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Solid-phase synthesis of 5′-triphosphate 2′-5′-oligoadenylates analogs with 3′-*O*-biolabile groups and their evaluation as RNase L activators and antiviral drugs

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ABSTRACT

5′-Triphosphate 2′-5′-oligoadenylate (2–5A) is the central player in the 2–5A system that is an innate immunity pathway in response to the presence of infectious agents. Intracellular endoribonuclease RNase L activated by 2–5A cleaves viral and cellular RNA resulting in apoptosis. The major limitations of 2–5A for therapeutic applications is the short biological half-life and poor cellular uptake. Modification of 2–5A with biolabile and lipophilic groups that facilitate its uptake, increase its in vivo stability and release the parent 2–5A drug in an intact form offer an alternative approach to therapeutic use of 2–5A. Here we have synthesized the trimeric and tetrameric 2–5A species bearing hydrophobic and enzymolabile pivaloyloxymethyl groups at 3′-positions and a triphosphate at the 5′-end. Both analogs were able to activate RNase L and the production of the trimer 2–5A (the most active) was scaled up to the milligram scale for antiviral evaluation in cells infected by influenza virus or respiratory syncytial virus. The trimer analog demonstrated some significant antiviral activity.

1. Introduction

In response to the presence of pathogens such as viruses, bacteria, parasites or tumor cells, mammalian cells trigger an innate immune response mainly mediated by interferons (IFNs).[1] One well-characterized mechanism of interferon activation to suppress viral infections is the 2–5A system[2] in which unusual short 5′-triphosphate (TP) 2′-5′-phosphodiester-linked oligoadenylates (2–5A) play a key role to modulate RNA degradation in cells.[3] These oligomers of adenosine (mostly trimers and tetramers) are synthesized from ATP by several cellular 2′-5′ oligoadenylate synthetases (OAS) induced by IFN upon activation by double-stranded viral RNA during infection. The 2–5A binds to an intracellular endoribonuclease RNase L converting it from an inactive monomer to its active dimer to cleave viral and cellular single-stranded RNA.[4–6] This RNA degradation inhibits protein synthesis resulting in cell apoptosis and thereby suppression of viral replication. The effects of 2–5A are transient since natural 2–5A are unstable in cells due to the activities of a 5′-phosphatase and a specific 2′-5′-phosphodiesterase that cleave 2–5A to AMP and ATP.[7]

With the aim to develop antiviral therapeutic agents based on the 2–5A/RNase L pathway, numerous 2–5A analogs have been designed and prepared to obtain RNase L activators with improved properties. Natural 2–5A cannot be used directly as a drug due to its lack of in vivo stability and cell permeability. A number of modifications of the nucleobase,[8,9] ribose,[10,11] internucleotide linkage[12–16] and 5′-phosphoryl group[17,18] have been introduced in the 2–5A to enhance the enzymatic stability and the cellular uptake of these analogs while maintaining or improving the ability of natural 2–5A to activate RNase L. Structure–activity studies on these diverse 2–5A analogs could provide knowledge on the structural requirements of RNase L for the binding to the oligoadenylate molecule. Among these requirements, the presence of a 5′-phosphoryl group, a minimum of three adenosine residues linked by 2′-5′-internucleotidic bonds are critical for the binding to RNase L[19] but to date, no accurate rationale could be ascertained to design efficient 2–5A analogs with permanent modifications.

An alternative approach to design modified 2–5A is founded on the prodrug strategy which consists of the temporary modification of the parent 2–5A by biolabile groups. Protection of 2–5A is aimed at improving its enzymatic stability in biological fluids, its hydrophobicity and therefore its internalization in cells while preserving its activity after the release of the native 2–5A drug in an intact form. For many years, the prodrug approach with oligonucleotides[20] has

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been mainly developed with the protection of the internucleoside phosphate linkage to mask the negative charge[21–24] and cell uptake has been improved in several examples.[22,25,26] Our initial research in this field was also focused on the phosphate modification by the *S*-acylthioethyl group[27] but more recently the ribose 2′-OH in RNA has received our attention to be modified by enzymolabile acyloxymethyl groups.[28–30] In the same way Damha and co-workers investigated the 2′-*O*-acetal levulinic ester (ALE) and various 2′-*O*-(amino acid) acetalesters as prodrug moieties.[31] Our preliminary results demonstrated that such 2′-*O*-acyloxymethyl uridylates fulfilled the criteria of nuclease resistance and esterase hydrolysis.[28] Among the different evaluated protecting groups, the pivaloyloxymethyl (PivOM) was selected for its chemical stability and its lipophilic character to improve cellular uptake. Following this, some 2′-OH of mixed-nucleobase RNA have been masked by the PivOM group to obtain RNA prodrugs. One of them was evaluated in a siRNA assay and the delivery of such modified siRNA was more effective than the natural one. For the first time, these data provided a proof-of concept for a prodrug-based approach for the delivery of RNA to cells.[30]

These previous data offer an excellent guidance to design and synthesize stable and lipophilic 2–5A analogs bearing biolabile PivOM groups at the 3′-position as exogenous RNase L activators for broad spectrum antiviral activity. It is noteworthy that recently Lönnberg and co-workers reported on the use of the PivOM for the synthesis of a 2′-5′-dinucleoside and a fully protected 2′-5′-adenylate trimer in a prodrug strategy to enhance 2–5A cellular uptake.[15,16] These molecules were evaluated neither as RNase L activators nor antiviral inhibitors but only their enzymatic deprotection was investigated. Here, we report the original synthesis of 5′-*O*-triphosphate 2–5A trimer and tetramer analogs with 3′-OH masked by PivOM (Fig. 1) and their biological properties. First their RNase L activating ability was tested using a Fluorescence Resonance Energy Transfer (FRET)-based assay and the trimer analog exhibited the best activation when compared to tetramer. Therefore, its synthesis was further improved and scaled up in order to evaluate its antiviral effects on two viral strains, influenza virus and respiratory syncytial virus (RSV) in cells.

The synthesis of 3′-*O*-PivOM 2–5A analogs was performed on solid support by the phosphoramidite chemistry with a DNA synthesizer. For this purpose a new strategy was developed because the PivOM as well as the phosphate and nucleobases protecting groups (cyanoethyl (CNE) and acyl, respectively) used in standard RNA synthesis are base-labile. Therefore, the final ammonia treatment could not be applied for deprotection of 2–5A analogs to preserve 3′-*O*-PivOM modification. Thus two strategies were possible: either these analogs would have to be synthesized with different protecting groups avoiding RNA deprotection in basic conditions or a new method of deprotection would have to be developed while keeping CNE and acyl as standard groups. The first strategy previously described which consisted in replacing acyl by silyl groups in nucleobases required a fluoride ions treatment to release deprotected RNA.[30] The major drawback of this strategy was the tedious multi-step preparation of the corresponding phosphoramidites. Consequently in this work CNE, acyl groups and succinyl linker to the solid support were selected and we have examined the appropriate conditions for their removal while retaining the PivOM intact.

