycin I
Arylazo coupling reaction
Antitumor antibiotic
Antiretroviral activity

ABSTRACT

The azo coupling of the antibiotic olivomycin I (**1**) with aryl diazonium tetrafluoroborates produced 5-aryldiazenyl-6-*O*-deglycosyl derivatives of **1**. The structures of new compounds were confirmed by ^{1}H NMR and mass spectrometry analysis. A quantum-chemical study was performed to analyze the possible directions of electrophilic substitution of **1** and the easiness of 6-*O*-disaccharide hydrolysis in the course of azo coupling. The antiproliferative and anti-retroviral activities of novel derivatives were studied.

1. Introduction

Olivomycin I (olivomycin A, Scheme 1, **1**) is a member of the aureolic acid family of antibiotics, a group of agents that also includes mithramycin and chromomycin A3.[1] The members of the aureolic acid family interact with high GC content regions in the DNA minor groove in a non-intercalative way; this interaction requires Mg^{2+}. Olivomycin I (discovered at Gause Institute of New Antibiotics) demonstrated promising potency in anticancer clinical trials.[2] Unfortunately, clinical use of **1** was limited due to high general toxicity. Although chemical modification of the natural antibiotics is a common-used method of the obtaining of novel derivatives that can demonstrate some advantages over the parent antibiotic, no systematic efforts to modify the structure of **1** and other aureolic acid type antibiotics have been reported. It is known that deglycosylation of **1** produces the aglycon olivin that lacks antiproliferative properties.[3] Some novel mithramycin derivatives with high antiproliferative activity were obtained using gene engineering methods or chemical modification at the 2′-carbonyl group of **1**.[4–6] Recently, we have synthesized a series of O-substituted 2′-oximes of **1**, in which individual compounds demonstrated reasonable antitumor activity.[7] In the present study, we report the reaction of electrophilic substitution in the aromatic part of the aglycon of **1**, that is, the azo-coupling of **1** with aryldiazonium salts, and the analysis of the unusual 5-aryldiazenyl-6-*O*-deglycosyl products of these reactions. The described modification shifted the range of cytotoxicity from nanomolar (as in the case of **1**) to micromolar.

2. Results

2.1. Chemistry

In the reaction of **1** with aryl diazonium tetrafluoroborates (2 equiv NaOH, 5 °C, 5–7 min) a series of 5-aryldiazenyl-6-*O*-deglycosyl-olivomycin I derivatives **2–6** were obtained in 35–65% yields after column chromatographic purification (Scheme 1). To our surprise the reaction was accompanied by the loss of the 6-*O*-disaccharide moiety, whereas incubation of **1** in the alkaline reaction mixture (used for azo-coupling in the absence of aryldiazonium salt) did not lead to the hydrolysis of the disaccharide. The structures of **2–6** were confirmed by high resolution mass spectrometry and NMR. The molecular ion signals in the mass spectra corresponded to 5-aryldiazenyl-6-O-deglycosylated compounds.

^{1}H NMR spectral data for the aromatic part of the compounds **2**, **4**, and **6** are presented in Table 1, the full ^{1}H NMR spectrum for **6** is given in Supplementary data. The concentrations of the solutions

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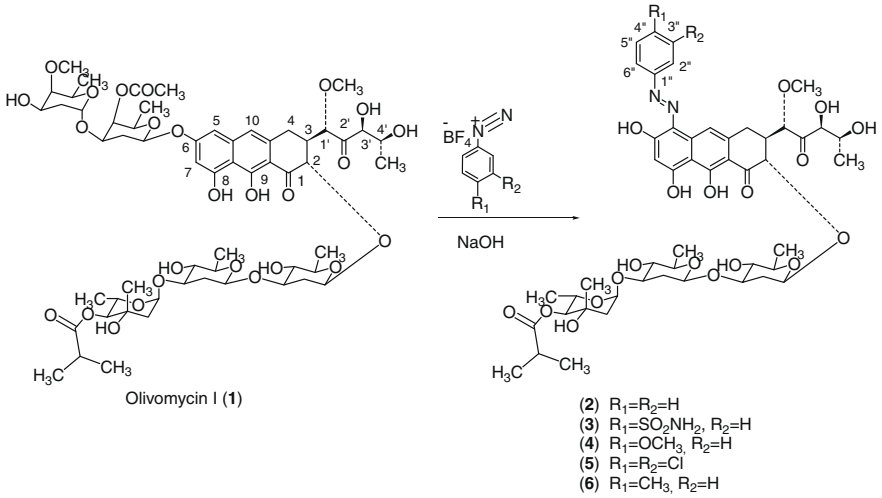
doi:10.1016/j.bmc.2009.05.076



Scheme 1. Synthesis of phenyldiazenyl derivatives of **1**.

