Ramified derivatives of 5-(perylen-3-ylethynyl) uracil-1-acetic acid and their antiviral properties†

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The propargylamide of N3-Pom-protected 5-(perylen-3-ylethynyl)uracil acetic acid, a universal precursor, was used in a CuAAC click reaction for the synthesis of several derivatives, including three ramified molecules with high activities against tick-borne encephalitis virus (TBEV). Pentaerythritol-based polyazides were used for the assembly of molecules containing 2···4 antiviral 5-(perylen-3-ylethynyl) uracil scaffolds, the first examples of polyvalent perylene antivirals. Cluster compounds showed enhanced absorbance, however, their fluorescence was reduced due to self-quenching. Due to the solubility issues, Pom group removal succeeded only for compounds with one peryleneethynyluracil unit. Four compounds, including one ramified cluster 9f, showed remarkable 1···3 nM EC50 values against TBEV in cell culture.

Introduction

5-(Perylen-3-ylethynyl)-deoxy-uridine dUY11 (Fig. 1), prepared initially as a fluorescent nucleoside,‡ had unexpectedly shown remarkably broad spectrum antiviral properties.§ Nucleoside derivative dUY11 and its arabino analog aUY11 effectively suppressed reproduction of a number of enveloped viruses in cell cultures, thus constituting a new class of antivirals with a non-nucleoside mechanism of action.‡§ The presumable target for these compounds is the lipid membrane bilayer of the virion envelope. Compounds dUY11 and aUY11 are rigid amphiphatic fusion inhibitors (RAFIs)‡§ that target the viral envelope either by a shape-determined biophysical mechanism‡§‡§ or by photosensitization.‡§ Both shape-determined and photophysical properties of the conjugated aryl part of the molecule are probably crucial for the antiviral properties, and the carbohydrate part seems to be less important. Very recently,§ we prepared 5-(perylen-3-ylethynyl)uracil-1-acetic acid [cm1UY11, Fig. 1] with a carboxymethyl group replacing the pentose moiety. The acid showed a pronounced antiviral activity against tick-borne encephalitis virus (TBEV),§ herpes simplex virus type 1 (HSV-1),§ and African swine fever virus (ASFV).§ Moreover, its 3-Pom-modified precursor cm1pUY11 (Fig. 1) and amides were also active against TBEV‡ and HSV-1.⁷

Thus, the 5-(perylen-3-ylethynyl)uracil scaffold seems to be responsible for the antiviral activity of perylene RAFIs. Lipophilic perylene residue is expected to reside in the lipid bilayer upon binding between a RAFI molecule and an enveloped virion. Therefore, one can expect cooperative enhancement of lipid membrane anchoring for compounds containing 2···4 perylene residues, or other effects of clustering perylenes.

The concept of clustered/multivalent (or dendrimeric) antivirals has been developed for decades,§ both for specific and non-specific compounds and functional groups. Recent promising examples include polyanions,§§ polycations,§§ as well as dendrimers carrying amino acids,§§ peptides,§§ phenols,§§ terpenes,§§ mono-§§ oligosaccharides,§§ and neuraminidase inhibitors.§§ The approach, however, has been never applied to RAFIs.

Fig. 1 Structures of broad spectrum antiviral peryleneethynyluracil compounds.
To study the possible effects of combining several perylene cores in a single molecule on the antiviral activity, we synthesized small clusters of 5-(perylen-3-ylethynyl)-1-(carboxymethyl)uracil, quantified their fluorescence properties, and measured the efficiency of TBEV reproduction inhibition by these clusters in PEK cell culture.

