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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: Gentil, 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 RNA¹. 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 level⁴⁻⁹. In line with these investigations and to obtain useful molecular tools¹⁰ 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

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.

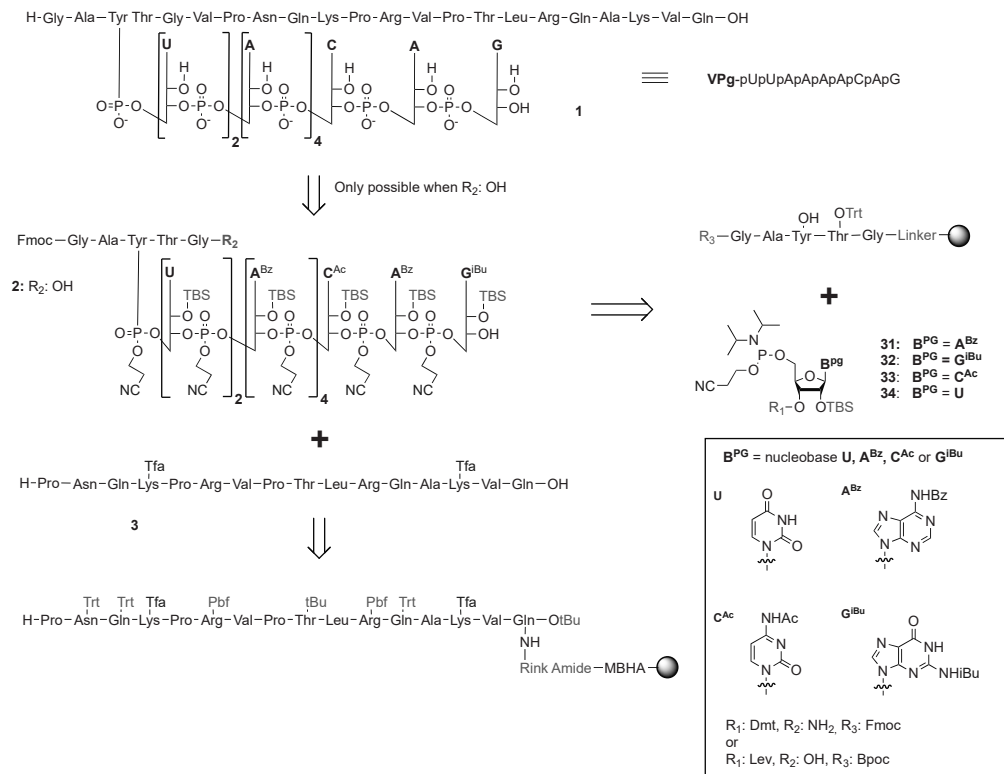


Figure 2. Retrosynthesis of the coxackie VPg using Lev as a temporary protective group.

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.

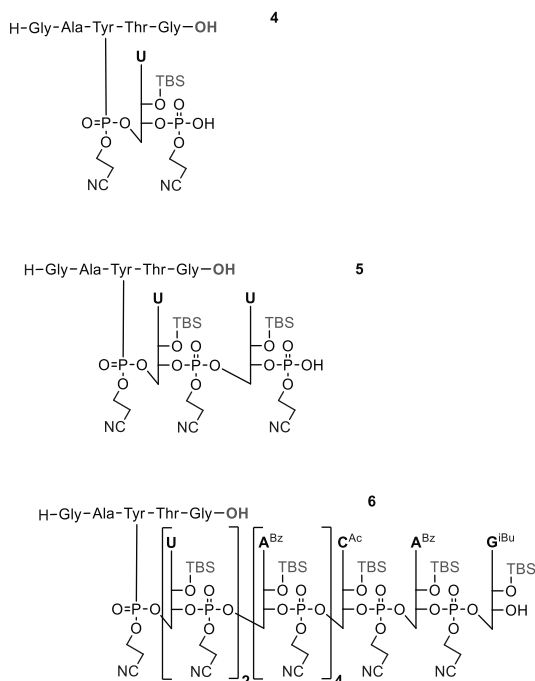
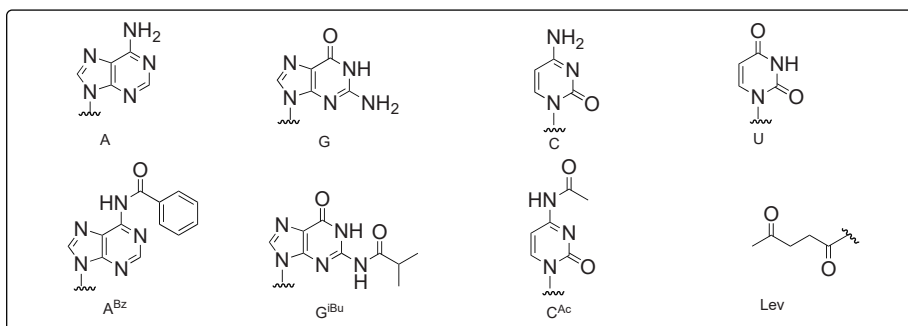
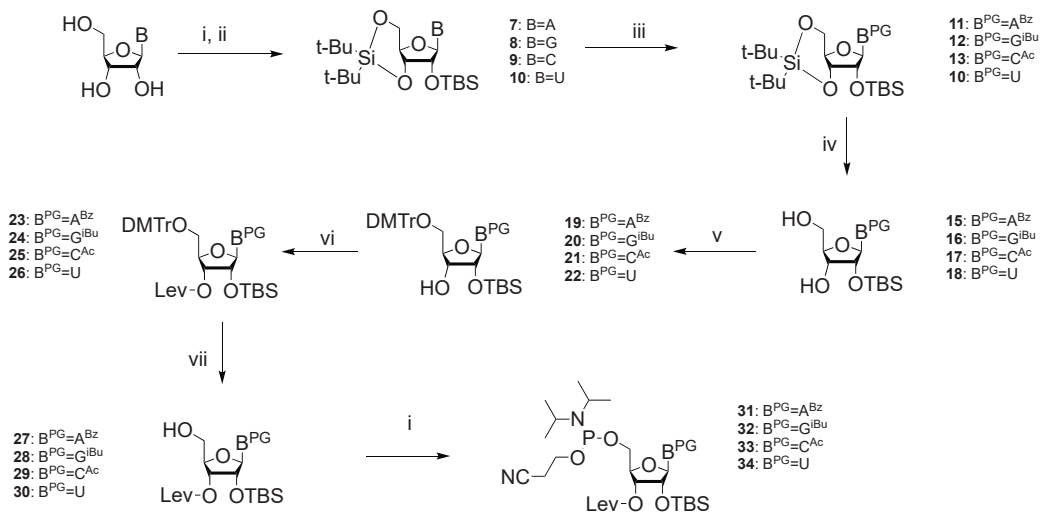


Figure 3. Target compounds. Protected nucleopeptide intermediate towards coxackievirus VPg.

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**. *N*2-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.

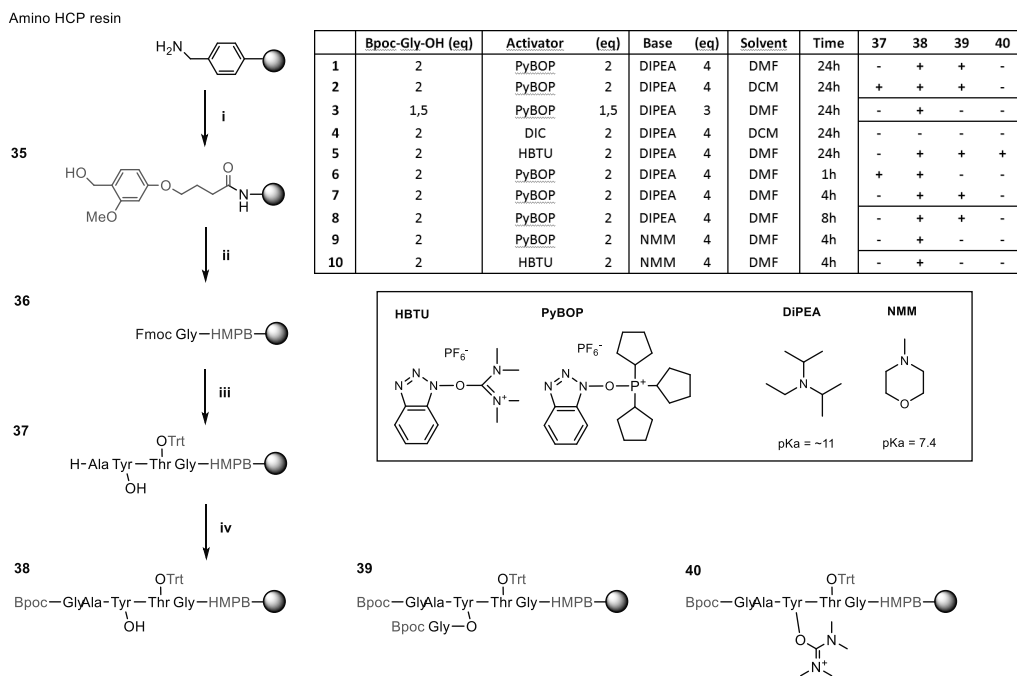


Scheme 1. (i) **7,8,9**: (t-Bu)₂Si(OTf)₂, DMF, 0 °C, 30 min; **10**: (t-Bu)₂SiCl₂, AgNO₃, DMF, 0 °C, 30 min (ii) **7,8,9**: TBS-Cl, Imidazole, 0 °C to rt, o.n.; **10**: TBS-Cl, Pyridine, AgNO₃, 0 °C to rt, 2 hrs, (iii) **11**: Bz-Cl, Pyr/DCM, rt, 3 h, then conc. NH₄OH, -10 °C to rt, o.n. **12**: iBu-Cl, Pyr, -20 °C, 2 h **13**: Ac₂O, 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 DMTr 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.