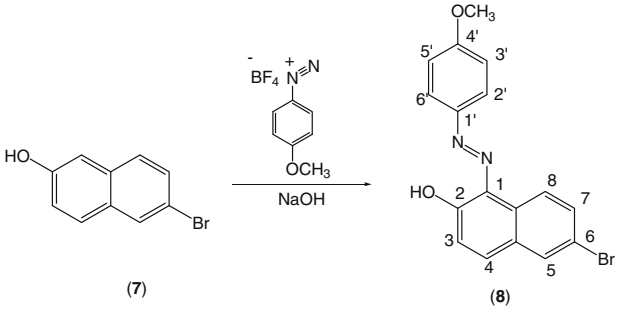
Table 1
^{1}H NMR data for **1** and its novel derivatives **2**, **4** and **6**

Compound (R_2 = H)	Atom number, type of signal										
	5-H	7-H	10-H	6-OH	8-OH	9-OH	$OCOCH_3$	OCH_3	2″-H, 6″-H	3″-H, 5″-H	4″- R_1
1[a]	6.60d[d]	6.53d[d]	6.74d	—	9.59s	15.62s	2.14s	3.49s, 3.58s	—	—	—
1[b]	6.64d[d]	6.51d[d]	6.80d	—	9.60s	15.68s	2.15s	3.49s, 3.56s	—	—	—
2 R_1 = H	—	6.12s	7.73s[c]	16.86s	10.10s	14.95s	—	3.48s	7.59d	7.44t	7.73t (H)
4 R_1 = OCH_3	—	6.24s	7.80s[c]	16.80s	10.11s	15.21s	—	3.48s	7.64m	6.99m	3.86s (OCH_3)
6 R_1 = CH_3	—	6.17s	7.77s[c]	16.95s	10.10s	15.04s	—	3.48s	7.52m	7.25m	2.39s (CH_3)

[a] Our data.
[b] Literature data.[8,9]
[c] $W_{1/2}$ ~2 Hz.
[d] Reverse assignment of the signals is possible.

were ~6 mg/mL, the broadening of the signals in the ^{1}H NMR spectra of **2**, **4**, and **6** was observed with the increase of the concentration of the solution. The analysis of ^{1}H NMR data demonstrated the absence of the disaccharide branch in the spectra of **2**, **4**, **6**. First, the signal of the $OCOCH_3$ group (at δ 2.14 ppm in **1**) was absent, and the number of signals of the methoxy groups was decreased: only one signal in the spectra of **2**, **4**, **6** instead of two signals in **1**, and seven signals of the methyl groups versus nine in **1**. Second, a signal of the 6-OH group appeared in the spectra of **2**, **4**, **6** at δ 16.8–17.0 ppm additionally to the signals at δ 14.9–15.1 ppm and δ 9.9–10.1 ppm that were analogous to the signals at δ 15.62 and at 9.59 ppm, corresponding to 9-OH and 8-OH, respectively, in the spectrum of unmodified **1**.

Low field position of 6-OH hydrogen atom in **2**, **4**, and **6** demonstrated the participation of this group in a strong hydrogen bond that can be formed with N_β atom only when the substituents at the N=N bond are in the *E*-configuration. This may occur if the substitution takes place both at the positions 5 and 7. To clarify this issue we studied NMR spectra of a model compound 6-bromonaphthalene-2-ol (**7**) and its azo-coupling product, 6-bromo-1-[(4-methoxyphenyl)diazenyl]naphthalene-2-ol (**8**) (Scheme 2). Compound **8** was synthesized by the interaction of **7** with (4-methoxyphenyl)diazonium tetrafluoroborate in 70% yield. The ^{1}H NMR spectrum of **8** was compared with the respective spectra of aryldiazenyl derivatives **2**, **4**, and **6** (Table 2).



Scheme 2. Synthesis of 6-bromo-1-((methoxyphenyl)diazenyl)naphthalene-2-ol (**7**).

Table 2
^{1}H NMR data for model compounds **7** and **8**

Compound	^{1}H NMR data										
	δ, ppm							J, Hz			
	1-H	3-H	4-H	5-H	7-H	8-H	2-OH	1-H,3-H	3-H,4-H	5-H,7-H	7-H,8-H
7	7.10d	7.10m	7.64d	7.90d	7.47dd	7.53d	5.08br s	2.4	9.5	2.1	8.8
8[a]	—	7.08d	7.65d	7.83d	7.61dd	8.57d	15.51br s	—	9.2	2.1	8.9

[a] For compound **8** $\delta_{2'\text{-H}(6'\text{-H})}$: 7.82m; $\delta_{3'\text{-H}(5'\text{-H})}$: 7.01m; $\delta_{4'\text{-}OCH_3}$: 3.88s.

