Self adjuvanting immunopeptides : design and synthesis

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Chapter 6: Towards convergent synthesis of viral VPg proteins linked to RNA

Presented at Europic2018, 3-7 June 2018, Egmond aan Zee, The Netherlands: Gential, G. P. P. *et al.* General methodology for the chemical synthesis of polynucleotidylated picornaviral genome-linked proteins, *Poster C06*.

Introduction

Nucleoproteins are naturally occurring polymers in which an hydroxy amino acid in a protein or peptide is covalently linked via a phosphodiester bond to the terminal hydroxyl of DNA or RNA[1]. Representatives of this class of hybrid biopolymers are widely found among different families of viruses, such as picornaviruses [2,3]. Members of the family of picornaviruses, such as polio- and coxsackievirus, have vertebrates as host and are associated with a number of diseases. Picornaviruses are RNA viruses the initiation of translation of which proceeds via a unique mechanism. Nucleoproteins of picornaviruses termed VPg (viral protein genome-linked) act as primer of RNA synthesis and a lot of research has been devoted to elucidate this complex mechanism at a molecular level[4–9]. In line with these investigations and to obtain useful molecular tools[10] attention was directed to the development of synthetic procedures to fragments of nucleoproteins. The main issue toward the assembly of fragments of nucleoproteins is to make compatible the chemistry of oligo(deoxy)nucleotides and oligopeptides. The automated synthesis of both oligonucleotides and oligopeptides are at a high level and within certain limits of size and composition all oligopeptides and oligonucleotides can be prepared. However, both the basic conditions inherent to the oligonucleotide synthesis and the acidic conditions belonging to oligopeptide synthesis can cause side reactions, which are detrimental for the nucleopeptide. Several synthetic approaches to nucleopeptides, including solution and solid phase approaches have been reported. Obviously, a solid phase procedure is most convenient to acquire nucleopeptides in which both the peptide and nucleotide part have a length larger than two amino acids and two nucleotides. The synthesis of DNA nucleopeptides is extensively investigated and several procedures have been published. For instance, the group of Grandas reported a solid phase synthesis of DNA nucleopeptides with both an extended peptide and oligodeoxynucleotide

part[11–13]. Nucleopeptides, varying in length and composition, in which the oligopeptide was covalently linked via a phosphodiester bond to the terminal 3' of the oligodeoxynucleotide, were prepared via an on-line solid phase approach. In contrast, the synthesis of RNA nucleopeptides is less explored. In one approach nucleotide amino acid building blocks were applied toward the solid phase synthesis of RNA nucleopeptides in which the oligopeptide is provided with a mono- or dinucleotide[14–18]. Recently van der Heden van Noort *et al* reported an automated sequential solid phase approach towards viral RNA-nucleopeptides[19]. In viral RNA nucleopeptides a hydroxy amino acid in the peptide is covalently linked via a phosphodiester bond to the terminal 5'-hydroxyl of RNA. As shown in Figure 1, to attain an on-line solid phase synthesis of a viral RNA nucleopeptide, first the oligopeptide was assembled on a HMBA resin followed by extension of the immobilized peptide with the RNA fragment. While common Fmoc protected amino acid building blocks were used for the peptide synthesis unconventional RNA building blocks were applied for the ensuing RNA synthesis. In these building blocks, the DMTr group, as a temporary protective group, is positioned at the 3' hydroxyl while the 2-cyanoethyl phosphoramidite function is installed at the 5' hydroxyl. A virus derived pentapeptide bearing a 9-mer oligonucleotide on the tyrosine was prepared [20].

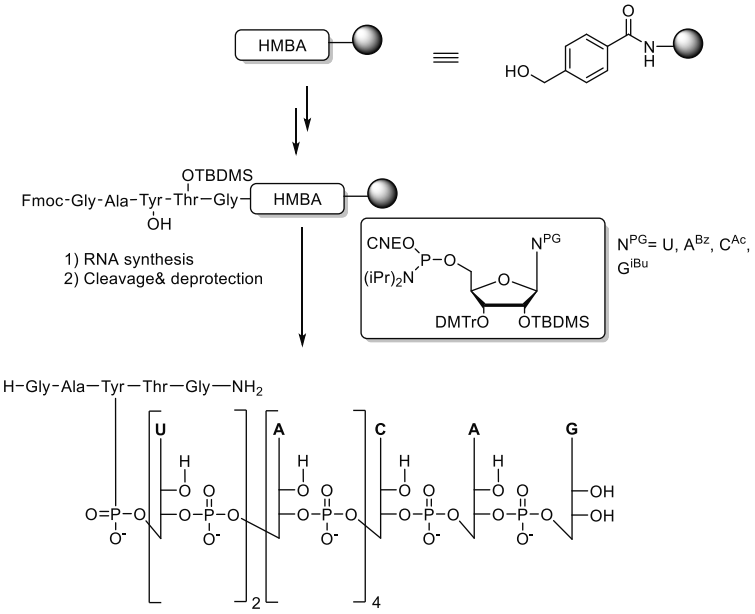


Figure 1. Oligonucleopeptide synthesis on peptide side chain using DMTr as a temporary protective group by van der Heden van Noort[20].

Although this method proved to be very powerful, the repetitive acid mediated cleavage of the temporary DMTr protective group forbid the use of an acid cleavable linker on the resin and therefor only nucleopeptides having carboxamide on the C-terminus are accessible. Nucleopeptides with a C-terminal carboxylic acid are more favorable and allow a solution phase block condensation with a separate oligopeptide to construct ultimately the complete native VPg nucleopeptide of picornaviruses. Bearing this goal in mind it was decided to adjust the method of van der Heden van Noort *et al.*[20] and coxsackievirus VPg **1** was chosen as potential target compound (Figure 2). Retrosynthetic analysis shows that complete VPg of this virus can be obtained by solution phase block condensation of peptide **3** and nucleopeptide **2**, both of which can be assembled by automated solid phase synthesis. Suitable Fmoc amino acid building blocks

for the solid phase synthesis of nucleopeptide **3** should be minimal protected to avoid acidic deprotection conditions incompatible with RNA. Consequently, lysine side chains are protected as TFA amides, which can be cleaved by ammonia treatment, a commonly used deprotection reagent for oligonucleotides. The oligonucleotide in nucleopeptide **2** was appended using nucleotide building blocks (**31-34**) in which the orthogonal acid cleavable DMT ether was replaced by the hydrazine cleavable levulinic ester (Lev-group). This modification of protective group strategy permits the application of HMBP resin that is provided with a mild acid cleavable linker, leading upon deprotection at the end of the synthesis to a native carboxylic acid on C terminus of the (nucleo)peptide.

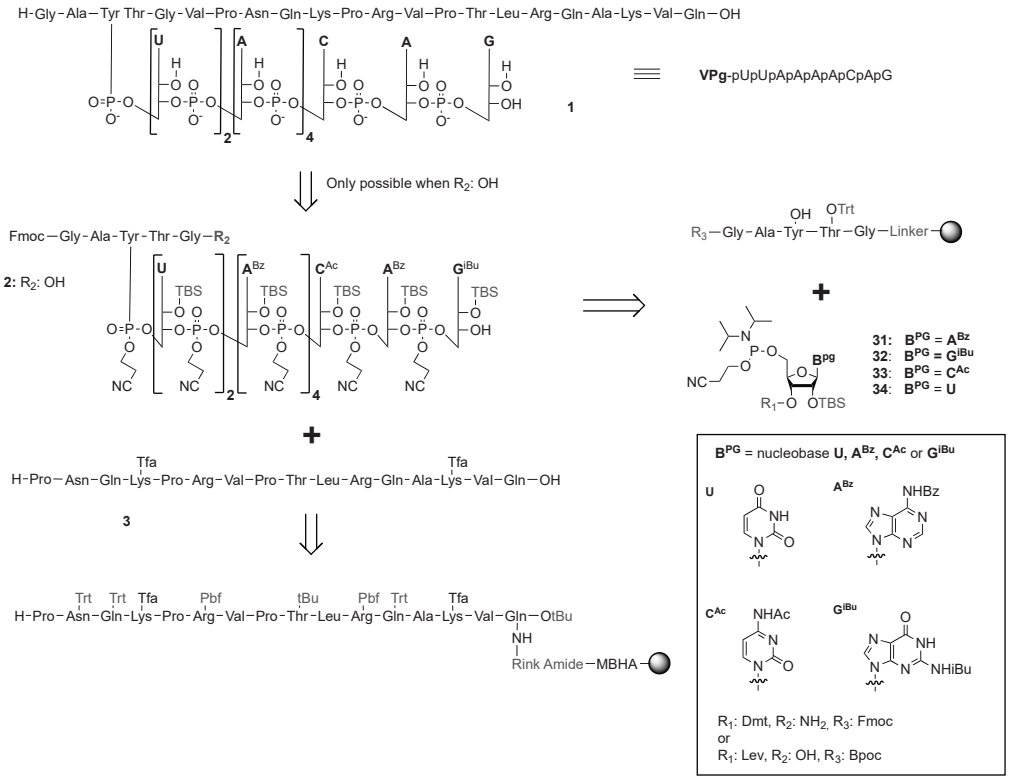


Figure 2. Retrosynthesis of the coxackie VPg using Lev as a temporary protective group.

This chapter describes the synthesis of nucleotide building blocks (**31-34**, Figure 2) and the application of these building blocks in the synthesis of partially protected nucleopeptides (**4-6**, Figure 3)**.** Two of the three obtained nucleopeptides were used in a block coupling with peptide **3** to give nucleopeptides **4** and **5** with extended peptide part.

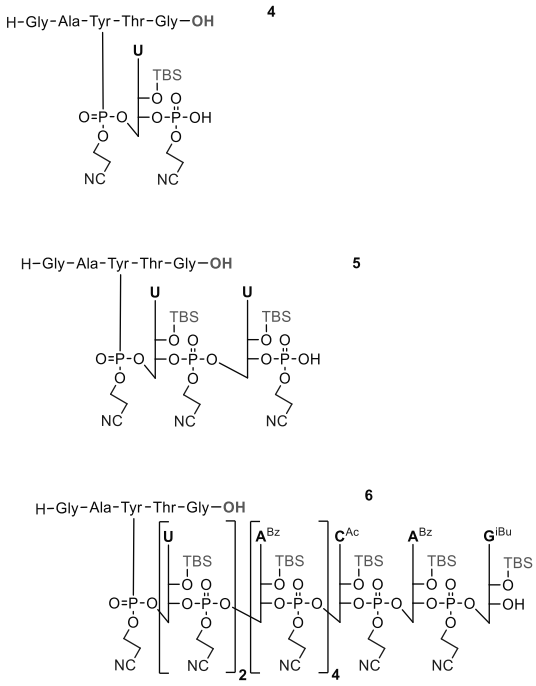
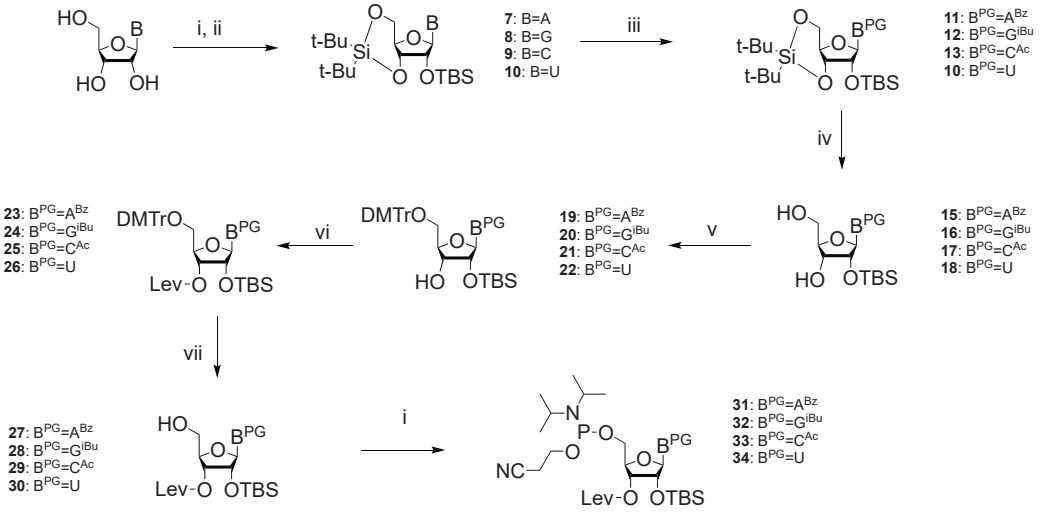
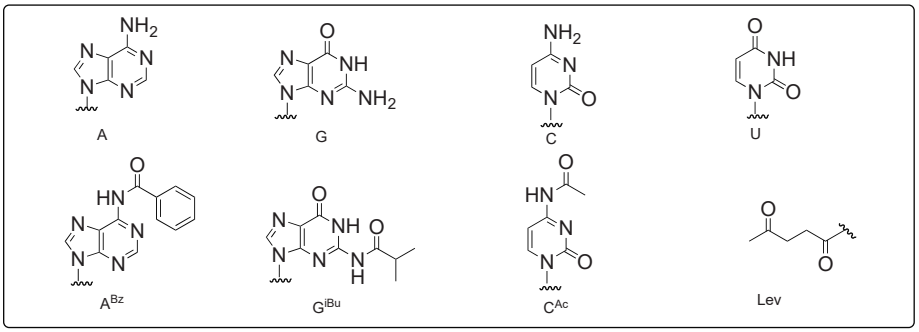


Figure 3. Target compounds. Protected nucleopeptide intermediate towards coxackie virus VPg.

Result and discussion

The syntheses of the 5'-phosphoramidite nucleoside building blocks, having the lev group as temporary protection (**31-34**) are depicted in Scheme 1. Starting from the commercially available standard ribonucleosides adenosine, guanosine, cytosine and uridine (A,G, C, U) two different silyl groups were introduced in a one-pot procedure to protect all the hydroxyl groups in the ribose moiety. The 5'- and 3'- hydroxyl functions were silylated by a reaction of the ribonucleoside with di-*tert*-butyl silyl dichloride under influence of silver nitrate. Upon completion of this reaction additional amounts of silver nitrate, pyridine and tert-butyldimethylsilyl chloride were added to install the tert-butyldimethylsilyl (TBS) ether at the 2'- hydroxyl function. In an alternative one-pot procedure to introduce the same protection group pattern di-tert-butylsilyl bis(trifluoromethanesulfonate) was applied to protect the 5'- and 3'- hydroxyl functions, while the combination tert-butyldimethylsilyl chloride and imidazole was used to introduce the TBS at the 2'-position. The exocyclic amino functions of adenosine, guanosine and cytosine were protected as amides. Benzoylation of adenosine **7** with benzoyl chloride and pyridine led to N,N'-dibenzoyl adenosine and subsequent ammonia treatment gave the required fully protected adenosine derivative **11**. N2-Isobutyryl-guanosine derivative **12** was obtained by reaction of the exocyclic amine in **8** with isobutyryl chloride (iBuCl) in pyridine. The exocyclic amine function in cytosine derivative **9** was acetylated with acetic anhydride in pyridine. The fully protected ribonucleosides derivatives (**10-13**) were further processed by the following sequence of reactions. The 5' and 3' hydroxyl group in **10-13** were deprotected using HF-Pyridine at 0°C. Depending on the nucleobase this reaction took 1 to 2h and in all cases no unwanted TBDMS cleavage was observed. Selective introduction of the DMTr group at the 5' hydroxyl was followed by carbodiimide mediated esterification of 3' hydroxyl with levulinic acid. The introduction of the Lev group proceeded more efficient with DIC than with EDC.HCl and more importantly migration of TBDMS was not observed.



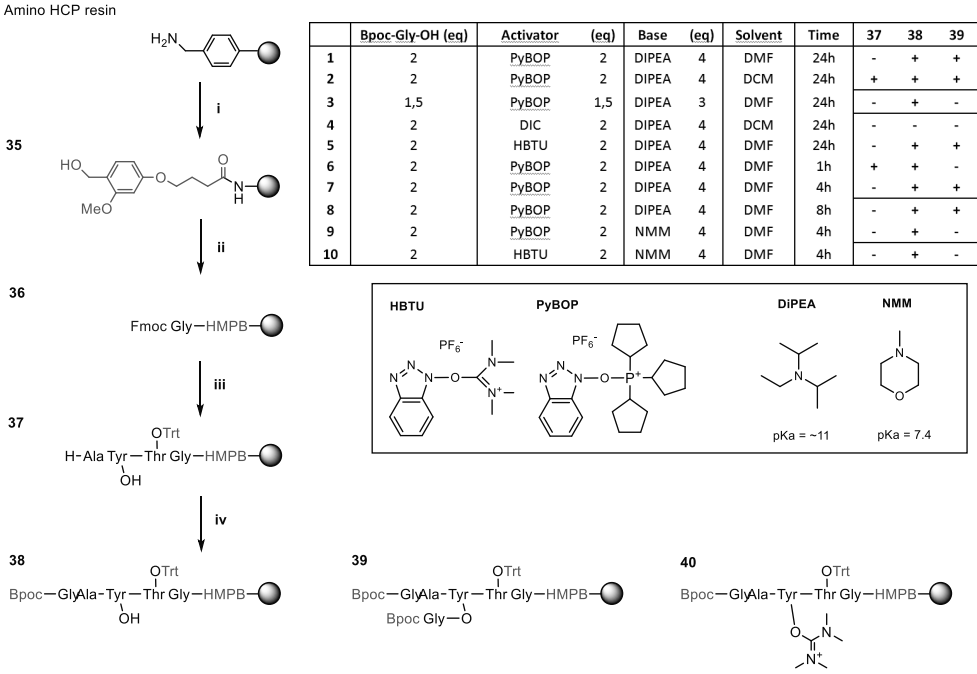


Scheme 1. (i) **7,8,9**: $(t\text{-Bu})_2Si(OTf)_2$, DMF, 0 °C, 30 min; **10**: $(t\text{-Bu})_2SiCl_2$, $AgNO_3$, DMF, 0 °C, 30 min (ii) **7,8,9**: TBS-Cl, Imidazole, 0 °C to rt, o.n. ; **10**: TBS-Cl, Pyridine, $AgNO_3$, 0 °C to rt, 2 hrs, (iii) **11**: Bz-Cl, Pyr/DCM, rt, 3 h, then conc. NH_4OH, -10 °C to rt, o.n. **12**: iBu-Cl, Pyr, -20 °C, 2 h **13**: Ac_2O, Pyr, 0 °C to rt, 2 h (iv) HF-Pyr, DCM, 0 °C, **15**, **18**: 1h, **16**, **17**: 2h; (v) DMTr-Cl, Pyr, 0 °C, o.n. (vi) Lev-OH, DIC, DMAP cat., DCM, rt, o.n. (vii) TsOH, DCM/MeOH, 0 °C, 10 min, (viii) PAM-Cl, TEA, DCM, rt, 15 min

To prevent TBDMS migration a catalytic amount of DMAP as a nucleophilic catalyst and DCM as solvent were used instead of DMF and bases such as triethylamine. The final two steps to the four nucleotide building blocks (**31-34**) comprise removal of the DMT group with diluted solution of *p*TsA in DCM/MeOH and reaction of the free 5' hydroxyl with 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite in the presence of N,N-diisopropylethylamine. Summarizing all 5'-phosphoramidite nucleoside building blocks, having the lev group as temporary protection (**31-34**) were prepared in an efficient manner and on sufficient scale to execute the automated solid phase synthesis.

Optimisation of the first nucleotide synthesis cycle.

Having the respective protected amino acid and nucleotide building blocks available, attention was directed to assessment of the appropriateness of these building blocks for an efficient automated solid phase synthesis of nucleopeptides.