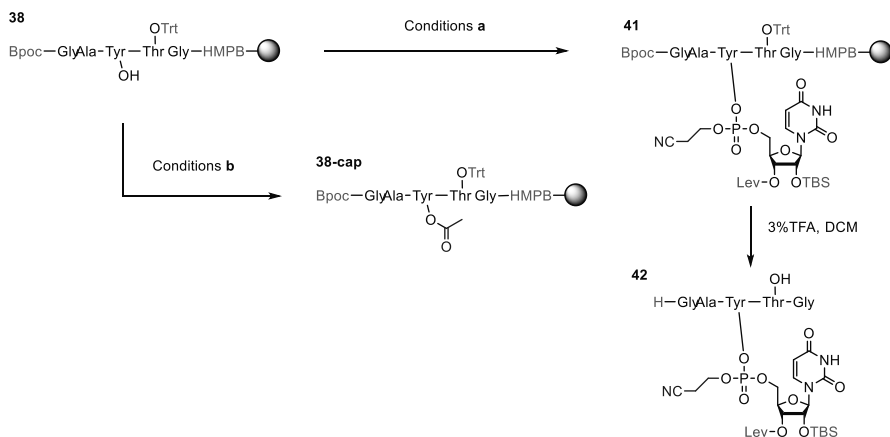
Having the respective protected amino acid and nucleotide building blocks available, attention was directed to assessment of the appropriateness of these building blocks for an efficient automated solid phase synthesis of nucleopeptides.



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) Ac₂O, DIPEA, NMP, rt, 1 min; (iv) table.

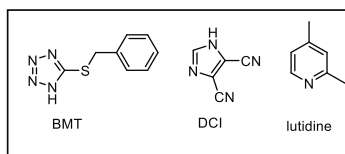
In the first instance the viability of the peptide synthesis was evaluated as the applied Fmoc amino acid building blocks were minimally protected not only to avoid harsh acidic deprotection conditions but also to provide an unprotected tyrosine hydroxyl group in the immobilized oligopeptide for oligonucleotide 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 DIPEA 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



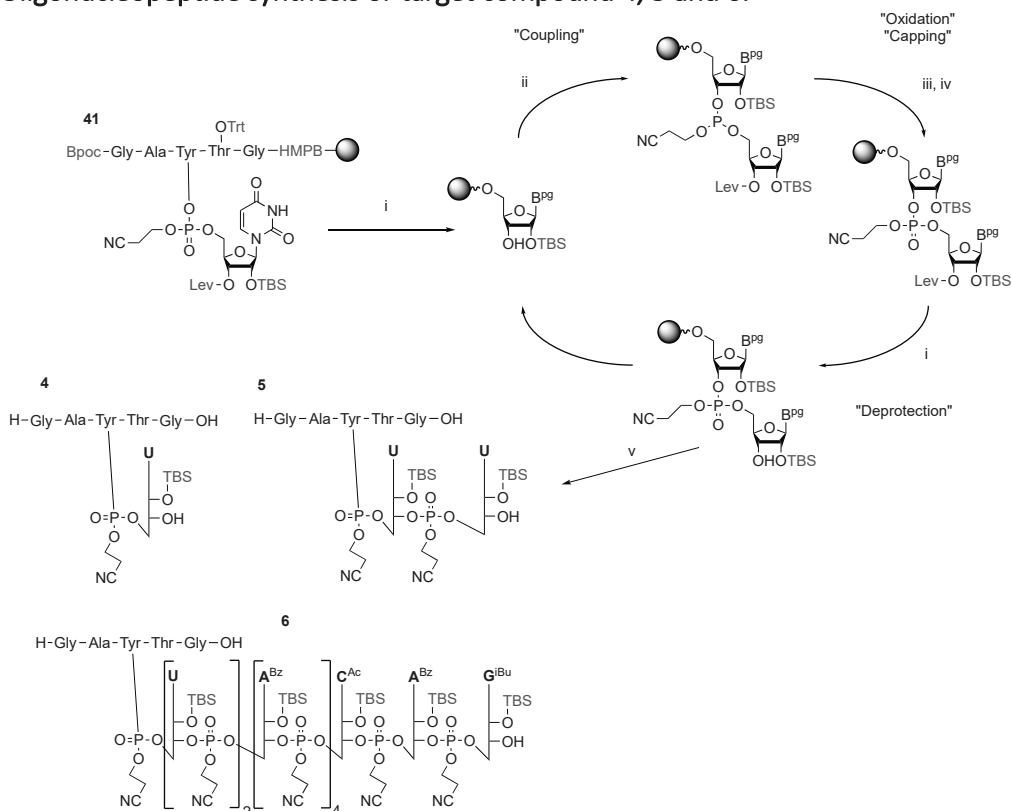
b	reagent (eq)	Activator (eq)	Base (eq)	Solvent	Time	38	38-cap
1	Acetic anhydride 318	Me-Im 376	Lutidine 388	THF	20min	++	-
2	Acetic anhydride 318	Me-Im 376	Lutidine 388	THF	60min	++	-
3	Acetic anhydride 318	Me-Im 376	Lutidine 388	THF	120min	++	-
4	Acetic anhydride 80		DIPEA 24	DMF	10min	+	+
5	Acetic 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 Ac₂O 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.

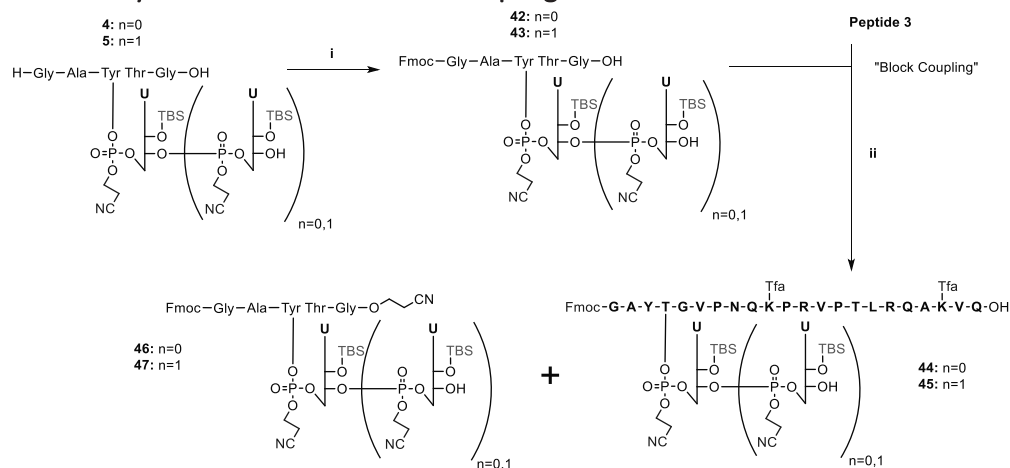
Oligonucleotide synthesis or target compound 4, 5 and 6.



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) I₂, THF/Pyr/H₂O, rt, 1.5 min; (iv) Ac₂O, Melm, 2,6-lutidine, NMP, rt, 30 sec (v) 3% TFA/DCM, rt, 5 min.

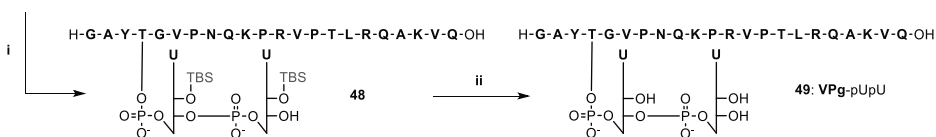
In order to prepare partially protected nucleopeptide **4** (Scheme 4), provided with one uridine moiety, the lev group at the 3' position in immobilized **41** should be removed without harming the integrity of the TBS at the 2' position. After extensive optimization the lev group at the 3' position 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.