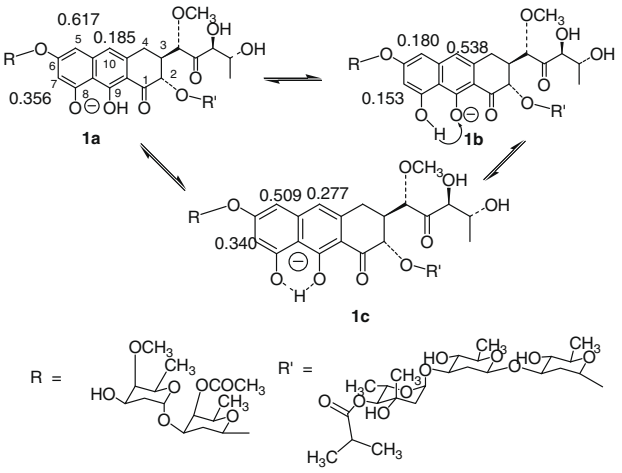
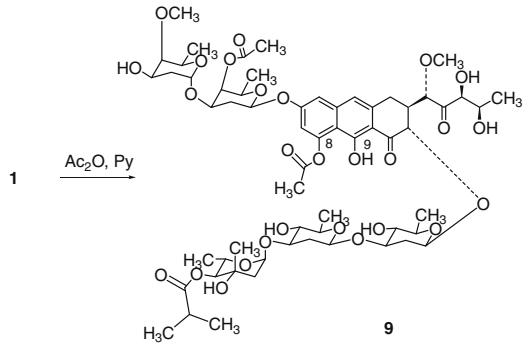


Figure 1. Anions (conjugated bases) of olivomycin (**1**). *Calculated Fukui *f*-indexes for HOMO are presented near to the corresponding atom.

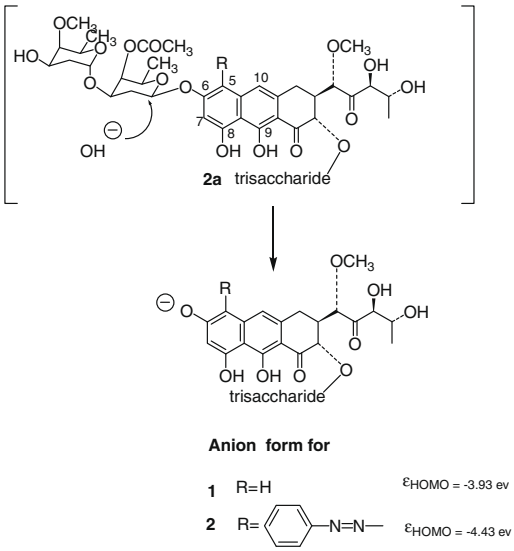
We found that the effects of the aryl diazenyl substituent on the chemical shifts of the hydrogen atom in the *peri*-position of **2**, **4**, **6** and the model naphthalene derivative **8** were very similar, and the difference in chemical shifts of the 8-H atom in **7** and **8** $\Delta\delta = \delta_{\mathbf{7}} - \delta_{\mathbf{8}} = -1.04$ coincided with the differences in chemical shifts of 10-H in **1** and its aryl diazenyl derivatives **2**, **4**, **6** ($\Delta\delta = -0.99$ for **2**, $\Delta\delta = -1.02$ for **4**, $\Delta\delta = -1.02$ for **6**). These calculations presume that the aryl diazenyl substituents in the naphthalene derivative **8** and in compounds **2–6** are closely positioned relative to the hydrogen atom in the *peri*-position, namely, in **2–6** these substituents are at the position 5.

Using a quantum-chemical method we investigated the alternative directions of the electrophilic attack on the anions of **1** (**1a**, **1b**, and **1c**) (Fig. 1). As S_E reactions in aromatic compounds with mild electrophils occur under orbital control, we used quantum-chemical semi-empirical method AM1 to calculate the values of frontier electronic density (Fukui *f*-indexes) on the highest occupied molecular orbital (HOMO) at the positions of possible electrophilic attack of **1a** by aryldiazonium cation. Fukui f_{HOMO} indexes in **1a** had a higher value at C-5 (0.617) that corresponded to higher nucleophilicity of the C-5-position in comparison to the C-7-position (0.356); for C-10 the value of f_{HOMO} was minimal (0.185). In contrast, the C-10 position was the most nucleophilic ($f_{HOMO} = 0.538$) for anion **1b**, whereas f_{HOMO} for C-5 and C-7 were 0.153 and 0.180, respectively. The calculation of the total energies of anions **1a** and **1b** showed that E_{tot}**1a** > E_{tot} **1b** (**1b** was more stable than **1a** by 40 kcal/mol). Probably, the abovementioned contradiction can be explained by assuming the presence of the intermediate **1c** with an averaged location of a proton between the O-atoms and delocalization of charges between the atoms O–H–O. The calculated f_{HOMO}-indexes of C-5, C-7 and C-10 for the hypothetical structure **1c** were 0.509, 0.340 and 0.277, respectively (nearly the same as for anion **1a**).

