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In search of Flavivirus inhibitors: evaluation of different tritylated nucleoside analogues

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ABSTRACT

Following up on a hit that was identified in a large scale cell-based antiviral screening effort, a series of triphenylmethyl alkylated nucleoside analogues were synthesized and evaluated for their *in vitro* antiviral activities against the dengue virus (DENV) and the yellow fever virus (YFV). Hereto, trityl moieties were attached at various positions of the sugar ring combined with subtle variations of the heterocyclic base. Several triphenylmethyl modified nucleosides were uncovered being endowed with submicromolar *in vitro* antiviral activity against the YFV. The most selective inhibitor in this series was 3',5'-bis-*O*-tritylated-5-chlorouridine (**1b**) affording a selectivity index of over 90, whereas the 3',5'-bis-*O*-tritylated inosine congener (**5b**) displayed the highest activity, but proved more toxic. The finding of these lipophilic structures being endowed with high antiviral activity for flaviviruses, should stimulate the interest for further structure-activity research.

1. Introduction

Several flaviviruses cause severe disease in man. Important members of this genus (family *Flaviviridae*) include the tick-borne encephalitis virus, the West Nile virus, the yellow fever virus (YFV) and the dengue virus (DENV) [1]. There is today no therapy available for the treatment of these potentially life-threatening viral infections [2,3]. We here report on a series of tritylated nucleoside analogues of which some inhibit *in vitro* DENV and YFV replication. Chemical synthesis of these congeners and antiviral evaluation herewith is reported.

DENV is a flavivirus transmitted by mosquitoes (*Aedes aegypti*). Today the virus is endemic in virtually all tropical and subtropical regions of the world [4]. Currently about 2.5 billion people are at risk for developing dengue fever and it is estimated that 50 to 100 million infections occur every year. Half a million people develop dengue haemorrhagic fever annually, of which ~ 25,000 of these cases are fatal [5]. Dengue haemorrhagic fever and dengue shock syndrome are leading causes of hospitalization and death in children in several South-East Asian countries [6]. Currently, there is neither vaccine nor an antiviral drug available, even though dengue is the fastest progressing vector-borne disease worldwide. An antiviral agent could be of good use in the prevention of epidemics and to avert worsening of disease severity [4, 5, 7].

YFV is another mosquito-borne flavivirus, endemic in tropical Africa and South America. It is estimated that 200,000 infections occur annually, leading to 30,000 deaths each year (<http://www.who.int/mediacentre/factsheets/fs100/en/> (November 2011)). A case fatality rate of 20 to 50% has been observed in the severely infected. Although a highly effective and

safe vaccine (17D) is available [1], an antiviral drug would prove useful for treating the severe infections that may occur in the non-vaccinated, especially during epidemics [8, 9].

A regularly used strategy for development of antiviral drugs is the screening of large compound libraries (*in silico* and/or *in vitro*) to identify hit molecules [10] followed by a hit-optimization process. Efforts in our group to discover inhibitors of flavivirus replication resulted in the identification of 2',5'-bis-*O*-tritylated uridine as a hit. This discovery was followed by synthesis of a series of close analogues in which different regio-isomers of trityl containing nucleosides were synthesized as a first attempt for setting up a structure-activity relationship (SAR) as reported here. In another paper [11], the *in vitro* anti-flavivirus activity of the most selective compound identified in this series, i.e. 3',5'-bis-*O*-tritylated uridine, is described in more detail and the mechanism of action, i.e. inhibition of the viral RNA polymerase, is reported.

2. Results and Discussion

2.1. Synthesis and analysis of bis-*O*-tritylated congeners

Synthesis of 5-chlorouridine (**9**) was initially attempted on unprotected uridine (**6**) using ceric(IV)ammonium nitrate (CAN) and concentrated HCl in MeOH at 70°C, according to the procedure described by Asakura and Robins, Figure 1 [12]. Although **9** was obtained in 79% yield, neutralization of the reaction mixture with NaOH proved necessary, resulting in significant amounts of inorganic salts with concomitant solubility problems in several organic solvents, yielding problems on subsequent tritylation attempts. Hence, synthesis of **9** was reinitiated according to another protocol by Asakura using acetylated uridine (**7**) [12]. The supplementary protection simplified workup and product isolation and avoided the use of HCl. Treatment of **7** with ceric ammonium nitrate (CAN) and LiCl in anhydrous MeCN at 80°C gave 89% of **8**, which was deprotected to afford **9** (85%).