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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 treatment. 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 levulinic ester protective group at the 3'-position is described. Application of minimally 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 protected 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 AgNO₃ and 1.4 mmol (0.30 mL, 0.30g) of (t-Bu)₂SiCl₂ 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 AgNO₃ 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. KHSO₄ followed by Brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 9.746 (s, 1H, NH), 7.260 (CHCl₃), 7.257 (d, *J* = 8.0 Hz, 1H, H⁶), 5.751 (dd, *J* = 8.1, 1.3 Hz, 1H, H⁵), 5.653 (s, 1H, H¹), 4.493 (dd, *J* = 9.2, 5.1 Hz, 1H, H^{5a}), 4.288 (d, *J* = 4.6 Hz, 1H, H²), 4.158 (td, *J* = 10.2, 5.1 Hz, 1H, H⁴), 3.968 (t, *J* = 9.4 Hz, 1H, H^{5b}), 3.863 (dd, *J* = 9.6, 4.7 Hz, 1H, H³), 1.040, 1.012 (2x s, 18H, 5'-3'-O-Si-tBu₂), 0.922 (s, 9H, 2'-O-Si-tBu), 0.177, 0.132 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K) δ: 163.70 (CO, C²), 149.91 (CO, C⁴), 139.55 (C⁶), 102.47 (C⁵), 94.07 (C¹), 77.48, 77.16, 76.84 (CHCl₃), 76.14 (C³), 75.43 (C²), 74.63 (C⁴), 67.68 (C⁵), 27.58, 27.07 (2x CH₃, 5'-3'-O-Si-tBu₂), 25.95 (CH₃, 2'-O-Si-tBu), 22.89, 20.45, 18.36 (3x C_q, Si-tBu), -4.19, -4.92 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₂₃H₄₂N₂O₆Si₂+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 H₂O, sat. NaHCO₃ followed by Brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 8.988 (s, 1H, NH), 7.594 (d, *J* = 8.1 Hz, 1H, H⁶), 7.260 (CHCl₃), 5.758 (dd, *J* = 8.1, 1.9 Hz, 1H, H⁵), 5.586 (d, *J* = 5.2 Hz, 1H, H¹), 4.596 (t, *J* = 5.2 Hz, 1H, H²), 4.206 (dd, *J* = 8.4, 4.0 Hz, 1H, H³), 4.145 (dd, *J* = 5.2, 2.2 Hz, 1H, H⁴), 3.956 (d, *J* = 12.1 Hz, 1H, H^{5a}), 3.813 (dd, *J* = 11.9, 4.6 Hz, 1H, H^{5b}), 2.986 (d, *J* = 3.7 Hz, 1H, OH⁵), 2.710 (d, *J* = 4.1 Hz, 1H, OH³), 0.898 (s, 9H, 2'-O-Si-tBu), 0.097, 0.084 (2x s, 6H, 2'-O-SiMe₂). **¹³C NMR (101 MHz, CDCl₃, 297.3K)δ:** 163.14 (CO, C²), 150.40 (CO, C⁴), 142.49 (C⁶), 102.81 (C⁵), 93.07 (C¹), 85.52 (C⁴), 77.48, 77.16, 76.84 (CHCl₃), 74.24 (C²), 70.96 (C³), 62.30 (C⁵), 25.77 (CH₃, 2'-O-Si-tBu), 18.10 (C_q, 2'-O-Si-tBu), -4.71, -5.00 (2x CH₃, 2'-O-Si-Me₂). **IR:** 2930, 1694, 1462, 1379, 1258, 1157, 1061, 837, 783, 758.

HRMS: [C₁₅H₂₆N₂O₆Si+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. NaHCO₃ followed by Brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 9.779 (s, 1H, NH), 7.961 (d, *J* = 8.2 Hz, 1H, H⁶), 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 (CHCl₃), 6.849 (d, *J* = 8.9 Hz, 4H, H^{arom}, DMTr), 5.966 (d, *J* = 2.9 Hz, 1H, H¹), 5.314 (d, *J* = 8.1 Hz, 1H, H⁵), 4.36 (m, 2H, H², H³), 4.12 (m, 1H, H⁴), 3.793 (s, 6H, OMe, DMTr), 3.57-3.45 (m, 2H, H^{5a}, H^{5b}), 2.644 (s, 1H, OH³), 0.913 (s, 9H, 2'-O-Si-tBu), 0.198, 0.169 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K)δ: 163.72 (CO, C²), 158.80, 158.72 (2x C_q, DMTr), 150.50 (CO, C⁴), 144.38 (C_q, DMTr), 140.33 (C⁶), 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 (C⁵), 88.79 (C¹), 87.25 (C_q, DMTr), 83.58 (C⁴), 77.48, 77.16, 76.85 (CHCl₃), 76.41 (C²), 70.48 (C³), 62.36 (C⁵), 55.31 (OMe, DMTr), 25.75 (CH₃, 2'-O-Si-tBu), 18.09 (C_q, 2'-O-Si-tBu), -4.55, -5.13 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₃₆H₄₄N₂O₈Si+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 (R_f around 0.65 in 50% EtOAc/PE). The organic layer was washed with sat. NaHCO₃ followed by Brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 9.941 (br, 1H, NH), 7.905 (d, *J* = 8.2 Hz, 1H, H⁶), 7.40-7.20 (m, 9H, H^{arom}, H^{ortho}, H^{meta}, H^{para}, DMTr), 7.260 (CHCl₃), 6.854 (d, *J* = 8.7 Hz, 4H, H^{arom}, DMTr), 6.002 (d, *J* = 4.8 Hz, 1H, H¹), 5.349 (t, *J* = 4.6 Hz, 1H, H³'), 5.311 (d, *J* = 8.1 Hz, 1H, H⁵), 4.521 (t, *J* = 4.8 Hz, 1H, H²), 4.26 (m, 1H, H⁴), 3.787 (s, 6H, OMe, DMTr), 3.539 (d, *J* = 11.2, 2.0 Hz, 1H, H^{5a}), 3.448 (dd, *J* = 10.8, 1.2 Hz, 1H, H^{5b}), 2.9-2.5 (m, 4H, R¹-CH₂CH₂-R², Lev), 2.194 (s, 3H, CH₃, Lev), 0.870 (s, 9H, 2'-O-Si-tBu), 0.106, 0.088 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K)δ: 206.22 (CO, Lev), 171.82 (CO, Lev), 163.63 (CO, C²), 158.76, 158.72 (2x C_q, DMTr), 150.66 (CO, C⁴), 144.19 (C_q, DMTr), 140.07 (C⁶), 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 (C⁵), 88.43 (C¹), 87.42 (C_q, DMTr), 81.16 (C⁴), 77.48, 77.16, 76.85 (CHCl₃), 74.45 (C²), 72.22 (C³), 62.28 (C⁵), 55.31 (OMe, DMTr), 37.65 (R¹-CH₂CH₂-R², Lev), 29.86 (CH₃, Lev), 27.80 (R¹-CH₂CH₂-R², Lev), 25.50 (CH₃, 2'-O-Si-tBu), 17.88 (C_q, 2'-O-Si-tBu), -5.00, -5.24 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₄₁H₅₀N₂O₁₀Si+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 (R_f around 0.45 in 100% EtOAc). Finally, the mixture was quenched using sat. NaHCO₃. The mixture was partitioned and the organic layer was washed with Brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 9.517 (s, 1H, NH), 7.808 (d, *J* = 8.1 Hz, 1H, H⁶), 7.260 (CHCl₃), 5.740 (d, *J* = 8.0 Hz, 1H, H⁵), 5.662 (d, *J* = 4.8 Hz, 1H, H¹), 5.172 (t, *J* = 4.6 Hz, 1H, H³'), 4.562 (t, *J* = 4.8 Hz, 1H, H²), 4.210 (m, 1H, H⁴), 3.929 (d, *J* = 12.1 Hz, 1H, H^{5a}), 3.764 (d, *J* = 12.2 Hz, 1H, H^{5b}), 2.5-2.9 (m, 4H, R¹-CH₂CH₂-R², Lev), 2.191 (s, 3H, CH₃, Lev), 0.840 (s, 9H, 2'-O-Si-tBu), 0.039, 0.028 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K)δ: 206.81 (CO, Lev) 172.44 (CO, Lev), 163.67 (CO, C²), 150.50 (CO, C⁴), 141.71 (C⁶), 102.51 (C⁵), 91.75 (C¹), 82.93 (C⁴), 77.47, 77.16, 76.83 (CHCl₃), 73.50 (C²), 72.04 (C³), 61.49