As an experimental proof of the above results we showed that, in alkaline conditions, the anion **1a** was formed, which is supported by the fact that acetylating of **1** in pyridine using acetic anhydride led to 8-*O*-acetyl-olivomycin I (**9**) in good yield (Scheme 3). Thus, the results of our calculations are in agreement with the initial assumption about the deprotonation of the 8-OH group in alkaline conditions and the attack of the aryldiazenyl cation on C-5. It can be suggested that the HO^- group in the product of azo-coupling is attacked, resulting in the loss of the disaccharide moiety (Scheme 4). Using quantum-chemical semi-empirical method AM1, we compared the energetic parameters of the hydrolysis of the 6-*O*-glycosyl bond for **1** and its hypothetic intermediate derivative substituted with the phenyldiazenyl moiety at C-5 (**2a**), assuming that this is



Scheme 3. Acetylation of **1**.



Scheme 4. Tentative mechanism of alkaline hydrolysis of the intermediate product **2a** in the azo-coupling reaction.

the usual S_N2-type of reaction (Scheme 4). Supposedly, the HO^- anion attacks the carbon atom linked to the phenoxy group, leading to the hydrolysis and elimination of the disaccharide.[10] Quantum-chemical studies showed favorable enthalpy and free energy for hydrolysis of **2a** ($\Delta H_{298^\circ} = -100.53$ kcal/mol and $\Delta G_{298^\circ} = -101.26$ kcal/mol) in contrast to hydrolysis of **1** ($\Delta H_{298^\circ} = -91.54$ kcal/mol and $\Delta G_{298^\circ} = -9.77$ kcal/mol). Probably, this difference is due to the stabilizing role of the Ph–N=N– group (electron withdraw –M– effect). Therefore, 5-substituted anion of the aromatic aglycon is likely to be a better nucleofugal group than the unsubstituted anion of **1**. The ε_{HOMO} of the anion for **2a** was smaller than the ε_{HOMO} of the anion for **1** (Scheme 4). The above calculations of energetic characteristics of alkaline hydrolysis are in line with our experimental data: the alkaline hydrolysis readily occurred for phenyldiazenyl derivative **2a** whereas incubation of **1** in the alkaline reaction mixture (used for azo-coupling in the absence of the aryldiazonium salt) caused no hydrolysis of the disaccharide.

2.2. Biology

The cytotoxicity of compounds **2–5** and **9** was investigated, with **1** as a reference drug. Table 3 shows the antiproliferative potencies of tested compounds against the murine leukemia L1210 and the human T-lymphocyte Molt4/C8 and CEM cell lines. 8-*O*-Acetyl-olivomycin (**9**) had comparable cytotoxicity as the parent antibiotic **1**. Removal of the disaccharide at the position 6

Table 3
Antiproliferative activity of compounds **1**, **2–5** and **9** against L1210, Molt4/C8 and CEM cell lines

Compound	IC_{50}[a] (μM)		
	L1210	Molt4/C8	CEM
Olivomycin I (**1**)	0.034 ± 0.002	0.0040 ± 0.0004	0.0025 ± 0.001
2	6.1 ± 1.3	1.6 ± 0.0	1.8 ± 0.0
3	>100	>100	>100
4	20 ± 3	7.3 ± 0.3	26 ± 4
5	5.6 ± 0.3	1.9 ± 0.1	2.6 ± 0.8
9	0.0080 ± 0.0055	0.022 ± 0.0004	0.0024 ± 0.0006

[a] The concentration that caused a 50% growth inhibition of cells after 48 h (L1210) or 72 h (other cell lines) of continuous drug exposure.

Table 4
Anti HIV-1 and HIV-2 activity of compound **1** and its derivatives **2–5**

Compound	EC_{50}[a] (μM)	
	HIV-1	HIV-2
Olivomycin I (**1**)	0.005 ± 0.003	0.004 ± 0.001
2	0.06 ± 0.03	⩾0.04
3	50 ± 1	>100
4	0.50 ± 0.42	⩾4
5	0.50 ± 0.42	⩾0.8

[a] The concentration required to protect CEM cells against HIV cytopathogenicity by 50%.

of the cyclic core of **1** resulted in a substantially decreased cell killing potency in **2** and **5**. The cytotoxicity of these compounds shifted to the low micromolar range of concentrations. Compound **6** was similarly potent as **2** and **5** (IC_{50} for K562 tumor cell line for **1** was 0.044 ± 0.012 μM, for HCT116 tumor cell line 0.055 ± 0.010 μM, for **6**—2.0±0.3 μM and 2.4±0.5 μM correspondingly). Compound **3** evoked no cytotoxic effect, being the least active among other aryl diazenyl derivatives.