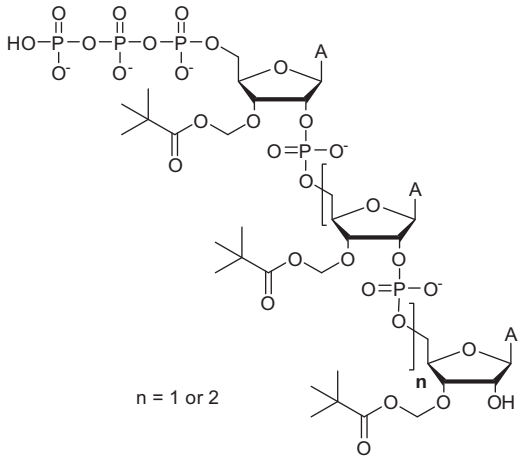


Figure 1. Structure of 5′-TP 3′-*O*-PivOM 2–5A analogs trimer and tetramer.

2. Chemistry

2.1. Synthesis of 2′-*O*-phosphoramidite 3′-*O*-PivOM adenosine 4

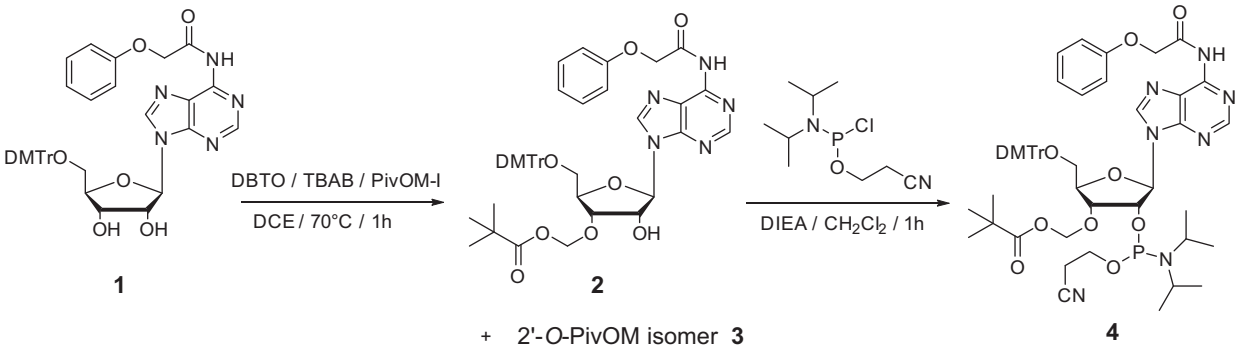
First we prepared the appropriate phosphoramidite **4** from the 3′-*O*-PivOM monomer **2** obtained as a side product during the non-regiospecific synthesis of 2′-*O*-PivOM adenosine **3** previously described (Scheme 1).[32] Starting from 5′-*O*-dimethoxytrityl (DMTr) N^6-phenoxyacetyl (Pac) adenosine **1**, the 2′ and 3′-hydroxyls were protected with PivOM via a 2′,3′-O-dibutylstannylidene intermediate which was treated with PivOM-chloride to give a mixture of the 3′-*O*-PivOM **2** and 2′-*O*-PivOM **3** derivatives (1/1). The desired slow eluting 3′-isomer **2** was isolated with 30% yield after silica gel chromatography. The tritylated 3-PivOM isomer **2** was converted to the corresponding 2′-amidite **4** with 68% yield by using 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite.

2.2. Evaluation of 2′-*O*-phosphoramidite 3′-*O*-PivOM adenosine 4 in the synthesis of a natural 5′-triphosphate 2–5 tetraadenylate

With the phosphoramidite **4** in hand, the natural 5′-TP 2–5A **5** was prepared on an automated DNA synthesizer to test the efficiency of the assembly. The synthesis was performed on a 1 μmol scale by using the commercially available controlled pore glass (LCAA-CPG) linked to 5′-*O*-DMTr 2′-*O*-acetyl N^6-Pac adenosine through a 3′-*O*-succinyl linker (Table 1, entry 1). The same elongation conditions (coupling times 180 s and 5′benzylmercaptotetrazole 0.3 M) as used in the case of 2′-PivOM amidites were applied and the average stepwise yield (99%) was comparable. Upon completion of the assembly, the tetramer still anchored to the support was 5′-functionnalized by a triphosphate following a procedure published earlier.[33] The 5′-OH was first converted in 5′-*H*-phosphonate which was simultaneously oxidized and activated as a phosphoroimidazolide derivative. Subsequent substitution of the imidazole by pyrophosphate gave rise to the fully protected and supported 5′-TP 2–5A. Then a DBU treatment was first applied to eliminate CNE from phosphates followed by an ammonia treatment to remove Pac and PivOM groups and to release the desired 5′-TP 2–5A **5**.[32] Ion-exchange HPLC and MALDI-TOF-MS analysis of the crude **5** revealed efficient elongation and 5′-functionnalization (major peak 49%). To the best of our knowledge this is the first chemical synthesis of the 5′ triphosphorylated 2–5A trimer on the solid support (Table 1, entry 1).

2.3. Study of suitable deprotection conditions to preserve PivOM groups using a 5′-TP 2′-PivOM tetrauridylate model 6

As mentioned above, one of the aims of this work was to find RNA deprotection conditions compatible with the preservation of PivOM groups. In the literature anhydrous organic amines were described to cleave and deprotect synthetic oligonucleotides, such



Scheme 1. Synthesis of 5′-*O*-DMTr 3′-*O*-PivOM N^6-phenoxyacetyl adenosine 2′-*O*-phosphoramidite **4**.

Table 1
Data for synthesized short 5′-TP 2–5A, 5′-TP 3′-PivOM uridylate and 5′-TP 2–5A 3′-PivOM analogs

Entry	ON	5′-Sequence-3′	Synthesis scale[a]	Linker to support	Crude purity[b] (%)	Crude material[c]	Purified material[d]	Molecular formula	Calcd[e] *m*/*z*	Found[e] *m*/*z*
1	**5**	$pppA_{2-5}A_{2-5}A_{2-5}A$	0.5	Succinyl	49	154	n.d[f]	$C_{40}H_{51}N_{20}O_{31}P_6$	1493.79	1491
2	**6**	$pppU_PU_PU_PU$	1.0	Succinyl	64	292	n.d[f]	$C_{54}H_{74}N_8O_{70}P_6$	1743.51	1744
3	**7**	$ppp(A_P)_{2-5}(A_P)_{2-5}A_{Piv}$	1.0	Succinyl	78	157	72	$C_{47}H_{64}N_{15}O_{30}P_5$	1473.96	1473
4	**8**	$ppp(A_P)_{2-5}(A_P)_{2-5}(A_P)_{2-5}A_{Piv}$	1.0	Succinyl	73	132	70	$C_{63}H_{85}N_{20}O_{38}P_6$	1916.33	1917
5	**7**	$ppp(A_P)_{2-5}(A_P)_{2-5}A_{Piv}$	10.0	Succinyl	51	740	654	$C_{47}H_{64}N_{15}O_{30}P_5$	1473.96	1474
6	**7**	$ppp(A_P)_{2-5}(A_P)_{2-5}A_{Piv}$	10.0	Q-linker	70	4590	1665	$C_{47}H_{64}N_{15}O_{30}P_5$	1473.96	1476

ppp = triphosphate; *P* = PivOM; *Piv* = pivaloyl; 2-5: 2′-5′ internucleotidic bond.
[a] Synthesis scale (μmol).
[b] Percentage purity of short 5′-TP-RNA in the crude as calculated from the integration of the IEX chromatogram.
[c] nmol of total crude material released from support and eluted in the TEAB fraction.
[d] nmol of isolated purified material (from TEAB and THF solutions).
[e] MALDI-TOF mass spectrometry in negative mode.
[f] n.d = not determined because not purified.