(C⁵), 37.84 (R¹-CH₂CH₂-R², Lev), 29.93 (CH₃, Lev), 27.89 (R¹-CH₂CH₂-R², Lev), 25.59 (CH₃, 2'-O-Si-tBu), 17.98 (C_q, 2'-O-Si-tBu), -5.05, -5.12 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₂₀H₃₂N₂O₈Si+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% NaHCO₃. The mixture was partitioned and the organic layer was washed with Brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 9.34 (br, 1H, NH), 7.84, 7.78 (d+d, 1H, J = 8.2 Hz, H⁶) 7.260 (CHCl₃), 5.99, 5.98 (d+d, 1H, J = 6.1 + 5.7 Hz, H¹), 5.73, 5.71 (d+d, 1H, J = 3.6 + 3.7 Hz, H⁵), 5.21, 5.12 (t+dd, 1H, J = 4.4 + 4.9, 3.4 Hz, H³), 4.34-4.23 (m, 2H, H² + H⁴), 4.00-3.70 (m, 4H, H⁵, NC-CH₂-CH₂-OR), 3.65-3.50 (m, 2H, CH, iPr₂NR), 2.9-2.5 (m, 6H, R¹-CH₂CH₂-R², Lev, NC-CH₂-CH₂-OR), 2.18, 2.17 (s+s, 3H, CH₃, Lev), 1.22-1.15 (m, 12H, CH₃, iPr₂NR) 0.82, 0.81 (s+s, 9H, 2'-O-Si-tBu), 0.01--0.02 (m, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K)δ: 206.39, 206.33 (CO, Lev) 171.94, 171.89 (CO, Lev), 163.39, 163.35 (C²), 150.60, 150.58 (C⁴), 140.04, 139.93 (C⁶), 117.49, 117.41 (CN), 102.76, 102.66 (C⁵), 88.25, 88.05 (C¹), 81.79, 81.72, 81.70, 81.62 (C⁴), 77.48, 77.16, 76.84 (CHCl₃), 74.32, 74.18 (C²), 72.50, 72.06 (C³), 63.08, 62.92, 62.83, 62.67 (C⁵), 58.70, 58.60, 58.48, 58.38 (NC-CH₂-CH₂-OR), 43.36, 43.26, 43.23, 43.14 (2x CH, iPr₂NR), 37.79, 37.74 (R¹-CH₂CH₂-R², Lev), 29.92 (CH₃, Lev), 27.90, 27.86 (R¹-CH₂CH₂-R², Lev), 25.50 (CH₃, 2'-O-Si-tBu), 24.92, 24.88, 24.85, 24.81, 24.77, 24.70 (4x CH₃, iPr₂NR), 20.57, 20.49, 20.45, 20.38 (NC-CH₂-CH₂-OR), 17.91, 17.90 (C_q, Si-tBu), -5.08, -5.11, -5.20, -5.25 (2x CH₃, 2'-O-Si-Me₂).

³¹P NMR (162 MHz, CDCl₃) 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: [C₃₇H₅₄N₇O₈PSi+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)₂Si(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 H₂O was added to the mixture and extracted three times using Et₂O. The organic layer was dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 8.278 (s, 1H, H²), 7.824 (s, 1H, H⁸), 7.260 (CHCl₃), 6.780 (s, 2H, NH₂), 5.901 (s, 1H, H¹), 4.600 (d, J = 4.7Hz, 1H, H²), 4.511 (dd, J = 9.5, 4.7 Hz, 1H, H³), 4.460 (dd, J = 9.1, 5.1 Hz, 1H, H^{5a}), 4.189 (td, J = 10.1, 5.1 Hz, 1H, H⁴), 4.008 (dd, J = 10.3, 9.3 Hz, 1H, H^{5b}), 1.043, 1.011 (2x s, 18H, 5'-3'-O-Si-tBu₂), 0.896 (s, 9H, 2'-O-Si-tBu), 0.132, 0.116 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K)δ: 156.06 (C⁶), 153.16 (C²), 149.21 (C⁴), 138.60 (C⁸), 120.23 (C⁵), 92.42 (C¹), 77.48, 77.16, 76.84 (CHCl₃), 75.87 (C³), 75.50 (C²), 74.70 (C⁴), 67.86 (C⁵), 27.54, 27.08 (2x CH₃, 5'-3'-O-Si-tBu₂), 25.96 (CH₃, 2'-O-Si-tBu), 22.79, 20.39, 18.36 (3x C_q, Si-tBu), -4.24, -4.91 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₂₄H₄₃N₅O₄Si₂+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. NH₄OH 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 H₂O and 20 mL of DCM were added before partitioning layers. The organic layer was washed with sat. NaHCO₃ followed by Brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 9.807 (br, 1H, NH), 8.689 (s, 1H, H²), 8.019 (s, 1H, H⁸), 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 (CHCl₃), 5.969 (s, 1H, H¹), 4.593 (d, J = 4.6 Hz, 1H, H²), 4.474 (dd, J = 9.2, 5.1 Hz, 1H, H³), 4.429 (dd, J = 9.6, 4.6 Hz, 1H, H^{5a}), 4.218 (td, J = 10.1, 5.1 Hz, 1H, H⁴), 4.007 (dd, J = 10.0, 9.2 Hz, 1H, H^{5b}), 1.048, 1.018 (2x s, 18H, 5'-3'-O-Si-tBu₂), 0.913 (s, 9H, 2'-O-Si-tBu), 0.153, 0.132 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K)δ: 165.27 (CO, Bz), 152.64 (C²), 151.12 (C⁶), 149.92 (C⁴), 141.05 (C⁸), 133.63 (C_q^{ipso}, Bz), 132.72, 128.71, 128.11 (C^{ortho}, C^{meta}, C^{para}, Bz), 123.66 (C⁵), 92.47 (C¹), 77.48, 77.16, 76.84 (CHCl₃), 75.89 (C³), 75.51 (C²), 74.76 (C⁴), 67.78 (C⁵), 27.49, 27.03 (2x CH₃, 5'-3'-O-Si-tBu₂), 25.91 (CH₃, 2'-O-Si-tBu), 22.77, 20.36, 18.32 (3x C_q, 3x Si-tBu), -4.24, -4.96 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₃₁H₄₇N₅O₅Si₂+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. NaHCO₃ followed by brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 9.568 (br, 1H, NH), 8.706 (s, 1H, H²), 8.029 (s, 1H, H⁸), 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 (CHCl₃), 5.904 (d, J = 10.9 Hz, 1H, OH⁵), 5.793 (d, J = 7.1 Hz, 1H, H¹), 5.029 (dd, J = 7.1, 4.8 Hz, 1H, H²), 4.307 (d, J = 4.8 Hz, 1H, H³), 4.261 (s, 1H, H⁴), 3.891 (d, J = 12.7 Hz, 1H, H^{5a}), 3.695 (t, J = 11.7 Hz, 1H, H^{5b}), 3.085 (s, 1H, OH³), 0.719 (s, 9H, 2'-O-Si-tBu), -0.242, -0.437 (2x s, 6H, 2'-O-SiMe₂). **¹³C NMR (101 MHz, CDCl₃, 297.3K)δ:** 164.83 (CO, Bz), 152.29 (C²), 150.51 (C⁶), 150.47 (C⁴), 142.95 (C⁸), 133.46 (C_q^{ipso}, Bz), 132.86, 128.76, 127.97 (C^{ortho}, C^{meta}, C^{para}, Bz), 124.19 (C⁵), 91.01 (C¹), 87.42 (C⁴), 77.48, 77.16, 76.84 (CHCl₃), 74.46 (C²), 72.54 (C³), 63.03 (C⁵), 25.45 (CH₃, 2'-O-Si-tBu), 17.75 (C_q, Si-tBu), -5.34, -5.44 (2x CH₃, 2'-O-Si-Me₂).