Results and discussion

Synthesis of compounds

Recently, we used the azide–alkyne click reaction for the synthesis of dUY11 derivatives with enhanced activity. Here we report the application of Huisgen–Meldal–Sharpless reaction (Cu(I)-catalyzed alkyne–azide cycloaddition, CuAAC) for the synthesis of 5-(perylen-3-ylethynyl)-1-(carboxymethyl)uracil derivatives. First, we prepared a set of branched azides 2–5 (Scheme 1). The starting tetraol 1 was mesylated with a controlled excess of mesyl chloride (MsCl) in DCM in the presence of triethylamine as a base. Tuning the mesyl chloride/hydroxyl ratio leads to the desired distribution of products in the reaction. Earlier, we prepared azides 4 and 5 using 1 : 3 molar ratio of 1 to mesyl chloride and used them for the assembly of complex oligonucleotide conjugates. Here we report the synthesis of azides 2 and 3 as main products. Mesylation with 1 : 1.2 molar ratio (1 : MsCl), and subsequent nucleophilic substitution with sodium azide affords compounds 2 and 3 in 32% and 27% yield, respectively, together with some amount of triazole 4.

We used pivaloyloxymethyl (Pom) protection for N3 position of the nucleobase for solubility reasons. 3-Pom-5-(perylen-3-ylethynyl)uracil acetic acid 6 was prepared from 5-iodouracil and the desired products were insoluble in alcohol. Therefore, we compared their fluorescence properties, and measured their e.ects of combining several perylene cores in a single molecule on the antiviral activity, we synthesized small clusters of 5-(perylen-3-ylethynyl)-1-(carboxymethyl)uracil, quantified their fluorescence properties, and measured the efficiency of TBEV reproduction inhibition by these clusters in PEK cell culture.

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self-quenching, fluorescence intensity decreases with the increase in the number of fluorophores comprising the 5-(perylen-3-ylethynyl)uracil groups. There were no drastic changes in fluorescence spectra for compounds 9c–f and 10c, except for relative enhancement of 550 nm band, and proximal long wavelength emission for polychromophore molecules 9d–f, suggesting weak excimer fluorescence from perylene residues located close to each other.

Antiviral properties

All prepared compounds, containing one (7–9b, 10a–c) or several (9d–f) perylene residues, were tested for cytotoxicity and for ability to inhibit TBEV (strain Absettarov) reproduction in PEK cell culture as measured by plaque reduction assay (Table 1). All experimental conditions were the same as in our previous studies.2,6,19,25

All the compounds showed little to no cytotoxicity on PEK cells and selectivity indices (SI = CC_{50}/EC_{50}) up to 50 000. The alkyne precursor 7 appeared to be the most potent compound in the series, showing nanomolar EC_{50}. Its unprotected analogue 8 and acid precursor 6 showed slightly lower activity, thus revealing low influence of small substituents in the uracil moiety, similarly to one of the previous studies,25a but contrary to another.26 Although cluster compounds 9d–f inhibited TBEV reproduction in one or two digit nanomolar concentrations, their single-perylene analogues were more potent. Remarkably, Pom deprotection of branched compound 9c, affording 10c, led to a considerable activity decrease.

Molecular modeling

While the abbreviation RAFl stands for Rigid Amphipathic Fusion Inhibitor, the compounds synthesized here contain a large number of rotatable bonds. Thus, their antiviral activity and membrane interactions may be largely defined by their conformational space. To obtain some insight into this conformational space, we performed a simple conformational analysis for molecular models of the compounds, seeking the global minima of the compound energies using the Confort method implemented in SYBYL-X 2.1.27 The optimized structures are shown in Fig. 4, along with the molecular surfaces colored by the hydrophobic potential.

As the most important part of the RAFl molecules is the perylene moiety,2,6,19a its exposure and ability to interact with the