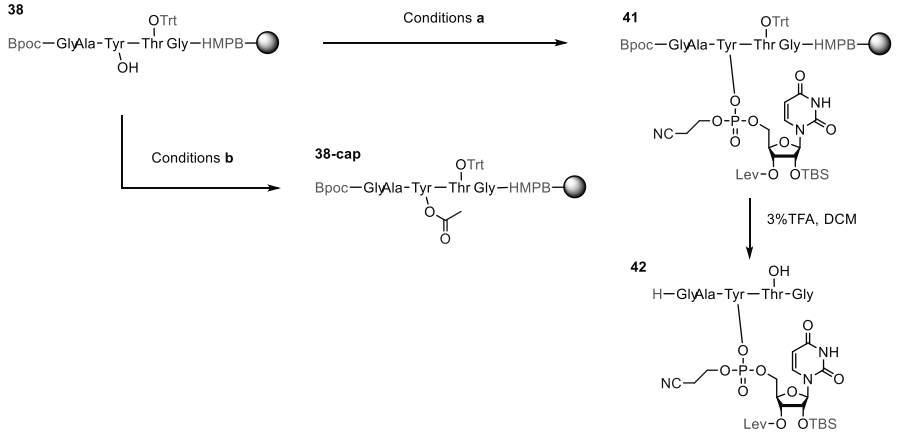


	Bpoc-Gly-OH (eq)	Activator	(eq)	Base	(eq)	Solvent	Time	37	38	39	40
1	2	PyBOP	2	DIPEA	4	DMF	24h	-	+	+	-
2	2	PyBOP	2	DIPEA	4	DCM	24h	+	+	+	-
3	1,5	PyBOP	1,5	DIPEA	3	DMF	24h	-	+	-	-
4	2	DIC	2	DIPEA	4	DCM	24h	-	-	-	-
5	2	HBTU	2	DIPEA	4	DMF	24h	-	+	+	+
6	2	PyBOP	2	DIPEA	4	DMF	1h	+	+	-	-
7	2	PyBOP	2	DIPEA	4	DMF	4h	-	+	+	-
8	2	PyBOP	2	DIPEA	4	DMF	8h	-	+	+	-
9	2	PyBOP	2	NMM	4	DMF	4h	-	+	-	-
10	2	HBTU	2	NMM	4	DMF	4h	-	+	-	-

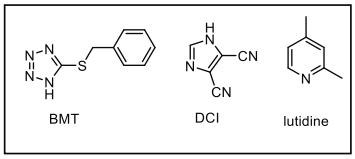
Scheme 2. Synthesis (i) HMPB-linker, PyBOP, HOBt, DiPEA, DMF, rt, 5 hrs, then (ii) Fmoc-Gly-OH, DIC, DMAP, DCM, rt, o.n.; (iii) SPPS: (a) piperidine/NMP (1:4, v:v), rt, 5 min; (b) Fmoc–AA–OH, HCTU, DiPEA, rt, 1 hr; (c) Ac_2O, DiPEA, NMP, rt, 1 min; (iv) table.

In the first instance the viability of the peptide synthesis was evaluated as the applied Fmoc amino acid building blocks were minimally protected not only to avoid harsh acidic deprotection conditions but also to provide an unprotected tyrosine hydroxyl group in the immobilized oligopeptide for oligonucleotide extension (Scheme 2). Moreover, to allow the projected block coupling of the nucleopeptide and peptide, protective group manipulations are required. Immobilized tetrapeptide Ala-Tyr-Thr-Gly (**37**) was prepared with the aid of commercially available Fmoc amino acids using HMPB resin and standard peptide chemistry (from **35** to **37**, Scheme 2). Although the tyrosine with an unprotected phenolic hydroxyl function was incorporated, the immobilized tetrapeptide **37** was synthesized without noticeable difficulties. A necessity to allow extension of the immobilized nucleopeptide with an oligoribonucleotide moiety comprises the replacement of the Fmoc group by the Bpoc group because the Fmoc will not survive the repeated cleavage of the Lev group at the 3' position during the RNA synthesis. However, the coupling of **37** with Bpoc-glycine to give **38** went problematic and gave **39** as the result of a double incorporation of Bpoc-glycine together with uronium side product **40** as identified after cleavage from the solid support (Scheme 2).

A number of different coupling condition were tested and monitored by HPLC after analytical cleavage. As depicted in the table of Scheme 2, using only 1.5 eq of both protected glycine and coupling agents (conditions 3) prevented any side reaction to happen. Using the less basic NMM instead of the common D*i*PEA also improved the rate of the reaction and the quality of the target peptide.



a	Nucleotide	(eq)	Activator	(eq)	Time	38	41
1	34	6	BMT	18	20min	+	++
2	34	6	BMT	18	60min	+	++
3	34	3	BMT	9	60min	++	++
4	34	6	DCI	18	20min	+	++
5	34	7	BMT	24	20min	-	+++
- = not observed, + = minor, ++ = major, +++= single product							

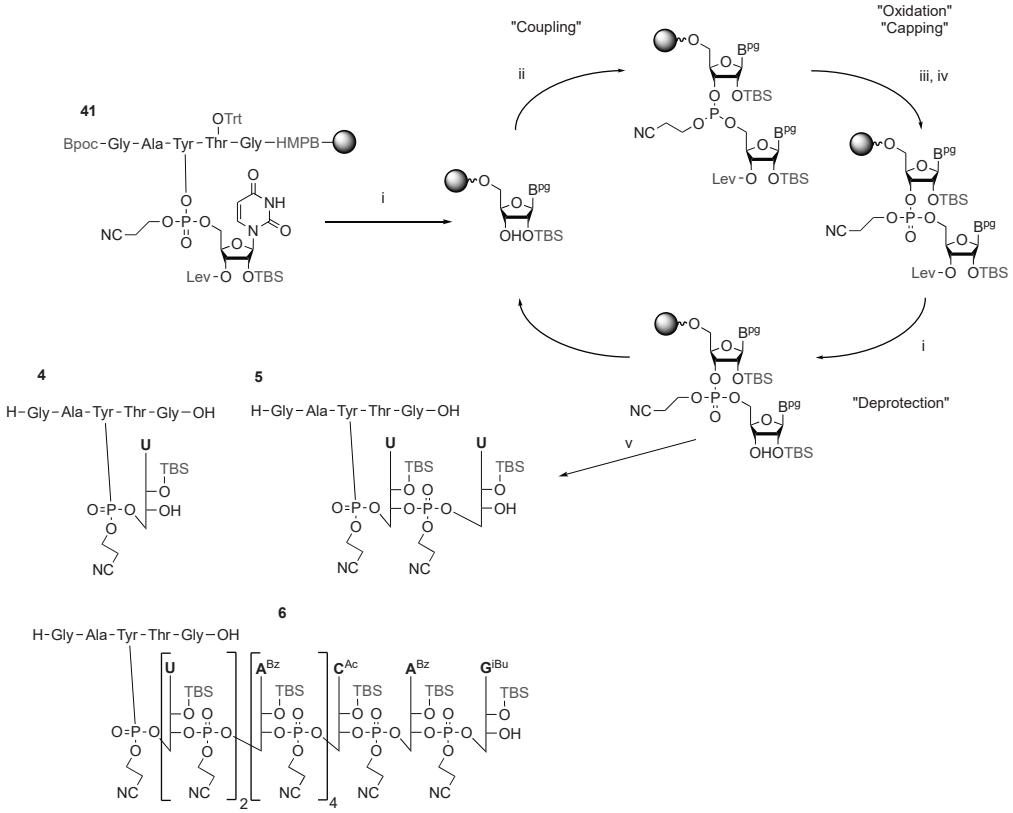


b	reagent	(eq)	Activator	(eq)	Base	(eq)	Solvent	Time	38	38-cap
1	Acetic anhydride	318	Me-Im	376	Lutidine	388	THF	20min	++	-
2	Acetic anhydride	318	Me-Im	376	Lutidine	388	THF	60min	++	-
3	Acetic anhydride	318	Me-Im	376	Lutidine	388	THF	120min	++	-
4	Acetic anhydride	80			DIPEA	24	DMF	10min	+	+
5	Acetic anhydride	80			DIPEA	24	DMF	30min	+	+
6	Acetic anhydride	80			DIPEA	24	DMF	60min	+	+
7	Acetic anhydride	80	DMAP	.cat	DIPEA	24	DMF	30min	-	++
8	Acetyl Chloride	20			DIPEA	48	DMF	30min	-	++
- = not observed, + = observed, ++ = single product										

Scheme 3. Coupling of the first nucleoside on the peptide side chain

After the quality of immobilized peptide **38** was established the extension of the free tyrosine hydroxyl group with nucleotides was investigated using phosphoramidite building blocks **31-34**. The number of equivalents of building block **31-34**, the nature and the number of equivalents of the activator (BMT or CM) and the reaction time were varied (Scheme 3) The quality of the products was established by HPLC analysis after cleavage from the solid support. It turned out that 7 eq of nucleotide amidite were necessary using BMT as activator for complete conversion toward nucleopeptide **41** (table a, condition 5). In line with these results it appeared that the capping step did not proceed without difficulties. As illustrated in the table b, various conditions were assessed of which condition 7 using Ac_2O in the presence of DMAP and DiPEA or condition 8 using AcCl with DiPEA proved to be the most effective. At the end of the synthesis nucleopeptide **41** was released from the solid support and the Bpoc and Trt groups were removed by treatment with 3% TFA in DCM to give partially protected nucleopeptide **42** as analyzed by HPLC-MS.

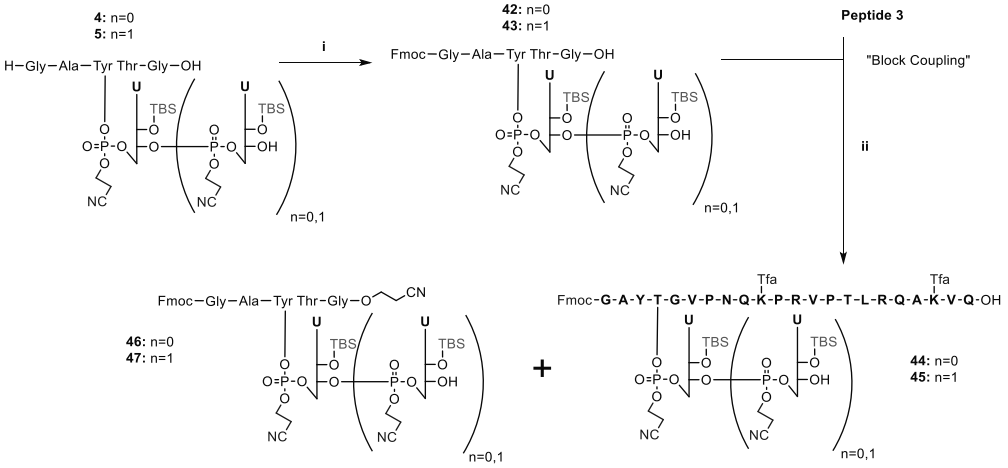
Oligonucleopeptide synthesis or target compound 4, 5 and 6.



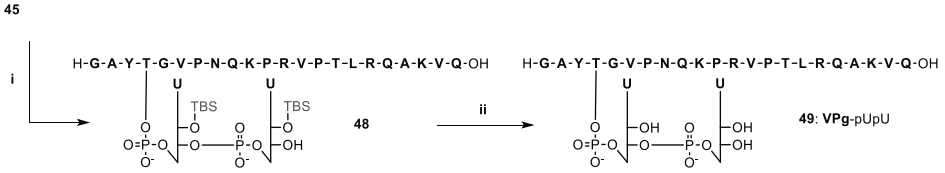
Scheme 4. (i) Hydrazine, THF/Pyr/AcOH, rt, 20 min; (ii) **31, 32, 33, 34**, BMT, dioxane/ACN, rt, 20/30/30/20 min; (iii) I_2, THF/Pyr/H2O, rt, 1.5 min; (iv) Ac_2O, MeIm, 2,6-lutidine, NMP, rt, 30 sec (v) 3% TFA/DCM, rt, 5 min.

In order to prepare partialy protected nucleopeptide **4** (Scheme 4), provided with one uridine moiety, the lev group at the 3' position in immobilized **41** should be removed without harming the intrigity of the TBS at the 2' position. After extensive optimization the lev group at the 3' position was removed using hydrazine monohydrate in a THF/pyridine/acetic acid solvent mixture for 20 min. It was established that under these conditions migration of TBDMS group from 2' to 3' hydroxyl did not take place. Subsequent mild acidic treatment with 3%TFA in DCM cleaved the product from the solid support and removed the Trt and Bpoc groups to provide the partially deprotected nucleopeptide **4**. Further extension of the RNA chain was investigated and the synthesis of partially protected nucleopeptides **5** and **6** was undertaken. Removal of the lev group from immobilized **41** was followed by the elongation of the oligonucleotide, comprising amidite coupling, oxidation of the intermediate phosphite and capping of the remaining hydroxyl groups. Depending on the nature of the nucleobase the coupling time was adjusted, being 20 min for the uridine and adenosine building blocks (**31** and **34**) and 30 min for the cytosine and guanosine building blocks (**32** and **33**). Partially protected target nucleopeptide **5** and **6** were obtained after treatment with 3% TFA in DCM analyzed by LCMS.

Preliminary studies on further block coupling



Scheme 5. (i) Fmoc-OSu. DiPEA, DMF, rt, o.n.; (ii) (a) PyBOP, HOBt, DiPEA, DMF, rt, 25 min; then (b) peptide **3**, see Figure 2, DMF, rt, o.n.



Scheme 6. (i) NH_4OH aq./dioxane (1:1, v:v), rt, 3 days; (ii) (a) TEA/TEA*3HF/DMF (2:3:4, v:v:v), rt, o.n; then (b) NH_4HCO_3 aq.

Finally, the viability of the projected block coupling was investigated by the condensation of nucleopeptide **5** (slightly contaminated with **4**) with 17 mer oligopeptide **3** (Scheme 5). This oligopeptide (see Figure 2), in which the side chain amino groups of the lysine moieties are protected with trifluoroacetyl groups, was obtained by standard solid phase peptide synthesis, using Tentagel S RAM**,** followed by purification with HPLC. The free N terminus of nucleopeptides **5** was reprotected with the Fmoc group using FmocOSu and DiPEA to give **43** that was used without further purification. Block coupling was achieved by preactivation of the glycine moiety in the nucleopeptides **43** using PyBOP for 25 min, followed by the addition of oligopeptide **3**. The reaction was monitored by LCMS and after overnight **45** was isolated, along with the cyanoethanol ester of the starting product (i.e. **47**). Complete deprotection was achieved using aqueous ammonia followed by TEA*HF and NH_4HCO_3 treatement. HPLC purification yielded compound **49** (**VPgpUpU**) as determined by mass-spectroscopic and chromatographic methods.

Conclusion

An efficient synthesis of 5' phosphoramidite ribonucleotide (A, C, G and U) building blocks having an orthogonal levunilic ester protective group at the 3'-position is described. Application of mimimally protected amino acid building blocks together with the newly prepared 5'-phosphoramidite ribonucleotide building blocks in an optimized automated solid support

synthesis makes available partially propected nucleopeptides comprising up to a pentapeptide and nonanucleotide. The viability of the partially protected nucleopeptides to participate in a block coupling with a partially protected peptide was ascertained by block condensation to give VPgpUpU (**49**).

Experimental

5',3'-Si(tBu)$_2$-2'-TBDMS-Uridine (10)

0.995 mmol (0.243g) of uridine was, after co-evaporation with 1,4-dioxane, dissolved in 5 mL of dry DMF, 2.44 mmol (0.414g) of $AgNO_3$ and 1.4 mmol (0.30 mL, 0.30g) of $(t\text{-}Bu)_2SiCl_2$ were added respectively and the mixture was stirred for 30 minutes at 0°C. Reaction completion was checked by TLC (Rf around 0.3 in 5% MeOH/DCM). Then 2.94 mmol (0.500g) of $AgNO_3$ and 5.0 mmol (0.40 mL, 0.39g) of Pyridine were added and the mixture was stirred for 15 minutes at 0 °C before adding 1.50 mmol (0.226g) of TBDMS-Cl and stirring for another 2 hours. Reaction completion was checked by TLC (Rf around 0.75 at 25% EtOAc/DCM, 0.85 at 50% EtOAc/DCM). 30 mL of EtOAc was added to the mixture and filtrated through celite (20mL of EtOAc was added during this process). The organic layer was extracted two times using sat. $KHSO_4$ followed by Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 0:100 to 20:80 EtOAc/DCM eluent, resulting in 0.444g (0.890mmol, 89.0%) of Compound **10** as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.746 (s, 1H, NH), 7.260 ($CHCl_3$), 7.257 (d, *J* = 8.0 Hz, 1H, H^6), 5.751 (dd, *J* = 8.1, 1.3 Hz, 1H, H^5), 5.653 (s, 1H, $H^{1'}$), 4.493 (dd, *J* = 9.2, 5.1 Hz, 1H, $H^{5'a}$), 4.288 (d, *J* = 4.6 Hz, 1H, $H^{2'}$), 4.158 (td, *J* = 10.2, 5.1 Hz, 1H, $H^{4'}$), 3.968 (t, *J* = 9.4 Hz, 1H, $H^{5'b}$), 3.863 (dd, *J* = 9.6, 4.7 Hz, 1H, $H^{3'}$), 1.040, 1.012 (2x s, 18H, 5'-3'-O-Si-tBu_2), 0.922 (s, 9H, 2'-O-Si-tBu), 0.177, 0.132 (2x s, 6H, 2'-O-SiMe_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K) δ: 163.70 (CO, C^2), 149.91 (CO, C^4), 139.55 (C^6), 102.47 (C^5), 94.07 ($C^{1'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 76.14 ($C^{3'}$), 75.43 ($C^{2'}$), 74.63 ($C^{4'}$), 67.68 ($C^{5'}$), 27.58, 27.07 (2x CH_3, 5'-3'-O-Si-tBu_2), 25.95 (CH_3, 2'-O-Si-tBu), 22.89, 20.45, 18.36 (3x C_q, Si-tBu), -4.19, -4.92 (2x CH_3, 2'-O-Si-Me_2).

IR: 2932, 2886, 2859, 1690, 1454, 1261, 1165, 1055, 1001, 826, 777, 750, 650.

HRMS: $[C_{23}H_{42}N_2O_6Si_2+H]^+$: found 499.2650, calculated 499.2654.

5'-OH-3'-OH-2'-TBDMS-Uridine (18)

0.523 mmol (0.261g) of Compound **10** was dissolved in 2.5 mL of DCM, 2.8 mmol (0.50mL) of diluted HF-Pyridine* was added dropwise at 0 °C and the mixture was stirred for 2 hours. Reaction completion was checked by TLC (Rf around 0.45 in 75% EtOAc/DCM, 0.60 in 100% EtOAc). The mixture was washed two times with H_2O, sat. $NaHCO_3$ followed by Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 50% to 100% EtOAc/DCM eluent resulting in 0.172g (0.480 mmol, 91.8%) of Compound **18** as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 8.988 (s, 1H, NH), 7.594 (d, *J* = 8.1 Hz, 1H, H^6), 7.260 ($CHCl_3$), 5.758 (dd, *J* = 8.1, 1.9 Hz, 1H, H^5), 5.586 (d, *J* = 5.2 Hz, 1H, $H^{1'}$), 4.596 (t, *J* = 5.2 Hz, 1H, $H^{2'}$), 4.206 (dd, *J* = 8.4, 4.0 Hz, 1H, $H^{3'}$), 4.145 (dd, *J* = 5.2, 2.2 Hz, 1H, $H^{4'}$), 3.956 (d, *J* = 12.1 Hz, 1H, $H^{5'a}$), 3.813 (dd, *J* = 11.9, 4.6 Hz, 1H, $H^{5'b}$), 2.986 (d, *J* = 3.7 Hz, 1H, $OH^{5'}$), 2.710 (d, *J* = 4.1 Hz, 1H, $OH^{3'}$), 0.898 (s, 9H, 2'-O-Si-tBu), 0.097, 0.084 (2x s, 6H, 2'-O-SiMe_2). **^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ**: 163.14 (CO, C^2), 150.40 (CO, C^4), 142.49 (C^6), 102.81 (C^5), 93.07 ($C^{1'}$), 85.52 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.24 ($C^{2'}$), 70.96 ($C^{3'}$), 62.30 ($C^{5'}$), 25.77 (CH_3, 2'-O-Si-tBu), 18.10 (C_q, 2'-O-Si-tBu), -4.71, -5.00 (2x CH_3, 2'-O-Si-Me_2). **IR:** 2930, 1694, 1462, 1379, 1258, 1157, 1061, 837, 783, 758.