IR: 3289, 2949, 2928, 2886, 1699, 1609, 1582, 1456, 1249, 1088, 835, 779, 706, 644 **HRMS:** [C₂₃H₃₁N₅O₅Si+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/H₂O. The organic layer was washed with sat. NaHCO₃ followed by brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 9.053 (s, 1H, NH), 8.735 (s, 1H, H²), 8.236 (s, 1H, H⁸), 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, meta}, DMTr), 7.260 (CHCl₃), 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, H¹), 5.028 (t, J = 5.1 Hz, 1H, H²), 4.369 (dd, J = 8.0, 4.0 Hz, 1H, H³), 4.290 (q, J = 3.4 Hz, 1H, H⁴), 3.780 (s, 6H, OMe, DMTr), 3.550 (dd, J = 10.7, 3.1 Hz, 1H, H^{5a}), 3.399 (dd, J = 10.7, 3.4 Hz, 1H, H^{5b}), 2.735 (d, J = 4.1 Hz, 1H, OH³), 0.843 (s, 9H, 2'-O-Si-tBu), -0.003, -0.142 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K)δ: 164.62 (CO, Bz), 158.72 (C_q, DMTr), 152.98 (C²), 151.79 (C⁶), 149.69 (C⁶), 144.63 (C_q, DMTr), 141.83 (C²), 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 (C⁵), 113.36 (CH^{arom}, DMTr), 88.56 (C^{1'}), 86.84 (C_q, DMTr), 84.42 (C^{4'}), 77.48, 77.16, 76.84 (CHCl₃), 75.83 (C^{2'}), 71.68 (C^{3'}), 63.43 (C^{5'}), 55.37 (OMe, DMTr), 25.70 (CH₃, 2'-O-Si-tBu), 18.03 (C_q, Si-tBu), -4.80, -5.02 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₄₄H₄₉N₅O₇Si+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. NaHCO₃ followed by Brine and dried using MgSO₄. Purification was performed with neutralized silica column chromatography using a 30% to 45% EtOAc/PE eluent resulting in 1.165g (1.315 mmol, 84.9%) of Compound **23** as a white foam.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 9.241 (s, 1H, NH), 8.737 (s, 1H, H²), 8.235 (s, 1H, H⁸), 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 (CHCl₃), 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, R¹-CH₂-CH₂-R², Lev), 2.201 (s, 3H, CH₃, Lev), 0.726 (s, 9H, 2'-O-Si-tBu), -0.007 (TMS), -0.024, -0.265 (2x s, 6H, 2'-O-Si-Me₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K)δ: 206.34 (CO, Lev), 171.80 (CO, Lev), 164.74 (CO, Bz), 158.72 (C_q, DMTr), 152.95 (C²), 151.93 (C⁶), 149.73 (C⁴), 144.48 (C_q, DMTr), 141.55 (C⁸), 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 (C⁵), 113.39 (CH^{arom}, DMTr), 88.08 (C^{1'}), 87.06 (C_q, DMTr), 82.43 (C^{3'}), 77.48, 77.16, 76.84 (CHCl₃), 74.49 (C^{2'}), 73.26 (C^{4'}), 63.23 (C^{5'}), 55.34 (OMe, DMTr), 37.81 (R¹-CH₂-CH₂-R², Lev), 29.99 (CH₃, Lev), 27.91 (R¹-CH₂-CH₂-R², Lev), 25.46 (CH₃, 2'-O-Si-tBu), 17.83 (C_q, Si-tBu), -5.11, -5.29 (2x CH₃, 2'-O-Si-Me₂).

IR: 2951, 2930, 2899, 2856, 2837, 1744, 1715, 1607, 1580, 1506, 1456, 1248, 1175, 1153, 1030, 835, 779, 704 **HRMS:** [C₄₉H₅₅N₅O₉Si+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. NaHCO₃. The organic layer was washed with sat. NaHCO₃ followed by Brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 9.284 (s, 1H, NH), 8.760 (s, 1H, H²), 8.043 (s, 1H, H⁸), 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 (CHCl₃), 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, R¹-CH₂-CH₂-R², Lev), 2.184 (s, 3H, CH₃, Lev), 0.661 (s, 9H, 2'-O-Si-tBu), 0.041 (TMS), -0.153, -0.479 (2x s, 6H, 2'-O-Si-Me₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K)δ: 206.36 (CO, Lev), 171.79 (CO, Lev), 164.59 (CO, Bz), 152.43 (C²), 150.57 (C⁶), 150.48 (C⁴), 143.03 (C⁸), 133.52 (C_q^{ipso}, Bz), 133.02, 128.95, 127.97 (C^{ortho}, C^{meta}, C^{para}, Bz), 124.37 (C⁵), 91.26 (C^{1'}), 86.00 (C^{3'}), 77.48, 77.16, 76.84 (CHCl₃), 74.28 (C^{2'}), 73.08 (C^{4'}), 62.89 (C^{5'}), 37.85 (R¹-CH₂-CH₂-R², Lev), 29.92 (CH₃, Lev), 27.87 (R¹-CH₂-CH₂-R², Lev), 25.39 (CH₃, 2'-O-Si-tBu), 17.76 (C_q, Si-tBu), -5.28, -5.84 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₂₈H₃₇N₅O₇Si+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% NaHCO₃. The mixture was partitioned and the organic layer was dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 9.222 (br, 1H, NH), 8.780 (s, 1H, H⁸), 8.466, 8.415 (s+s, 1H, H⁸), 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 (CHCl₃), 6.159, 6.135 (d+d, J = 6.3 + 5.7 Hz, 1H, H¹), 5.400, 5.342 (dd+dd, J = 4.9, 3.4 + 5.0, 2.6 Hz, 1H, H³), 4.888, 4.814 (t+t, J = 5.6 + 5.4 Hz, 1H, H²), 4.40-4.35 (m, 1H, H⁴), 4.0-3.7 (m, 4H, H⁵, NC-CH₂-CH₂-OR), 3.7-3.5 (m, 2H, CH, *i*Pr₂NR), 2.9-2.5 (m, 6H, R¹-CH₂CH₂-R², Lev, NC-CH₂-CH₂-OR), 2.182 (s, 3H, CH₃, Lev), 1.22-1.16 (m, 12H, CH₃, *i*Pr₂NR) 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-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K)δ: 206.21, 206.18 (CO, Lev) 171.84 (CO, Lev), 164.71 (CO, Bz), 152.91 (C²), 151.98, 151.83 (C⁶), 149.65 (C⁴), 141.71, 141.44 (C⁸), 133.85 (C_q^{ipso}, Bz), 132.77, 128.87, 127.92 (C^{ortho}, C^{meta}, C^{para}, Bz), 123.08, 123.02 (C⁵), 117.57 (CN), 88.34, 87.97 (C¹), 82.56, 82.47, 82.39 (C⁴), 77.48, 77.16, 76.84 (CHCl₃), 75.01, 74.94 (C²), 73.28, 72.70 (C³), 63.08, 62.90, 62.72, 62.57 (C⁵), 58.78, 58.57 (NC-CH₂-CH₂-OR), 43.36, 43.24, 43.12 (2x CH, *i*Pr₂NR), 37.80, 37.77 (R¹-CH₂CH₂-R², Lev), 29.90 (CH₃, Lev), 27.89, 27.86 (R¹-CH₂CH₂-R², Lev), 25.42 (CH₃, 2'-O-Si-tBu), 24.90, 24.84, 24.79, 24.72 (4x CH₃, *i*Pr₂NR), 20.48, 20.42, 20.41, 20.35 (NC-CH₂-CH₂-OR), 17.80 (C_q, Si-tBu), -5.19, -5.28, -5.35, -5.40 (2x CH₃, 2'-O-SiMe₂).

³¹P NMR (162 MHz, CDCl₃) δ: 149.20, 149.05.

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

HRMS: [C₃₇H₅₄N₇O₄PSi+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-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 Imidazole 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 H₂O, Brine and dried using MgSO₄. 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.

¹H NMR (399 MHz, CDCl₃, 330K) δ: 8.67 (br, 1H, NH₂), 7.34 (s, 1H, H⁶), 7.26 (CHCl₃), 6.50 (br, 1H, H⁵), 5.65 (s, 1H, H¹), 4.47 (dd, J = 8.1, 4.7 Hz, 1H, H^{5a}), 4.24 - 4.12 (m, 2H, H², H⁴), 3.95 (t, J = 9.5 Hz, 1H, H^{5b}), 3.77 (d, J = 6.0 Hz, 1H, H³), 1.01, 1.00 (2x s, 18H, 5'-3'-O-Si-tBu₂), 0.90 (s, 9H, 2'-O-Si-tBu), 0.13, 0.10 (2x s, 6H, 2'-O-Si-Me₂).

¹³C NMR (100 MHz, CDCl₃, 330K) δ: 163.86 (C²), 153.64 (C⁴), 140.16 (C⁶), 96.64 (C⁵), 93.98 (C¹), 77.48, 77.16, 76.84 (CHCl₃), 76.09 (C³), 75.69 (C²), 74.79 (C⁴), 67.74 (C⁵), 27.60, 27.08 (2x CH₃, 5'-3'-O-Si-tBu₂), 25.97 (CH₃, 2'-O-Si-tBu), 22.81, 20.39, 18.30 (3x C_q, Si-tBu), -4.19, -4.84 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₂₃H₄₃N₃O₅Si₂+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 °C and the mixture was stirred for

2 hours while 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 with sat. NH₄Cl, sat. NaHCO₃, Brine and dried using MgSO₄. Purification was performed with silica column chromatography using a 0% to 2% MeOH/DCM eluent resulting in 0.614g (1.137 mmol, 98.1%) of Compound **13** as a white solid.