Table 1 TBEV (strain Absettarov) reproduction inhibition efficiency (EC_{50}) and cytotoxicity (CC_{50}, PEK cells) of the compounds 6–10

|  | EC_{50}, μM | CC_{50}, μM |
|---|---|---|
| 6 | 0.00121 ± 0.00025 | >50 | >50 |
| 7 | 0.00097 ± 0.00015 | >50 | >50 |
| 8 | 0.002 ± 0.001 | >50 | <50 |
| 9a | 0.046 ± 0.017 | >50 | >50 |
| 9b | 0.0010 ± 0.0003 | >50 | >50 |
| 9c | 0.008 ± 0.001 | >50 | 18 |
| 9d | 0.024 ± 0.007 | >50 | <50 |
| 9e | 0.015 ± 0.001 | >50 | <50 |
| 9f | 0.0033 ± 0.0011 | >50 | >50 |
| 10a | 0.0081 ± 0.0017 | >50 | >50 |
| 10b | 0.066 ± 0.013 | >50 | >50 |
| 10c | 0.10 ± 0.05 | >50 | 37 ± 13 |

Data for compound 6 are from ref. 6. Morphological changes of cell membranes were observed.
viral membrane should play a crucial role in the potency of the compounds. It can be seen that compounds 6–8, as well as aUY11, do not show substantial flexibility, and the perylene moiety of these compounds is completely exposed. Molecules 9 and 10, bearing longer and more flexible moieties, show less uniform antiviral activity, which is more strongly affected by possible conformational behavior. For example, the influence of Pom is opposite in the pairs 9a–10a and 9b–10b; in the former, the benzyl moiety may interact with perylene, whereas in the latter the hydroxyethyl group should prefer a more hydrophilic environment. Using coloring by the hydrophobic potential, one can see the amphipathic nature of perylenylethynyluracil compounds. Perylenylethynyluracil units are probably responsible for lipid bilayer anchoring and for the general antiviral effect. The polar part of the molecules seems to have a modulating (within two orders of magnitude) effect on the inhibition of a viral replication. Remarkably, in the most active compounds, the polar part of the molecule has a small hydrophobic unit, Pom or benzyl, but not both together. Although neither the target, nor the mechanism of antiviral action of RAFls was directly proven to date, this simple observation can be used in a future design of antivirals.

**Experimental**

**General methods**

Reagents and solvents were from commercial suppliers and used as received, except DMF, DMSO, CH2Cl2, CHCl3, EtOH and methanol were used freshly distilled from CaH2. Azides 4, 5, tetrakis(5-hydroxy-2-oxapentyl)methane and 3-Pom-5-(perylene-3-ylethynyl)uracil-1-acetic acid 6 were prepared as described. 500 MHz 1H and 125.7 MHz 13C NMR spectra were recorded on Bruker AMX-400 or Bruker Avance 500 spectrometers and referenced to DMSO-d6 (2.50 ppm for 1H and 39.5 ppm for 13C) or CDCl3 (7.26 ppm for 1H and 77.16 ppm for 13C). 1H NMR coupling constants are reported in hertz (Hz) and refer to apparent multiplicities. Electrospray ionization high resolution mass spectra (ESI HRMS) of low molecular weight compounds were recorded using a Thermo Scientific Orbitrap Exactive mass spectrometer (positive or negative ion mode). UV spectra were recorded on a Varian Cary 100 spectrophotometer. Fluorescence spectra were recorded using a PerkinElmer LS 55 fluorescence spectrometer. Analytical thin layer chromatography was performed on Kieselgel 60 F254 precoated aluminum plates (Merck). Silica gel column chromatography was performed using Merck Kieselgel 60 0.040–0.063 mm.

**Cell culture and viruses**

Porcine embryo kidney (PEK) cell line was maintained at 37 °C in medium 199 (FSBSI “Chumakov FSC R&D IBP RAS”, Russia) supplemented with 5% fetal bovine serum (Gibco). Tick-borne encephalitis virus strain Absettarov (GenBank access no. KU885457) was from the laboratory collection of FSBSI “Chumakov FSC R&D IBP RAS”.