HRMS: $[C_{15}H_{26}N_2O_6Si+H]^+$: found 359.1634, calculated 359.1633.

**70% HF-Pyridine contains 1mol HF per 28.57g at d=1.1g/mL, or 38.5M. 6:1 dilution in pyridine is 5.5M, or 0.1818mL/mmol.*

5'-DMTr-3'-OH-2'-TBDMS-Uridine (22)

0.480 mmol (0.172g) of Compound **18** was dissolved in 2 mL of dry pyridine, 0.576 mmol (0.195g) of 4,4'-Dimethoxytritylchloride was added at -10 °C and the mixture was stirred for 5 nights. Reaction completion was checked by neutralized TLC (Rf around 0.25 in 25% EtOAc/PE). The mixture was concentrated using rotary evaporation before being redissolved in EtOAc. The organic layer was washed with sat. $NaHCO_3$ followed by Brine and dried using $MgSO_4$. Purification was performed with neutralized silica column

chromatography using an 0% to 50% EtOAc/PE eluent resulting in 0.250g (0.378 mmol, 78.9%) of Compound **22** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.779 (s, 1H, NH), 7.961 (d, *J* = 8.2 Hz, 1H, H^6),), 7.384 (d, *J* = 7.2 Hz, 2H, $H^{arom, ortho}$, DMTr), 7.34-7.20 (m, 9H, H^{arom}, H^{ortho}, H^{meta}, H^{para}, DMTr), 7.260 ($CHCl_3$), 6.849 (d, *J* = 8.9 Hz, 4H, H^{arom}, DMTr), 5.966 (d, *J* = 2.9 Hz, 1H, $H^{1'}$), 5.314 (d, *J* = 8.1 Hz, 1H, H^5), 4.36 (m, 2H, $H^{2'}$, $H^{3'}$), 4.12 (m, 1H, $H^{4'}$), 3.793 (s, 6H, OMe, DMTr), 3.57-3.45 (m, 2H, $H^{5'a}$, $H^{5'b}$), 2.644 (s, 1H, $OH^{3'}$), 0.913 (s, 9H, 2'-O-Si-tBu), 0.198, 0.169 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 163.72 (CO, C^2), 158.80, 158.72 (2x C_q, DMTr), 150.50 (CO, C^4), 144.38 (C_q, DMTr), 140.33 (C^6), 135.24, 135.04 (2x C_q, DMTr), 130.24, 130.14 (2x CH^{arom}, DMTr), 128.16 (CH^{arom}, DMTr), 128.11 (CH^{arom}, DMTr), 127.28 (CH^{arom}, DMTr), 113.39 (2x CH^{arom}, DMTr), 102.37 (C^5), 88.79 ($C^{1'}$), 87.25 (C_q, DMTr), 83.58 ($C^{4'}$), 77.48, 77.16, 76.85 ($CHCl_3$), 76.41 ($C^{2'}$), 70.48 ($C^{3'}$), 62.36 ($C^{5'}$), 55.31 (OMe, DMTr), 25.75 (CH_3, 2'-O-Si-tBu), 18.09 (C_q, 2'-O-Si-tBu), -4.55, -5.13 (2x CH_3, 2'-O-Si-Me_2).

IR: 2951, 2928, 1684, 1506, 1456, 1250, 1175, 1115, 1034, 827, 779, 756, 700.

HRMS: $[C_{36}H_{44}N_2O_8Si+Na]^+$: found 683.2757, calculated 683.2759.

5'-DMTr-3'-Lev-2'-TBDMS-Uridine (26)

0.378 mmol (0.250g) of Compound **22** was dissolved in 2mL of dry DCM, a catalytic amount of 4-dimethylaminopyridine, 0.5 mmol (0.05mL, 0.06g) of levulinic acid and 0.64 mmol (0.10mL, 0.081g) of Diisopropylcarbodiimide were added respectively and the mixture was stirred overnight. Reaction completion was checked by normal TLC (Rf around 0.65 in 50% EtOAc/PE). The organic layer was washed with sat. $NaHCO_3$ followed by Brine and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 25% to 100% EtOAc/PE eluent resulting in 0.249g (0.328 mmol, 86.8%) of Compound **26** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.941 (br, 1H, NH), 7.905 (d, *J* = 8.2 Hz, 1H, H^6), 7.40-7.20 (m, 9H, H^{arom}, H^{ortho}, H^{meta}, H^{para}, DMTr), 7.260 ($CHCl_3$), 6.854 (d, *J* = 8.7 Hz, 4H, H^{arom}, DMTr), 6.002 (d, *J* = 4.8 Hz, 1H, $H^{1'}$), 5.349 (t, *J* = 4.6 Hz, 1H, H3'), 5.311 (d, *J* = 8.1 Hz, 1H, H^5), 4.521 (t, *J* = 4.8 Hz, 1H, $H^{2'}$), 4.26 (m, 1H, $H^{4'}$), 3.787 (s, 6H, OMe, DMTr), 3.539 (d, *J* = 11.2, 2.0 Hz, 1H, $H^{5'a}$), 3.448 (dd, *J* = 10.8, 1.2 Hz, 1H, $H^{5'b}$), 2.9-2.5 (m, 4H, R^1-C$\underline{H}_2$C$\underline{H}_2$-R^2, Lev), 2.194 (s, 3H, CH_3, Lev), 0.870 (s, 9H, 2'-O-Si-tBu), 0.106, 0.088 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 206.22 (CO, Lev), 171.82 (CO, Lev), 163.63 (CO, C^2), 158.76, 158.72 (2x C_q, DMTr), 150.66 (CO, C^4), 144.19 (C_q, DMTr), 140.07 (C^6), 134.95, 134.83 (2x C_q, DMTr), 130.21, 130.09 (2x CH^{arom}, DMTr), 128.08 (CH^{arom}, DMTr), 127.26 (CH^{arom}, DMTr), 113.36 (2x CH^{arom}, DMTr), 102.50 (C^5), 88.43 ($C^{1'}$), 87.42 (C_q, DMTr), 81.16 ($C^{4'}$), 77.48, 77.16, 76.85 ($CHCl_3$), 74.45 ($C^{2'}$), 72.22 ($C^{3'}$), 62.28 ($C^{5'}$), 55.31 (OMe, DMTr), 37.65 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 29.86 (CH_3, Lev), 27.80 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.50 (CH_3, 2'-O-Si-tBu), 17.88 (C_q, 2'-O-Si-tBu), -5.00, -5.24 (2x CH_3, 2'-O-Si-Me_2).

IR: 2953, 2928, 2855, 1684, 1506, 1456, 1250, 1175, 1153, 1032, 829, 778, 756, 702.

HRMS: $[C_{41}H_{50}N_2O_{10}Si+Na]^+$: found 781.3128, calculated 781.3127.

5'-OH-3'-Lev-2'-TBDMS-Uridine (30)

8.554 mmol (6.492g) of Compound **26** was dissolved in 50mL DCM/MeOH (7:3 v:v), 85.54 mmol (16.27g, monohydrate) of diluted *p*-toluenesulfonic acid (8.14 wt% in DCM/MeOH(7:3 v:v))* was added at 0 °C and the mixture was stirred for 10 minutes. Reaction completion was checked by TLC (Rf around 0.45 in 100% EtOAc). Finally, the mixture was quenched using sat. $NaHCO_3$. The mixture was partitioned and the organic layer was washed with Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 20% to 100% EtOAc/PE eluent resulting in 3.678g (8.056 mmol, 94.2%) of Compound **30** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.517 (s, 1H, NH), 7.808 (d, *J* = 8.1 Hz, 1H, H^6), 7.260 ($CHCl_3$), 5.740 (d, *J* = 8.0 Hz, 1H, H^5), 5.662 (d, *J* = 4.8 Hz, 1H, $H^{1'}$), 5.172 (t, *J* = 4.6 Hz, 1H, H3'), 4.562 (t, *J* = 4.8 Hz, 1H, $H^{2'}$), 4.210 (m, 1H, $H^{4'}$), 3.929 (d, *J* = 12.1 Hz, 1H, $H^{5'a}$), 3.764 (d, *J* = 12.2 Hz, 1H, $H^{5'b}$), 2.5-2.9 (m, 4H, R^1-C$\underline{H}_2$C$\underline{H}_2$-R^2, Lev), 2.191 (s, 3H, CH_3, Lev), 0.840 (s, 9H, 2'-O-Si-tBu), 0.039, 0.028 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 206.81 (CO, Lev) 172.44 (CO, Lev), 163.67 (CO, C^2), 150.50 (CO, C^4), 141.71 (C^6), 102.51 (C^5), 91.75 ($C^{1'}$), 82.93 ($C^{4'}$), 77.47, 77.16, 76.83 ($CHCl_3$), 73.50 ($C^{2'}$), 72.04 ($C^{3'}$), 61.49

($C^{5'}$), 37.84 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 29.93 (CH_3, Lev), 27.89 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.59 (CH_3, 2'-O-Si-tBu), 17.98 (C_q, 2'-O-Si-tBu), -5.05, -5.12 (2x CH_3, 2'-O-Si-Me_2).

IR: 3055, 2953, 2928, 2884, 2857, 1684, 1462, 1387, 1258, 1155, 1109, 1088, 835, 779, 760.

HRMS: $[C_{20}H_{32}N_2O_8Si+H]^+$: found 457.1998, calculated 457.2001.

**The solution was prepared using 85.54 mmol (16.27g) p-toluenesulfonic acid monohydrate in 200mL DCM/MeOH (7:3 v:v), resulting in a 8.14 wt% solution.*

5'-PAM(CNE)-3'-Lev-2'-TBDMS-Uridine (34)

1.80 mmol (0.821g) of Compound **30** was dissolved in 20mL of dry DCM, 2.9 mmol (0.29g, 0.40mL) of Triethylamine and 2.2 mmol (0.53g, 0.50mL) of 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite were added respectively and the mixture was stirred for 10 minutes. Reaction completion was checked by normal TLC (Rf around 0.85 in 100% EtOAc). Finally, the mixture was quenched using aqueous 5wt% $NaHCO_3$. The mixture was partitioned and the organic layer was washed with Brine and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 1:20:79 to 1:50:49 TEA:EtOAc:Hex eluent resulting in 1.162g (1.769 mmol, 98.2%) of Compound **34** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.34 (br, 1H, NH), 7.84, 7.78 (d+d, 1H, *J* = 8.2 Hz, H^6) 7.260 ($CHCl_3$), 5.99, 5.98 (d+d, 1H, *J* = 6.1 + 5.7 Hz, $H^{1'}$), 5.73, 5.71 (d+d, 1H, *J* = 3.6 + 3.7 Hz, H^5), 5.21, 5.12 (t+dd, 1H, *J* = 4.4 + 4.9, 3.4 Hz, $H^{3'}$), 4.34-4.23 (m, 2H, $H^{2'}$ + $H^{4'}$), 4.00-3.70 (m, 4H, $H^{5'}$, NC-$C\underline{H_2}$-CH_2-OR), 3.65-3.50 (m, 2H, CH, $\underline{iPr_2}$NR), 2.9-2.5 (m, 6H, R^1-$C\underline{H_2}C\underline{H_2}$-$R^2$, Lev, NC-$CH_2$-$C\underline{H_2}$-OR), 2.18, 2.17 (s+s, 3H, CH_3, Lev) , 1.22-1.15 (m, 12H, CH_3, $\underline{iPr_2}$NR) 0.82, 0.81 (s+s, 9H, 2'-O-Si-tBu), 0.01--0.02 (m, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 206.39, 206.33 (CO, Lev) 171.94, 171.89 (CO, Lev), 163.39, 163.35 (C^2), 150,60, 150.58 (C^4), 140.04, 139.93 (C^6), 117.49, 117.41 (CN), 102.76, 102.66 (C^5), 88.25, 88.05 ($C^{1'}$), 81.79, 81.72, 81.70, 81.62 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.32, 74.18 ($C^{2'}$), 72.50, 72.06 ($C^{3'}$), 63.08, 62.92, 62.83, 62.67 ($C^{5'}$), 58.70, 58.60, 58.48, 58.38 (NC-$\underline{C}H_2$-CH_2-OR), 43.36, 43.26, 43.23, 43.14 (2x CH, $\underline{iPr_2}$NR), 37.79, 37.74 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 29.92 (CH_3, Lev), 27.90, 27.86 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.50 (CH_3, 2'-O-Si-tBu), 24.92, 24.88, 24.85, 24.81, 24.77, 24.70 (4x CH_3, $\underline{iPr_2}$NR), 20.57, 20.49, 20.45, 20.38 (NC-CH_2-$\underline{C}H_2$-OR), 17.91, 17.90 (C_q, Si-tBu), -5.08, -5.11, -5.20, -5.25 (2x CH_3, 2'-O-Si-Me_2).

^{31}P NMR (162 MHz, $CDCl_3$) δ: 149.62, 148.60.

IR: 2965, 2930, 2859, 1744, 1717, 1684, 1456, 1379, 1364, 1253, 1200, 1180, 1155, 1125, 1103, 1076, 1045, 978, 858, 837, 808, 779, 729, 677, 640.

HRMS: $[C_{37}H_{54}N_7O_8PSi+H]^+$: found 657.3077, calculated 657.3079.

5',3'-Si(tBu)$_2$-2'-TBDMS-Adenosine (7)

10 mmol (2.672g) of Adenosine was dissolved in 20 mL of dry DMF, 12.3 mmol (4.0 mL, 5.40g) of (t-Bu)$_2$Si(OTf)$_2$ was added at 0 °C and the mixture was stirred for 30 minutes. Reaction completion was checked by TLC (Rf around 0.3 at 6% MeOH/DCM). Then 50.7 mmol (3.45g) of Imidazole and 12.3 mmol (1.85g) of TBDMS-Cl were added and the mixture was stirred overnight while warming to room temperature. Reaction completion was checked by TLC (Rf around 0.3 at 75% EtOAc/PE). 120 mL of H_2O was added to the mixture and extracted three times using Et_2O. The organic layer was dried using $MgSO_4$. Purification was performed with silica column chromatography using a 30:80 to 100:0 EtOAc/PE eluent, resulting in 3.77g (7.22mmol, 72.2%) of Compound **7** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 8.278 (s, 1H, H^2), 7.824 (s, 1H, H^8), 7.260 ($CHCl_3$), 6.780 (s, 2H, NH_2), 5.901 (s, 1H, $H^{1'}$), 4.600 (d, *J* = 4.7Hz, 1H, $H^{2'}$), 4.511 (dd, *J* = 9.5, 4.7 Hz, 1H, $H^{3'}$), 4.460 (dd, *J* = 9.1, 5.1 Hz, 1H, $H^{5'a}$), 4.189 (td, *J* = 10.1, 5.1 Hz, 1H, $H^{4'}$), 4.008 (dd, *J* = 10.3, 9.3 Hz, 1H, $H^{5'b}$), 1.043, 1.011 (2x s, 18H, 5'-3'-O-Si-tBu$_2$), 0.896 (s, 9H, 2'-O-Si-tBu), 0.132, 0.116 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 156.06 (C^6), 153.16 (C^2), 149.21 (C^4), 138.60 (C^8), 120.23 (C^5), 92.42 ($C^{1'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 75.87 ($C^{3'}$), 75.50 ($C^{2'}$), 74.70 ($C^{4'}$), 67.86 ($C^{5'}$), 27.54, 27.08 (2x CH_3, 5'-3'-O-Si-tBu$_2$), 25.96 (CH_3, 2'-O-Si-tBu), 22.79, 20.39, 18.36 (3x C_q, Si-tBu), -4.24, -4.91 (2x CH_3, 2'-O-Si-Me_2).

IR: 3312, 3157, 2932, 2887, 2859, 1672, 1601, 1128, 1063, 1003, 827, 754, 651.

HRMS: $[C_{24}H_{43}N_5O_4Si_2+H]^+$: found 522.2922, calculated 522.2926.

5',3'-Si(tBu)$_2$-2'-TBDMS-Adenosine(Bz) (11)

7.22 mmol (3.59g) of Compound **7** was dissolved in 50mL of dry pyridine/DCM (1:4 v:v), 30.2 mmol (3.5mL, 4.24g) of Benzoylchloride was added and the mixture was stirred overnight. Reaction completion was checked by TLC (Rf around 0.9 in 50% EtOAc/PE). Then 20 mL of conc. NH_4OH was added at -10 °C and the

mixture was stirred overnight. Reaction completion was checked by TLC (Rf around 0.6 in 50% EtOAc/PE). Finally, the mixture pH was lowered to 5 using 18mL of concentrated HCl. 60 mL of H_2O and 20 mL of DCM were added before partitioning layers. The organic layer was washed with sat. $NaHCO_3$ followed by Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 1:5 to 2:5 EtOAc/Hex eluent resulting in 3.404g (5.44mmol, 75.3%) of Compound **11** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.807 (br, 1H, NH), 8.689 (s, 1H, H^2), 8.019 (s, 1H, H^8), 8.010 (d, *J* = 8.7 Hz, 2H, $H^{arom,\ ortho}$, Bz), 7.535 (t, *J* = 7.4 Hz, 1H, $H^{arom,\ para}$, Bz) 7.445 (t, *J* = 7.6 Hz, 2H, $H^{arom,\ meta}$, Bz), 7.260 ($CHCl_3$), 5.969 (s, 1H, $H^{1'}$), 4.593 (d, *J* = 4.6 Hz, 1H, $H^{2'}$), 4.474 (dd, *J* = 9.2, 5.1 Hz, 1H, $H^{3'}$), 4.429 (dd, *J* = 9.6, 4.6 Hz, 1H, $H^{5'a}$), 4.218 (td, *J* = 10.1, 5.1 Hz, 1H, $H^{4'}$), 4.007 (dd, *J* = 10.0, 9.2 Hz, 1H, $H^{5'b}$), 1.048, 1.018 (2x s, 18H, 5'-3'-O-Si-tBu_2), 0.913 (s, 9H, 2'-O-Si-tBu), 0.153, 0.132 (2x s, 6H, 2'-O-SiMe_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 165,27 (CO, Bz), 152.64 (C^2), 151.12 (C^6), 149.92 (C^4), 141.05 (C^8), 133.63 (C_q^{ipso}, Bz), 132.72, 128.71, 128.11 (C^{ortho}, C^{meta}, C^{para}, Bz), 123.66 (C^5), 92.47 ($C^{1'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 75.89 ($C^{3'}$), 75.51 ($C^{2'}$), 74.76 ($C^{4'}$), 67.78 ($C^{5'}$), 27.49, 27.03 (2x CH_3, 5'-3'-O-Si-tBu_2), 25.91 (CH_3, 2'-O-Si-tBu), 22.77, 20.36, 18.32 (3x C_q, 3x Si-tBu), -4.24, -4.96 (2x CH_3, 2'-O-SiMe_2).

IR: 2932, 2886, 2859, 1697, 1609, 1582, 1454, 1250, 1138, 1057, 826, 752, 652.

HRMS: $[C_{31}H_{47}N_5O_5Si_2+H]^+$: found 626.3190, calculated 626.3188.