DNA or RNA molecules.[34,35] The amine function is preferably either a primary or secondary amine with the ability to nucleophilically attack a base labile group on the nucleotide. The amine reagent is used neat or dissolved in an organic solvent that not substantially dissolve the oligonucleotide from the solid support. These findings revealed that the reagent could be removed from the synthesis column leaving the oligonucleotide retained upon the support then it was recovered in a buffer without the need of evaporation. This attractive deprotection method prompted us to test several amines and consequently to apply a mixture of butylamine in THF (1/1) to a protected and support bound RNA decamer with 2′-*O*-PivOM groups as a model. MALDI-TOF-MS analysis did not reveal the presence of the expected fully deprotected RNA but a peak at $m/z = 4166$ matched with the decamer without the CNE and acyl groups and with PivOM groups still in the 2′-position. This data indicated that these deprotection conditions could be suitable for the PivOM preservation.

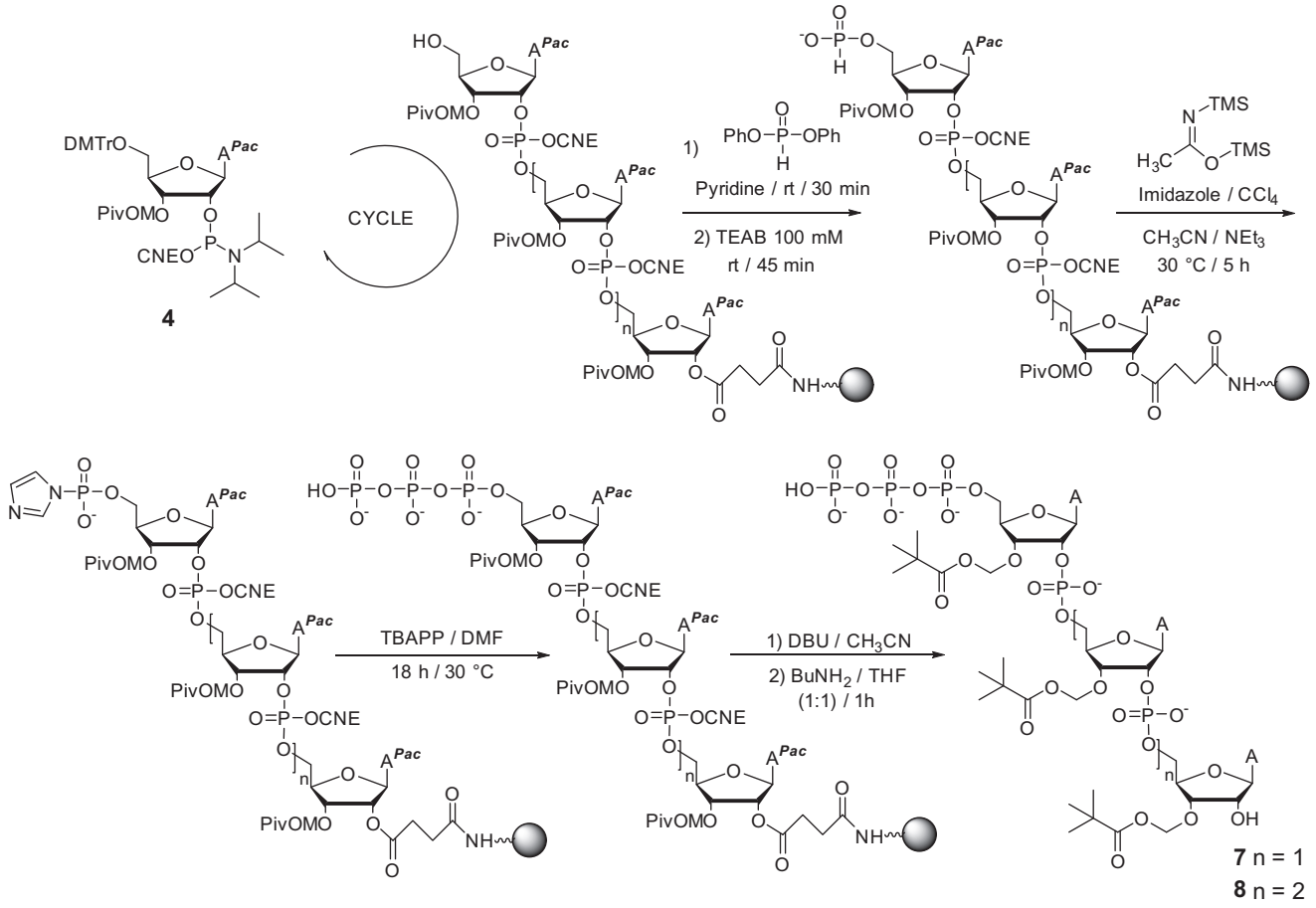
Thus we prepared on a 1 μmol scale a 3′-5′ tetrauridylate model from the commercially available (Chemgenes) 2′-*O*-PivOM uridine phosphoramidite and LCAA-CPG linked to 5′-*O*-DMTr 2′-*O*-Ac U. This short RNA was functionalized with a 5′-TP to mimic the targeted 2–5A analog in order to set up the correct conditions for the best deprotection and recovery. After assembly and 5′-triphosphorylation, the uridylate model compound was first treated with 1 M DBU for 3 min then with a butylamine/THF solution (1/1) at room temperature for 1 h. After washing the support with THF to remove residual amine reagent, the proper 5′-TP 2′-PivOM tetrauridylate **6** was eluted with a 50 mM TEAB solution. The crude material was efficiently recovered (292 nmol) with an estimated purity of 64% (IEX-HPLC analysis) and **6** was characterized by MALDI-TOF MS ($m/z = 1744$) (Table 1, entry 2).

2.4. Solid-phase synthesis of 5′-TP 3′-PivOM 2–5A analogs trimer 7 and tetramer 8 on a 1 μmol scale (Table 1, entries 3 and 4)

Early studies have shown the requirement of at least three 2′,5′-linked adenine residues and a 5′-phosphoryl group for the efficient activation of RNase L.[19] The best activation was obtained with the trimer or the tetramer forms thus we prepared two 5′-TP 2–5A analogs (3- and 4-mers) bearing PivOM groups as prodrugs molecules to evaluate their biological properties.