**Synthetic procedures**

6,6-Bis(5-hydroxy-2-oxapentyl)-4,8-dioxa-11-hydroxyundec-1-yl azide (2). Mesyl chloride (7.06 g, 60 mmol) was added dropwise to a mixture of tetrakis(5-hydroxy-2-oxapentyl)methane (17.26 g, 50 mmol) and triethylamine (11.1 mL, 80 mmol) in dry DCM (300 mL). The reaction was monitored by TLC (10% EtOH in CHCl3; starting alcohol: Rf 0.33). After the consumption of all starting material, the reaction mixture was washed with distilled water (2×100 mL), brine (3×50 mL) and dried over Na2SO4. Evaporation of the solvent in vacuum gave yellowish oily liquid. It was dissolved in dry DMSO (60 mL), and sodium azide (10 g; 154 mmol) was added under magnetic stirring. After 12 h, water (120 mL) was added and the mixture of products was extracted from the aqueous layer with EtOAc (4×100 mL); organic fractions were combined, washed with distilled water (5×100 mL), brine (3×100 mL) and dried over Na2SO4. After evaporation of the solvent in vacuum, the residue was chromatographed on silica gel (gradient elution with 20 → 45% of 96% ethanol in CHCl3). Compound 2c (5.24 g, 32% yield) was

![Fig. 4 Structures of compounds optimized using SYBYL-X 2.1 software; molecular surfaces are colored by the hydrophobic potential (left scale).](image-url)
obtained as a colorless viscous liquid. $R_f$ 0.34 (5% EtOH in CHCl$_3$).$^1$H NMR (500 MHz; CDCl$_3$) δ 6.37 (t, 6H, J = 5.3 Hz), 3.55 (t, 6H, J = 5.5 Hz), 3.47 (br s, 3H), 3.44 (t, 2H, $J = 5.97$ Hz), 3.38 (s, 6H), 3.36–3.32 (m, 4H), 1.84–1.74 (m, 8H). $^{13}$C NMR (125 MHz; CDCl$_3$) δ 70.9, 70.5, 70.4, 68.1, 61.5, 48.5, 44.8, 31.8, 29.0. HRMS (ESI, m/z): calcd for C$_{21}$H$_{24}$N$_{6}$O$_{8}$: [M + H$^+$]: 394.2548, found: 394.2557. Earlier eluting diazide 3 (4.7 g; 27%) and triazole 4 (1.43 g; 8%) were obtained as colorless viscous liquids. Their analytical data matched with ones reported earlier.\cite{20}

[3-(Pivaloyloxymethyl)-5-(perylene-3-ylthienyl)uracil-1-yl]-N-(1-benzyl-1,2,3-triazol-4-yl)acetamide (9a).

Click reaction between alkyne 7 and mono-azides, a general procedure

Azide (0.30 mmol) and propargyl amide 7 (0.26 mmol) were dissolved in DMSO (10 mL) under vigorous magnetic stirring. The solution was flushed with argon for degassing. 0.1 M solution of CuSO$_4$·5H$_2$O and TBTA ligand (1 : 1) in 55% DMSO was added (260 mL). Then ascorbic acid (100 mg mL$^{-1}$) in H$_2$O (91 mL) was quickly loaded and the flask was tightly closed. After 12 h TLC showed full consumption of the starting alkyne 7. Reaction mixture was quenched with brine (50 mL) and lumpy solid precipitate was isolated by centrifugation. The crude product was washed with distilled water (2 × 10 mL). The residue was applied on the silica gel using evaporation of the acetone solution with small portion of the solvent. The obtained powder was carefully spilled on the column filled with silica gel in CH$_2$Cl$_2$. The following compounds were isolated.