Tritylation of unprotected ribonucleosides (uridine, chlorouridine, inosine, Fig. 1 and 2, respectively) is straightforward [13, 14] and was done at 80°C overnight using 2.5 to 3 eq. of triphenylmethyl chloride, affording the 5'-monotritylated (**1a**, **4a**, **5a**) and the 2',5'- and 3',5'-bis-*O*-tritylated analogues (**1b-c**, **4b-c**, **5b-c**). The latter regioisomers were separated tediously by column chromatography, and their structure was proven using 2D-NMR techniques (COSY, HSQC, HMBC), with the 2',5'-analogues proving the slightly more lipophilic ones. Assignment for all compounds starts with unambiguous assignment of the ¹H signals, using COSY NMR (H-H correlation spectroscopy) starting from the easily identifiable H1'. As an example, for **1b** (3',5'-bis-*O*-trityl uridine derivative) the H1' signal is coupled to the H2' signal at 3.67 ppm. Fortunately, also the OH signal at 2.90 ppm is coupled to the signal at 3.67 ppm, indicating already C2'-OH to be unalkylated (Fig. 4A). HSQC further allows

assignment of the carbon signals (Fig. 4B). Finally, HMBC analysis shows a correlation between the quaternary carbons of the trityl moieties around 88 ppm with the proton signals for respectively H3' and H5', and absence of such correlation for H2'. Hence, the structure for **1b** can be unambiguously identified (Fig. 4C). The same strategy was adapted for **1c** (Fig. 4D) and for all other tritylated species (additional spectra can be found in the supplementary file).

In analogy, concomitant mono- and bis-*O*-tritylation of respectively thymidine and 2'-deoxyuridine proved straightforward using 1.8 eq. of trityl chloride yielding compounds **2a-b**, **3a-b**. To obtain the ribonucleoside congeners monotritylated at a secondary alcohol, the more labile monomethoxytrityl (MTr) moiety was used to temporarily protect the 5'-position, followed by tritylation of one of the remaining alcohol moieties. Finally, acidic treatment with TFA in DCM preferentially removed the more labile MTr moiety to afford **1d-e** and **4d**. During the tritylation reaction however, the in situ formed pyridinium hydrochloride combined with the increased temperature removed part of the MTr protecting group and allowed substitution for a trityl moiety, resulting in low yields of the desired compounds. Hence, selective 3'-tritylation of thymidine and 2'-deoxyuridine therefore was accomplished following monobenzoylation of the primary alcohol (Figure 3). Hereto, the 5'-benzoylated analogues were heated overnight with 1.6 eq. of trityl chloride at 85°C, and deprotected with LiOH in aqueous methanol to afford the analogues **2c** and **3c**.

2.2. Assessment of the antiviral activity

The lipophilic uridine analogue **1c** was found in an antiviral screen of an in house available library of nucleosidic compounds to exert selective inhibition of YFV replication

[11] and was used as a lead compound for exploration of its structure activity relationship properties. The 2',5'-bis-*O*-trityl uridine (**1c**) therefore was resynthesized to confirm its activity and at the same time the regioisomeric structures with lipophilic trityl (triphenylmethyl, Tr) moieties attached at different positions on the sugar ring were synthesized and evaluated. The hit compound **1c** inhibited YFV replication with an EC₅₀ of 1.2 μM, and did not affect normal cell morphology at concentrations up to 100 μM, resulting in a calculated selectivity index (SI) of around 80. A 25-fold weaker but still selective activity (SI of ~3) was noticed as well for DENV. Hence, a series of nucleoside derivatives was synthesized with different heterocyclic bases and with one or two lipophilic triphenylmethyl moieties attached at different positions of the sugar ring. An overview of all new compounds tested is given in Fig. 5, and the results are gathered in Table 1.