The synthesis of these analogs **7** and **8** with all the 3′-OH protected by a PivOM group required first the preparation of a support LCAA-CPG linked to 5′-*O*-DMTr 3′-*O*-PivOM N^6-Pac adenosine through a 2′-*O*-succinyl linker following a literature procedure.[36] An efficient loading of 61 μmol/g was obtained. The assembly of 2–5A analogs was achieved on a 1 μmol scale from this support and the phosphoramidite **4** as described above (Scheme 2). Then their 5′-triphosphorylation was performed followed by their deprotection with DBU then butylamine. IEX-HPLC analysis of the two crude materials **7** and **8** showed a major peak representing a purity of 78% and 73%, respectively (Fig. 2, Table 1, entries 3 and 4). MALDI-TOF MS analyses revealed in both cases one peak at m/z 1501 for **7** and at m/z 1944 for **8** corresponding to the desired analogs and a second peak with a difference of 30 Da related to a side product. This difference was assigned to the mass of a formaldehyde molecule which would correspond to the loss of the oxymethyl linker (CH_2O) of PivOM group in adenosine at the 3′-end of 2–5A sequence.

Actually upon butylamine treatment once the succinyl linker was cleaved, free 2′-OH in *cis*-position of 3′-PivOM could lead to a transesterification reaction of pivaloyl (Piv) moiety with loss of formaldehyde and a mixture of 3′-Piv and 2′-Piv was probably



Scheme 2. Solid-phase automated synthesis of 5′-TP 3′-*O*-PivOM 2–5A analogs trimer **7** and tetramer.

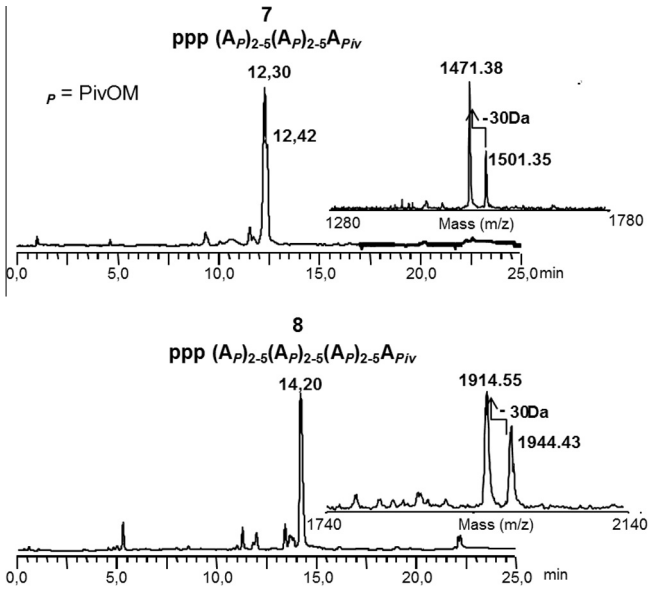
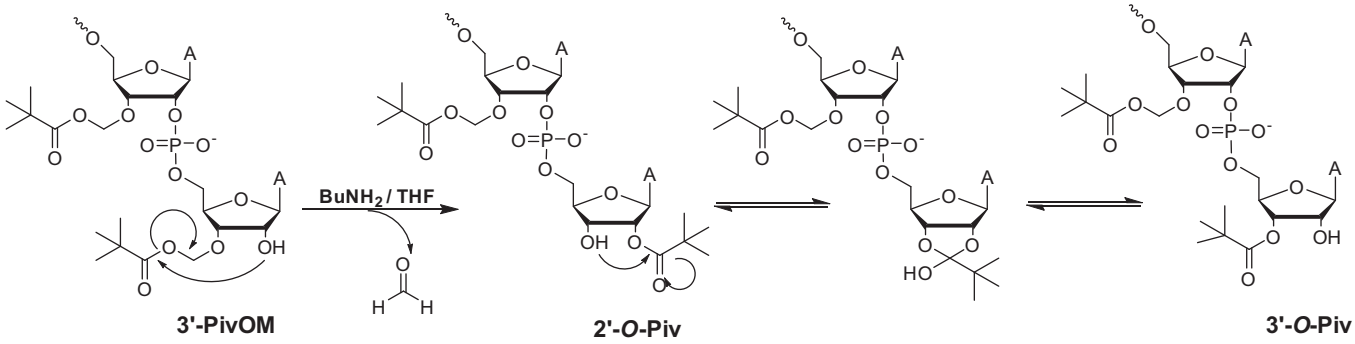


Figure 2. IEX-HPLC and MALDI-TOF MS analyses of the crude material 2–5A trimer **7** and tetramer **8** analogs after deprotection upon butylamine/THF treatment.

obtained at the 3′-end (Scheme 3). However these different species 2′-Piv, 3′-Piv and 3′-PivOM have close lipophilicity and could not be separated by HPLC. The major peak of each crude material was isolated by IEX-HPLC semi-preparative with 96% purity and 70 nmol were recovered. Their MALDI-TOF MS analysis showed a unique peak at *m*/*z* 1916.75 for the tetramer analog **8** attesting the exclusive presence of Piv groups on 2′ or 3′-OH of the 3′-terminal adenosine. For the trimer **7** a major peak at *m*/*z* 1473 was observed but a second peak at *m*/*z* 1502 indicated also the presence of the PivOM group (Fig. 3, Table 1, entry 3). Nevertheless the alteration of 3′-PivOM in 2′ or 3′-Piv should not affect the prodrug properties of the 2–5A analogs since the pivaloyl ester should be also cleaved by cell esterases. Both 2–5 analogs **7** and **8** were tested in a FRET-based assay for their ability to activate RNase L (see Section 3).

2.5. Solid-phase synthesis of 5′-TP 3′-PivOM 2–5A analogs trimer 7 on a 10 μmol scale (Table 1, entries 5 and 6)

With the aim to obtain sufficient amount of the trimer analog **7** for antiviral evaluation in cells, its automated production from **4** was scaled up to 10 μmol. After assembly completion, the 5′-functionalization with triphosphate moiety was performed in the same manner as above. Then a DBU solution was applied to remove CNE of the trimer followed by a dry solution of butylamine in THF to cleave the succinyl linker and to deprotect nucleobases. The crude material (740 nmol) was recovered by elution with a TEAB solution and IEX-HPLC analysis revealed a purity of 51% for the trimer analog **7** in the crude material (Table 1, entry 5). The recovery yield (7.4%) was very low therefore we checked the content of the washing THF solution (2143 nmol) to find some desired material (with 24% purity) which was worthwhile to purify. Both THF and TEAB solutions were separately IEX-HPLC purified to afford larger total



Scheme 3. Transesterification side-reaction on the 3′-terminal adenosine of 2–5A analogs upon butylamine treatment.