[3-(Pivaloyloxymethyl)-5-(perylene-3-ylthienyl)uracil-1-yl]-N-(1-benzyl-1,2,3-triazol-4-yl)acetamide (9a). [3-(Pivaloyloxymethyl)-5-(perylene-3-ylthienyl)uracil-1-yl]-N-(1-benzyl-1,2,3-triazol-4-yl)acetamide (9a) was prepared using benzyl azide. Gradient elution with EtOH (1 → 5%) in CH$_2$Cl$_2$ and evaporation gave pure compound as orange solid, yield 137 mg (75%). $R_f$ 0.32 (5% EtOH in CH$_2$Cl$_2$). UV-Vis (20% DMSO in 96% EtOH) $\lambda$$_{\text{max}}$ nm: (411 388 600), (470 48 000), $\lambda$$_{\text{min}}$ nm (m, 1 cm$^{-1}$ cm$^{-1}$ cm$^{-1}$): 441 (388 600), 547 (26 800). Fluorescence (96% EtOH, $\lambda$$_{\text{em}}$ 530 nm, excitation $\lambda$$_{\text{max}}$ nm): 334, 440, 467; (96% EtOH, $\lambda$$_{\text{em}}$ 420 nm, emission $\lambda$$_{\text{max}}$ nm): 476, 509. $^1$H NMR (500 MHz, DMSO-d$_6$) δ 8.70–8.71 (m, 1H), 8.38–8.28 (m, 2H), 7.83–7.78 (m, 2H), 7.7–7.73 (m, 1H, 2H), 7.43–7.54 (t, 2H, $J = 7.62$ Hz), 7.39–7.28 (m, 6H), 8.76 (s, 2H), 5.58 (s, 2H), 4.54 (s, 3H, 2H), 3.33–3.27 (m, 2H), 1.19 (s, 9H). $^{13}$N NMR (125 MHz, DMSO-d$_6$) δ 176.4, 166.1, 166.5, 149.5, 149.4, 144.5, 136.0, 134.2, 133.7, 131.3, 130.3, 130.5, 129.8, 128.7, 128.3, 128.3, 128.1, 128.0, 127.9, 127.7, 127.7, 127.7, 126.8, 126.9, 125.6, 121.4, 121.2, 121.0, 119.1, 96.8, 90.8, 90.7, 87.8, 87.5, 76.3, 65.1, 50.8, 38.3, 28.1, 26.7. HRMS (ESI, m/z): calcd for C$_{21}$H$_{24}$N$_{6}$O$_{8}$Na [(M + Na$^+$)]: 618.1999, found: 618.2000.

[3-(Pivaloyloxymethyl)-5-(perylene-3-ylthienyl)uracil-1-yl]-N-(1-[2-hydroxyethyl]-1,2,3-triazol-4-yl)acetamide (9b).

[3-(Pivaloyloxymethyl)-5-(perylene-3-ylthienyl)uracil-1-yl]-N-(1-[2-hydroxyethyl]-1,2,3-triazol-4-yl)acetamide (9b) was prepared using $\text{2-azidothanol}$. Gradient elution with MeOH (1 → 6%) in CHCl$_3$ and evaporation gave pure compound as orange solid, yield 120 mg (67%). $R_f$ 0.6 (10% MeOH in CHCl$_3$). UV-Vis (20% DMSO in 96% EtOH) $\lambda$$_{\text{max}}$ nm: (411 388 600), (470 48 000), 547 (26 800). Fluorescence (96% EtOH, $\lambda$$_{\text{em}}$ 530 nm, excitation $\lambda$$_{\text{max}}$ nm): (344, 440, 467; 96% EtOH, $\lambda$$_{\text{em}}$ 420 nm, emission $\lambda$$_{\text{max}}$ nm): 476, 509. $^1$H NMR (500 MHz, DMSO-d$_6$) δ 8.59–8.60 (m, 1H), 8.47–8.48 (m, 2H), 7.82–7.83 (m, 3H), 8.23 (d, 1H, $J = 7.55$ Hz), 8.36–8.27 (m, 3H), 8.25 (d, 1H, $J = 8.23$ Hz), 7.82–7.77 (m, 2H), 7.68–7.62 (m, 2H), 7.53 (t, 2H, $J = 7.68$ Hz), 5.86 (s, 2H), 4.53 (s, 2H), 3.99–3.94 (m, 2H), 3.2–3.18 (m, 1H), 1.14 (s, 9H). $^{13}$N NMR (125 MHz; DMSO-d$_6$) δ 176.6, 166.0, 160.5, 149.7, 149.3, 134.1, 133.7, 131.3, 130.5, 130.1, 129.8, 128.6, 128.3, 127.8, 127.7, 127.6, 127.0, 126.9, 125.6, 121.4, 121.4, 120.2, 120.2, 119.1, 96.8, 90.9, 90.7, 87.8, 80.6, 73.5, 65.1, 50.8, 38.3, 28.1, 26.7. HRMS (ESI, m/z): calcd for C$_{21}$H$_{24}$N$_{6}$O$_{8}$Na [(M + Na$^+$)]: 705.2042, found: 705.2043.
Click reaction between alkyne 7 and polyazides 3–5, general procedure