5'-OH-3'-OH-2'-TBDMS-Adenosine(Bz) (15)

5.232 mmol (3.275g) of compound **11** was dissolved in 25 mL of DCM, 22 mmol (4.0mL) of diluted HF-Pyridine* was added dropwise at 0 °C and the mixture was stirred for 60 minutes. Reaction completion was checked by TLC (Rf around 0.3 in 100% EtOAc). The mixture was washed with sat. $NaHCO_3$ followed by brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 0% to 10% MeOH/EtOAc eluent resulting in 2.499g (5.146 mmol, 98.4%) of Compound **15** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.568 (br, 1H, NH), 8.706 (s, 1H, H^2), 8.029 (s, 1H, H^8), 7.969 (d, *J* = 4.7 Hz, 2H, $H^{arom,\ ortho}$, Bz), 7.521(t, *J* = 7.4 Hz, 1H, $H^{arom,\ para}$, Bz), 7.428 (t, *J* = 7.6 Hz, 2H, $H^{arom,\ meta}$, Bz), 7.260 ($CHCl_3$), 5.904 (d, *J* = 10.9 Hz, 1H, $OH^{5'}$) 5.793 (d, *J* = 7.1 Hz, 1H, $H^{1'}$), 5.029 (dd, *J* = 7.1, 4.8 Hz, 1H, $H^{2'}$), 4.307 (d, *J* = 4.8 Hz, 1H, $H^{3'}$), 4.261 (s, 1H, $H^{4'}$), 3.891 (d, *J* = 12.7 Hz, 1H, $H^{5'a}$), 3.695 (t, *J* = 11.7 Hz, 1H, $H^{5'b}$), 3085 (s, 1H, $OH^{3'}$), 0.719 (s, 9H, 2'-O-Si-tBu), -0.242, -0.437 (2x s, 6H, 2'-O-SiMe_2). **^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ**: 164.83 (CO, Bz)), 152.29 (C^2), 150.51 (C^6), 150.47 (C^4), 142.95 (C^8), 133.46 (C_q^{ipso}, Bz), 132.86, 128.76, 127.97 (C^{ortho}, C^{meta}, C^{para}, Bz), 124.19 (C^5), 91.01 ($C^{1'}$), 87.42 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.46 ($C^{2'}$), 72.54 ($C^{3'}$), 63.03 ($C^{5'}$), 25.45 (CH_3, 2'-O-Si-tBu), 17.75 (C_q, Si-tBu), -5.34, -5.44 (2x CH_3, 2'-O-Si-Me_2).

IR: 3289, 2949, 2928, 2886, 1699, 1609, 1582, 1456, 1249, 1088, 835, 779, 706, 644 **HRMS:** $[C_{23}H_{31}N_5O_5Si+H]^+$: found 486.2167, calculated 486.2167

**70% HF-Pyridine contains 1mol HF per 28.57g at d=1.1g/mL, or 38.5M. 6:1 dilution in pyridine is 5.5M, or 0.1818mL/mmol.*

5'-DMTr-3'-OH-2'-TBDMS-Adenosine(Bz) (19)

2.158 mmol (1.048g) of compound **15** was dissolved in 5 mL of dry pyridine, 2.68 mmol (0.907g) of dimethoxytritylchloride was added at -10 °C and the mixture was stirred overnight. Reaction completion was checked by neutralized TLC (Rf around 0.75 in 100% EtOAc). Finally, the reaction was quenched using 0.5mL of MeOH. The mixture was concentrated using rotary evaporation before being extracted in DCM/H_2O. The organic layer was washed with sat. $NaHCO_3$ followed by brine and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 30% to 100% EtOAc/PE eluent resulting in 1.613g (2.046 mmol, 94.9%) of compound **19** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.053 (s, 1H, NH), 8.735 (s, 1H, H^2), 8.236 (s, 1H, H^8), 8.028 (d, *J* = 7.3 Hz, 2H, $H^{arom,\ ortho}$, Bz), 7.607(t, *J* = 7.4 Hz, 1H, $H^{arom,\ para}$, Bz) 7.523 (t, *J* = 7.5 Hz, 2H, $H^{arom,\ meta}$, Bz), 7.450 (d, *J* = 7.2 Hz, 2H, $H^{arom,\ ortho}$, DMTr), 7.339 (d, *J* = 8.5 Hz, 4H, H^{arom}, DMTr), 7.276 (t, *J* = 7.0 Hz, 2H, H^{arom}, DMTr^{meta}), 7.260 ($CHCl_3$), 7.216 (t, *J* = 7.1 Hz, 1H, $H^{arom,\ para}$, DMTr), 6.816 (d, *J* = 8.9 Hz, 4H, H^{arom}, DMTr), 6.111 (d, *J* = 5.3 Hz, 1H, $H^{1'}$), 5.028 (t, *J* = 5.1 Hz, 1H, $H^{2'}$), 4.369 (dd, *J* = 8.0, 4.0 Hz, 1H, $H^{3'}$), 4.290 (q, *J* = 3.4 Hz, 1H, $H^{4'}$), 3.780 (s, 6H, OMe, DMTr), 3.550 (dd, *J* = 10.7, 3.1 Hz, 1H, $H^{5'a}$), 3.399 (dd, *J* = 10.7, 3.4 Hz, 1H, $H^{5'b}$), 2.735 (d, *J* = 4.1 Hz, 1H, $OH^{3'}$), 0.843 (s, 9H, 2'-O-Si-tBu), -0.003, -0.142 (2x s, 6H, 2'-O-SiMe_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 164.62 (CO, Bz), 158.72 (C_q, DMTr), 152.98 (C^2), 151.79 (C^6), 149.69 (C^6), 144.63 (C_q, DMTr), 141.83 (C^2), 135.71 (C_q, DMTr), 133.84 (C_q^{ipso}, Bz), 132.92 (CH^{arom}, Bz), 130.20 (CH^{arom}, DMTr), 129.03 (CH^{arom}, Bz), 128.26, 128.06, 127.94 (3x CH^{arom}, 2x DMTr, 1x Bz), 127.21 (CH^{arom},

DMTr), 123.33 (C^5), 113.36 (CH^{arom}, DMTr), 88.56 ($C^{1'}$), 86.84 (C_q, DMTr), 84.42 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 75.83 ($C^{2'}$), 71.68 ($C^{3'}$), 63.43 ($C^{5'}$), 55.37 (OMe, DMTr), 25.70 (CH_3, 2'-O-Si-tBu), 18.03 (C_q, Si-tBu), -4.80, -5.02 (2x CH_3, 2'-O-Si-Me_2).

IR: 2951, 2930, 2905, 2857, 2835, 1699, 1607, 1580, 1506, 1456, 1246, 1175, 1029, 883, 781, 752, 702, 644

HRMS: $[C_{44}H_{49}N_5O_7Si+H]^+$: found 788.3480, calculated 788.3474.

5'-DMTr-3'-Lev-2'-TBDMS-Adenosine(Bz) (23)

1.548 mmol (1.220g) of compound **19** was dissolved in 8 mL of dry DCM, 0.17 mmol (0.021g) of 4-dimethylaminopyridine, 2.0 mmol (0.20mL, 0.23g) of levulinic acid and 1.9 mmol (0.30mL, 0.24g) of Diisopropylcarbodiimide were added respectively and the mixture was stirred overnight. Reaction completion was checked by neutralized TLC (Rf around 0.75 in 100% EtOAc). The organic layer was washed with sat. $NaHCO_3$ followed by Brine and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 30% to 45% EtOAc/PE eluent resulting in 1.165g (1.315 mmol, 84.9%) of Compound **23** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.241 (s, 1H, NH), 8.737 (s, 1H, H^2), 8.235 (s, 1H, H^8), 8.038 (d, J = 7.4 Hz, 2H, $H^{arom,\ ortho}$, Bz), 7.594 (t, J = 7.4 Hz, 1H, $H^{arom,\ para}$, Bz) 7.510 (t, J = 7.5 Hz, 2H, $H^{arom,\ meta}$, Bz), 7.442 (d, J = 7.3 Hz, 2H, $H^{arom,\ ortho}$, DMTr), 7.331 (dd, J = 8.8, 1.5 Hz, 4H, H^{arom}, DMTr),7.282 (t, J = 7.0 Hz, 2H, $H^{arom,\ meta}$, DMTr), 7.260 ($CHCl_3$), 7.220 (t, J = 7.2 Hz, 1H, $H^{arom,\ para}$, DMTr),6.821 (d, J = 8.5 Hz, 4H, H^{arom}, DMTr), 6.126 (d, J = 6.4 Hz, 1H, $H^{1'}$), 5.476 (dd, J = 5.0 Hz, 1H, $H^{3'}$), 5.115 (dd, J = 6.2, 5.3 Hz, 1H, $H^{2'}$), 4.335 (q, J= 2.9 Hz, 1H, $H^{4'}$), 3.777 (s, 6H, OMe, DMTr),3.560 (dd, J = 10.7, 3.1 Hz, 1H, $H^{5'a}$), 3.418 (dd, J = 10.7, 3.3 Hz, 1H, $H^{5'b}$), 2.9-2.5 (m, 4H, R^1-$C\underline{H_2}C\underline{H_2}$-$R^2$, Lev), 2.201 (s, 3H, CH_3, Lev) , 0.726 (s, 9H, 2'-O-Si-tBu), -0.007 (TMS), -0.024, -0.265 (2x s, 6H, 2'-O-SiMe_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 206.34 (CO, Lev) 171.80 (CO, Lev), 164.74 (CO, Bz), 158.72 (C_q, DMTr), 152.95 (C^2), 151.93 (C^6), 149.73 (C^4), 144.48 (C_q, DMTr), 141.55 (C^8), 135.50 (C_q, DMTr), 133.82 (C_q^{ipso}, Bz), 132.87 (CH^{arom}, Bz), 130.20 (CH^{arom}, DMTr), 128.95 (CH^{arom}, Bz), 128.23, 128.10, 127.99 (3x CH^{arom}, 2x DMTr, 1x Bz), 127.17 (CH^{arom}, DMTr), 123.06 (C^5), 113.39 (CH^{arom}, DMTr), 88.08 ($C^{1'}$), 87.06 (C_q, DMTr), 82.43 ($C^{3'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.49 ($C^{2'}$), 73.26 ($C^{4'}$), 63.23 ($C^{5'}$), 55.34 (OMe, DMTr), 37.81 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 29.99 (CH_3, Lev), 27.91 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.46 (CH_3, 2'-O-Si-tBu), 17.83 (C_q, Si-tBu), -5.11, -5.29 (2x CH_3, 2'-O-Si-Me_2).

IR: 2951, 2930, 2899, 2856, 2837, 1744, 1715, 1607, 1580, 1506, 1456, 1248, 1175, 1153, 1030, 835, 779, 704 **HRMS:** $[C_{49}H_{55}N_5O_9Si+H]^+$: found 886.3849, calculated 886.3842.

5'-OH-3'-Lev-2'-TBDMS-Adenosine(Bz) (27)

1.315 mmol (1.003g) of Compound **23** was dissolved in 10mL DCM/MeOH (7:3 v:v), 13.19 mmol (2.509g, monohydrate) of diluted *p*-toluenesulfonic acid (6.27 wt% in DCM/MeOH(7:3 v:v))* was added at 0 °C and the mixture was stirred for 10 minutes. Reaction completion was checked by TLC (Rf around 0.4 in 100% EtOAc). Finally, the mixture was quenched using sat. $NaHCO_3$. The organic layer was washed with sat. $NaHCO_3$ followed by Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 80% to 100% EtOAc/PE eluent resulting in 0.663g (1.138 mmol, 86.6%) of Compound **27** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.284 (s, 1H, NH), 8.760 (s, 1H, H^2), 8.043 (s, 1H, H^8), 8.002 (d, J = 7.5 Hz, 2H, $H^{arom,\ ortho}$, Bz), 7.577 (t, J = 7.4 Hz, 1H, $H^{arom,\ para}$, Bz) 7.485 (t, J = 7.6 Hz, 2H, $H^{arom,\ meta}$, Bz), 7.260 ($CHCl_3$), 5.994 (d, J = 10.1 Hz, 1H, $OH^{5'}$), 5.815 (d, J = 7.7 Hz, 1H, $H^{1'}$), 5.476 (d, J = 5.2 Hz, 1H, $H^{3'}$), 5.114 (dd, J = 7.6, 5.2 Hz, 1H, $H^{2'}$), 4.303 (s, 1H, $H^{4'}$), 3.939 (d, J = 12.9 Hz, 1H, $H^{5'a}$), 3.779 (dd, J = 11.5, 9.8 Hz, 1H, $H^{5'b}$), 2.9-2.5 (m, 4H, R^1-$C\underline{H_2}C\underline{H_2}$-$R^2$, Lev), 2.184 (s, 3H, CH_3, Lev), 0.661 (s, 9H, 2'-O-Si-tBu), 0.041 (TMS), -0.153, -0.479 (2x s, 6H, 2'-O-SiMe_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 206.36 (CO, Lev) 171.79 (CO, Lev), 164.59 (CO, Bz), 152.43 (C^2), 150.57 (C^6), 150.48 (C^4), 143.03 (C^8), 133.52 (C_q^{ipso}, Bz), 133.02, 128.95, 127.97 (C^{ortho}, C^{meta}, C^{para}, Bz), 124.37 (C^5), 91.26 ($C^{1'}$), 86.00 ($C^{3'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.28 ($C^{2'}$), 73.08 ($C^{4'}$), 62.89 ($C^{5'}$), 37.85 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 29.92 (CH_3, Lev), 27.87 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.39 (CH_3, 2'-O-Si-tBu), 17.76 (C_q, Si-tBu), -5.28, -5.84 (2x CH_3, 2'-O-Si-Me_2).

IR: 3254, 3167, 3123, 3065, 2953, 2928, 2859, 1695, 1609, 1580, 1454, 1152, 1093, 1080, 860, 837, 775, 727, 700, 650, 635

HRMS: $[C_{28}H_{37}N_5O_7Si+H]^+$: found 584.2535, calculated 584.2535

**The solution was prepared using 13.19 mmol (2.509g) p-toluenesulfonic acid monohydrate in 40mL DCM/MeOH (7:3 v:v), resulting in a 6.27 wt% solution.*

5'-PAM(CNE)-3'-Lev-2'-TBDMS-Adenosine(Bz) (31)

1.138 mmol (0.663g) of Compound **27** was dissolved in 10mL of dry DCM, 2.5 mmol (0.25g, 0.35mL) of Triethylamine and 1.8 mmol (0.42g, 0.40mL) of 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite were added respectively and the mixture was stirred for 10 minutes. Reaction completion was checked by neutralized TLC (Rf around 0.80 in 100% EtOAc, 0.30 in 50% EtOAc/PE, 0.75 in DCM). Finally, the mixture was quenched using aqueous 5wt% $NaHCO_3$. The mixture was partitioned and the organic layer was dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 1:99 TEA:DCM eluent resulting in 0.727g (0.927 mmol, 81.5%) of Compound **31** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.222 (br, 1H, NH), 8.780 (s, 1H, H^2), 8.466, 8.415 (s+s, 1H, H^8), 8.010 (d, *J* = 7.4 Hz, 2H, $H^{arom,\ ortho}$, Bz), 7.569 (t, *J* = 7.8 Hz, 1H, $H^{arom,\ para}$, Bz) 7.488 (t, *J* = 6.7 Hz, 2H, $H^{arom,\ meta}$, Bz), 7.260 ($CHCl_3$), 6.159, 6.135 (d+d, *J* = 6.3 + 5.7 Hz, 1H, $H^{1'}$), 5.400, 5.342 (dd+dd, *J* = 4.9, 3.4 + 5.0, 2.6 Hz, 1H, $H^{3'}$), 4.888, 4.814 (t+t, *J* = 5.6 + 5.4 Hz, 1H, $H^{2'}$), 4.40-4.35 (m, 1H, $H^{4'}$), 4.0-3.7 (m, 4H, $H^{5'}$, NC-$\underline{CH_2}$-CH_2-OR), 3.7-3.5 (m, 2H, CH, $\underline{iPr_2}$NR), 2.9-2.5 (m, 6H, R^1-$\underline{CH_2}\underline{CH_2}$-$R^2$, Lev, NC-$CH_2$-$\underline{CH_2}$-OR), 2.182 (s, 3H, CH_3, Lev) , 1.22-1.16 (m, 12H, CH_3, $\underline{iPr_2}$NR) 0.727, 0.707 (s+s, 9H, 2'-O-Si-tBu), -0.032 (TMS), -0.064, -0.091, -0.267, -0.282 (2x s+s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 206.21, 206.18 (CO, Lev) 171.84 (CO, Lev), 164.71 (CO, Bz), 152.91 (C^2), 151.98, 151.83 (C^6), 149.65 (C^4), 141.71, 141.44 (C^8), 133.85 (C_q^{ipso}, Bz), 132.77, 128.87, 127.92 (C^{ortho}, C^{meta}, C^{para}, Bz), 123.08, 123.02 (C^5), 117.57 (CN), 88.34, 87.97 ($C^{1'}$), 82.56, 82.47, 82.39 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 75.01, 74.94 ($C^{2'}$), 73.28, 72.70 ($C^{3'}$), 63.08, 62.90, 62.72, 62.57 ($C^{5'}$), 58.78, 58.57 (NC-$\underline{C}H_2$-CH_2-OR), 43.36, 43.24, 43.12 (2x CH, $\underline{iPr_2}$NR), 37.80, 37.77 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 29.90 (CH_3, Lev), 27.89, 27.86 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.42 (CH_3, 2'-O-Si-tBu), 24.90, 24.84, 24.79, 24.72 (4x CH_3, $\underline{iPr_2}$NR), 20.48, 20.42, 20.41, 20.35 (NC-CH_2-$\underline{C}H_2$-OR), 17.80 (C_q, Si-tBu), -5.19, -5.28, -5.35, -5.40 (2x CH_3, 2'-O-Si-Me_2).

^{31}P NMR (162 MHz, $CDCl_3$) δ: 149.20, 149.05.

IR: 2965, 2930, 2886, 2858, 1744, 1716, 1609, 1582, 1454, 1250, 1155, 1028, 837, 779, 708, 679

HRMS: $[C_{37}H_{54}N_7O_4PSi+H]^+$: found 784.3615 , calculated 784.3619.

5',3'-Si(tBu)$_2$-2'-TBDMS-Cytidine (9)

1.99 mmol (0.485g) of cytidine was, after two co-evaporations in 1,4-dioxane, dissolved in 10 mL of dry DMF, 2.30 mmol (0.75 mL, 1.01g) of $(t\text{-}Bu)_2Si(OTf)_2$ was added at 0 °C and the mixture was stirred for 100 minutes. Reaction completion was checked by TLC (Rf around 0.40 at 10% MeOH/DCM). Then 10.1 mmol (0.691g) of Imidazole and 3.09 mmol (0.465g) of TBDMS-Cl were added and the mixture was stirred overnight while warming to room temperature. Reaction completion was checked by TLC (Rf around 0.50 at 10% MeOH/DCM, 0.30 at 100% EtOAc, 0.20 at 80% EtOAc/DCM). Reaction was not fully completed, so 1.1 mmol (0.076g) of Imodazole and 0.989 mmol (0.149g) of TBDMS-Cl were added and the mixture was stirred for another 4 hours with no result. The mixture was quenched with 1 mL MeOH, and concentrated, before being redissolved in 30 mL DCM and washed with 20 mL H_2O, Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using using an 80:20 to 100:0 EtOAc:DCM eluent resulting in 0.895g (1.80 mmol, 89.9%) of compound **9** as a white solid.