Whereas the 5' monotritylated analogue **1a** proved 5-fold more potent against DENV, cytotoxicity increased drastically. Both **1d** and **1e**, having a single trityl group attached at either the 2' or 3' hydroxyl position, likewise proved to be relatively toxic molecules. Remarkably, the inhibitory activity of the 3',5'-bis-*O*-tritylated regioisomer **1b** for DENV increased more than 15-fold in comparison with the lead compound **1c** (EC₅₀ of 1.75 and 30 μM, respectively), but cytotoxicity likewise increased. In contrast, both **1b** and **1c** exhibited substantial selectivity for inhibition of YFV replication with a SI of >80.

Removal of the remaining 2' hydroxyl moiety in **1b** resulting in the bis-*O*-tritylated 2'-deoxyuridine **2a** endowed the compound with analogous inhibitory properties, resulting in a SI above 17 for inhibition of DENV (EC₅₀ 3.7 μM) and above 70 for YFV (EC₅₀ 0.9 μM). The monotrityl analogues **2b** and **2c** on the other hand resulted in more adverse effects on the host cell and had lost activity against both the DENV and YFV. Further modification of the

base moiety with a 5-methyl group resulted in the thymidine series **3a-c** which proved considerably less active. Only the bis-*O*-tritylated derivative **3a** resulted in some selectivity for YFV with an EC₅₀ of 10 μM (a 10-fold reduction in activity in comparison with **2a**) and a CC₅₀ of 70 μM.

Chlorination of the uracil moiety as in the series **4a-d** resulted in rather unexpected results. While none or very weak inhibitory properties were recorded in the DENV screen, all three analogues, the mono- and bis-*O*-tritylated nucleosides **4a-c**, were endowed with substantial antiviral effect against YFV. The 2',5'-bis-*O*-tritylated analogue **4c** proved most selective with an EC₅₀ of 0.75 μM and a SI above 90; herein **4c** is markedly less toxic than its congeners **4a** and **4b** and thus results in a higher SI. .

Next, the effect of expanding the base moiety to a two-ring heterocycle was evaluated by analysing tritylated inosine analogues. Herewith, the O⁶ position of the hypoxanthine base could be imagined as a possible mimic of the O⁴ position on the uracil moiety as in **1a-c**. All inosine analogues **5a-c** proved relatively toxic. Only the 3',5'-bis-*O*-tritylated inosine **5b** resulted in a pronounced antiviral effect (EC₅₀ of <0.4 μM against YFV). In analogy with the uridine series, again the 3',5'-modified nucleoside resulted in the best inhibitory properties but was more cytotoxic than the uridine congener **1b**. Finally, a series of cytidine derivatives either unprotected at the base moiety or N⁴-benzoylated, were prepared. None of these molecules inhibited viral replication (data not shown).

To conclude, a general comment to the structure of these compounds is warranted. Obviously the molecular weight is very high (>700) and the log P of some of the presented compounds is very high, which are not the optimal properties for drug-like compounds one might expect. As shown however, a nice inhibitory effect is noticed in a cellular assay and as

discussed by De Burghgraeve et al. [11], the polymerase can be clearly pinpointed as the real target. Apparently uptake is thus not an issue in this cellular assay. In addition, while the trityl group is known as a sugar protecting group, its acid stability is relatively high and hence should not pose too many degradation problems. Nevertheless, we are currently exploring strategies to substitute for one or both of the trityl moieties.

3. Conclusion

In summary, several triphenylmethyl modified nucleosides were discovered that were endowed with relatively pronounced *in vitro* anti-YFV activity. While the lead compound **1c** displayed an EC₅₀ for YFV of 1.2 μM, the congener **1b** inhibited YFV already at slightly lower concentration and the inhibitory properties against DENV were even more in favor of **1b**. Likewise, **4b**, **4c** and **5b** proved submicromolar inhibitors for YFV proliferation. Antiviral activity against DENV mostly proved less strong. These lipophilic compounds are exerting their effect through inhibition of the RNA polymerase of the Flaviviridae. Overall, in view of these simple lipophilic structures being endowed with strong YFV inhibiting properties, further structure-activity research is warranted.