Azide (0.025 mmol) and propargyl amide (0.1 M solution of CuSO4) were dissolved in DMSO (3 mL) under vigorous magnetic stirring. The solution was flushed with argon for degassing and then solution of CuSO4·5H2O and TBTA ligand was added (5% excess for each azido group). After 48 hours, the reaction mixture was quenched with 5% excess for each azido group. The precipitate was isolated using centrifugation. The obtained powder was carefully spilled on the column filled with silica gel in CH2Cl2. The following compounds were obtained.

11-(bis[5-hydroxy-2-oxapentyl]-1,1-(bis[5-(3-pivaloyloxy)methyl]-5-(prenyl-3-ylthynyl)uracil-1-yl]acetyl)-amino)-1,2,3-triazol-1-yl)-2-oxapent-1-yl)methane (9d), Tetrakis[5-(4-[N-(3-pivaloyloxy)methyl]-5-(prenyl-3-ylthynyl)uracil-1-yl]acetyl]-amino)-1,2,3-triazol-1-yl)-2-oxapent-1-yl)methane (9f)

Tetrakis[5-(4-[N-(3-pivaloyloxy)methyl]-5-(prenyl-3-ylthynyl)uracil-1-yl]acetyl]-amino)-1,2,3-triazol-1-yl)-2-oxapent-1-yl)methane (9f) was prepared using azide 5. Gradient elution with ETOH (1 → 25%) in CH2Cl2 and evaporation gave pure compound as orange solid, yield 19 mg (38%).

Pom group removal, a general procedure

Pom-protected compound (66 % in CH2Cl2 and evaporation gave pure compound as orange solid, yield 19 mg (38%). The precipitate was centrifuged thoroughly and washed with water (4 × 3 mL).

[5-(Prenyl-3-ylthynyl)uracil-1-yl]-N-propargylacetamide (8).

Yield 13 mg (41%).

Fluorescence (96% ETOH, λmax 530 nm, excitation λmax nm): 455, 440, 468; (96% ETOH, λmax 420 nm, emission λmax nm): 477, 511.

1H NMR (400 MHz, DMSO-d6) δ 8.77–8.79 (m, 4H), 7.93 (s, 4H), 7.81–7.76 (m, 8H), 7.66–7.6 of 30 min TLC showed full consumption of the starting material. The reaction mixture was poured into 10% citric acid (6 mL). The precipitate was centrifuged thoroughly and washed with water (4 × 3 mL).
EtOH, λ<sub>ex</sub> 420 nm, emission λ<sub>max</sub> nm): 450, 475, 508. <sup>1</sup>H NMR (500 MHz, DMSO-<i>d</i><sub>6</sub>) δ 11.83 (br s, 1H), 8.22–8.78 (m, 1H), 8.43 (d, 1H, <i>J</i> = 7.32 Hz), 8.4–8.3 (m, 3H), 8.27 (d, 1H, <i>J</i> = 8.24 Hz), 7.84–7.79 (m, 2H), 7.71–7.63 (m, 2H), 7.58–7.52 (m, 2H), 7.47 (s, 2H), 3.97–3.91 (m, 2H), 3.18 (t, 1H, <i>J</i> = 2.44 Hz). <sup>13</sup>C NMR (100 MHz, DMSO-<i>d</i><sub>6</sub>) δ 166.4, 161.2, 150.0, 149.9, 144.1, 134.2, 133.7, 131.1, 131.0, 130.4, 130.1, 129.8, 128.6, 128.3, 127.8, 127.6, 127.0, 126.9, 125.7, 122.9, 121.6, 121.4, 121.3, 120.3, 119.5, 97.3, 90.6, 88.6, 80.7, 73.5, 49.8, 28.1. HRMS (ESI, m/z): calculated for C<sub>34</sub>H<sub>25</sub>N<sub>6</sub>O<sub>10</sub> [M + H]<sup>+</sup>: 587.3974, found: 587.3973.