¹H NMR (300 MHz, CDCl₃, 293.7K) δ: 10.46 (s, 1H, NH), 7.71 (d, *J* = 7.6 Hz, 1H, H⁶), 7.44 (d, *J* = 7.6 Hz, 1H, H⁵), 7.26 (CHCl₃), 5.71 (s, 1H, H¹), 4.55 (dd, *J* = 9.2, 5.2 Hz, 1H, H^{5a}), 4.34 – 4.21 (m, 2H, H², H⁴), 4.00 (t, *J* = 10.3 Hz, 1H, H^{3b}), 3.79 (dd, *J* = 9.7, 4.3 Hz, 1H, H³), 2.32 (s, 3H, CH₃, Ac), 1.02, 1.02 (2x s, 18H, 5'-3'-O-Si-tBu₂), 0.94 (s, 9H, 2'-O-Si-tBu), 0.22, 0.15 (2x s, 6H, 2'-O-Si-Me₂).

¹³C NMR (75 MHz, CDCl₃, 293.7K) δ: 171.68 (CO, Ac), 163.39 (C²), 154.71 (C⁴), 143.30 (C⁶), 97.01 (C⁵), 94.38 (C¹), 77.59, 77.16, 76.74 (CHCl₃), 75.82 (C³), 75.46, 74.87 (C², C⁴), 67.88 (C⁵), 27.61, 27.08 (2x CH₃, 5'-3'-O-Si-tBu₂), 26.01 (CH₃, 2'-O-Si-tBu), 25.11 (CH₃, Ac), 22.92, 20.47, 18.34 (3x C_q, Si-tBu), -4.16, -4.80 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₂₅H₄₅N₃O₆Si₂+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 with sat. NH₄Cl, sat. NaHCO₃, Brine and dried using MgSO₄. Purification was performed with silica column chromatography using a 0% to 10% MeOH/DCM eluent resulting in 0.307g (0.768 mmol, 82.1%) of Compound **17** as a clear solid.

¹H NMR (400 MHz, CDCl₃, 293.7K) δ: 10.27 (s, 1H, NH), 8.35 (d, *J* = 7.5 Hz, 1H, H⁶), 7.39 (d, *J* = 7.5 Hz, 1H, H⁵), 7.26 (CHCl₃), 5.67 (d, *J* = 2.3 Hz, 1H, H¹), 4.57 (s, 1H, OH⁵), 4.45 (m, 1H, H²), 4.24 (dd, *J* = 11.2, 5.9 Hz, 1H, H⁴), 4.08 (d, *J* = 6.0 Hz, 1H, H³), 3.99 (d, *J* = 11.7 Hz, 1H, H^{5a}), 3.84 (d, *J* = 8.5 Hz, 1H, H^{5b}), 3.04 (d, *J* = 6.7 Hz, 1H, OH³), 2.21 (s, 3H, CH₃, Ac), 0.87 (s, 9H, 2'-O-Si-tBu), 0.12, 0.07 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 293.7K) δ: 171.40 (CO, Ac), 163.14 (C²), 155.58 (C⁴), 146.67 (C⁶), 97.07 (C⁵), 93.29 (C¹), 85.10 (C²), 77.48, 77.16, 76.84 (CHCl₃), 75.34 (C⁴), 69.19 (C³), 60.54 (C⁵), 25.77 (CH₃, 2'-O-Si-tBu), 24.84 (CH₃, Ac), 18.04 (C_q, Si-tBu), -4.58, -5.20 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₁₇H₂₉N₃O₆Si+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 a 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.

¹H NMR (400 MHz, CDCl₃, 293.7K) δ: 10.47 (s, 1H, NH), 8.47 (d, *J* = 7.5 Hz, 1H, H⁶), 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 (CHCl₃), 7.15 (d, *J* = 7.5 Hz, 1H, H⁵), 6.88 (d, *J* = 8.8 Hz, 4H, H^{arom}, DMTr), 5.91 (d, *J* = 0.9 Hz, 1H, H¹), 4.38 (s, 1H, H³), 4.29 (dd, *J* = 4.6, 0.7 Hz, 1H, H²), 4.11 (d, *J* = 7.9 Hz, 1H, H⁴), 3.82 (2x s, *J* = 2.0 Hz, 6H, OMe, DMTr), 3.60 (dd, *J* = 11.1, 1.7 Hz, 1H, H^{5a}), 3.54 (dd, *J* = 11.2, 2.7 Hz, 1H, H^{5b}), 2.45 (d, *J* = 8.8 Hz, 1H, OH³), 2.29 (s, 3H, CH₃, Ac), 0.94 (s, 9H, 2'-O-Si-tBu), 0.30, 0.19 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 293.7K) δ: 171.13 (CO, Ac), 163.33 (C²), 158.77 (C_q, DMTr), 155.07 (C⁴), 144.90 (C⁶), 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 (C⁵), 90.76 (C¹), 87.20 (C_q, DMTr), 83.16 (C⁴), 77.48, 77.16, 76.84 (CHCl₃), 76.76 (C²), 69.14 (C³), 61.60 (C⁵), 55.32 (CH₃, OMe, DMTr), 25.91 (CH₃, 2'-O-Si-tBu), 24.93 (CH₃, Ac), 18.15 (C_q, Si-tBu), -4.27, -5.35 (2x CH₃, 2'-O-Si-Me₂). **IR:** 2949, 2928, 2897, 2855, 2839, 1667, 1609, 1489, 1115, 814, 787.

HRMS: [C₃₈H₄₇N₃O₈Si+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. NaHCO₃ dried using MgSO₄ 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.

¹H NMR (400 MHz, CDCl₃, 293.7K) δ: 10.31 (s, 1H, NH), 8.44 (d, *J* = 7.5 Hz, 1H, H⁶), 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 (CHCl₃), 7.13 (d, *J* = 7.5 Hz, 1H, H⁵), 6.87 (d, *J* = 8.9 Hz, 4H, H^{arom}, DMTr), 5.92 (d, *J* = 2.1 Hz, 1H, H¹), 5.18 (dd, *J* = 7.7, 4.3 Hz, 1H, H³), 4.50 (dd, *J* = 4.2, 2.1 Hz, 1H, H²), 4.37 (d, *J* = 7.7 Hz, 1H, H⁴), 3.81 (2x s, 6H, OMe, DMTr), 3.63 (dd, *J* = 11.3, 2.0 Hz, 1H, H^{5a}), 3.41 (dd, *J* = 11.4, 2.2 Hz, 1H, H^{5b}), 2.85 – 2.45 (m, 4H, R¹-CH₂CH₂-R², Lev), 2.29 (s, 3H, CH₃, Ac), 2.19 (s, 3H, CH₃, Lev), 0.87 (s, 9H, 2'-O-Si-tBu), 0.18, 0.05 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 293.7K) δ: 206.05 (CO, Lev), 171.90 (CO, Lev), 171.09 (CO, Ac), 163.24 (C²), 158.80 (C_q, DMTr), 155.09 (C⁴), 144.71 (C⁶), 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 (C⁵), 91.08 (C¹), 87.40 (C_q, DMTr), 80.36 (C⁴), 77.48, 77.16, 76.84 (CHCl₃), 74.79 (C²), 70.89 (C³), 61.10 (C⁵), 55.36 (CH₃, OMe, DMTr), 37.69 (R¹-CH₂CH₂-R², Lev), 29.95 (CH₃, Lev), 27.80 (R¹-CH₂CH₂-R², Lev), 25.70 (CH₃, 2'-O-Si-tBu), 25.02 (CH₃, Ac), 18.03 (C_q, Si-tBu), -4.61, -5.43 (2x CH₃, 2'-O-Si-Me₂). **IR:** 2953, 2928, 2855, 1717, 1667, 1489, 1248, 1175, 1155, 1117, 1031, 1005, 829, 779.