4. Experimental protocols

4.1. General synthetic procedures

Starting nucleosides were obtained from ACROS. All other chemicals were provided by Aldrich or ACROS and were of the highest quality. ^1H and ^{13}C NMR spectra were determined with a 300 MHz Varian Gemini apparatus with tetramethylsilane as internal standard for the ^1H NMR spectra (s = singlet, d = doublet, dd = double doublet, t = triplet, br. s = broad signal, br. d = broad doublet, m = multiplet) and the solvent signal – DMSO- d_6 (δ = 39.6 ppm) or CDCl_3 (δ = 76.9 ppm) – for the ^{13}C NMR spectra. Exact mass measurements were performed with a quadrupole/orthogonal acceleration time-of-flight tandem mass spectrometer (qTOF2, Micromass, Manchester, UK) fitted with a standard electrospray ionization (ESI) interface. All solvents were carefully dried or bought as such. Detailed procedures can be found in the supplementary file.

4.2. Tritylated uridine analogues

Uridine (**6**) (1.1 g, 4.4 mmol) was coevaporated twice with dry pyridine and dissolved in dry pyridine (4.5 mL/mmol). Triphenylmethyl chloride (2.8 eq) was added and the solution was heated to 80°C overnight under argon. The reaction was quenched by adding methanol (3 mL) at room temperature for 30 min. The solution was then concentrated and diluted in dichloromethane. The organic solution was washed three times with a solution of saturated NaHCO_3 and the combined aqueous layers were extracted with dichloromethane. Combined organic layers were dried over Na_2SO_4 , filtered and concentrated under vacuum. The residue was purified by column chromatography on silica (going from 20 to 0% hexane in dichloromethane containing 0.5% triethylamine, followed by 0 to 2.5% MeOH/ DCM and

0.5% triethylamine) and afforded the tritylated compounds **1a** (1184 mg, 2.43 mmol, 56% yield), **1b** (262 mg, 0.36 mmol, 8% yield) and **1c** (517 mg, 0.71 mmol, 16% yield) as white foams.

5'-O-trityluridine (1a): ^1H NMR (DMSO- d_6 , 300 MHz): δ 3.20 (m, 1H, 5a'-H); 3.26 (m, 1H, 5b'-H); 3.96 (m, 1H, 4'-H); 4.09 (m, 2H, 2'-H and 3'-H); 4.16 (d, 1H, J = 5.1 Hz, OH); 5.32 (d, 1H, J = 5.7 Hz, 5-H); 5.50 (d, 1H, J = 4.4 Hz, OH); 5.75 (d, 1H, J = 4.0 Hz, 1'-H); 7.28-7.39 (m, 15H, h,i,j,k,l-H); 7.71 (d, 1H, J = 8.1 Hz, 6-H); 11.35 (br s, 1H, 3-NH). ^{13}C (DMSO- d_6 , 75 MHz): 63.23 (C5'); 59.51 (C3'); 73.33 (C2'); 82.30 (C4'); 88.91 (Ph₃-C and C1'); 101.43 (C5); 127.15, 127.97, 128.27, 143.40 (arom-C); 140.58 (C6); 150.46 (C2); 162.98 (C4). HRMS (thgly) calcd. for C₂₈H₂₆N₂O₆Na⁺ (M+Na⁺) 509.1683; found 509.1708.