^{1}H NMR (399 MHz, $CDCl_3$, 330K) δ: 8.67 (br, 1H, NH_2), 7.34 (s, 1H, H^6), 7.26 ($CHCl_3$), 6.50 (br, 1H, H^5), 5.65 (s, 1H, $H^{1'}$), 4.47 (dd, *J* = 8.1, 4.7 Hz, 1H, $H^{5'a}$), 4.24 - 4.12 (m, 2H, $H^{2'}$, $H^{4'}$), 3.95 (t, *J* = 9.5 Hz, 1H, $H^{5'b}$), 3.77 (d, *J* = 6.0 Hz, 1H, $H^{3'}$), 1.01, 1.00 (2x s, 18H, 5'-3'-O-Si-tBu_2), 0.90 (s, 9H, 2'-O-Si-tBu), 0.13, 0.10 (2x s, 6H, 2'-O-Si-Me_2).

^{13}C NMR (100 MHz, $CDCl_3$, 330K) δ: 163.86 (C^2), 153.64 (C^4), 140.16 (C^6), 96.64 (C^5), 93.98 ($C^{1'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 76.09 ($C^{3'}$), 75.69 ($C^{2'}$), 74.79 ($C^{4'}$), 67.74 ($C^{5'}$), 27.60, 27.08 (2x CH_3, 5'-3'-O-Si-tBu_2), 25.97 (CH_3, 2'-O-Si-tBu), 22.81, 20.39, 18.30 (3x C_q, Si-tBu), -4.19, -4.84 (2x CH_3, 2'-O-Si-Me_2).

IR: 2932, 2895, 2884, 2859, 17.22, 1645, 1472, 1055, 826, 779, 752, 650.

HRMS: $[C_{23}H_{43}N_3O_5Si_2+H]^+$: found 498.2810, calculated 498.2814

5',3'-Si(tBu)$_2$-2'-TBDMS-Cytidine(Ac) (13)

1.159 mmol (0.570g) of Compound **9** was, after one co-evaporation with pyridine, dissolved in 5mL of dry pyridine, 2.9 mmol (0.27 mL, 0.29g) of acetic anhydride was added at 0 °Cand the mixture was stirred for

2 hours whiel warming to room temperature. Reaction completion was checked by TLC (Rf around 0.70 at 10% MeOH/DCM). The mixture was concentrated, co-evaporated with toluene and washed withsat. NH_4Cl, sat. $NaHCO_3$, Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using using an 0% to 2% MeOH/DCM eluent resulting in 0.614g (1.137 mmol, 98.1%) of Compound **13** as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$, 293.7K) δ: 10.46 (s, 1H, NH), 7.71 (d, *J* = 7.6 Hz, 1H, H^6), 7.44 (d, *J* = 7.6 Hz, 1H, H^5), 7.26 ($CHCl_3$), 5.71 (s, 1H, $H^{1'}$), 4.55 (dd, *J* = 9.2, 5.2 Hz, 1H, $H^{5'a}$), 4.34 – 4.21 (m, 2H, $H^{2'}$, $H^{4'}$), 4.00 (t, *J* = 10.3 Hz, 1H, $H^{5'b}$), 3.79 (dd, *J* = 9.7, 4.3 Hz, 1H, $H^{3'}$), 2.32 (s, 3H, CH_3, Ac), 1.02, 1.02 (2x s, 18H, 5'-3'-O-Si-tBu_2), 0.94 (s, 9H, 2'-O-Si-tBu), 0.22, 0.15 (2x s, 6H, 2'-O-Si-Me_2).

^{13}C NMR (75 MHz, $CDCl_3$, 293.7K) δ: 171.68 (CO, Ac), 163.39 (C^2), 154.71 (C^4), 143.30 (C^6), 97.01 (C^5), 94.38 ($C^{1'}$), 77.59, 77.16, 76.74 ($CHCl_3$), 75.82 ($C^{3'}$), 75.46, 74.87 ($C^{2'}$, $C^{4'}$), 67.88 ($C^{5'}$), 27.61, 27.08 (2x CH_3, 5'-3'-O-Si-tBu_2), 26.01 (CH_3, 2'-O-Si-tBu), 25.11 (CH_3, Ac), 22.92, 20.47, 18.34 (3x C_q, Si-tBu), -4.16, -4.80 (2x CH_3, 2'-O-Si-Me_2).

IR: 2951, 2934, 2895, 2859, 1659, 1493, 1248, 1165, 1053, 997, 827, 779, 650.

HRMS: $[C_{25}H_{45}N_3O_6Si_2+H]^+$: found 540.2916, calculated 540.2920

5'-OH-3'-OH-2'-TBDMS-Cytidine(Ac) (17)

0.936 mmol (0.505g) of Compound **13** was dissolved in 5.0 mL of DCM, 3.85 mmol (0.70mL) of diluted HF-Pyridine* was added dropwise at 0 °C and the mixture was stirred for 2 hours. Reaction completion was checked by TLC (Rf around 0.45 in 10% MeOH/DCM). The mixture was washed withsat. NH_4Cl, sat. $NaHCO_3$, Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 0% to 10% MeOH/DCM eluent resulting in 0.307g (0.768 mmol, 82.1%) of Compound **17** as a clear solid.

^{1}H NMR (400 MHz, $CDCl_3$, 293.7K) δ: 10.27 (s, 1H, NH), 8.35 (d, *J* = 7.5 Hz, 1H, H^6), 7.39 (d, *J* = 7.5 Hz, 1H, H^5), 7.26 ($CHCl_3$), 5.67 (d, *J* = 2.3 Hz, 1H, $H^{1'}$), 4.57 (s, 1H, $OH^{5'}$), 4.45 (m, 1H, $H^{2'}$), 4.24 (dd, *J* = 11.2, 5.9 Hz, 1H, $H^{4'}$), 4.08 (d, *J* = 6.0 Hz, 1H, $H^{3'}$), 3.99 (d, *J* = 11.7 Hz, 1H, $H^{5'a}$), 3.84 (d, *J* = 8.5 Hz, 1H, $H^{5'b}$), 3.04 (d, *J* = 6.7 Hz, 1H, $OH^{3'}$), 2.21 (s, 3H, CH_3, Ac), 0.87 (s, 9H, 2'-O-Si-tBu), 0.12, 0.07 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 293.7K) δ: 171.40 (CO, Ac), 163.14 (C^2), 155.58 (C^4), 146.67 (C^6), 97.07 (C^5), 93.29 ($C^{1'}$), 85.10 ($C^{2'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 75.34 ($C^{4'}$), 69.19 ($C^{3'}$), 60.54 ($C^{5'}$), 25.77 (CH_3, 2'-O-Si-tBu), 24.84 (CH_3, Ac), 18.04 (C_q, Si-tBu), -4.58, -5.20 (2x CH_3, 2'-O-Si-Me_2).

IR: 2951, 2930, 2889, 2857, 1647, 1491, 1248, 1113, 1059, 827, 779.

HRMS: $[C_{17}H_{29}N_3O_6Si+H]^+$: found 400.1889, calculated 400.1898

**70% HF-Pyridine contains 1mol HF per 28.57g at d=1.1g/mL, or 38.5M. 6:1 dilution in pyridine is 5.5M, or 0.1818mL/mmol.*

5'-DMTr-3'-OH-2'-TBDMS-Cytidine(Ac) (21)

7.544 mmol (3.015g) of Compound **17** was dissolved in 40 mL of dry Pyridine, 9.238 mmol (3.130g) of 4,4'-Dimethoxytritylchloride was added at 0 °C and the mixture was stirred overnight. Reaction completion was checked by TLC (Rf around 0.80 in 10% MeOH/DCM, 0.60 in 100% EtOAc). Finally the reaction was quenched by adding 1 mL MeOH. The mixture was concentrated using rotary evaporation. Purification was performed with neutralized silica column chromatography using an 2:20:78 to 2:98:0 TEA:EtOAc:PE eluent resulting in 4.802g (6.842 mmol, 90.7%) of Compound **21** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 293.7K) δ:10.47 (s, 1H, NH), 8.47 (d, *J* = 7.5 Hz, 1H, H^6), 7.44 (d, *J* = 7.3 Hz, 2H, H^{ortho}, DMTr), 7.40 – 7.20 (m, 7H, H^{arom}, H^{meta}, H^{para}, DMTr), 7.26 ($CHCl_3$), 7.15 (d, *J* = 7.5 Hz, 1H, H^5), 6.88 (d, *J* = 8.8 Hz, 4H, H^{arom}, DMTr), 5.91 (d, *J* = 0.9 Hz, 1H, $H^{1'}$), 4.38 (s, 1H, $H^{3'}$), 4.29 (dd, *J* = 4.6, 0.7 Hz, 1H, $H^{2'}$), 4.11 (d, *J* = 7.9 Hz, 1H, $H^{4'}$), 3.82 (2x s, *J* = 2.0 Hz, 6H, OMe, DMTr), 3.60 (dd, *J* = 11.1, 1.7 Hz, 1H, $H^{5'a}$), 3.54 (dd, *J* = 11.2, 2.7 Hz, 1H, $H^{5'b}$), 2.45 (d, *J* = 8.8 Hz, 1H, $OH^{3'}$), 2.29 (s, 3H, CH_3, Ac), 0.94 (s, 9H, 2'-O-Si-tBu), 0.30, 0.19 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 293.7K) δ: 171.13 (CO, Ac), 163.33 (C^2), 158.77 (C_q, DMTr), 155.07 (C^4), 144.90 (C^6), 144.36, 135.55, 135.29 (3x C_q, DMTr), 130.19, 128.24, 128.13, 127.25, 113.41 (5x CH^{arom}, DMTr), 96.98 (C^5), 90.76 ($C^{1'}$), 87.20 (C_q, DMTr), 83.16 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 76.76 ($C^{2'}$), 69.14 ($C^{3'}$), 61.60 ($C^{5'}$), 55.32 (CH_3, OMe, DMTr), 25.91 (CH_3, 2'-O-Si-tBu), 24.93 (CH_3, Ac), 18.15 (C_q, Si-tBu), -4.27, -5.35 (2x CH_3, 2'-O-Si-Me_2). **IR:** 2949, 2928, 2897, 2855, 2839, 1667, 1609, 1489, 1115, 814, 787.

HRMS: $[C_{38}H_{47}N_3O_8Si+H]^+$: found 702.3210, calculated 702.3205

5'-DMTr-3'-Lev-2'-TBDMS-Cytidine(Ac) (25)

5.733 mmol (4.038g) of Compound **21** was dissolved in 25mL of dry DCM, a catalytic amount of 4-dimethylaminopyridine, 7.7 mmol (0.78mL, 0.89g) of Levulinic acid and 7.34 mmol (1.15mL, 0.927g) of Diisopropylcarbodiimide were added respectively and the mixture was stirred for 5 hours. Reaction completion was checked by TLC (Rf around 0.80 in 100% EtOAc). The organic layer was washed with sat. $NaHCO_3$ dried using $MgSO_4$ and concentrated using rotary evaporation. The mixture was redissolved in THF, centrifuged and the solution was collected. Purification was performed with neutralized silica column chromatography using an 1:30:79 to 1:99:0 TEA:EtOAc:PE eluent resulting in 4.398g (5.497 mmol, 95.6%) of Compound **25** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 293.7K) δ: 10.31 (s, 1H, NH), 8.44 (d, *J* = 7.5 Hz, 1H, H^6), 7.40 (d, *J* = 8.7 Hz, 2H, $H^{arom,\ ortho}$, DMTr), 7.40 – 7.20 (m, 7H, H^{arom}, H^{meta}, H^{para}, DMTr), 7.26 ($CHCl_3$), 7.13 (d, *J* = 7.5 Hz, 1H, H^5), 6.87 (d, *J* = 8.9 Hz, 4H, H^{arom}, DMTr), 5.92 (d, *J* = 2.1 Hz, 1H, $H^{1'}$), 5.18 (dd, *J* = 7.7, 4.3 Hz, 1H, $H^{3'}$), 4.50 (dd, *J* = 4.2, 2.1 Hz, 1H, $H^{2'}$), 4.37 (d, *J* = 7.7 Hz, 1H, $H^{4'}$), 3.81 (2x s, 6H, OMe, DMTr), 3.63 (dd, *J* = 11.3, 2.0 Hz, 1H, $H^{5'a}$), 3.41 (dd, *J* = 11.4, 2.2 Hz, 1H, $H^{5'b}$), 2.85 – 2.45 (m, 4H, R^1-C<u>H$_2$C</u><u>H$_2$</u>-R^2, Lev), 2.29 (s, 3H, CH_3, Ac), 2.19 (s, 3H, CH_3, Lev), 0.87 (s, 9H, 2'-O-Si-tBu), 0.18, 0.05 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 293.7K) δ: 206.05 (CO, Lev), 171.90 (CO, Lev), 171.09 (CO, Ac), 163.24 (C^2), 158.80 (C_q, DMTr), 155.09 (C^4), 144.71 (C^6), 144.20, 135.28, 135.16 (3x C_q, DMTr), 130.23, 128.22, 128.18, 127.29, 113.45 (5x CH^{arom}, DMTr), 97.00 (C^5), 91.08 ($C^{1'}$), 87.40 (C_q, DMTr), 80.36 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.79 ($C^{2'}$), 70.89 ($C^{3'}$), 61.10 ($C^{5'}$), 55.36 (CH_3, OMe, DMTr), 37.69 (R^1-<u>C</u>H$_2$CH$_2$-R^2, Lev), 29.95 (CH_3, Lev), 27.80 (R^1-CH$_2$<u>C</u>H$_2$-R^2, Lev), 25.70 (CH_3, 2'-O-Si-tBu), 25.02 (CH_3, Ac), 18.03 (C_q, Si-tBu), -4.61, -5.43 (2x CH_3, 2'-O-Si-Me_2). **IR:** 2953, 2928, 2855, 1717, 1667, 1489, 1248, 1175, 1155, 1117, 1031, 1005, 829, 779.

HRMS: $[C_{43}H_{53}N_3O_{10}Si+H]^+$: found 800.3578, calculated 800.3573

5'-OH-3'-Lev-2'-TBDMS-Cytidine(Ac) (29)

5.363 mmol (4.290g) of Compound **25** was dissolved in 50mL DCM/MeOH (7:3 v:v), 58.88 mmol (11.20g, monohydrate) of diluted *p*-toluenesulfonic acid (7.80 wt% in DCM/MeOH (7:3 v:v))* was added at 0 °C and the mixture was stirred for 10 minutes. Reaction completion was checked by TLC (Rf around 0.45 in 100% EtOAc). Finally the mixture was quenched using sat. $NaHCO_3$. The mixture was partitioned and the organic layer was washed with Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 50:0 to 100:0 EtOAc:DCM eluent resulting in 2.450g (4.920 mmol, 91.8%) of Compound **29** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 293.7K) δ: 10.07 (s, 1H, NH), 8.22 (d, *J* = 7.5 Hz, 1H, H^6), 7.44 (d, *J* = 7.5 Hz, 1H, H^5), 7.26 ($CHCl_3$), 5.62 (d, *J* = 3.8 Hz, 1H, $H^{1'}$), 5.18 (t, *J* = 5.1 Hz, 1H, $H^{3'}$), 4.72 (t, *J* = 4.3 Hz, 1H, $H^{2'}$), 4.27 (d, *J* = 5.4 Hz, 1H, $H^{4'}$), 3.97 (d, *J* = 12.9 Hz, 1H, $H^{5'a}$), 3.75 (d, *J* = 12.7 Hz, 1H, $H^{5'b}$), 2.90 – 2.50 (m, 4H, R^1-C<u>H$_2$C</u><u>H$_2$</u>-R^2, Lev), 2.27 (s, 3H, CH_3, Ac), 2.18 (s, 3H, CH_3, Lev), 0.85 (s, 9H, 2'-O-Si-tBu), 0.05, 0.03 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 293.7K) δ: 206.54 (CO, Lev), 172.57 (CO, Lev), 171.26 (CO, Ac), 163.24 (C^2), 155.32 (C^4), 146.73 (C^6), 97.10 (C^5), 94.22 ($C^{1'}$), 83.17 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 73.34 ($C^{2'}$), 71.70 ($C^{3'}$), 61.20 ($C^{5'}$), 37.90 (R^1-<u>C</u>H$_2$CH$_2$-R^2, Lev), 29.92 (CH_3, Lev), 27.93 (R^1-CH$_2$<u>C</u>H$_2$-R^2, Lev), 25.69 (CH_3, 2'-O-Si-tBu), 25.03 (CH_3, Ac), 18.03 (C_q, Si-tBu), -4.85, -5.16 (2x CH_3, 2'-O-Si-Me_2).

IR: 3291, 2951, 2928, 2857, 1717, 1486, 1231, 1155, 1111, 837, 779.

HRMS: $[C_{22}H_{35}N_3O_8Si+H]^+$: found 498.2261, calculated 498.2266

**The solution was prepared using 58.88 mmol (11.20g) p-toluenesulfonic acid monohydrate in 120mL DCM/MeOH (7:3 v:v), resulting in a 9.33 wt% solution.*

5'-PAM(CNE)-3'-Lev-2'-TBDMS-Cytidine(Ac) (33)

1.00 mmol (0.500g) of Compound 2**9** was dissolved in 10mL of dry DCM, 1.4 mmol (0.15g, 0.20mL) of Triethylamine and 1.1 mmol (0.26g, 0.25mL) of 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite were added respectively and the mixture was stirred for 10 minutes. Reaction completion was checked by normal TLC (Rf around 0.60 in 100% EtOAc). Finally, the mixture was quenched using aqueous 5wt% $NaHCO_3$. The mixture was partitioned and the organic layer was washed with Brine and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 1:25:74 to 1:79:20 TEA:EtOAc:Hex eluent resulting in 0.576g (0.825 mmol, 82.5%) of Compound **33** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 293.7K) δ: 10.14, 10.09 (s+s, 1H, NH), 8.43, 8.36 (d+d, *J* = 7.5 + 7.6 Hz, 1H, H^6), 7.39 (d, *J* = 7.5 Hz, 1H, H^5), 7.26 ($CHCl_3$), 5.94, 5.91 (d+d, *J* = 2.9, 2.4 Hz, 1H, $H^{1'}$), 5.07, 4.99 (dd+dd, *J* = 7.2,

4.5 + 6.6, 4.6 Hz, 1H, $H^{3'}$), 4.46 – 4.34 (m, 2H, $H^{2'}$, $H^{4'}$), 4.14 – 3.72 (m, 4H, $H^{5'}$, NC-CH_2-CH_2-OR), 3.68 - 3.53 (m, 2H, CH, iPr_2NR), 2.85 – 2.48 (m, 6H, R^1-CH_2CH_2-R^2, Lev, NC-CH_2-CH_2-OR), 2.28 (s, 3H, CH_3, Ac), 2.18, 2.17 (s+s, 3H, CH_3, Lev), 1.27 – 1.12 (m, 12H, CH_3, iPr_2NR), 0.87, 0.86 (s+s, 9H, 2'-O-Si-tBu), 0.13, 0.12, 0.02, 0.01 (2x s+s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 293.7K) *δ*: 206.25, 206.13 (CO, Lev), 171.94, 171.87 (CO, Lev), 171.35 (CO, Ac), 163.21, 163.13 (C^2), 155.19, 155.14 (C^4), 144.95, 144.84 (C^6), 117.65, 115.59 (CN), 96.71, 96.62 (C^5), 91.04, 90.88 ($C^{1'}$), 80.95, 80.87 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.85, 74.78 ($C^{2'}$), 71.22, 70.53 ($C^{3'}$), 61.86, 61.69, 61.31, 61.14 ($C^{5'}$), 58.97, 58.76, 58.72, 58.51 (NC-CH_2-CH_2-OR), 43.43, 43.33, 43.31, 43.21 (2x CH, iPr_2NR), 37.82, 37.73 (R^1-CH_2CH_2-R^2, Lev), 29.96 (CH_3, Lev), 27.87, 27.82 (R^1-CH_2CH_2-R^2, Lev), 25.70 (CH_3, 2'-O-Si-tBu), 25.09, 25.07 (CH_3, Ac), 24.91, 24.89, 24.84, 24.82, 24.77, 24.74 (4x CH_3, iPr_2NR), 20.54, 20.46, 20.42, 20.34 (NC-CH_2-CH_2-OR), 18.05 (C_q, Si-tBu), -4.69, -5.36, -5.43 (2x CH_3, 2'-O-Si-Me_2).