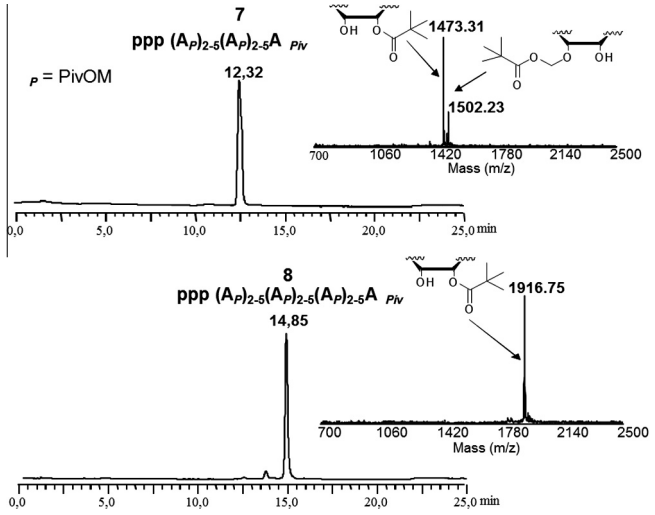


Figure 3. IEX-HPLC and MALDI-TOF MS analyses of purified 2–5A trimer **7** and tetramer **8** analogs.

quantities of **7** (654 nmol). When compared to the synthesis of the uridylate trimer **6**, the recovery of the crude material **7** was about 2–4 times lower (29.2%, 15.7% and 7.4%, see Table 1 entries 2, 3 and 5). The difference between these two syntheses was the 2′-OH PivOM (for **7**) or acetyl (for **6**) protection of the ribonucleoside linked through succinyl linker to the support. Acetyl group was removed by butylamine whereas PivOM remained on the 2′-OH which could not assist the cleavage of the succinyl linker. This hypothesis could explain the difference in the amount of recovered crude material. Even if the use of succinyl linkage allowed synthesis of the desired trimer analog **7**, we investigated the use of the hydroquinone-*O,O′*-diacetic acid (Q-linker) known to be more labile in basic conditions.[37] First we prepared CPG solid support derivatized with the 3′-PivOM adenosine **2** through the Q-linker with a 41 μmol/g loading. As previously with the succinyl linker, the trimer analog assembly was performed at 10 μmol scale then the functionalization at the 5′-end with the triphosphate moiety and finally the deprotection were carried out. The synthesis was more efficient since the purity of the desired trimer **7** was estimated by HPLC at 70% in the 2000 nmol of crude material released in TEAB solution (Table 1, entry 6). THF solution was analyzed and trimer **7** was present at 28% in the crude material representing 2590 nmol. The analog **7** was further isolated after HPLC purification with high purity and in satisfactory quantities (1665 nmol, 16.7%). As expected, recovery of material was more efficient with Q-linker than with succinyl linker upon butylamine treatment. Both batches of the trimer analog **7** were pooled to give rise 3.73 mg of total material for antiviral studies.

3. Biological evaluation of 2–5A 3′-PivOM analogs

3.1. RNase L activation evaluated by FRET-based assay

The synthesized trimer **7** and tetramer **8** analogs were evaluated in vitro for activation of RNase L using a FRET-based enzymatic assay.[38] The ability of the natural 2–5A trimer and 2–5A analogs **7** and **8** to activate RNase L was determined by monitoring the cleavage of a synthetic 5′-[FAM] 36-mer RNA-[BHQ-1]. This RNA substrate corresponds to a segment of the intergenic region of RSV genomic RNA containing several sites potentially cleaved by RNase L (UpUp and UpAp). Upon RNA cleavage the fluorescent FAM group is released from the BHQ quencher. Recombinant human RNase L expressed from a baculovirus vector in insect cells was used in this assay. Fluorescence was measured in a continuous mode at 535 nm for 50 min under excitation at 485 nm. Concentration of 2–5A molecules required for cleavage of half of the synthetic RNA substrate (EC_{50}) was determined. The EC_{50} of the natural 2–5A trimer was 6.1 nM whereas the EC_{50} values were 19.1 and 241 nM for the trimer **7** and the tetramer **8** analogs, respectively. The EC_{50} of both analogs were in the nmol range. Whereas the trimer **7** resulted only in a minimal decrease in activity, the tetramer **8** exhibited a moderate activation of RNase L with a 40-fold drop. However, it is noteworthy that this experiment did not take in account the advantage provided by the prodrug approach since no esterase was present to remove the PivOM groups and to reconstitute the parent 2–5A.

3.2. Antiviral activity of the 2–5A 3′-PivOM trimer

The antiviral activity of the trimer 2–5A analog was first determined by evaluating the inhibition of influenza A/WSN/33 (H1N1) virus in human lung epithelial cells. After 72 h incubation, the antiviral inhibitory effect was measured by the reduction of the luminescence signal from the determination of the viral RNA level in using a Real-Time PCR a one-step quantitative RT-PCR assay.[39,40] An EC_{50} value was obtained from the concentration of active compound causing a reduction of half of the viral RNA production and the cytotoxic effect (CC_{50}) was evaluated by spectrophotometry determination of ATP levels in a cell viability assay (Fig. 4). The efficacy of the trimer analog (EC_{50} = 14.6 μM) was quite similar to the efficacy of a control compound ALS-480 (EC_{50} = 3.2 μM) which serves as reference in Alios BioPharma assays. However the trimer analog exhibited marginal specific activity in cell culture because the window between EC_{50} and CC_{50} values was narrow. Indeed, the EC_{50} = 14.6 μM, CC_{50} = 47 μM window is only approximately 3-fold making it likely that cell killing is contributing significantly to the observed antiviral effect in the assay. In comparison the activity of ALS-480 is clearly not mediated via killing cells as the EC_{50} = 3.2 μM and CC_{50} = 194 μM window is large (selectivity index 60). Nevertheless, it is noteworthy that no transfection agent

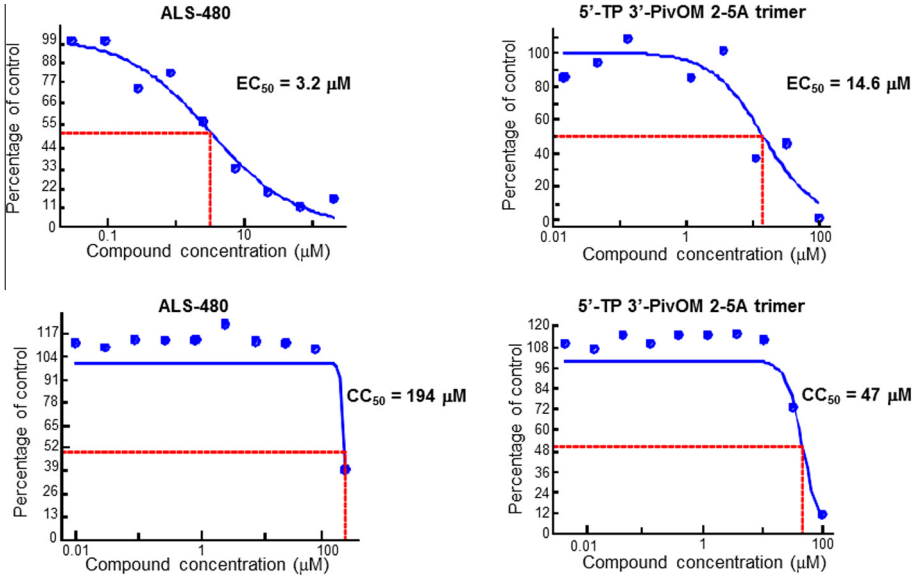


Figure 4. Determination of EC_{50} and CC_{50} of 5′-TP 3′-PivOM 2–5A trimer in human lung epithelial cells infected by influenza virus.

was used in this assay and the 2–5A 3′-PivOM analog could be active proving the benefit of the biolabile groups on the increase of the time life in cytoplasm and cell penetration.