In search for intracellular targets important for antitumor activities of the aureolic acid derivatives, other workers and we have found that these compounds in vitro can target the DNA dependent enzyme topoisomerase I (topo I).[7,11] We studied the ability of our novel 5-aryldiazenyl-6-*O*-deglycosyl derivatives to attenuate topo I mediated unwinding of supercoiled plasmid DNA by the method described in Ref. 7. As shown in Figure 2, compound **1** at 10–20 μM almost completely prevented the enzymatic unwinding of the plasmid as determined by the migration of DNA in the agarose gel. At 5 μM of **1** the topo I activity was only marginally affected (not shown). In striking contrast, compounds **2–4** had no effect on topo I mediated DNA relaxation even at 20 μM (Fig. 2). At this concentration **5** blocked plasmid relaxation whereas **6** had little effect. Thus, in our series of derivatives of **1**, the topo I inhibitory potency was substantially decreased except for 5-(3, 4-dichlorophenyl)diazenyl-6-*O*-deglycosylolivomycin I (**5**).

Finally, the antiviral activity of the novel derivatives against human immunodeficiency viruses HIV-1 and HIV-2 was tested (Table 4). Interestingly, whereas the anti-HIV activity of **1** was detectable at cytotoxic concentrations, several derivatives inhibited HIV-1 at concentrations that were well below their antiproliferative potency. The selectivity indices (IC_{50}/EC_{50} ratio) for HIV-1 were 30 (for **2**), >2 (for **3**), 52 (for **4**) and 5.2 (for **5**).

Compounds **2–5** were also tested against a variety of DNA and RNA viruses, including herpes simplex virus type 1 (HSV-1), HSV-2, vaccinia virus and vesicular stomatitis virus (VSV) in HEL cell cultures; VSV, Coxsackie virus B4 and respiratory syncytial virus in HeLa cell cultures; parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxsackie virus B4 and Punta Toro virus in Vero cell lines, and influenza virus A (H1N1; H3N2) and B in MDCK cells. All the derivatives **2–5** were inactive against these types of viruses at subtoxic concentrations (data not shown).

3. Discussion

The antibiotics of the aureolic family are attractive for development of clinically applicable drugs because of their exceptionally high cytocidal potency in cultured bacterial and tumor cells. Unfortunately, **1**, mithramycin and chromomycin A3 largely remain experimental agents due to general toxicity in vivo. Therefore, SAR studies within the aureolic acid family antibiotics might yield the compounds with an attenuated activity that would nevertheless retain clinically relevant properties. One way to diminish the activity might be structural modifications of the side chain in **1**. In this study we focused on the structural modifications of aglycon of **1** produced by the azo coupling of **1** with aryl diazonium tetrafluoroborates. The azo-coupling of **1** with aryl diazonium tetrafluoroborates accompanied by the loss of the disaccharide branch in the position 6 of the aglycon, produced 5-aryldiazenyl-6-*O*-deglycosyl derivatives of **1** with dramatically decreased cytotoxicity. The growth inhibitory concentrations of compounds **2** and **5** were 3 orders of magnitude higher than those of **1**. Most probably, the lack of the disaccharide branch makes **2–5** less cytotoxic that **1**, because both di- and trisaccharide moieties and the substituents in them are important for the interaction with DNA.[1,12] Thus, azo-coupling of the aglycon's side chain does not restore the cytotoxicity of the derivatives of **1** in the absence of the disaccharide branch.

Given that the aureolic acid derivatives are strong DNA minor groove ligands, it is tempting to hypothesize that these compounds can interfere with DNA dependent enzymes and template processes, thereby triggering the cytotoxic cascades in the cell.[1] Indeed, the formation of high affinity drug–DNA complexes by mithramycin, chromomycin A3 or **1** in the presence of Mg^{2+} can alter the duplex conformation and hamper gene transcription and replication.[13,14] Furthermore, these compounds and other classes of DNA binders may prevent the enzymatic modification of the duplex conformation by interfering with the enzyme–DNA

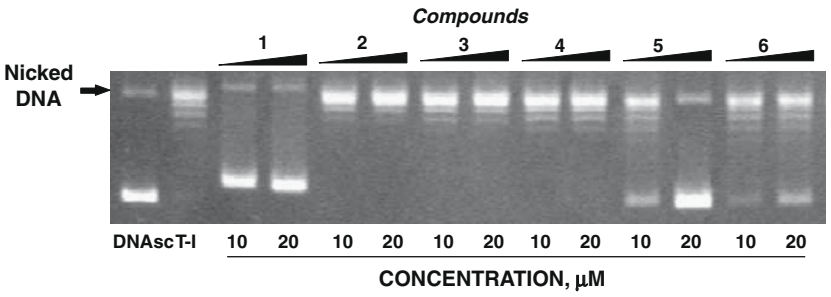


Figure 2. Electrophoretic mobility of p-Hot plasmid after topo I mediated relaxation in the presence of compounds **1–6**. *DNAsc, supercoiled DNA marker; T-I—Topo I track, no compound in the reaction mixture.