3'-5'-bis-O-trityluridine (1b): ^1H (CDCl₃, 500Mhz): δ = 2.90 (d, J = 6.9Hz, 1H, 2'-OH), 3.04 (dd, J = 2.6 and 10.9Hz, 1H, 5'-H), 3.36 (dd, J = 2.1 and 10.9Hz, 1H, 5'-H), 3.65-3.69 (m, 1H, 2'-H), 3.75-3.78 (m, 1H, 4'-H), 4.32 (dd, J = 4.0 and 5.0Hz, 1H, 3'-H), 5.25 (dd, J = 8.2 and 2.2Hz, 1H, 5-H), 5.99 (d, J = 5.1Hz, 1H, 1'-H), 7.18-7.44 (m, 30H, Tr), 7.59 (d, J = 8.2Hz, 1H, 6-H), 8.54 (s, 1H, 3-NH) ppm. ^{13}C (CDCl₃, 500 MHz): δ = 63.08 (C5'), 73.38 (C3'), 74.80 (C2'), 82.82 (C4'), 87.77 (4°C of Tr), 88.19 (4°C of Tr), 89.69 (C1'), 102.21 (C5), 127.40, 127.73, 127.94, 128.19, 128.72, 143.11, 143.38 (Tr), 140.35 (C6), 150.38 (C2), 162.78 (C4) ppm. HRMS (thgly) calcd. for C₄₇H₄₀N₂O₆Na⁺ (M+Na⁺): 751.2779; found 751.2776.

2',5'-bis-O-trityluridine (1c): ^1H (CDCl₃, 500Mhz): δ = 2.79 (d, J = 4.5Hz, 1H, 3'-H), 3.11 (d, J = 1.5Hz, 2H, 5'-H), 3.98 (s, 1H, 4'-H), 4.51 (dd, J = 4.5 and 7.5Hz, 1H, 2'-H), 5.11 (dd, J = 2.1 and 8.1Hz, 1H, 5-H), 6.57 (d, J = 7.5Hz, 1H, 1'-H), 7.05-7.31 (m, 30H, Tr), 7.70 (d, J = 8.1 Hz, 1H, 6-H), 8.85 (s, 1H, 3-NH) ppm. ^{13}C (CDCl₃, 500 MHz): δ = 64.37 (C5'), 70.87

(C3'), 77.77 (C2'), 84.32 (C4'), 86.22 (C1'), 87.84 (4°C of Tr), 87.98 (4°C of Tr), 102.68 (C5), 127.50, 127.81, 127.98, 128.28, 128.50, 128.55, 142.84, 143.14 (Tr), 140.98 (C6), 150.72 (C2), 163.00 (C4) ppm. HRMS (thgly) calcd. for C₄₇H₄₀N₂O₆Na⁺ (M+Na⁺): 751.2779, found 751.2772.

4.4. 5-Chlorouridine (**9**)

Uridine (**6**) (3.0 g, 12.3 mmol) was coevaporated with dry pyridine (2x10 mL) and dissolved in dry pyridine (20 mL). Acetic anhydride (4.1 mL, 43.5mmol) was added and the mixture was stirred at room temperature overnight under argon. Work-up afforded the acetylated uridine intermediate **7** (3.81 g, 10.3 mmol, 84% yield) as a white foam [ESI for C₁₅H₁₈N₂O₉ (M⁺): 258.8 (2,3,5-tri-*O*-acetylribose+H⁺, 37.5%), 370.7 (M+H⁺, 13%), 392.9 (M+Na⁺, 100%)]. The obtained compound **7** (3.81 g, 10.3 mmol) was coevaporated with dry acetonitrile (3x20 mL), followed by addition of acetonitrile (165 mL), LiCl (515 mg, 12.2 mmol) and dried ceric ammonium nitrate (CAN, 11.3 g, 20.6 mmol). The mixture was stirred at 80°C for 44h, after which it was concentrated and partitioned between ethyl acetate (400 mL) and brine (200 mL). Further work-up of the organic layer yielded the peracetylated-5-chlorouridine **8** as a white powder in 89% yield [ESI for C₁₅H₁₇ClN₂O₉ (M⁺): 258.8 (2,3,5-tri-*O*-acetylribose+H⁺, 36%), 404.6 (M+H⁺, 9%), 426.9 (M+Na⁺, 100%)]. This latter compound was dissolved in MeOH (25 mL) and ammonia (25% in H₂O, 25 mL). The mixture was stirred at room temperature for 90 min before an additional aliquot of ammonia (10 mL) was added. The solution was finally stirred overnight, concentrated and coevaporated with toluene (3x15 mL). The desired product **9** was obtained as a white foam (2.7g, 9.7 mmol,

94%) and used for tritylation without any further purification. NMR and MS data were in agreement with published results [12, 15]: ESI for $C_9H_{11}ClN_2O_6$ [(M-H)⁻]: 277.0 (100%).