Secondly, the antiviral inhibitory effect of the 3′-PivOM 2–5A trimer was evaluated in a Vero cell line (CCL-81) infected by the RSV strain A2.[41,42] In this cytopathic effect (CPE)-based assay the trimer 2–5A analog demonstrated similar activity to the positive control ALS-480. In both cases an antiviral activity was noticed from drug concentrations of 15 nM and above (Fig. 5). The control compound ALS-480 exhibited a higher cytotoxicity since tested alone the blue color disappeared at 33.3 μM whereas for the trimer analog as up to 100 μM compound concentrations, no reduction in cell viability was observed in the absence of RSV (blue color). However, in the presence of RSV the cellular cytotoxicity was synergistically enhanced for both ALS-480 (1.23 μM) and the trimer analog (33.3 μM) (Fig. 5, dotted red line). Nevertheless its activity window was larger than the one of the ALS-480 reference compound. This data suggests that the 5′-TP 3′-PivOM 2–5A trimer was able to activate RNase L in the Vero cell line and exhibited a noteworthy antiviral activity.

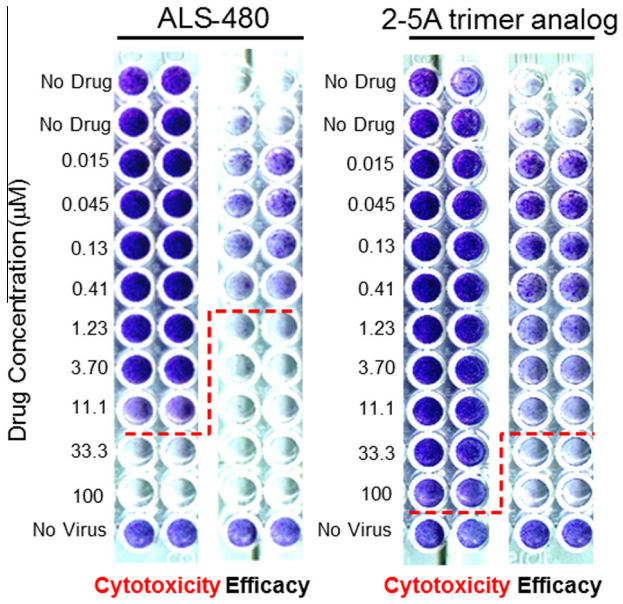


Figure 5. Inhibition of RSV A2 replication in Vero cell line in a CPE-based assay.

4. Conclusion

In conclusion, we succeeded in the synthesis of a prodrug of 2–5A trimer bearing biolabile groups with significant ability to activate RNase L and a noteworthy antiviral activity vs. RSV. This promising data is encouraging to pursue the prodrug approach to increase cellular delivery of 2–5A molecules.

5. Material and methods

5.1. General experimental procedures

CH_3CN, pyridine and triethylamine were distilled over calcium hydride. Dichloromethane and carbon tetrachloride were distilled over phosphorus pentoxide. All reactions were performed in anhydrous conditions under argon. The NMR experiments were accomplished on a Bruker DRX 400 spectrometer at 20 °C. HRMS analyses were obtained with electrospray ionization (ESI) in positive mode on a Q-TOF Micromass spectrometer.

Analytical and semi-preparative high performance liquid chromatographies were performed on a Dionex DX 600 HPLC system or a Dionex U 3000 HPLC system equipped with anion-exchange DNAPac PA 100 columns (4 × 250 mm for analysis or 9 × 250 mm, Dionex). The following HPLC solvent systems were used: 5% CH_3CN in 25 mM Tris–HCl buffer, pH 8 (buffer A) and 5% CH_3CN containing 400 mM $NaClO_4$ in 25 mM Tris–HCl buffer, pH 8 (buffer B). Flow rates were 1.5 and 5 mL min^{-1} for analysis and semi-preparative purposes, respectively. MALDI-TOF mass spectra were recorded on a Voyager-DE spectrometer (Perseptive Biosystems, USA) using a 10:1 (m/m) mixture of 2,4,6-trihydroxyacetophenone/ammonium citrate as a saturated solution in acetonitrile/water (1:1, v/v) for the matrix. Analytical samples were mixed with the matrix in a 1:5 (v/v) ratio, crystallized on a 100-well stainless steel plate and analyzed. UV quantitation of RNAs was performed on a Varian Cary 300 Bio UV/Visible spectrometer by measuring absorbance at 260 nm.

5.2. Chemistry

5.2.1. 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-pivaloyloxymethyl-N^6-phenoxyacetyl-adenosine 2

Compound **2** was prepared according to a reported procedure.[32] The crude mixture of both isomers **2** and **3** was subjected to silica

gel column chromatography with a step gradient of acetone (0–40%) in dichloromethane with 1% pyridine. The second-eluted isomer was the desired compound **2** and was obtained as yellow foam after evaporation of the solvent. ^{1}H NMR (400 MHz, HH-COSY, $CDCl_3$): δ 9.38 (s, 1H, N*H*), 8.70 (s,1H, *H*-2), 8.20 (s, 1H, *H*-8), 7.34-7.03 (m, 18 H, H ar, DMTr, Pac), 6.01 (d, $^3J_{H1'/H2'}$ = 5.6 Hz), 1H, *H*-1′), 5.38, 5.35 (2d$_{AB}$, J_{AB} = 6.4 Hz, 1H+1H, OC*H*$_2$O), 4.94 (t, $^3J_{H2'/H3',H1'}$ = 5.2 Hz, 1H, *H*-2′), 4.83 (s, 2H, NHCOC*H*$_2$Ph), 4.56 (dd, $^3J_{H3'/H2',H4'}$ = 4 Hz, *H*-3′), 4.32 (q, $^3J_{H4'/H5',H5'',H3'}$ = 3.7 Hz, *H*-4′), 3.46 (dd, $^2J_{H5'/H5''}$ = 10.4 Hz; $^3J_{H5'/H4}$ = 4 Hz, 1H, *H*-5′), 3.26 (dd, $^2J_{H5''/H5'}$ = 10.8 Hz; $^3J_{H5''/H4}$ = 4 Hz, 1H, *H*-5″), 1.17 (s, 9H, OCOC(C*H*$_3$)$_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.8 (OC=O), 166.6 (NH*C*O), 158.6, 157.0, (Cq, Car), 157.0 (Cq, Pac), 152.5 (C$_2$), 151.5 (C$_6$), 148.4, 144.4 (Cq, Car), 142.2 (C$_8$), 130.0, 129.9, 128.2, 128.0, 127.9, 126.9, 125.3, 122.5, 114.9, 113.2 (CH, Car), 123.1 (C$_5$), 89.4 (O*C*H$_2$O), 88.8 (C$_{1'}$), 86.6 (Cq, DMTr), 83.0 (C$_{4'}$), 79.0 (C$_{2'}$), 74.2 (C$_{3'}$), 68.2 (NHCOCH$_2$OPh), 62.9 (C$_{5'}$), 55.2 (OCH$_3$, DMTr), 38.8 (Cq, OCO*C*(CH$_3$)$_3$), 27.0 (OCOC(*C*H$_3$)$_3$). HRMS (ESI$^+$) *m*/*z* calcd for $C_{45}H_{48}N_5O_{10}$ (M+H)$^+$ 818.3400, found 818.3401.