interactions.[15,16] Addressing the question of whether **1** and its 5-aryldiazenyl-6-*O*-deglycosyl derivatives can target the DNA dependent enzyme topo I, and whether this effect is critical for cytotoxicity of these compounds, we showed here that **1** did attenuate the enzymatic relaxation of the supercoiled DNA, although at the concentrations ~2 orders of magnitude higher than those required for induction of cell death (compare the concentrations in Table 3 and Fig. 2). In line with the decreased cytotoxicity of **2**–**5** (Table 3), these compounds were even weaker topo I blockers than **1**, with **5** as the only exception whose topo I attenuation potency was comparable with that of **1** (Fig. 2). However, the cytotoxicity of **1** and **5** differed dramatically; also, the cytotoxicities of **2** and **5** were similar despite the fact that **2** lacked topo I inhibitory potency at least in the range of tested concentrations. These results provide evidence that the cytotoxic and topo I poisoning activities of **1** and its novel derivatives do not correlate directly, therefore, topo I inhibition is unlikely to be a critical target for cytotoxicity of these agents.

Interestingly, several derivatives that lacked the disaccharide chain showed selective anti-HIV activity. Instead, no activity was observed against a wide variety of other DNA and RNA viruses, suggesting the selectivity of these compounds for HIV. Investigations to reveal the mechanism of anti-HIV activity of the derivatives of **1** are in progress.

4. Conclusion

In summary, aiming at an attenuation of cytotoxicity of the aureolic acid family antibiotic **1**, we synthesized a series of 5-aryldiazenyl-6-*O*-deglycosyl derivatives by the azo-coupling of the aglycon of **1** with aryl diazonium tetrafluoroborates. The synthetic reactions were accompanied by hydrolysis of the disaccharide branch at the 6-*O* position of the aglycon. Compared to **1**, the resulting compounds were significantly less cytotoxic against the mammalian cell lines, and less potent inhibitors of HIV-mediated cytopathogenicity. Nevertheless, the cytotoxicity of **2** and **5**, as well as the anti-retroviral activities of **2**, **4**, and **5** remained within the micromolar range of concentrations. These studies demonstrate that further investigations of structure–cytotoxic and structure–antiviral activities relationships for aureolic acid derivatives may lead to selective and less toxic antitumor or antiretroviral compounds.

5. Experimental

5.1. Chemistry

5.1.1. General experimental procedures

Olivomycin I (**1**) was produced at the pilot plant of the Gause Institute of New Antibiotics, Moscow. All reagents and solvents were purchased from Aldrich, Fluka, and Merck except specified otherwise. The progress reaction products, column eluates and all final samples were analyzed by TLC and HPLC. TLC was performed on Merck $G60F_{254}$ precoated plates in the following systems: $CHCl_3$–MeOH, 9:1 (A); $CHCl_3$–MeOH–HCO_2H, 9:1:0.05 (B); $CHCl_3$–MeOH–H_2O–HCO_2H, 13:6:1:0.1 (C). Reaction products were purified by column chromatography on Merck Silica Gel 60 (0.063–0.200 mm). Melting points were determined on Buchi SMP-20 and are presented uncorrected. HPLC analyses were performed on a Shimadzu HPLC LC 50 instrument equipped with a Kromasil-100-C18 column (4.0 × 250 mm, 7 μk, BioChem Mack, Russia) and variable wavelength UV detector set at 270 nm with the injection volume 10 μL. Elutions were carried out at a flow rate of 100 μL/min by the 0.01 M H_3PO_4–MeCN mixture at 20 °C using MeCN gradient 40–80% (system A), 40–95% (system B) and 50–90% (system C). The sample concentrations were 0.05–0.2 mg/mL. The ^1H NMR spectra were recorded on a Unity 400 (Varian) spectrometer at 400 MHz using $CDCl_3$ as a solvent; the $CHCl_3$ signal in the solvent (δ 7.24) was used as an internal standard. The concentrations of the solutions were approximately 6 mg/mL. Mass spectra were determined by Matrix-assisted laser desorption/ionization (MALDI) on a Brucker BIFLEX III spectrometer (Germany), mass spectra determined by Electrospray Ionization (ESI) were recorded on a Finnigan MAT 900S spectrometer (Germany, Bremen). The data on the predominant monoisotope peak are presented. All solutions were dried over sodium sulfate and evaporated under reduced pressure on a Buchi rotary evaporator at temperature <35 °C.

Aryl diazonuim tetraflouoroborates were obtained from corresponding aryl diazonium chlorides by the addition of equimolar amount of HBF_4 (50% aqueous solution) and filtration of the precipitate.