HRMS: [C₄₃H₅₃N₃O₁₀Si+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. NaHCO₃. The mixture was partitioned and the organic layer was washed with Brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 293.7K) δ: 10.07 (s, 1H, NH), 8.22 (d, *J* = 7.5 Hz, 1H, H⁶), 7.44 (d, *J* = 7.5 Hz, 1H, H⁵), 7.26 (CHCl₃), 5.62 (d, *J* = 3.8 Hz, 1H, H¹), 5.18 (t, *J* = 5.1 Hz, 1H, H³), 4.72 (t, *J* = 4.3 Hz, 1H, H²), 4.27 (d, *J* = 5.4 Hz, 1H, H⁴), 3.97 (d, *J* = 12.9 Hz, 1H, H^{5a}), 3.75 (d, *J* = 12.7 Hz, 1H, H^{5b}), 2.90 – 2.50 (m, 4H, R¹-CH₂CH₂-R², Lev), 2.27 (s, 3H, CH₃, Ac), 2.18 (s, 3H, CH₃, Lev), 0.85 (s, 9H, 2'-O-Si-tBu), 0.05, 0.03 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 293.7K) δ: 206.54 (CO, Lev), 172.57 (CO, Lev), 171.26 (CO, Ac), 163.24 (C²), 155.32 (C⁴), 146.73 (C⁶), 97.10 (C⁵), 94.22 (C¹), 83.17 (C⁴), 77.48, 77.16, 76.84 (CHCl₃), 73.34 (C²), 71.70 (C³), 61.20 (C⁵), 37.90 (R¹-CH₂CH₂-R², Lev), 29.92 (CH₃, Lev), 27.93 (R¹-CH₂CH₂-R², Lev), 25.69 (CH₃, 2'-O-Si-tBu), 25.03 (CH₃, Ac), 18.03 (C_q, Si-tBu), -4.85, -5.16 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₂₂H₃₅N₃O₈Si+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 **29** 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% NaHCO₃. The mixture was partitioned and the organic layer was washed with Brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 293.7K) δ: 10.14, 10.09 (s+s, 1H, NH), 8.43, 8.36 (d+d, *J* = 7.5 + 7.6 Hz, 1H, H⁶), 7.39 (d, *J* = 7.5 Hz, 1H, H⁵), 7.26 (CHCl₃), 5.94, 5.91 (d+d, *J* = 2.9, 2.4 Hz, 1H, H¹), 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-CH₂-CH₂-OR), 3.68 - 3.53 (m, 2H, CH, *iPr*₂NR), 2.85 – 2.48 (m, 6H, R¹-CH₂CH₂-R², Lev, NC-CH₂-CH₂-OR), 2.28 (s, 3H, CH₃, Ac), 2.18, 2.17 (s+s, 3H, CH₃, Lev), 1.27 – 1.12 (m, 12H, CH₃, *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-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 293.7K) δ: 206.25, 206.13 (CO, Lev), 171.94, 171.87 (CO, Lev), 171.35 (CO, Ac), 163.21, 163.13 (C²), 155.19, 155.14 (C⁴), 144.95, 144.84 (C⁶), 117.65, 115.59 (CN), 96.71, 96.62 (C⁵), 91.04, 90.88 (C¹), 80.95, 80.87 (C⁴), 77.48, 77.16, 76.84 (CHCl₃), 74.85, 74.78 (C²), 71.22, 70.53 (C³), 61.86, 61.69, 61.31, 61.14 (C⁵), 58.97, 58.76, 58.72, 58.51 (NC-CH₂-CH₂-OR), 43.43, 43.33, 43.31, 43.21 (2x CH, *iPr*₂NR), 37.82, 37.73 (R¹-CH₂CH₂-R², Lev), 29.96 (CH₃, Lev), 27.87, 27.82 (R¹-CH₂CH₂-R², Lev), 25.70 (CH₃, 2'-O-Si-tBu), 25.09, 25.07 (CH₃, Ac), 24.91, 24.89, 24.84, 24.82, 24.77, 24.74 (4x CH₃, *iPr*₂NR), 20.54, 20.46, 20.42, 20.34 (NC-CH₂-CH₂-OR), 18.05 (C_q, Si-tBu), -4.69, -5.36, -5.43 (2x CH₃, 2'-O-Si-Me₂).

³¹P NMR (162 MHz, CDCl₃, 293.7K) δ: 149.83, 148.69.

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

HRMS: [C₃₁H₅₂N₅O₉PSi+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)₂Si(OTf)₂ was added at 0 °C and the mixture was stirred for 40 minutes. Reaction completion was checked by TLC (R_f 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 (R_f 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.

¹H NMR (400 MHz, DMSO, 297.3K)δ: 10.67 (s, 1H, NH), 7.91 (s, 1H, H⁸), 6.36 (s, 2H, NH₂), 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, H₂O), 2.50 (s, DMSO), 1.06, 1.00 (2x s, 18H, 5'-3'-O-Si-tBu₂), 0.86 (s, 9H, 2'-O-Si-tBu), 0.09, 0.07 (2x s, 6H, 2'-O-Si-Me₂).

¹³C NMR (101 MHz, DMSO, 297.3K)δ: 156.68 (CO, C⁶), 153.77 (C²), 150.77 (C⁴), 135.61 (C⁸), 116.56 (C⁵), 90.08 (C¹), 75.68 (C³), 74.73 (C²), 73.91 (C⁴), 66.97 (C^{5'}), 40.15, 39.94, 39.73, 39.52, 39.31, 39.10, 38.89 (DMSO), 27.33, 26.86 (2x CH₃, 5'-3'-O-Si-tBu₂), 25.72 (CH₃, 2'-O-Si-tBu), 22.25, 20.00, 18.05 (3x C_q, Si-tBu), -4.57, -5.11 (2x CH₃, 2'-O-Si-Me₂).

IR: 3474, 3292, 3159, 3140, 3088, 3019, 2936, 2884, 2856, 2816, 2778, 2714, 1688, 1167, 1049, 899, 833, 777. **HRMS:** [C₂₄H₄₃N₅O₅Si₂+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 isobutrylchloride as added at -20 °C and the mixture was stirred for 1 hours. Reaction completion was checked by TLC (R_f 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.

¹H NMR (400 MHz, CDCl₃, 297.3K)δ: 11.97 (s, 1H, NH), 7.96 (s, 1H, NH), 7.73 (s, 1H, H⁸), 7.26 (CHCl₃), 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, CH₃, *iBu*), 1.07, 1.04 (2x s, 18H, 5'-3'-O-Si-tBu₂), 0.94 (s, 9H, 2'-O-Si-tBu), 0.16, 0.15 (2x s, 6H, 2'-O-Si-Me₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K)δ: 177.88 (CO, *iBu*), 155.20 (CO, C⁶), 147.25, 147.25 (C⁴, C²), 136.71 (C⁸), 121.89 (C⁵), 91.99 (C¹), 77.48, 77.16, 76.84 (CHCl₃), 76.19 (C³), 76.00 (C²), 74.78 (C⁴), 67.96 (C^{5'}), 36.84 (CH, *iBu*), 27.62, 27.13 (2x CH₃, 5'-3'-O-Si-tBu₂), 26.06 (CH₃, 2'-O-Si-tBu), 22.97, 20.49 (2x C_q, Si-tBu), 19.16, 19.00 (2x CH₃, *iBu*), 18.54 (C_q, Si-tBu), -4.03, -4.81 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₂₈H₄₉N₅O₆Si₂+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 with sat. KHSO₄ and dried using MgSO₄. 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.

¹H NMR (400 MHz, DMSO, 297.3K) δ: 12.08 (s, 1H, NH), 11.69 (s, 1H, NH), 8.28 (s, 1H, H⁸), 5.86 (d, J = 6.8 Hz, 1H, H¹), 5.11 (t, J = 5.4 Hz, 1H, OH⁵), 5.06 (d, J = 4.6 Hz, 1H, OH³), 4.54 (dd, J = 6.7, 4.9 Hz, 1H, H²), 4.09 (td, J = 4.7, 2.2 Hz, 1H, H⁴), 3.98 – 3.94 (m, 1H, H⁴), 3.70 – 3.50 (m, 2H, H⁵), 3.36 (s, HOD, H₂O), 2.76 (hept, J = 6.8 Hz, 1H, CH, iBu), 2.50 (DMSO), 1.12, 1.10 (2x s, 6H, CH₃, iBu), 0.71 (s, 9H, 2'-O-Si-tBu), -0.08, -0.20 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, DMSO, 297.3K) δ: 180.16 (CO, iBu), 154.82 (CO, C⁶), 149.04 (C²), 148.22 (C⁴), 137.48 (C⁸), 119.95 (C⁵), 86.32 (C⁴), 85.90 (C¹), 76.43 (C²), 70.82 (C³), 61.42 (C⁵), 40.14, 39.94, 39.73, 39.52, 39.31, 39.10, 38.90 (DMSO), 34.75 (CH, iBu), 25.50 (CH₃, 2'-O-Si-tBu), 18.91, 18.80 (2x CH₃, iBu), 17.77 (C_q, Si-tBu), -4.96, -5.45 (2x CH₃, 2'-O-SiMe₂).