4.5. General tritylation procedures

The obtained chlorouridine **9** (298 mg, 1.07 mmol) was coevaporated twice with dry pyridine and dissolved in dry pyridine (4.5 mL/mmol). Trityl chloride (2.8 eq; TrCl) was added and the solution was heated to 80°C overnight under argon. The reaction was quenched by adding methanol (3 mL) and work-up including column chromatography on silica afforded the tritylated compounds **4a** (243 mg, 0.48 mmol, 45% yield), **4b** (90 mg, 0.12 mmol, 11% yield) and **4c** (174 mg, 0.23 mmol, 21% yield) as white foams.

An analogous procedure on inosine using a mixture of anhydrous pyridine and DMF for dissolution, afforded after three days of heating the tritylated derivatives **5a** (345 mg, 0.68 mmol, 34% yield), **5b** (55 mg, 0.07 mmol, 4% yield) and **5c** (240 mg, 0.32 mmol, 16% yield) as white foams. Synthesis of the tritylated uridine derivatives **1a-c** has been reported briefly in the accompanying paper. Analogous procedures yielded the tritylated 2'-deoxynucleoside derivatives. Selective tritylation at a secondary alcohol on the other hand was accomplished following protection of the primary 5'-hydroxyl moiety with either a monomethoxytrityl or a benzoyl moiety and followed by deprotection of the 5'-position.

Only for the tritylated uridine analogues complete NMR assignment has been provided as an example. For all other compounds only HRMS data are given, and all ¹H and ¹³C NMR data can be found in the supplementary files, including figures highlighting complete assignment for the bis-*O*-tritylated uridine analogues as well as their HPLC purity evaluation.

2'-*O*-Trityluridine (1d) [16]: HRMS (thgly) calcd. for C₂₈H₂₆N₂O₆Na [(M+Na)⁺]: 509.1683; found 509.1671.

3'-*O*-Trityluridine (1e) [16]: HRMS (thgly) calcd. for C₂₈H₂₆N₂O₆Na [(M+Na)⁺]: 509.1683; found 509.1689.

5',3'-di-*O*-Trityl-2'-deoxyuridine (2a) [17]: HRMS (thgly) calcd. for C₄₇H₃₉N₂O₅ [(M-H)⁻]: 711.2864; found 711.2880.

5'-*O*-Trityl-2'-deoxyuridine (2b) [18]: HRMS (thgly) calcd. for C₂₈H₂₅N₂O₅ [(M-H)⁻]: 469.1769; found 469.1772.

3'-*O*-Trityl-2'-deoxyuridine (2c): [17]: HRMS (thgly) calcd. for C₂₈H₂₆N₂O₅Na [(M+Na)⁺]: 493.1734, found 493.1745.

5',3'-di-*O*-Tritylthymidine (3a) [17, 19]: HRMS calcd. for C₄₈H₄₂N₂O₅Na [(M+Na)⁺]: 749.2986; found 749.2993.

5'-*O*-Tritylthymidine (3b) [20]: HRMS calcd. for C₂₉H₂₈N₂O₅Na [(M+Na)⁺]: 507.1890; found 507.1907.

3'-*O*-Tritylthymidine (3c): [17, 19]: HRMS (thgly) calcd. for C₂₉H₂₈N₂O₅Na [(M+Na)⁺]: 507.1890; found 507.1884.

5'-*O*-Trityl-5-chlorouridine (4a): HRMS (thgly) calcd. for C₂₈H₂₄ClN₂O₆ [(M-H)⁻]: 519.1328; found 519.1339.

3',5'-di-*O*-Trityl-5-chlorouridine (4b) [21]: HRMS (thgly) calcd. for C₄₇H₃₈ClN₂O₆ [(M-H)⁻]: 761.2424; found 761.2437.

2',5'-di-*O*-Trityl-5-chlorouridine (4c) [21]: HRMS (thgly) calcd. for C₄₇H₃₈ClN₂O₆ [(M-H)⁻]: 761.2424; found 761.2426.