5.1.2. 5-Phenyldiazenyl-6-*O*-deglycosylolivomycin I (2)

Solution of NaOH (3.3 mg, 0.084 mmol) in H_2O (1 mL) was added to the solution of **1** (50 mg, 0.042 mmol) in EtOH (1.5 mL) at 5 °C. Phenyl diazonium tetraflouroborate (15.6 mg, 0.084 mmol) was added and the reaction mixture was stirred for 5 min at 5 °C. The reaction mixture was poured into the mixture of ethyl acetate–5% HCl, organic fraction was separated, washed with brine until pH 7 was reached, then dried over $NaSO_4$ and evaporated. The residue was purified by column chromatography, elution was carried out with the mixture $CHCl_3$–MeOH (9:1). The resulting fractions containing **2** were combined and evaporated. The residue was dissolved in ethyl acetate; the addition of petroleum ether gave a precipitate which was filtered off, washed with petroleum ether and dried in vacuum to yield **2** as an amorphous dark red powder (25 mg, 60%), mp 169–171 °C (decomp.); R_f (system A) 0.35; HPLC (system A) t_R 23.86 min, purity 92% (percent of the main peak); MALDI *m/z* 1007.4006 (M^++Na), calculated for $C_{49}H_{64}N_2O_{19}$+Na 1007.4001.

5.1.3. 5-(4-Sulfamoylphenyl)diazenyl-6-*O*-deglycosylolivomycin I (3)

The compound **3** was obtained as described for **2**, starting from **1** (50 mg, 0.042 mmol) and 4-sulfamoylphenyl diazonium tetraflouroborate (22.8 mg, 0.084 mmol). Yield 20 mg (40%), mp 178–180 °C (decomp.), R_f (system A) 0.11, HPLC (system A) t_R 12.63 min, purity 93% (percent of the main peak); MALDI *m/z* 1086.3725 (M^++Na), calculated for $C_{49}H_{65}N_3O_{21}S$+Na 1086.3729.

5.1.4. 5-(4-Methoxyphenyl)diazenyl-6-*O*-deglycosylolivomycin I (4)

The compound **4** was obtained as reported for **2**, starting from **1** (100 mg, 0.084 mmol) and 4-methoxyphenyl diazonium tetraflouroborate (37 mg, 0.167 mmol). Yield 45 mg (53%), mp 188–190 °C (decomp.), R_f (system A) 0.33, HPLC (system B) t_R 27.81 min, purity 95% (percent of the main peak), MALDI *m/z* 1037.4111 (M^++Na), calculated for $C_{50}H_{66}N_2O_{20}$ 1014.4209.

5.1.5. 5-(3,4-Dichlorophenyl)diazenyl-6-*O*-deglycosylolivomycin I (5)

The compound **5** was obtained as described for **2**, starting from **1** (50 mg, 0.042 mmol) and 3,4-dichlorophenyl diazonium tetraflouroborate (22 mg, 0.084 mmol). Yield 20 mg (35%), mp 155–157 °C (decomp.), R_f (system A) 0.40, HPLC (system A) t_R 27.39 min, purity 93% (percent of the main peak); MALDI *m/z* 1075.3228 (M^++Na), calculated for $C_{49}H_{62}Cl_2N_2O_{19}$+Na 1075.3222.

5.1.6. 5-(4-Methylphenyl)diazenyl-6-*O*-deglycosylolivomycin I (6)

The compound **6** was obtained as described for **2**, starting from **1** (100 mg, 0.084 mmol) and *p*-tolyl diazonium tetraflouroborate (34 mg, 0.168 mmol). Yield 54 mg (65%), mp 178–180 °C

(decomp.), R_f (system A) 0.37; HPLC (system A) t_R 27.56 min, purity 91% (percent of the main peak), ESI-MS 999.4340 (M^++H), calculated for $C_{50}H_{66}N_2O_{19}$ 998.4260.

5.1.7. 6-Bromo-2-naphthol (7)

Compound **7** was obtained as described.[17] ^{1}H NMR data for this compound is presented in Table 2.

5.1.8. 6-Bromo-1-((4-methoxyphenyl)diazenyl)naphthalene-2-ol (8)

4-Methoxyphenyl diazonium tetraflouroborate (100 mg, 0.45 mmol) was added to the solution of 6-bromo-2-naphthol (**7**) (110 mg, 0.50 mmol) and NaOH (40 mg, 0.99 mmol) in H_2O (2 mL) at 5 °C. The reaction mixture was stirred for 15 min at 5 °C. The resulting precipitate was filtered off, washed with H_2O and dried. R_f 0.49 (system B), HPLC (system C) t_R 13.73 min; ESI-MS 357.0231 (M^++H), calculated for $C_{17}H_{13}BrN_2O_2$ 365.0160. ^{1}H NMR data for this compound is presented in Table 2.

5.1.9. 8-*O*-Acetyl-olivomycin I (9)

To the solution of **1** (50 mg, 0.043 mmol) in dry pyridine (1 mL) Ac_2O (4 μL, 0, 43 mmol) was added. The reaction mixture was stirred for 1 h at room temperature, evaporated to a minimum volume, then the residue was dissolved in ethyl acetate and petroleum ester was added to the solution. The resulting precipitate was filtered off and dried. Yield 41 mg (79%), mp 170–172 °C (decomp.), R_f 0.53 (system B), HPLC (system A) t_R 12.96 min, purity 95% (percent of the main peak); MALDI 1261.5260 (M^++Na), calculated for $C_{60}H_{86}O_{27}$+Na 1261.5254. ^{1}H NMR spectrum ($CDCl_3$) of **9** corresponded to the ^{1}H NMR spectrum of **1**,[7] except no signal at 9.60 pm (s, 1H, 8-OH) whereas the signal at 2.34 ppm was present (s, 3H, 8-$OCOCH_3$).