^{31}P NMR (162 MHz, $CDCl_3$, 293.7K) *δ*: 149.83, 148.69.

IR: 2965, 2930, 2886, 2857, 1719, 1667, 1492, 1364, 1231, 1155, 1117, 1043, 812, 779, 731.

HRMS: $[C_{31}H_{52}N_5O_9PSi+H]^+$: found 698.3343, calculated 698.3345

5',3'-Si(tBu)$_2$-2'-TBDMS- Guanosine (8)

10.1 mmol (3.04g) of guanosine hydrate was, after three co-evaporations in 1,4-dioxane, dissolved in 50 mL of dry DMF, 11.0 mmol (3.60 mL, 4.86g) of (t-Bu)$_2$Si(OTf)$_2$ was added at 0 °C and the mixture was stirred for 40 minutes. Reaction completion was checked by TLC (Rf around 0.45 at 10% MeOH/DCM). Then 50.1 mmol (3.41g) of Imidazole and 15.1 mmol (2.28g) of TBDMS-Cl were added and the mixture was stirred overnight while warming to room temperature. Reaction completion was checked by TLC (Rf around 0.55 at 10% MeOH/DCM). The mixture was quenched with 1mL MeOH and cooled to 0 °C. Purification was performed by filtering the suspension and washing the product residue with cold MeOH, resulting in 4.570g (8.498mmol, 84.2%) of Compound **8** as a white solid.

^{1}H NMR (400 MHz, DMSO, 297.3K)*δ*: 10.67 (s, 1H, NH), 7.91 (s, 1H, H^8), 6.36 (s, 2H, NH_2), 5.72 (s, 1H, $H^{1'}$), 4.56 (s, 1H, $H^{2'}$), 4.34 (dd, *J* = 7.6, 3.5 Hz, 1H, $H^{5'a}$), 4.28 (dd, *J* = 8.7, 5.2 Hz, 1H, $H^{3'}$), 4.02 – 3.88 (m, 2H, $H^{5'b}$, $H^{4'}$), 3.35 (s, HOD, H_2O), 2.50 (s, DMSO), 1.06, 1.00 (2x s, 18H, 5'-3'-O-Si-tBu_2), 0.86 (s, 9H, 2'-O-Si-tBu), 0.09, 0.07 (2x s, 6H, 2'-O-Si-Me_2).

^{13}C NMR (101 MHz, DMSO, 297.3K)*δ*:156.68 (CO, C^6), 153.77 (C^2), 150.77 (C^4), 135.61 (C^8), 116.56 (C^5), 90.08 ($C^{1'}$), 75.68 ($C^{3'}$), 74.73 ($C^{2'}$), 73.91 ($C^{4'}$), 66.97 ($C^{5'}$), 40.15, 39.94, 39.73, 39.52, 39.31, 39.10, 38.89 (DMSO), 27.33, 26.86 (2x CH_3, 5'-3'-O-Si-tBu_2), 25.72 (CH_3, 2'-O-Si-tBu), 22.25, 20.00, 18.05 (3x C_q, Si-tBu), -4.57, -5.11 (2x CH_3, 2'-O-Si-Me_2).

IR: 3474, 3292, 3159, 3140, 3088, 3019, 2936, 2884, 2856, 2816, 2778, 2714, 1688, 1167, 1049, 899, 833, 777. **HRMS:** $[C_{24}H_{43}N_5O_5Si_2+H]^+$: found 538.2873, calculated 538.2876

5',3'-Si(tBu)$_2$-2'-TBDMS- Guanosine(iBu) (12)

8.498 mmol (4.570g) of compound **8** was, after one co-evaporation with pyridine, dissolved in 80mL of dry Pyridine, 21.5 mmol (2.25 mL, 2.29g) of isobutyrylchloride as added at -20 °Cand the mixture was stirred for 1 hours. Reaction completion was checked by TLC (Rf around 0.80 at 10% MeOH/DCM). The mixture was quenched with 10 mL of MeOH and left to warm to room temperature for 1 hour. The mixture was concentrated before being redissolved in MeOH and cooled to 0°C. Purification was performed by filtering the suspension and washing the product residue with cold MeOH, resulting in 5.060g (8.324 mmol, 98.0%) of Compound **12** as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)*δ*: 11.97 (s, 1H, NH), 7.96 (s, 1H, NH), 7.73 (s, 1H, H^8), 7.26 ($CHCl_3$), 5.79 (s, 1H, $H^{1'}$), 4.50 (dd, *J* = 9.2, 4.8 Hz, 1H, $H^{5'a}$), 4.42 (s, 1H, $H^{2'}$), 4.30-4.15 (m, 2H, $H^{3'}$, $H^{4'}$), 4.00 (t, *J* = 9.6 Hz, 1H, $H^{5'b}$), 2.62 (dt, *J* = 13.6, 6.8 Hz, 1H, CH, iBu), 1.30, 1.28 (2x d, *J* = 3.3 + 3.2 Hz, 6H, CH_3, iBu), 1.07, 1.04 (2x s, 18H, 5'-3'-O-Si-tBu_2), 0.94 (s, 9H, 2'-O-Si-tBu), 0.16, 0.15 (2x s, 6H, 2'-O-Si-Me_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)*δ*: 177.88 (CO, iBu), 155.20 (CO, C^6), 147.25, 147.25 (C^4, C^2), 136.71 (C^8), 121.89 (C^5), 91.99 ($C^{1'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 76.19 ($C^{3'}$), 76.00 ($C^{2'}$), 74.78 ($C^{4'}$), 67.96 ($C^{5'}$), 36.84 (CH, iBu), 27.62, 27.13 (2x CH_3, 5'-3'-O-Si-tBu_2), 26.06 (CH_3, 2'-O-Si-tBu), 22.97, 20.49 (2x C_q, Si-tBu), 19.16, 19.00 (2x CH_3, iBu), 18.54 (C_q, Si-tBu), -4.03, -4.81 (2x CH_3, 2'-O-Si-Me_2).

IR: 3472, 3291, 3154, 3140, 3088, 3024, 2934, 2886, 2859, 2818, 2778, 2714, 1688, 1674, 1597, 1471, 1142, 1053, 829, 779, 673.

HRMS: $[C_{28}H_{49}N_5O_6Si_2+H]^+$: found 608.3293, calculated 608.3294

5'-OH-3'-OH-2'-TBDMS- Guanosine(iBu) (16)

0.181 mmol (0.110g) of Compound **12** was dissolved in 5.0 mL of DCM, 0.83 mmol (0.15mL) of diluted HF-Pyridine* was added dropwise at 0 °C and the mixture was stirred for 2 hours. Reaction completion was checked by TLC (Rf around 0.40 in 5% MeOH/DCM). The mixture was washed withsat. $KHSO_4$ and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 0% to 10% MeOH/DCM eluent resulting in 0.078g (0.167 mmol, 92.2%) of Compound **16** as a white solid.

^{1}H NMR (400 MHz, DMSO, 297.3K) *δ:* 12.08 (s, 1H, NH), 11.69 (s, 1H, NH), 8.28 (s, 1H, H^8), 5.86 (d, *J* = 6.8 Hz, 1H, $H^{1'}$), 5.11 (t, *J* = 5.4 Hz, 1H, $OH^{5'}$), 5.06 (d, *J* = 4.6 Hz, 1H, $OH^{3'}$), 4.54 (dd, *J* = 6.7, 4.9 Hz, 1H, $H^{2'}$), 4.09 (td, *J* = 4.7, 2.2 Hz, 1H, $H^{4'}$), 3.98 – 3.94 (m, 1H, $H^{4'}$), 3.70 – 3.50 (m, 2H, $H^{5'}$), 3.36 (s, HOD, H_2O), 2.76 (hept, *J* = 6.8 Hz, 1H, CH, iBu), 2.50 (DMSO), 1.12, 1.10 (2x s, 6H, CH_3, iBu), 0.71 (s, 9H, 2'-O-Si-tBu), -0.08, -0.20 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, DMSO, 297.3K) *δ:* 180.16 (CO, iBu), 154.82 (CO, C^6), 149.04 (C^2), 148.22 (C^4), 137.48 (C^8), 119.95 (C^5), 86.32 ($C^{4'}$), 85.90 ($C^{1'}$), 76.43 ($C^{2'}$), 70.82 ($C^{3'}$), 61.42 ($C^{5'}$), 40.14, 39.94, 39.73, 39.52, 39.31, 39.10, 38.90 (DMSO), 34.75 (CH, iBu), 25.50 (CH_3, 2'-O-Si-tBu), 18.91, 18.80 (2x CH_3, iBu), 17.77 (C_q, Si-tBu), -4.96, -5.45 (2x CH_3, 2'-O-Si-Me_2).

IR: 3474, 3291, 3154, 3142, 2930, 2882, 2859, 2779, 2714, 1682, 1601, 1252, 1142, 1090, 1049, 835, 781, 673. **HRMS:** $[C_{20}H_{33}N_5O_6Si+H]^+$: found 468.2267, calculated 468.2273

**70% HF-Pyridine contains 1mol HF per 28.57g at d=1.1g/mL, or 38.5M. 6:1 dilution in pyridine is 5.5M, or 0.1818mL/mmol.*

5'-DMTr-3'-OH-2'-TBDMS- Guanosine(iBu) (20)

1.43 mmol (0.680g) of Compound **16** was dissolved in 10 mL of dry Pyridine, 1.68 mmol (0.596g) of 4,4'-Dimethoxytritylchloride was added at 0 °C and the mixture was stirred overnight. Reaction completion was checked by TLC (Rf around 0.85 in 100% EtOAc). Finally the reaction was quenched by adding 0.5 mL MeOH. The (yellow) mixture was concentrated using rotary evaporation and co-evaporated with toluene (solution started to turn orange) before being redissolved in DCM (adding slight amount of sat. $NaHCO_3$ made it turn yellow again). The organic layer was washed with sat. $NaHCO_3$ and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 2:0:98 to 2:98:0 TEA:EtOAc:PE eluent resulting in 0.726g (0.943 mmol, 66.1%) of Compound **20** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K) *δ:* 12.04 (s, 1H, NH), 8.04 (s, 1H, NH), 7.86 (s, 1H, H^8), 7.56 (d, *J* = 7.0 Hz, 1H, H^{ortho}, DMTr), 7.41 (dd, *J* = 8.8, 7.5 Hz, 4H, H^{arom}, DMTr), 7.30 – 7.10 (m, 3H, H^{meta}, H^{para}, DMTr), 7.26 ($CHCl_3$), 6.79 (t, *J* = 8.9 Hz, 4H, H^{arom}, DMTr), 5.76 (d, *J* = 7.3 Hz, 1H, $H^{1'}$), 5.21 (dd, *J* = 7.3, 5.3 Hz, 1H, $H^{2'}$), 4.34 (dd, *J* = 5.2, 1.2 Hz, 1H, $H^{3'}$), 4.23 (s, 1H, $H^{4'}$), 3.76, 3.74 (2x s, 6H, OMe, DMTr), 3.56 (dd, *J* = 10.7, 1.6 Hz, 1H, $H^{5'a}$), 3.07 (dd, *J* = 10.8, 2.7 Hz, 1H, $H^{5'b}$), 2.91 (s, 1H, $OH^{3'}$), 1.53 (hept, *J* = 6.7 Hz, 1H, CH, iBu), 0.83 (d, *J* = 7.0 Hz, 3H, CH_3, iBu), 0.81 (s, 9H, 2'-O-Si-tBu), 0.60 (d, *J* = 6.9 Hz, 3H, CH_3, iBu), 0.01, -0.19 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K) *δ:* 178.75 (CO, iBu), 158.83 (C_q, DMTr), 155.57 (C^6), 148.40 (C^2), 147.33 (C^4), 145.22 (C_q, DMTr), 139.21 (C^8), 136.19, 135.65 (2x C_q, DMTr), 130.06, 128.17, 128.02, 127.26 (4x CH^{arom}, DMTr), 122.52 (C^5), 113.38 (CH^{arom}, DMTr), 88.23 ($C^{1'}$), 86.26 (C_q, DMTr), 84.59 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.47 ($C^{2'}$), 71.28 ($C^{3'}$), 63.78 ($C^{5'}$), 55.33 (CH_3, OMe, DMTr), 35.93 (CH, iBu), 25.59 (CH_3, 2'-O-Si-tBu), 18.52, 18.48 (2x CH_3, iBu), 17.93 (C_q, Si-tBu), -5.00, -5.04 (2x CH_3, 2'-O-Si-Me_2).

IR: 3368, 3157, 3057, 3036, 2949, 2928, 2855, 2835, 1674, 1605, 1508, 1248, 1175, 1142, 1096, 1034, 781, 831, 702.

HRMS: $[C_{41}H_{51}N_5O_8Si+H]^+$: found 770.3583, calculated 770.358

5'-DMTr-3'-Lev-2'-TBDMS- Guanosine(iBu) (24)

0.858 mmol (0.661g) of Compound **20** was dissolved in 5mL of dry DCM, a catalytic amount of 4-dimethylaminopyridine, 1.5 mmol (0.15mL, 0.17g) of Levulinic acid and 1.1 mmol (0.18mL, 0.15g) of

Diisopropylcarbodiimide were added respectively and the mixture was stirred overnight. Reaction completion was checked by normal TLC (Rf around 0.80 in 100% EtOAc). The organic layer was washed with sat. $NaHCO_3$ dried using $MgSO_4$ and concentrated using rotary evaporation. The mixture was redissolved in THF, centrifuged and the solution was collected. Purification was performed with neutralized silica column chromatography using an 1:30:79 to 1:99:0 TEA:EtOAc:PE eluent resulting in 0.586g (0.675 mmol, 78.6%) of Compound **24** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K) δ: 11.94 (s, 1H, NH), 7.79 (s, 1H, H^8), 7.58 (d, *J* = 7.0 Hz, 2H, $H^{arom,\ ortho}$, DMTr), 7.42 (t, *J* = 8.6 Hz, 4H, H^{ortho}, DMTr), 7.30 – 7.10 (m, 3H, H^{meta}, H^{para}, DMTr), 6.78 (dd, *J* = 11.8, 8.9 Hz, 4H, H^{arom}, DMTr), 5.69 (d, *J* = 7.7 Hz, 1H, $H^{1'}$), 5.50 (dd, *J* = 5.4, 1.5 Hz, 1H, $H^{3'}$), 5.33 (dd, *J* = 7.6, 5.5 Hz, 1H, $H^{2'}$), 4.19 (d, *J* = 1.3 Hz, 1H, $H^{4'}$), 3.76, 3.74 (2x s, 6H, OMe, DMTr), 3.57 (dd, *J* = 10.7, 1.5 Hz, 1H, $H^{5'a}$), 3.06 (dd, *J* = 10.8, 2.4 Hz, 1H, $H^{5'b}$), 2.9 – 2.50 (m, 4H, R^1-C$\underline{H_2}$C$\underline{H_2}$-R^2, Lev), 2.17 (s, 3H, CH_3, Lev), 1.31 (m, 1H, CH, iBu), 0.80 (d, *J* = 6.8 Hz, 3H, CH_3, iBu), 0.74 (s, 9H, 2'-O-Si-tBu), 0.55 (d, *J* = 6.9 Hz, 3H, CH_3, iBu), 0.02, -0.23 (2x s, 6H, 2'-O-SiMe_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K) δ: 206.40 (CO, Lev), 178.40 (CO, iBu), 171.72 (CO, Lev), 158.86 (C_q, DMTr), 155.53 (C^6), 148.25 (C^2), 147.08 (C^4), 145.30 (C_q, DMTr), 139.37 (C^8), 136.19, 135.54 (2x C_q, DMTr), 130.10, 128.20, 128.00, 127.30 (4x CH^{arom}, DMTr), 122.79 (C^5), 113.41 (CH^{arom}, DMTr), 88.45 ($C^{1'}$), 86.38 (C_q, DMTr), 82.51 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 72.76 ($C^{2'}$), 72.68 ($C^{3'}$), 63.41 ($C^{5'}$), 55.33 (CH_3, OMe, DMTr), 37.76 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 35.99 (CH, iBu), 29.94 (CH_3, Lev), 27.86 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.46 (CH_3, 2'-O-Si-tBu), 18.44, 18.43 (2x CH_3, iBu), 17.82 (C_q, Si-tBu), -5.00, -5.29 (2x CH_3, 2'-O-Si-Me_2

IR: 3150, 3059, 3036, 2949, 2928, 2887, 2857, 1674, 1605, 1508, 1250, 1175, 1152, 1090, 1032, 833, 781, 702. **HRMS:** $[C_{46}H_{57}N_5O_{10}Si+H]^+$: found 868.3953, calculated 868.3947

5'-OH-3'-Lev-2'-TBDMS- Guanosine(iBu) (28)

0.635 mmol (0.551g) of Compound **24** was dissolved in 5mL DCM/MeOH (7:3 v:v), 6.15 mmol (1.17g, monohydrate) of diluted *p*-toluenesulfonic acid (7.80 wt% in DCM/MeOH (7:3 v:v))* was added at 0 °C and the mixture was stirred for 10 minutes. Reaction completion was checked by TLC (Rf around 0.30 in 100% EtOAc). Finally the mixture was quenched using sat. $NaHCO_3$. The mixture was partitioned and the organic layer was washed with Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 50:50:0 (via 100:0:0) to 95:0:5 EtOAc:DCM:MeOH eluent resulting in 0.342g (0.604 mmol, 95.0%) of compound **28** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K) δ: 12.26 (s, 1H, NH), 9.22 (s, 1H, NH), 7.78 (s, 1H, H^8), 7.26 ($CHCl_3$), 5.66 (d, *J* = 7.3 Hz, 1H, $H^{1'}$), 5.42 (dd, *J* = 5.4, 0.8 Hz, 1H, $H^{3'}$), 5.21 (d, *J* = 8.2 Hz, 1H, $OH^{5'}$), 4.89 (dd, *J* = 7.1, 5.5 Hz, 1H, $H^{2'}$), 4.24 (s, 1H, $H^{4'}$), 3.93 (dd, *J* = 12.3, 1.8 Hz, 1H, $H^{5'a}$), 3.82 – 3.71 (m, 1H, $H^{5'b}$), 2.90 – 2.50 (m, 6H, R^1-C$\underline{H_2}$C$\underline{H_2}$-R^2, Lev, CH, iBu), 2.19 (s, 3H, CH_3, Lev), 1.24, 1.23 (2x d, *J* = 3.0, 2.8 Hz, 6H, CH_3, iBu), 0.69 (s, 9H, 2'-O-Si-tBu), -0.14, -0.36 (2x s, 6H, 2'-O-Si-Me_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K) δ: 206.52 (CO, Lev), 179.27 (CO, iBu), 171.94 (CO, Lev), 155.33 (C^6), 147.98 (C^2), 147.32 (C^4), 139.26 (C^8), 122.70 (C^5), 90.35 ($C^{1'}$), 84.69 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 73.84 ($C^{3'}$), 73.30 ($C^{2'}$), 62.54 ($C^{5'}$), 37.86 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 36.36 (CH, iBu), 29.94 (CH_3, Lev), 27.89 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.43 (CH_3, 2'-O-Si-tBu), 19.16, 18.91 (2x CH_3, iBu), 17.81 (C_q, Si-tBu), -5.25, -5.60 (2x CH_3, 2'-O-Si-Me_2).