5.2.2. 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-pivaloyloxymethyl-2′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) N^6-phenoxyacetyl-adenosine 4

Compound **2** (2 g, 2.45 mmol, 1 equiv) was dried by 3 coevaporations with anhydrous CH_3CN. Then the residue was dissolved in anhydrous CH_2Cl_2 (25 mL) and a mixture of *N*,*N*-diisopropylethylamine (770 μL, 4.4 mmol, 1.8 equiv), 2-cyanoethyl *N*,*N*-diisoproplylchlorophosphoramidite (814 μL, 3.67 mmol, 1.5 equiv) in CH_2Cl_2 (2 mL) was added dropwise. The mixture was stirred under argon at room temperature for 2 h. After completion, ethyl acetate was added, the reaction mixture was poured into saturated $NaHCO_3$ solution and AcOEt extractions were carried out. The mixture obtained after drying of the extract over Na_2SO_4 and removal of the solvent was purified by silica gel column chromatography with an isocratic gradient of CH_2Cl_2 and AcOEt (1:1) with 1% pyridine. The desired phosphoramidite **4** was obtained as white foam after evaporation of the solvent. (1.7 g, 1.67 mmol, 68%). ^{31}P NMR (121 MHz, $CDCl_3$): δ 151.39, 151.09. HRMS (ESI)$^+$ *m*/*z* calcd for $C_{54}H_{65}N_7O_{11}P$ (M+H)$^+$ 1018.4467, found 1018.4480.

5.2.3. 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-pivaloyloxymethyl-2′-*O*-succinyl-CPG N^6-phenoxyacetyl-adenosine

To a dried microwave vial was added succinylated LCAA-CPG (650 mg), adenosine derivative **2** (213 mg, 0.26 mmol, 1 equiv), 4-dimethylaminopyridine (32 mg, 0.26 mmol, 1 equiv), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (100 mg, 52 mmol, 2 equiv) in anhydrous CH_3CN (5 mL). The reaction mixture was heated over microwave irradiation at 60 °C for 1 h. The CPG was filtered off, washed with CH_2Cl_2 and dried.

For the capping step: in a 50 mL tube flask, CPG was mixed with 2.5 mL of a solution of 5% phenoxyacetic anhydride in tetrahydrofuran and pyridine and 2.5 mL of a solution 10% methylimidazole in THF. The flask was shaken at room temperature for 1 h. The CPG was filtered off, washed with CH_2Cl_2 and dried over P_2O_5. Nucleoside loading was determined by DMTr analysis at 498 nm and was 62 μmol/g.

5.2.4. 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-pivaloyloxymethyl-2′-*O*-hydroquinone-*O*,*O*′-diacetylhemiester-N^6-phenoxyacetyl-adenosine

Adenosine derivative **2** (1.67 g, 2 mmol, 1 equiv) was dried by 3 coevaporations with anhydrous pyridine. Then the residue **2**, hydroquinone-*O*,*O*′-diacetic acid (543 mg, 2.4 mmol, 1.2 equiv), 4-dimethylaminopyridine (24 mg, 0.2 mmol, 0.2 equiv), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (382 mg, 2 mmol, 1 equiv) and triethylamine (200 μL) were dissolved in anhydrous pyridine (14 mL) and stirred at room temperature for 6 h. Then pyridine solution was evaporated to oil, redissolved in CH_2Cl_2 and H_2O extractions were carried out. The mixture obtained after drying of the extract over Na_2SO_4 and removal of the solvent to yield crude yellow foam. HRMS (ESI)$^+$ *m*/*z* calcd for $C_{55}H_{56}N_5O_{15}$ (M+H)$^+$ 1026.3759, found 1026.3773.

5.2.5. 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-pivaloyloxymethyl-2′-*O*-Q-CPG-N^6-phenoxyacetyl-adenosine

To a dried 50 mL tube flask was added LCAA-CPG (1 g), unpurified 2′-*O*-hydroquinone-*O*,*O*′-diacetylhemiester derivative (551 mg, 0.5 mmol, 1 equiv), 4-dimethylaminopyridine (12.4 mg, 0.1 mmol, 0.2 equiv), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (192 mg, 1 mmol, 2 equiv) in anhydrous pyridine (10 mL). The flask was shaken at room temperature for 6 h. The CPG was filtered off, washed with CH_2Cl_2 and dried.

For the capping step: in a 50 mL tube flask, CPG was mixed with 5 mL of a solution of 5% phenoxyacetic anhydride in tetrahydrofuran and pyridine and 5 mL of a solution 10% methylimidazole in THF. The flask was shaken at room temperature for 1 h. The CPG was filtered off, washed with CH_2Cl_2 and dried over P_2O_5. Nucleoside loading was determined by DMTr analysis at 498 nm and was 40 μmol/g.

5.2.6. Synthesis of 2–5A sequences on solid-support

RNA synthesis was performed on an ABI 394 synthesizer (Applied Biosystems) at 1 or 10 μmol scale using the prepared solid-supports with a succinyl or a Q-Linker. 2–5A sequences were assembled in Twist oligonucleotide synthesis columns (Glen research) with the phosphoramidite building block **4**. Phosphoramidite **4** was vacuum dried prior to its dissolution in extra dry acetonitrile (Biosolve) at 0.1 M. For the coupling reaction, the activator was 5-benzylmercaptotetrazole (BMT, Chemgenes) used at 0.3 M concentration. Dichloroacetic acid (3% in CH_2Cl_2) (Glen research) was the detritylation reagent. The capping step was performed with a mixture of 5% phenoxyacetic anhydride (Pac_2O) in THF and 10% *N*-methylimidazole in THF (Link Technologies). The oxidizing solution was 0.1 M iodine in THF/pyridine/H_2O (78:20:2; v/v/v) (Link Technologies). After 2–5A assembly completion, the column was removed from the synthesizer and dried under a stream of argon.

5.2.7. 5′-Triphosphorylation of 5–10

The solid-supported 1 or 10 μmol 5′-*H*-phosphonate, 5′-phosphoroimidazolide derivatives, 5′-triphosphate as well as tris(tri-*n*-butylammonium) hydrogen pyrophosphate were synthesized as described previously.[33]

5.2.8. Deprotection and release of 5′-TP sequences 6–10

5.2.8.1. 1 μmol Synthesis scale (6–8). 2 mL of a 1 M DBU solution in anhydrous CH_3CN was applied to the column for 3 min. Then the solution was removed and the solid-support was washed with anhydrous CH_3CN followed by a 1 min flush with argon and dried under vacuum over P_2O_5 for 2 h. Then 2 mL of a dry solution of butylamine/THF 1:1 (v/v) was applied to the synthesis column using two glass syringes filled of 4 Å molecular sieves (5 beads each). The solution was pushed back and forth through the synthesis column for 2 min and left to react 2 h at 30 °C. The solution was removed and the column was rinsed with 1 mL of dry THF followed by a 1 min gently flush with argon. The crude material was eluted with 2 mL of 50 mM TEAB in a 50 mL round-bottomed flask. The mixture was evaporated under reduced pressure to dryness and the residue was co-evaporated three times with 1 mL of water. The residue was dissolved in water (1.5 mL divided in three portions for flask rinse: 0.8, 0.4, 0.3 mL) and transferred to 2 mL Eppendorf-vials then lyophilized from water.