5.2. Quantum-chemical calculations

All structures were calculated by semi-empiric AM1 method using the GAUSSIAN 98 program package with full geometry optimization.

5.3. Biology

5.3.1. Cytotoxicity measurements

Propagation of the murine leukemia L1210, the human T-lymphocyte Molt4/C8 and CEM cell lines, as well as cytotoxicity and anti-retroviral activity assays were performed as described earlier.[7,18,19] Novel compounds were evaluated against the following viruses: herpes simplex virus type 1 (HSV-1) strain KOS, the thymidine kinase deficient (TK^-) HSV-1 KOS strain resistant to ACV (ACV^r), herpes simplex virus type 2 (HSV-2) strain G, vaccinia virus Lederle strain, respiratory syncytial virus (RSV) strain Long, vesicular stomatitis virus (VSV), Coxsackie B4, Parainfluenza 3, Reovirus-1, Sindbis, Punta Toro, influenza virus type A (H1N1, H3N2), and type B. The antiviral assays other than anti-HIV were based on inhibition of virus-induced cytopathicity in human embryonic lung (HEL) fibroblasts, African green monkey cells (Vero), human epithelial cervix carcinoma cells (HeLa), or Madin-Darby canine kidney cells (MDCK). Confluent cell cultures in microtiter 96-well plates were inoculated with 100 $CCID_{50}$ of virus (1 $CCID_{50}$ being the virus dose that infects 50% cells). After 1–2 h the residual virus was removed, and the cells were incubated in the presence of varying concentrations of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cultures in the absence of test compounds. Antiviral activity was expressed as the EC_{50}, that is, the concentration required to reduce virus-induced cytopathicity by 50%. Cytotoxicity of the test compounds was expressed as the minimal cytotoxic concentration (MCC), that is, the concentration that caused a microscopically detectable alteration of cell morphology.

5.3.2. Antiviral activity

To assess the inhibition of HIV-induced cytopathicity, human CEM cells ($\sim 3 \times 10^5$ cells/mL) were infected with ~100 CCID50 HIV-1(III_B) or HIV-2(ROD) per mL and seeded in 96-well (200 μL/well) microtiter plates containing appropriate dilutions of the test compounds. After 4 days of incubation at 37 °C, formation of syncytia was examined microscopically. The EC_{50} values were determined as described above. The cytostatic concentration of the compound was calculated as the CC_{50}, that is, the concentration required to reduce cell proliferation by 50%; proliferation of untreated cells was regarded as 100%.

5.3.3. Topo I assay

The ability of compounds **1–6** to modulate the activity of topo I in a cell free solution was determined in a DNA relaxation assay.[7] Briefly, 1 unit of purified topo I (Promega) were incubated with 0.25 μg of supercoiled pBR322 plasmid DNA (Fermentas, Lithuania) in the buffer (35 mM Tris–HCl, pH 8.0, 72 mM KCl, 5 mM $MgCl_2$, 5 mM DDT, 2 mM spermidine, 0.1 mg/mL bovine serum albumin) in the absence (tracks 'DNAsc' and 'T-I', Fig. 2) or presence of compounds **1–6** (at indicated concentrations) at 37 °C for 30 min. The reaction was terminated by the addition of 1% sarcosyl. Proteinase K (50 μg/mL) was added, and DNA topoisomers were resolved by electrophoresis in 1% agarose gel (3 h, 70 V) in the buffer containing 40 mM Tris-acetate, pH 7.6, 1 mM ethylenediamine tetraacetic acid disodium salt. After electrophoresis the gels were stained with 0.5 μg/mL ethidium bromide and photographed under UV light.

Acknowledgments

This study was supported by the Russian Foundation for Basic Research (Grant 06-04-08127), the grant of the President of Russian Federation for the support of young scientists (MK-5422.2007.4) and the grant from the Centers of Excellence (No. 05/15) of the K.U.Leuven. The authors are grateful to Drs. V. Zbarsky (Gause Institute of New Antibiotics, Moscow) for providing the olivomycin I batches, M. Reznikova and N. Malyutina (both from Gause Institute of New Antibiotics, Moscow) for HPLC analysis, Valeria A. Glazunova (Blokhin Cancer Center, Moscow) for cytotoxicity assays (K562 and HCT116 tumor cell lines), L. van Berckelaer and L. Ingels (Leuven, Belgium) for their assistance in the cytotoxicity and antiviral activity assays.

Supplementary data

Supplementary data (^{1}H NMR spectrum of compound **6**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.05.076.

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