IR: 3159, 2930, 2886, 2857, 1674, 1603, 1557, 1402, 1252, 1153, 1096, 837, 779.

HRMS: $[C_{25}H_{39}N_5O_8Si+H]^+$: found 566.2637, calculated 566.2641

**The solution was prepared using 6.15 mmol (1.17g) p-toluenesulfonic acid monohydrate in 15mL DCM/MeOH (7:3 v:v), resulting in a 7.80 wt% solution.*

5'-PAM(CNE)-3'-Lev-2'-TBDMS- Guanosine(iBu) (32)

0.604 mmol (0.342g) of compound **28** was dissolved in 6mL of dry DCM, 1.1 mmol (0.11g, 0.15mL) of triethylamine and 0.72 mmol (0.17g, 0.16mL) of 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite were added respectively and the mixture was stirred for 10 minutes. Reaction completion was checked by normal TLC (Rf around 0.45 in 100% EtOAc). Finally the mixture was quenched using aqueous 5wt% $NaHCO_3$. The mixture was partitioned and the organic layer was washed with Brine and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 1:20:79 to 1:99:0 TEA:EtOAc:Hex eluent resulting in 0.360g (0.469 mmol, 77.7%) of Compound **32** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K) δ: 12.06 (br, 1H, NH), 8.90 (br, 1H, NH), 8.05, 8.00 (s+s, 1H, H^8), 7.26 ($CHCl_3$), 5.77 (d, *J* = 6.6 Hz, 1H), 5.44 – 5.38 (m, 1H, $H^{3'}$), 4.89, 4.69 (dd+t, *J* = 6.8, 5.3 + 5.8 Hz, 1H, $H^{2'}$), 4.32 – 4.25 (m, 1H, $H^{4'}$), 4.00 – 3.75 (m, 4H, $H^{5'}$, NC-CH_2-CH_2-OR), 3.67 – 3.54 (m, 2H, CH, iPr_2NR), 2.90 – 2.50 (m, 7H, R^1-CH_2CH_2-R^2, Lev, NC-CH_2-CH_2-OR, CH, iBu), 2.21 (s, 3H, CH_3, Lev), 1.29 – 1.09 (m, 18H, CH_3, iPr_2NR, CH_3, iBu), 0.73, 0.71 (s+s, 9H, CH_3, 2'-O-Si-tBu), -0.07, -0.08 (s+s, 3H, 2'-O-Si-Me), -0.28 (s, 3H, 2'-O-Si-Me).
^{13}C NMR (101 MHz, $CDCl_3$, 297.3K) δ: 206.65, 206.54 (CO, Lev), 178.77, 178.72 (CO, iBu), 171.97, 171.90 (CO, Lev), 155.72, 155.67 (C^6), 148.61, 148.48 (C^2), 147.72, 147.69 (C^4), 138.00 (C^8), 121.72, 121.66 (C^5), 117.85, 117.77 (CN), 88.01, 87.85 ($C^{1'}$), 82.57, 82.52, 82.48, 82.43 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.97, 74.46 ($C^{2'}$), 73.95, 73.12 ($C^{3'}$), 63.64, 63.47, 63.29, 63.14 ($C^{5'}$), 58.56, 58.44, 58.35, 58.22 (NC-CH_2-CH_2-OR), 43.48, 43.35, 43.30, 43.17 (2x CH, iPr_2NR), 37.89 (R^1-C$H_2$$CH_2$-$R^2$, Lev), 36.44, 36.41 (CH, iBu), 30.00 (CH_3, Lev), 27.86 (R^1-CH_2CH_2-R^2, Lev), 25.44 (CH_3, 2'-O-Si-tBu), 24.96, 24.89, 24.86, 24.84, 24.77 (4x CH_3, iPr_2NR), 20.67, 20.59, 20.57, 20.50 (NC-CH_2-CH_2-OR), 19.19, 19.17, 18.98, 18.95 (2x CH_3, iBu), 17.87, 17.86 (C_q, Si-tBu), -5.11, -5.21, -5.40, -5.42 (2x CH_3, 2'-O-Si-Me_2).
^{31}P NMR (162 MHz, $CDCl_3$) δ: 149.06, 148.98.
IR: 3078, 2965, 2930, 2886, 2857, 1719, 1667, 1624, 1557, 1493, 1364, 1310, 1231, 1180, 1155, 1117, 1043, 999, 978, 829, 779, 731.
HRMS: $[C_{34}H_{56}N_7O_9PSi+H]^+$: found 766.3719, calculated 766.3719

Compound 35: **HO-HMPB-HCP**

210-263 µmol (1.051 g) of Amino HCP resin (200-250 µmol/g) was put in a 20mL filter-syringe and swelled in 4 mL of dry DMF. Then a mixture of 649 µmol (156mg) of HMPB-linker, 659 µmol (343 mg) of PyBOP and 799 µmol (108mg) of HOBt dissolved in 6 mL of dry DMF was added followed by 1.44 mmol (186mg, 0.250mL) of DIPEA and the mixture was shaken for 5 hours at room temperature. The solution was drained from the resin and the resin was washed two times with DMF and three times with DCM.

Compound 36: **Fmoc-Gly-HMPB-HCP**

210-263 µmol of HO-HMPB-HCP resin **35** was put in a 10mL filter-syringe and swelled in 10 mL of dry DCM. Then 1.05 mmol (313mg) of Fmoc-Gly-OH, 1.29 mmol (163mg, 0.200mL) DIC and a catalytic amount (4 flakes) of DMAP were added and the mixture was shaken for one night at room temperature. The solution was drained from the resin and the resin was washed two times with DCM, three times with DMF and three times with DCM yielding 1.228g (95.6% via mass analysis) of Fmoc-Gly-HMPB-HCP resin **36**, with an estimated load (via mass analysis) of 239 µmol/g. **Analysis**:***Mass-based***: The dry resin appeared to have a mass of 1.228 g, which is an increase of 177 mg. The increase of resin should ideally result in a load of 294 µmol on 1.228 g, which is a load of 239 µmol/g. ***Fmoc-based UV/Vis***: 2.26 mg of Fmoc-Gly-HMPB-HCP resin **36** was taken and dissolved in 1mL of Piperidine/DMF (1:4, v:v). The mixture was left for 30 minutes at room temperature before adding 9 mL of MeOH. The resulting solution was filtrated to remove resin particles possibly interfering with the measurement. Three measurements were performed at 300nm;1,2: Sample vs Solution Sample(piperidine/DMF/MeOH (1:4:45)) resulting in A values of 0.548 and 0.552;3: Sample vs Sample (in reference cuvette) resulting in an A value of -0.005. The correct A value should be approximately (0.548 + 0.552) / 2 – (-0.005) = 0.555. To obtain the load from this A value we used the formula:

$$\mathbf{L}_{(mmol/g)} = (\mathbf{A} * \mathbf{Volume}_{(mL)}) / (\mathbf{C}_{(fmoc\ =\ 7800)} * \mathbf{Mass}_{(g)})$$

Using this formula we obtained a load of $\mathbf{L}_{(mmol/g)}$ = (0.555 * 10 mL) / (7.8 * 2.26 mg) = 0.315 mmol/g.

Compound 37: **H-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP**

98.9 µmol (414 mg) of Fmoc-Gly-HMPB-HCP resin **36** (239 µmol/g) elongated using Solid Phase Peptide Synthesis (SPPS) yielding 97.9 µmol (464 mg) of H-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP resin **37** (211 µmol/g). **Analysis**: **H-Ala-Tyr-Thr-Gly-OH** 2.5 µmol (10 mg) of H-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP resin **37** was taken and put in a 2mL filter syringe. 300 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 5 mL of cold Et_2O, followed by 300 µL 3%TFA/DCM and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitatewas observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 90 → 40% ; ACN: 0 → 50% ; aq. 0.5% TFA: 10%) **LC:**

R_t4.94 min **MS:**$[C_{18}H_{26}N_4O_7+H]^+$: found 411.07, calculated 411.19. $[2C_{18}H_{26}N_4O_7+H]^+$: found 821.13, calculated 821.37. $[3C_{18}H_{26}N_4O_7+H]^+$: found 1230.93, calculated 1231.55.

SPPS Prodcedure: Solid Phase Peptide Synthesis was performed using Fmoc-based strategy, with each cycle containing an *Fmoc deprotection*, *amino acid coupling* and *capping* step. Amino acids used: Fmoc-Thr(OTrt)-OH, Fmoc-Tyr-OH and Fmoc-Ala-OH. After each step the resin washed multiple times with NMP. An extra Fmoc deprotection cycle was introduced at the end to remove the final Fmoc protective group. As a final step the resin was washed multiple times with DCM and dried in open air. **Fmoc deprotection**: Four consecutive times 8mL of a solution of 20% Piperidine in NMP per gram resin (≈200 µmol) wes added to the resin and shaken for 4 minutes each. **Amino acid coupling**: 5 equivalents of AA were preactivated using 5 equivalents of HCTU (0.25M in NMP), 10 equivalents of DiPEA (1.0M in NMP) and NMP (4 mL / gram resin (≈200 µmol)) before being added to the resin and shaken for 1 hour. **Capping**: 20 equivalents of Ac_2O (0.5M in NMP) and 10 equivalents of DiPEA (1.0M in NMP) were added to the resin and shaken for 3 minutes.

Compound 38: Bpoc-Gly-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP

180 µmol (853 mg) of H-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCPresin **37** (211 µmol/g) was taken and put in a 20mL filter syringe and 374 µmol (185 mg, DHCA salt) Bpoc-Gly-OH was added as a dry powder. 375 µmol (195 mg) of PyBOP and 737 µmol (74.5 mg, 81.0 µl) of NMM as a solution in 10 ml dry DMF were added and the mixture was shaken for 6 hours at room temperature. The solution was drained from the resin and the resin was washed three times with DMF and three times with DCM yielding 166 µmol (833 mg of Bpoc-Gly-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP resin **38** (199 µmol/g).

Analysis:**H-Gly-Ala-Tyr-Thr-Gly-OH** 2.0 µmol (10 mg) of Bpoc-Gly-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP resin **38** was taken and put in a 2mL filter syringe. 300 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 5 mL of cold Et_2O, followed by 300 µL 3%TFA/DCM and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. **LC-MS analysis:**(Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 90 → 40% ; ACN: 0 → 50% ; aq. 0.5% TFA: 10%)**LC:**R_t4.41 min **MS:**$[C_{20}H_{29}N_5O_8+H]^+$: found 468.13, calculated 468.21. $[2C_{20}H_{29}N_5O_8+H]^+$: found 935.13, calculated 935.41. $[3C_{20}H_{29}N_5O_8+H]^+$: found 1402.07, calculated 1402.61. $[4C_{20}H_{29}N_5O_8+H]^+$: found 1870.13, calculated 1869.81.

Testing procedure: Reagents and conditions 5.3 µmol (25 mg) of H-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP resin **37** was taken and put in a 2 mL filter syringe and **a** equivalents of Bpoc-Gly-OH were added as a dry powder. Then **b** equivalents of **B** and **c** equivalents of **C** as a 1 mL solution in **d** were added and the mixture was shaken for **e** hours at room temperature. The solution was drained from the resin and the resin was washed three times with DMF and three times with DCM.

	a (eq)	B	b(eq)	C	c(eq)	d	e	37	38	39	40
1	2	PyBOP	2	DIPEA	4	DMF	24h	-	+	+	-
2	2	PyBOP	2	DIPEA	4	DCM	24h	+	+	+	-
3	1,5	PyBOP	1,5	DIPEA	3	DMF	24h	-	+	-	-
4	2	DIC	2	DIPEA	4	DCM	24h	-	-	-	-
5	2	HBTU	2	DIPEA	4	DMF	24h	-	+	+	+
6	2	PyBOP	2	DIPEA	4	DMF	1h	+	+	-	-
7	2	PyBOP	2	DIPEA	4	DMF	4h	-	+	+	-
8	2	PyBOP	2	DIPEA	4	DMF	8h	-	+	+	-
9	2	PyBOP	2	NMM	4	DMF	4h	-	+	-	-
10	2	HBTU	2	NMM	4	DMF	4h	-	+	-	-

Compound 41: Bpoc-Gly-Ala-Tyr(OpUlev)-Thr(Otrt)-Gly-HMPB-HCP

49.8 µmol (250 mg) ofBpoc-Gly-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP resin **38** (199 µmol/g) was taken and put in a 10 mL filter syringe. ACN was added and the resin was allowed to swell while being shaken for 10

minutes. The solvent was drained and the resin was washed three times with ACN. Then 346 µmol (227 mg) of uridine **34** (4.0 mL of a 0.087 M solution in ACN/Dioxane (1:1, v:v)) and 1.2 mmol (0.23 g) of BMT (4.0 mL of a 0.30M solution in ACN) were added and the mixture was shaken for 30 minutes at room temperature. The solution was drained from the resin and the resin was washed three times with ACN. Then two consecutive times 375 µmol of I_2 (7.5 mL of a 0.05M solution in THF/Pyr/H_2O (7:2:1, v:v:v)) was added and the mixture was shaken for 60 seconds at room temperature before draining the solution from the resin. The resin was washed three times with ACN and three times with DCM yielding 49.8 µmol of Bpoc-Gly-Ala-Tyr(OpUlev)-Thr(OTrt)-Gly-HMPB-HCP resin **41**.

Analysis: **H-Gly-Ala-Tyr(OpUlev)-Thr-Gly-OH** 10 mg (≈2.0 µmol) of Bpoc-Gly-Ala-Tyr(OpUlev)-Thr(OTrt)-Gly-HMPB-HCP resin **41** was taken and put in a 2mL filter syringe. 300 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 5 mL of cold Et_2O, followed by 300 µL 3%TFA/DCM and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 90 → 40% ; ACN: 0 → 50% ; aq. 0.5% TFA: 10%) **LC:** R_t8.62 min **MS:** $[C_{43}H_{63}N_8O_{18}PSi+H]^+$: found 1039.40, calculated 1039.38.

Testing procedure: Reagents and conditions 5.0 µmol (25 mg) of Bpoc-Gly-Ala-Tyr(OH)-Thr(Otrt)-Gly-HMPB-HCP resin **38** (199 µmol/g) was taken and put in a 2 mL filter syringe. ACN was added and the resin was allowed to swell while being shaken for 10 minutes. The solvent was drained and the resin was washed three times with ACN. Then **a** equivalents of **A** and **b** equivalents of **B** were added as stock solutions of 0.3M and 0.1M respectively and the mixture was shaken for **c** minutes at room temperature. The solution was drained from the resin and the resin was washed three times with ACN. Then two consecutive times **d** equivalents of **D** were added as stock solution of 0.05M and the mixture was shaken for **e** seconds at room temperature. The solution was drained from the resin and the resin was washed three times with ACN and three times wih DCM.

	A	a(eq)	B	b(eq)	c	38	41
1	34	6	BMT	18	20min	+	++
2	34	6	BMT	18	60min	+	++
3	34	3	BMT	9	60min	++	++
4	34	6	DCI	18	20min	+	++
5	34	7	BMT	24	20min	-	+++
- = not observed, + = minor, ++ = major, +++= single product							

Compound 38-cap: Bpoc-Gly-Ala-Tyr(OAc)-Thr(Otrt)-Gly-HMPB-HCP

2.0 µmol (10 mg) ofBpoc-Gly-Ala-Tyr(OH)-Thr(Otrt)-Gly-HMPB-HCP resin **38** (199 µmol/g) was taken and put in a 2 mL filter syringe. ACN was added and the resin was allowed to swell while being shaken for 10 minutes. The solvent was drained and the resin was washed three times with ACN. Then 190 µmol (19.4mg, 18 µl) of Ac_2O and 48 µmol (6.2 mg, 8.4 µl) of DiPEA (0.5 mL of a freshly made DMF/Ac_2O/DiPEA (500:19:9, v:v:v) solution) were added and the mixture was shaken for 60 minutes at room temperature.The solution was drained from the resin and the resin was washed three times with ACN and three times wih DCM yielding 2.0 µmol of Bpoc-Gly-Ala-Tyr(OAc)-Thr(Otrt)-Gly-HMPB-HCP resin **38-cap**.

Analysis:**H-Gly-Ala-Tyr(OAc)-Thr-Gly-OH**

2.0 µmol of Bpoc-Gly-Ala-Tyr(OAc)-Thr(Otrt)-Gly-HMPB-HCP resin **38-cap** was taken and put in a 2mL filter syringe. 300 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 5 mL of cold Et_2O, followed by 300 µL 3%TFA/DCM and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 90 → 40% ; ACN: 0 → 50% ; aq. 0.5% TFA: 10%) **LC:** R_t6.03 min

MS:$[C_{22}H_{31}N_5O_9+H]^+$: found 510.20, calculated 510.22. $[2C_{22}H_{31}N_5O_9+H]^+$: found 1019.20, calculated 1019.43. $[3C_{22}H_{31}N_5O_9+H]^+$: found 1529.07, calculated 1528.64.

Testing procedure: Reagents and conditions 2.0 µmol (10 mg) of Bpoc-Gly-Ala-Tyr(OH)-Thr(Otrt)-Gly-HMPB-HCP resin **35** (199 umol/g) was taken and put in a 2 mL filter syringe. ACN was added and the resin was allowed to swell while being shaken for 10 minutes. The solvent was drained and the resin was washed three times with ACN. Then **a** equivalents of **A**, **b** equivalents of **B** and **c** equivalents of **C** were added as stock solutions in a volume of **d** in **e** and the mixture was shaken for **f** minutes at room temperature.The solution was drained from the resin and the resin was washed three times with ACN and three times wih DCM.

	B	b(eq)	C	c(eq)	A	a(eq)	d (mL)	e	f	38	38-cap
1	Acetic anhydride	318	Me-Im	376	Lutidine	388	0.6	THF	20min	++	-
2	Acetic anhydride	318	Me-Im	376	Lutidine	388	0.6	THF	60min	++	-
3	Acetic anhydride	318	Me-Im	376	Lutidine	388	0.6	THF	120min	++	-
4	Acetic anhydride	80			DIPEA	24	0.5	DMF	10min	+	+
5	Acetic anhydride	80			DIPEA	24	0.5	DMF	30min	+	+
6	Acetic anhydride	80			DIPEA	24	0.5	DMF	60min	+	+
7	Acetic anhydride	80	DMAP	.cat	DIPEA	24	0.5	DMF	30min	-	++
8	Acetyl Chloride	20			DIPEA	48	0.5	DMF	30min	-	++
	- = not observed, + = observed, ++ = single product										

Compound 4: H-Gly-Ala-Tyr(OpU)-Thr-Gly-OH

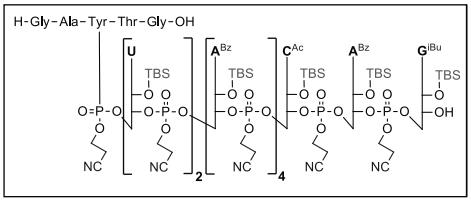
25 mg (≈ 5.0 µmol)ofBpoc-Gly-Ala-Tyr(OpUlev)-Thr(OTrt)-Gly-HMPB-HCP resin **41** was taken and put in a 2 mL filter syringe. ACN was added and the resin was allowed to swell while being shaken for 20 minutes. The solvent was drained and the resin was washed three times with ACN. Then 375 µmol of hydrazine (1.5 mL of a 0.25 M solution in THF/Pyr/AcOH (5:3:2, v:v:v)) was added and the mixture was shaken for 20 minutes at room temperature. The solution was drained from the resin and the resin was washed three times with ACN and three times with DCM. **Analysis**: Around 5.0 µmol Bpoc-Gly-Ala-Tyr(OpU)-Thr(OTrt)-Gly-HMPB-HCP resin **41** was taken and put in a 2mL filter syringe. 300 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 5 mL of cold Et_2O, followed by 300 µL 3% TFA/DCM and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. The 800 µL of product containing solution and remaining LC-MS solution were combined and transfered to an eppendorf. The eppendorf was cooled using liquid nitrogen for 2 minutes and freezedried overnight resulting in 1.18 mg (1.25 µmol, 25%) of Compound **4** as a white fluffy solid. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80→ 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:**R_t5.36 min **MS:**$[C_{38}H_{57}N_8O_{16}PSi+H]^+$: found 941.33, calculated 941.35. $[2C_{38}H_{57}N_8O_{16}PSi+H]^+$: found 1882.20, calculated 1881.69.