**Cell toxicity assay**

A cytotoxicity test in porcine embryo kidney (PEK) cells was performed as described previously.<sup>26a</sup> In brief, PEK cells were seeded and incubated for 72 h at 37°C. Stock solutions of the compounds with the concentration of 5 mM were prepared in 100% DMSO (Sigma). Two-fold dilutions of studied compounds were prepared in medium 199 on Earle solution to obtain final concentrations starting from 50 μM. Equal volumes of compound dilutions were added in four replicates to the cells. Cell control was treated with the same sequential concentrations of DMSO as in compounds dilutions in four replicates. After incubation at 37°C on days 1 or 7 C<sub>50</sub> values were calculated according to the Kerber method.<sup>28</sup>

**Antiviral activity assays**

Plaque reduction test was performed as previously described<sup>26a</sup> on tick-borne encephalitis virus strain Absettarov. Stock solutions of the compounds with concentration of 5 mM were prepared in 100% DMSO (Sigma). Compounds were added to PEK cells simultaneously with virus and incubated at 37°C for 1 h with gentle shaking every 15–20 min. Then, each well was overlaid with 1 mL of 1.26% methylcellulose (Sigma) containing 2% FBS (Gibco). After incubation at 37°C in CO<sub>2</sub> incubator for 6 days cells were fixed with 96% ethanol. Plaques were stained with 0.4% gentian violet. C<sub>50</sub> values were calculated according to Reed-and-Muench method.<sup>29</sup>

**Statistical analysis**

Data are expressed as means ± standard deviations. The statistical significance was analyzed using Student’s t test for at least three independent experiments.

**Molecular modelling**

The molecules were drawn in InstantJChem<sup>30</sup> and transferred to SYBYL-X 2.1 (ref. 27) in MOL format. MMFF94 charges<sup>31</sup> were assigned to the atoms and the Powell<sup>32</sup> optimization (10 000 steps, gradient termination, threshold 0.05 kcal (mol<sup>−1</sup> x A<sup>−1</sup>)) was performed in MMFF94s force field.<sup>31a</sup> Then Confort<sup>33</sup> global minimization was performed, sampling 2000 conformations (program maximum) and optimizing them with termination by negative charge. Precision parameter was set to 0.001. Electrostatics was considered. The number of acyclic rotors concurrently sampled was set to 200, rotors in cycles were not sampled due to the aromaticity of all the studied cycles. Compound 9f contains more atoms than can be treated by Confort and thus was not optimized using this method. Fast Connolly surfaces<sup>33</sup> for the optimized structures were calculated.
by MOLCAD in SYBYL-X 2.1 and colored according to hydrophobic potential.

**Conclusions**

In summary, we used pentaerythritol-based azides for the preparation of clusters containing up to four residues of 5-(perylen-3-ylethenyl)uracil, an antiviral scaffold. UV-Vis, fluorescence, and anti-TBEV properties of compounds were studied. For the first time, the antiviral activity of RAFI clusters was demonstrated. Four compounds, including tetramer 9, showed one-digit nanomolar activity against TBEV, being among the most potent anti-TBEV molecules to date. Some structural features of the most active compounds can be used in further design of antivirals.

**Conflicts of interest**

There are no conflicts of interest to declare.

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