2'-*O*-Trityl-5-chlorouridine (4d): HRMS (thgly) calcd. for C₂₈H₂₅ClN₂O₆Na [(M+Na)⁺]: 543.1293; found 543.1271.

5'-*O*-Tritylinosine (5a) [22, 23]: HRMS (thgly) calcd. for C₂₉H₂₇N₄O₅ [(M+H)⁺]: 511.1976; found 511.1997.

3',5'-di-*O*-Tritylinosine (5b) [24, 25]: HRMS (thgly) calcd. for C₄₈H₄₁N₄O₅ [(M+H)⁺]:
753.3071; found 753.3074.

2',5'-di-*O*-Tritylinosine (5c) [24, 25]: HRMS (thgly) calcd. for C₄₈H₄₁N₄O₅ [(M+H)⁺]:
753.3071; found 753.3089.

4.6. Antiviral assays and activity determination

The YFV-17D vaccine strain (Stamaril) (Aventis Pasteur MSD, Brussels, Belgium) was passaged once in Vero-B cells to prepare a working virus stock and stored at -80°C until further use. DENV serotype 2 New Guinea C (NGC) strain (kindly provided by V. Deubel [formerly at Institut Pasteur, Lyon, France]) was cultivated in C6/36 mosquito cells (ATCC: **CRL-1660**). Green monkey kidney cells [Vero-B cells (ECACC for DENV assays and ATCC CCL-81 for YFV assays)] were grown in minimum essential medium MEM Rega-3 (Gibco, Merelbeke, Belgium) supplemented with 10% foetal calf serum (FCS), 1% L-glutamine and 1% sodium bicarbonate. Antiviral assays were performed in medium with 2% FCS. Vero-B cells were seeded at a density of 7×10^3 (DENV assay) or 2×10^4 (YFV assay) cells/well in 100 μl assay medium and allowed to adhere overnight. To each well of a 96-well plate, 100 μl of culture medium containing 100 50% cell culture infectious doses (i.e., CCID_{50}) of virus was added to each well, after which serial dilutions of the compounds were added. Following a one-week incubation, culture medium was discarded and cultures were fixed with ethanol and stained with 1% methylene blue. In parallel, cell cultures were incubated in the presence of compound and absence of virus. The 50% effective concentration (EC_{50}), which is defined as the compound concentration that is required to inhibit the virus-induced CPE by 50%, and 50% cytotoxic concentration (CC_{50}), which is defined as the compound concentration that is required to inhibit the cell growth by 50%, was visually determined. Ribavirin was included as a reference compound. Two independent experiments were carried out.

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Figure captions

Figure 1. Synthesis of the tritylated uridine and 5-chlorouridine analogues. Reaction conditions: i) Acetic anhydride, pyridine, RT; ii) CAN, LiCl, CH₃CN, 80°C; iii) aq. Ammonia – methanol, RT; iv) 2.5 eq. TrCl, pyridine, 80°C overnight; v) 2.5 eq. TrCl, pyridine, 80°C, 48h; 3% TFA in DCM, 2h at °C. Ac: acetyl; Tr: trityl or triphenylmethyl; other abbreviations see text.

Figure 2. Synthesis of the tritylated inosine congeners. Reaction conditions: 2.5 eq. TrCl, pyridine, 80°C 48h.

Figure 3. Synthesis of the tritylated deoxynucleosides. Reaction conditions: i) 1.8 eq. TrCl, pyridine, 80°C overnight; ii) for **2c** and **3c**: Benzoyl chloride, pyridine, 0°C; TrCl, pyridine, 18h 85°C; LiOH 1M in MeOH-water, 5h at RT. Trityl (Tr) structure is depicted at right.

Figure 4. NMR analysis is shown, corroborating the structure for **1b** and **1c**. Panel A shows the COSY spectrum for **1b** with assignment of the sugar proton signals. The HSQC spectrum in Panel B allows assignment of the sugar carbon signals, with in Panels C and D the HMBC correlation between the quaternary trityl signals and the corresponding sugar proton for respectively compound **1b** and **1c**.

Figure 5. Structural overview of all evaluated tritylated nucleoside analogues.