Compound 5: H-Gly-Ala-Tyr(OpUpU)-Thr-Gly-OH

25 mg (≈ 5.0 µmol)ofBpoc-Gly-Ala-Tyr(OpUlev)-Thr(OTrt)-Gly-HMPB-HCP resin **41** was taken and put in a 2 mL filter syringe. ACN was added and the resin was allowed to swell while being shaken for 20 minutes. The solvent was drained and the resin was washed three times with ACN. Then 375 µmol of hydrazine (1.5

mL of a 0.25M solution in THF/Pyr/AcOH (5:3:2, v:v:v)) was added and the mixture was shaken for 20 minutes at room temperature. The solution was drained from the resin and the resin was washed three times with ACN. Then 79 µmol (52 mg) of uridine **34** (0.60 mL of a 0.13 M solution in ACN/Dioxane (1:1, v:v)) and 90 µmol (15 mg) of BMT (0.30 mL of a 0.30M solution in ACN) were added and the mixture was shaken for 20 minutes at room temperature. The solution was drained from the resin and the resin was washed four times with ACN. Then two consecutive times 75 µmol of I_2 (1.5 mL of a 0.05 M solution in THF/Pyr/H_2O (7:2:1, v:v:v)) was added and the mixture was shaken for 60 seconds at room temperature before draining the solution from the resin. The resin was washed three times with ACN and three times with DCM.Then 375 µmol of hydrazine (1.5 mL of a 0.25 M solution in THF/Pyr/AcOH (5:3:2, v:v:v)) was added and the mixture was shaken for 20 minutes at room temperature. The solution was drained from the resin and the resin was washed three times with ACN and three times with DCM yielding a mixture of Bpoc-Gly-Ala-Tyr(OpU)-Thr(OTrt)-Gly-HMPB-HCP resin and Bpoc-Gly-Ala-Tyr(OpUpU)-Thr(OTrt)-Gly-HMPB-HCP resin.

Analysis: The mixture of Bpoc-Gly-Ala-Tyr(OpU)-Thr(OTrt)-Gly-HMPB-HCP resin and Bpoc-Gly-Ala-Tyr(OpUpU)-Thr(OTrt)-Gly-HMPB-HCP resin was taken and put in a 2mL filter syringe. 300 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 5 mL of cold Et_2O, followed by 300 µL 3%TFA/DCM and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. The 800 uL product containing solution and remaining LC-MS solution were combined and transfered to an eppendorf. The eppendorf was cooled using liquid nitrogen for 2 minutes and freezedried overnight yielding 3.48 mg (estimated 2.0 - 2.5 µmol, 40% - 50%) of a mixture of Compound **4** and Compound **5** as a white fluffy solid.**LC-MS analysis:**(Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 90→ 40% ; ACN: 0 → 50% ; aq. 0.5% TFA: 10%) Compound **4**: **LC:**R_t8.12 min **MS:** $[C_{38}H_{57}N_8O_{16}PSi+H]^+$: found 941.27, calculated 941.35. $[2C_{38}H_{57}N_8O_{16}PSi+H]^+$: found 1882.07, calculated 1881.69.Compound **5**: **LC:**R_t9.74 min **MS:**$[C_{56}H_{85}N_{11}O_{24}P_2Si_2+H]^+$: found 1414.40, calculated 1414.49.



Compound 6: H-Gly-Ala-Tyr(OpUpUpApApApApCpApG)-Thr-Gly-OH

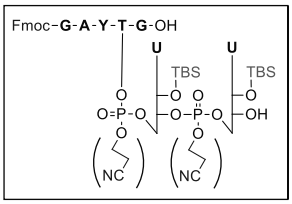
50 mg (≈10 µmol) of Bpoc-Gly-Ala-Tyr(OpUlev)-Thr(Otrt)-Gly-HMPB-HCPresin **41** was loaded in the DNA/RNA-synthesizer and elongated in 8 cycles to a 5'-pUpUpApApApApCpApG-3' sequence, with each cycle containing a *Levulinyl deprotection step*, *nucleotide coupling step, oxidation step*, and *capping step*. Nucleotides used: uridine **34** ,adenosine **31**, cytidine **33** and guanosine**32**. After each step the resin washed multiple times with ACN. An extra levulinyl deprotection cycle was introduced at the end to remove the final Levulinyl protective group. As a final step the resin was washed multiple times with DCM and dried in open air.

Sequence no.	**a** (times)	**b**	**B** (solvent)
1	2	PAM-U	ACN/ Dioxane (1:1, v:v)
2, 3, 4, 5, 7	2	PAM-A	Dioxane
6	3	PAM-C	ACN/ Dioxane (1:1, v:v)
8	3	PAM-G	ACN/ Dioxane (1:1, v:v)

Nucleotide coupling: **a** consecutive times the resin was brought in contact for 10 minutes with 30 µmol of nucleotide **b** (0.3 mL of a 0.1M solution in **B**)and 90 µmol of BMT activator (0.3 mL of a 0.3M solution in ACN). **Oxidation:**Three consecutive times the resin was brought in contact for 30 seconds with 50 µmol of I_2 (1.0 mL of a 0.05 M solution in THF/Pyr/H_2O (7:2:1, v:v:v)). **Capping**: Two consecutive times the resin was brought in contact for 30 seconds with 1.2 mmol N-Methyl-Imidazole (0.5 mL of a 20% solution in THF) and 1 mmol *tert*-butylphenoxyacetic anhydride (or alternatively Ac_2O) + 1.2 mmol lutidine (0.5mL of a 20% + 30% solution in THF).

Analysis: 20 mg (≈4.0µmol) resin was taken and put in a 2mL filter syringe. 600 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 10mL of cold Et_2O/pentane (1:1, v:v), followed by 600 µL 3%TFA/DCM and 1 mL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 5 minutes at -20 °C and centrifuged for 5 minutes.

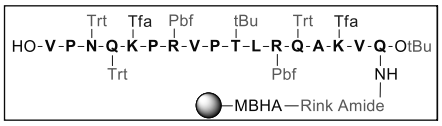
The supernatant was removed and the residue was dissolved in 2 mL of 0.1 M NH_4OAc/ACN (1:1, v:v) (noted the residue is not soluble in either H_2O or ACN, only a mixture of both). 100 µL was taken and diluted with 1mL of H_2O for LC-MS analysis.The 1.9 mL solution and remaining LC-MS solution were combined and transfered to an eppendorf. The eppendorf was cooled using liquid nitrogen for 2 minutes and freezedried overnight yielding 7.5 mg (estimated 1.4µmol, 35%) of a mixture of fragment intermediates and Compound **6** as a white fluffy solid. **LC-MS analysis:** (Gemini 3u C_{18} 110A analytical column, linear gradient in 12.5minutes; H_2O: 40 → 0% ; ACN: 50 → 90% ; aq. 0.1M NH_4OAc: 10%) **LC:** R_t8.4 – 10.6 min. **MS:** $[C_{229}H_{316}N_{51}O_{75}P_9Si_9+H]^{3+}$: m/z found 1839.3, calculated 1838.6



Compound 43: Fmoc-Gly-Ala-Tyr(OpUpU)-Thr-Gly-OH An eppendorf containing 2.8µmol (3.2 mg) of Compound **5*** was taken and 5.5 µmol (1.9 mg) of Fmoc-OSu (250 µL of a freshly made 22 mM solution in DMF) followed by 11.0 µmol (1.42 mg, 1.82 µl) of DiPEA (250 µL of a 44 mM solution in DMF) and the mixture was left to react overnight at room temperature. 5 µL was taken and added to50 µL of H_2O/ACN/tBuOH (1:1:1, v:v:v) for LC-MS analysis. The LC-MS showed succesfull protection of the N-terminus amine, resulting in full conversion of Compound **5** into Compound **43****.**LC-MS analysis:**(Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80→ 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) Compound **44'**: **LC:** R_t6.83 min **MS:** $[C_{50}H_{64}N_7O_{18}PSi+H]^+$: found 1110.33, calculated 1110.39. Compound **44**: **LC:** R_t7.21 min **MS:**$[C_{53}H_{67}N_8O_{18}PSi+H]^+$: found 1163.33, calculated 1163.42. Compound **45'**: **LC:**R_t7.76 min **MS:** $[C_{56}H_{85}N_{11}O_{26}P_2Si_2+H]^+$: found 1583.27, calculated 1583.53.Compound **45**: **LC:** R_t7.88 min **MS:** $[C_{71}H_{95}N_{11}O_{26}P_2Si_2+H]^+$: found 1636.40, calculated 1636.55.

As a mixture of Compound **4 and **5** consisting primarily of **5**. The amount of µmol was determined by mass as if it was solely Compound **5***

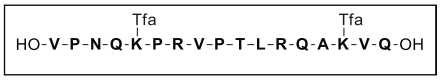
As a mixture of Compound **42'** (= **42** – 1 cyanoethyl), **42**, Compound **43'** (= **43** – 1 cyanoethyl) and Compound **43



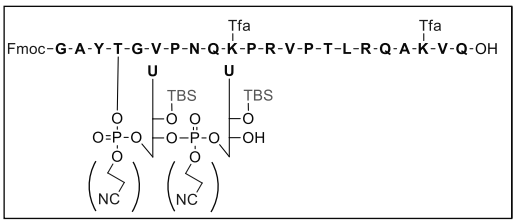
H-Val-Pro-Asn(Trt)-Gln(Trt)-Lys(Tfa)-Pro-Arg(Pbf)-Val-Pro-Thr(tBu)-Leu-Arg(Pbf)-Gln(Trt)-Ala-Lys(Tfa)-Gln(NH-Rink Amide-MBHA-PS)-OtBu 99.8 µmol (128 mg) of H_2N-Rink Amide-MBHA-PS (780 µmol/g) elongated using Solid Phase Peptide Synthesis (SPPS) yielding 89.3 µmol (425 mg, 89.5%) of H-Val-Pro-Asn(Trt)-Gln(Trt)-Lys(Tfa)-Pro-Arg(Pbf)-Val-Pro-Thr(tBu)-Leu-Arg(Pbf)-Gln(Trt)-Ala-Lys(Tfa)-Gln(NH-Rink Amide-MBHA-PS)-OtBu (210 µmol/g).

SPPS procedure: Solid Phase Peptide Synthesis was performed using Fmoc based strategy, with each cycle containing an *Fmoc deprotection*, *amino acid coupling* and *capping* step. Amino acids used: Fmoc-Glu-OtBu, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Thr(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Pro-OH and Fmoc-Lys(Tfa)-OH. After each step the resin washed multiple times with NMP. An extra Fmoc deprotection cycle was introduced at the end to remove the final Fmoc protective group. As a final step the resin was washed multiple times with DCM and dried in open air. **Fmoc deprotection**: Four consecutive times 3.2mL of a solution of 20% Piperidine in NMP per100 mg resin (78 µmol) was added to the resin and shaken for 4 minutes each. **Amino acid coupling**: 5 equivalents of AA were preactivated using 5 equivalents of HCTU (0.25M in NMP), 10 equivalents of DiPEA (1.0M in NMP) and NMP (1.56 mL / 100 mg resin (78 µmol)) before being added to the resin and shaken for 1 hour. **Capping**: 20 equivalents of Ac_2O (0.5M in NMP) and 10 equivalents of DiPEA (1.0M in NMP) were added to the resin and shaken for 3 minutes.

Analysis: 2.1 µmol (10 mg) of peptide resin was taken and put in a 2 mL filter syringe. 500 µL TFA/TIS/H_2O (95/2.5/2.5, v:v:v) was added and left for 30 minutes. The solvent was dropped into at tube containing 10 mL of cold Et_2O, followed by 500 µL TFA/TIS/H_2O (95:2.5:2.5, v:v:v) and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:** R_t4.48 min **MS:** $[C_{90}H_{149}F_6N_{29}O_{25}+2H]^{2+}$: found 1076.60, calculated 1076.57.



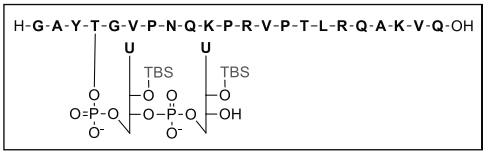
H-Val-Pro-Asn-Gln-Lys(Tfa)-Pro-Arg-Val-Pro-Thr-Leu-Arg-Gln-Ala-Lys(Tfa)-Gln-OH (3) 87.2 µmol (415 mg) of resin bound Compound **5** was taken and put in a 20mL filter syringe. 5.0 mL TFA:TIS:H_2O (95:2.5:2.5, v:v:v) was added and left for 30 minutes. The solvent was dropped into at tube containing 40 mL of cold Et_2O, followed by 5 mL TFA:TIS:H_2O (95:2.5:2.5, v:v:v) which was dropped into a second tube containing 40 mL of cold Et_2O. Crashing out of compound was observed in both tubes, though as expected much more in the first tube. The tubes were cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residues were partially dissolved in 5 mL of H_2O, resulting in a slurry. 100 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. The remaining slurry was stored at -20 °C. **Purification:**HPLC purification was performed by diluting the slurry 1:1 with DMSO allowing the slurry to form a properly dissolved clear solution. The solution was then purified by HPLC and the obtained fractions transferered to 20 mL tubes, cooled using liquid nitrogen for 2 minutes and freezedried overnight yielding Compound **3** as a white fluffy solid.**HPLC**: (linear gradient in15 minutes;H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC**: R_t 11 min. **Pre-HPLC LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80→ 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%)**LC:**R_t4.48 min **MS:**$[C_{90}H_{149}F_6N_{29}O_{25}+2H]^{2+}$: found 1076.60, calculated 1076.57. **Post-HPLC LC-MS analysis:**(Gemini 3u C_{18} 110A analytical column, linear gradient in 12.5minutes; H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.1M NH_4OAc: 10%) **LC:**R_t4.3 min. **MS**:$[C_{90}H_{149}F_6N_{29}O_{25}+H]^{+}$: found 2151.5, calculated 2152.13. $[C_{90}H_{149}F_6N_{29}O_{25}+2H]^{2+}$: found 1076.9, calculated 1076.57.



Compound 45: An eppendorf containing Compound **43*** was taken and 5.69 µmol (2.96 mg) PyBOP, 5.6 µmol (0.76 mg) HOBt (100 µl of a freshly made solution of 57 mM PyBOP and 56 mM HOBtin DMF) and 5.5 µmol (0.76 mg, 0.91 µl) of DiPEA (125 µL of a 44 mM solution in DMF) were added and the mixture was allowed to preactivate for 25 minutes before adding 5.6 µmol (12 mg) of peptide **3** predissolved in 200 µL DMF. The final mixture was left to react overnight at room temperature, but showed no notable difference after 2 hours. 5 µL was taken and added to 50 µL of H_2O/ACN/tBuOH (1:1:1, v:v:v) for LC-MS analysis. The LC-MS analysis showed moderate conversion of Compound **43** into Compound **45****. **Purification:** The resulting mixture was diluted with a mixture of H_2O/ACN/tBuOH (1:1:1, v:v:v) before being injected in the **HPLC.** Fractions containing Compound **45'** and **45** were collected and put together. Finally the product containing solution was concentrated using rotary evaporation. **Pre-HPLC LC-MS analysis:**(Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80→ 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%). Compound **46'**: **LC:** R_t7.12 min **MS:** $[C_{53}H_{67}N_8O_{18}PSi+H]^{+}$: found 1163.40, calculated 1163.42. Compound **46**: **LC:** R_t 7.45 min **MS:** $[C_{56}H_{70}N_9O_{18}PSi+H]^{+}$: found 1216.47, calculated 1216.44. Compound **47'**: **LC:** R_t8.04 min **MS:**$[C_{71}H_{95}N_{11}O_{26}P_2Si_2+H]^{+}$: found 1636.40, calculated 1636.55.Compound **47**: **LC:** R_t8.04 min **MS:**$[C_{71}H_{95}N_{11}O_{26}P_2Si_2+H]^{+}$: found 1689.33, calculated 1689.58.Compound **44'**: **LC:** R_t6.09 min **MS:**$[C_{140}H_{211}F_6N_{36}O_{42}PSi+2H]^{2+}$: found 1622.73, calculated 1622.75. Compound **44**: **LC:** R_t6.26 min **MS:**$[C_{143}H_{243}F_6N_{37}O_{42}PSi+2H]^{2+}$: found 1649.27, calculated 1648.76. Compound **45'**: **LC:** R_t6.64 min **MS:**$[C_{158}H_{239}F_6N_{39}O_{50}P_2Si_2+2H]^{2+}$: found 1859.07, calculated 1859.32.Compound **45**: **LC:** R_t6.70 min **MS:**$[C_{161}H_{242}F_6N_{40}O_{50}P_2Si_2+2H]^{2+}$: found 1885.80, calculated 1885.33.

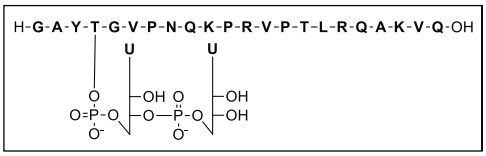
As a mixture of Compound **42, Compound **43'** (= **45** – 1 cyanoethyl) and Compound **43**.*

***As a mixture of Compound **44'** (= **44** – 1 cyanoethyl), **44**, Compound **45'** (= **45** – 1 cyanoethyl) and Compound **45**.*



Compound 48: VPg-pUpU Compound **45***, was dissolved in 0.5 mL of of 30-33% sat. NH_4OH and left for 4 days at room temperature. Finally the mixture concentrated, redissolved in aq. 0.1M NH_4OAc and concentrated again. Finally it was redissolved 1 mL of H_2O and 50 uL was taken for LC-MS analysis showing

full conversion of Compound **45** and **45'** into Compound **48**. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80→ 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) Compound **48**: **LC:** R_t5.09 min **MS:** $[C_{136}H_{228}N_{38}O_{46}P_2Si_2+2H]^{2+}$: found 1625.73, calculated 1625.29. $[C_{136}H_{228}N_{38}O_{46}P_2Si_2+H+F_3CCOOH]^{2+}$: found 1681.73, calculated 1682.29.
As a mixture of compound **45' (= **47** – 1 cyanoethyl) and compound **45**.*



Compound 49: Previously obtained Compound **48** was treated with 3.1 mmol of HF (0.5 mL of TEA/TEA*3HF/DMF (2:3:4, v:v:v)) and the mixture was left overnight at room temperature. Then 2.9 mmol of $NH_4^+HCO_3^-$ (1.5 mL of a 15 wt% solution) was added dropwise with increasing speed. **Purification:** The solution was desalted using ion exchange column chromatography using an aq. NH_4HCO_3 eluent. After LC-MS analysis the obtained fractions were concentrated, redissolved in 1 mL of H_2O/ACN/tBuOH (1:1:1, v:v:v) and freezedried to remove NH_4HCO_3 salt. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80→ 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) Compound **49**: **LC:** R_t5.10 min **MS:** $[C_{124}H_{200}N_{38}O_{46}P_2+2H]^{2+}$: found 1511.53, calculated 1511.20 $[C_{124}H_{200}N_{38}O_{46}P_2+H+F_3CCOOH]^{2+}$: found 1566.87, calculated 1568.20 $[C_{124}H_{200}N_{38}O_{46}P_2+3H]^{3+}$: found 1007.67, calculated 1007.81.

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