5.2.8.2. 10 μmol Synthesis scale (7). The same procedure as above was applied with 5 mL of DBU solution and 5 mL of butylamine/THF (1:1) solution. The solution was removed from the column and was collected in a 100 ml round bottom flask filled with 10 mL of 50 mM TEAB. A first elution was performed with 2 mL of dry THF in this same flask, followed by a 1 min gently flush with argon. Then a second elution was performed with 5 mL of 50 mM TEAB in a 50 mL flask. Both mixtures were evaporated under reduced pressure to dryness and the residues were co-evaporated three times with 1 mL of water. Each residue was dissolved in water (1.5 mL divided in three portions for flask rinse: 0.8, 0.4, 0.3 mL) and transferred to 2 mL Eppendorf-vials then lyophilized from water.

5.2.9. Purification and desalting of 5′-TP sequences 5–8

The crude 5′-TP sequences (**5**–**8**) were analyzed by anion exchange HPLC using a 0–30% linear gradient of buffer B in buffer A, and they were characterized by MALDI-TOF spectrometry. The crude mixtures (**7**–**8**) were then purified by semi-preparative IEX-HPLC with a 0–30% linear gradient of buffer B in buffer A. The pure fractions of 5′-TP 2–5A were pooled in a 100 mL round-bottomed flask and were concentrated to dryness under reduced pressure. The residues were dissolved in 100 mM TEAB buffer, pH 8 (8 mL divided in three portions for flask rinse: 5, 2, 1 mL) and were loaded on a C_{18} cartridge (Waters, Sep-Pak®). Elution was performed with 10 mL of 50 mM TEAB then with 10 mL of 50% CH_3CN in 12.5 mM TEAB. The second fraction containing the desired compound was collected in a 100 mL round-bottomed flask and was freeze-dried. The residue was dissolved in 1.5 mL water (divided in 3 portions of 0.8, 0.4, 0.3 mL for flask rinse) and transferred to a 2 mL Eppendorf-vial and lyophilized from water. Lyophilized 5′ TP-RNAs were stored at −20 °C for several months without any degradation.

5.3. FRET assay

The activation of RNase L endonuclease activity can be detected in a real-time Förster resonance energy transfer (FRET) assay. This assay utilizes a 36 nucleotide synthetic oligoribonucleotide substrate: 6-FAM-UUA UCA AAU UCU UAU UUG CCC CAU UUU UUU GGU UUA-BHQ-1 with a fluorescence emitter (6-FAM) at the 5′-end and a quencher (BHQ-1) at the 3′-end. The RNA sequence corresponds to a segment of the intergenic region of respiratory syncytial virus (RSV) genomic RNA, chosen because it contains several cleavage sites for RNase L (UU or UA). Upon RNA cleavage, the fluorescent group is released from the quencher. The recombinant human RNase L expressed from a baculovirus vector in insect cells was used in this assay at an effective concentration of 20 nM, together with 200 nM FRET RNA in a final volume of 10 μL cleavage buffer [25 mM Tris–HCl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 50 μM ATP, 7 mM 2-mercaptoethanol, and 0.005% tween 20]. 2–5A compounds were added with a 384-well black polypropylene plate. Fluorescence was measured in a continuous mode up to 50 min with a Wallac 1420 Victor^{3}V multilabel counter (PerkinElmer Life Sciences, Shelton, CT)(excitation 485 nm; emission 535 nm). False positives were eliminated by screening the compounds in parallel in the absence of RNase L. Measured EC_{50} in the RNase L activation assay is defined as the concentration at which the emitted fluorescence reaches half of its maximum rate (V_{max}). The overall catalytic rate of the enzyme, expressed as k_{cat}, is directly calculated by dividing V_{max} by the enzyme concentration. Compounds **7** and **8** were run in duplicate with natural 2–5A.

5.4. Antiviral activity

5.4.1. Influenza virus assay

Human lung epithelial cells (A549) are routinely cultured in Dulbeco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and penicillin–streptomycin (100 U/mL) and maintained at 37 °C, 5% CO_2. The laboratory-adapted influenza strain A/WSN/33 (H1N1) was obtained from (ATCC). This virus has been previously propagated by infecting 10-day-old embryonated chicken eggs. Allantoic fluid containing virus was harvested 48 h post-inoculation. Titer of virus stock was determined by plaque assay on MDCK cell monolayers.

A549 cells were prepared and plated in 24-well culture plates at a density of approximately 1×10^5 cells/well and incubated for 24 h at 37 °C. The next day the cells were washed twice with PBS and then dosed with serially diluted compound (1:3), after incubating at 37 °C for 1 h, the cells were challenged with influenza A/WSN/33 virus. Cells were then incubated at 37 °C for 72 h. After 72 h incubation the A549 cell supernatant media was collected and centrifuged to remove cellular debris. The supernatant was then divided into aliquots and stored at −80 °C. Influenza viral RNA was extracted from the A549 cell supernatant using the QIAamp viral RNA mini kit (Qiagen, GmbH, Hilden, Germany) and viral RNA quantity was assessed by OD_{260}. Viral RNA was then stored at −80 °C. To assess the level of influenza virus RNA in the cellular supernatant a Real-Time PCR a one-step quantitative RT-PCR assay was employed, this assay makes use of minor groove binder (MGB) probe technology. Real-Time PCR using primers and probe targeting a highly conserved region of the matrix gene (M2) of influenza type A viruses were used. The required concentration of compound to reduce the 50% viral copy number (EC_{50}) was calculated by regression analysis of the dose–response curves generated from these data.

Cellular cytotoxicity was assessed using an ATP-based assay readout. A549 cells (5000 cells/well) were grown in 96-well plates for 24 h. The medium was then replaced with that containing serially diluted compounds and the cells are further incubated for 72 h. The cytotoxicity of the compounds was measured using a Cell Titer-Glo® Luminescent Cell Viability Assay (ATP-based cell viability assay) (Promega, Madison, WI). Cellular efficacy and toxicity of oseltamivir was tested in parallel as control for all experiments.

5.4.2. Respiratory syncytial virus CPE assay

The host cell line Vero (CCL-81, ATCC) was propagated in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) with 5% Fetal Bovine Serum and 200 Units/ml Penicillin/Streptomycin at 37 °C, 5% CO_2. The day before the experiment, the cells were trypsinized, counted and plated at 30,000 cell/well in 96-well plates. On the day of the experiment, test compounds were serially diluted in serum free DMEM media and then added to the plated cells. The wells designated for positive control (cells only without virus) and negative control (cells with virus but no drug protection) were not dosed. After 2 h pre-incubation with compounds, RSV A2 (VR-1540, ATCC) at the MOI of 0.05 was added to the cells, except for the positive controls. The plate was then incubated at 37 °C, 5% CO_2 and observed daily for the cytopathic effects (CPE) in the cells. When the negative controls achieved full or near full CPE, the culture media was removed from the wells and Hucker's Crystal Violet Solution (Fisher Scientific) was added to the cells at 50 μL per well and further incubated for 5 min at room temperature. The stained plate was then washed extensively to remove the background stain, air dried and scanned. The cytotoxicity assay of compounds was performed simultaneously with a CPE assay and was carried out the same way as respective CPE assay except that no virus was added to the cells.

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