IR: 3474, 3291, 3154, 3142, 2930, 2882, 2859, 2779, 2714, 1682, 1601, 1252, 1142, 1090, 1049, 835, 781, 673. HRMS: [C₂₀H₃₃N₅O₆Si+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. NaHCO₃ made it turn yellow again). The organic layer was washed with sat. NaHCO₃ and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K) δ: 12.04 (s, 1H, NH), 8.04 (s, 1H, NH), 7.86 (s, 1H, H⁸), 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 (CHCl₃), 6.79 (t, J = 8.9 Hz, 4H, H^{arom}, DMTr), 5.76 (d, J = 7.3 Hz, 1H, H¹), 5.21 (dd, J = 7.3, 5.3 Hz, 1H, H²), 4.34 (dd, J = 5.2, 1.2 Hz, 1H, H³), 4.23 (s, 1H, H⁴), 3.76, 3.74 (2x s, 6H, OMe, DMTr), 3.56 (dd, J = 10.7, 1.6 Hz, 1H, H^{5a}), 3.07 (dd, J = 10.8, 2.7 Hz, 1H, H^{5b}), 2.91 (s, 1H, OH³), 1.53 (hept, J = 6.7 Hz, 1H, CH, iBu), 0.83 (d, J = 7.0 Hz, 3H, CH₃, iBu), 0.81 (s, 9H, 2'-O-Si-tBu), 0.60 (d, J = 6.9 Hz, 3H, CH₃, iBu), 0.01, -0.19 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K) δ: 178.75 (CO, iBu), 158.83 (C_q, DMTr), 155.57 (C⁶), 148.40 (C²), 147.33 (C⁴), 145.22 (C_q, DMTr), 139.21 (C⁸), 136.19, 135.65 (2x C_q, DMTr), 130.06, 128.17, 128.02, 127.26 (4x CH^{arom}, DMTr), 122.52 (C⁵), 113.38 (CH^{arom}, DMTr), 88.23 (C¹), 86.26 (C_q, DMTr), 84.59 (C⁴), 77.48, 77.16, 76.84 (CHCl₃), 74.47 (C²), 71.28 (C³), 63.78 (C⁵), 55.33 (CH₃, OMe, DMTr), 35.93 (CH, iBu), 25.59 (CH₃, 2'-O-Si-tBu), 18.52, 18.48 (2x CH₃, iBu), 17.93 (C_q, Si-tBu), -5.00, -5.04 (2x CH₃, 2'-O-SiMe₂).

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

HRMS: [C₄₁H₅₁N₅O₈Si+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. NaHCO₃ dried using MgSO₄ 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.

¹H NMR (400 MHz, CDCl₃, 297.3K) δ: 11.94 (s, 1H, NH), 7.79 (s, 1H, H⁸), 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, H¹), 5.50 (dd, *J* = 5.4, 1.5 Hz, 1H, H³), 5.33 (dd, *J* = 7.6, 5.5 Hz, 1H, H²), 4.19 (d, *J* = 1.3 Hz, 1H, H⁴), 3.76, 3.74 (2x s, 6H, OMe, DMTr), 3.57 (dd, *J* = 10.7, 1.5 Hz, 1H, H^{5a}), 3.06 (dd, *J* = 10.8, 2.4 Hz, 1H, H^{5b}), 2.9 – 2.50 (m, 4H, R¹-CH₂CH₂-R², Lev), 2.17 (s, 3H, CH₃, Lev), 1.31 (m, 1H, CH, iBu), 0.80 (d, *J* = 6.8 Hz, 3H, CH₃, iBu), 0.74 (s, 9H, 2'-O-Si-tBu), 0.55 (d, *J* = 6.9 Hz, 3H, CH₃, iBu), 0.02, -0.23 (2x s, 6H, 2'-O-SiMe₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K) δ: 206.40 (CO, Lev), 178.40 (CO, iBu), 171.72 (CO, Lev), 158.86 (C_q, DMTr), 155.53 (C⁶), 148.25 (C²), 147.08 (C⁴), 145.30 (C_q, DMTr), 139.37 (C⁸), 136.19, 135.54 (2x C_q, DMTr), 130.10, 128.20, 128.00, 127.30 (4x CH^{arom}, DMTr), 122.79 (C⁵), 113.41 (CH^{arom}, DMTr), 88.45 (C¹), 86.38 (C_q, DMTr), 82.51 (C⁴), 77.48, 77.16, 76.84 (CHCl₃), 72.76 (C²), 72.68 (C³), 63.41 (C⁵), 55.33 (CH₃, OMe, DMTr), 37.76 (R¹-CH₂CH₂-R², Lev), 35.99 (CH, iBu), 29.94 (CH₃, Lev), 27.86 (R¹-CH₂CH₂-R², Lev), 25.46 (CH₃, 2'-O-Si-tBu), 18.44, 18.43 (2x CH₃, iBu), 17.82 (C_q, Si-tBu), -5.00, -5.29 (2x CH₃, 2'-O-Si-Me₂)

IR: 3150, 3059, 3036, 2949, 2928, 2887, 2857, 1674, 1605, 1508, 1250, 1175, 1152, 1090, 1032, 833, 781, 702. **HRMS:** [C₄₆H₅₇N₅O₁₀Si+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. NaHCO₃. The mixture was partitioned and the organic layer was washed with Brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K) δ: 12.26 (s, 1H, NH), 9.22 (s, 1H, NH), 7.78 (s, 1H, H⁸), 7.26 (CHCl₃), 5.66 (d, *J* = 7.3 Hz, 1H, H¹), 5.42 (dd, *J* = 5.4, 0.8 Hz, 1H, H³), 5.21 (d, *J* = 8.2 Hz, 1H, OH⁵), 4.89 (dd, *J* = 7.1, 5.5 Hz, 1H, H²), 4.24 (s, 1H, H⁴), 3.93 (dd, *J* = 12.3, 1.8 Hz, 1H, H^{5a}), 3.82 – 3.71 (m, 1H, H^{5b}), 2.90 – 2.50 (m, 6H, R¹-CH₂CH₂-R², Lev, CH, iBu), 2.19 (s, 3H, CH₃, Lev), 1.24, 1.23 (2x d, *J* = 3.0, 2.8 Hz, 6H, CH₃, iBu), 0.69 (s, 9H, 2'-O-Si-tBu), -0.14, -0.36 (2x s, 6H, 2'-O-Si-Me₂).

¹³C NMR (101 MHz, CDCl₃, 297.3K) δ: 206.52 (CO, Lev), 179.27 (CO, iBu), 171.94 (CO, Lev), 155.33 (C⁶), 147.98 (C²), 147.32 (C⁴), 139.26 (C⁸), 122.70 (C⁵), 90.35 (C¹), 84.69 (C⁴), 77.48, 77.16, 76.84 (CHCl₃), 73.84 (C³), 73.30 (C²), 62.54 (C^{5a}), 37.86 (R¹-CH₂CH₂-R², Lev), 36.36 (CH, iBu), 29.94 (CH₃, Lev), 27.89 (R¹-CH₂CH₂-R², Lev), 25.43 (CH₃, 2'-O-Si-tBu), 19.16, 18.91 (2x CH₃, iBu), 17.81 (C_q, Si-tBu), -5.25, -5.60 (2x CH₃, 2'-O-Si-Me₂).

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

HRMS: [C₂₅H₃₉N₅O₈Si+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% NaHCO₃. The mixture was partitioned and the organic layer was washed with Brine and dried using MgSO₄. 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.

¹H NMR (400 MHz, CDCl₃, 297.3K) δ: 12.06 (br, 1H, NH), 8.90 (br, 1H, NH), 8.05, 8.00 (s+s, 1H, H⁸), 7.26 (CHCl₃), 5.77 (d, *J* = 6.6 Hz, 1H), 5.44 – 5.38 (m, 1H, H³), 4.89, 4.69 (dd+t, *J* = 6.8, 5.3 + 5.8 Hz, 1H, H²), 4.32 – 4.25 (m, 1H, H⁴), 4.00 – 3.75 (m, 4H, H⁵, NC-CH₂-CH₂-OR), 3.67 – 3.54 (m, 2H, CH, *i*Pr₂NR), 2.90 – 2.50 (m, 7H, R¹-CH₂CH₂-R², Lev, NC-CH₂-CH₂-OR, CH, *i*Bu), 2.21 (s, 3H, CH₃, Lev), 1.29 – 1.09 (m, 18H, CH₃, *i*Pr₂NR, CH₃, *i*Bu), 0.73, 0.71 (s+s, 9H, CH₃, 2'-O-Si-*t*Bu), -0.07, -0.08 (s+s, 3H, 2'-O-Si-Me), -0.28 (s, 3H, 2'-O-Si-Me).

¹³C NMR (101 MHz, CDCl₃, 297.3K) δ: 206.65, 206.54 (CO, Lev), 178.77, 178.72 (CO, *i*Bu), 171.97, 171.90 (CO, Lev), 155.72, 155.67 (C⁶), 148.61, 148.48 (C²), 147.72, 147.69 (C⁴), 138.00 (C⁸), 121.72, 121.66 (C⁵), 117.85, 117.77 (CN), 88.01, 87.85 (C¹), 82.57, 82.52, 82.48, 82.43 (C⁴), 77.48, 77.16, 76.84 (CHCl₃), 74.97, 74.46 (C²), 73.95, 73.12 (C³), 63.64, 63.47, 63.29, 63.14 (C⁵), 58.56, 58.44, 58.35, 58.22 (NC-CH₂-CH₂-OR), 43.48, 43.35, 43.30, 43.17 (2x CH, *i*Pr₂NR), 37.89 (R¹-CH₂CH₂-R², Lev), 36.44, 36.41 (CH, *i*Bu), 30.00 (CH₃, Lev), 27.86 (R¹-CH₂CH₂-R², Lev), 25.44 (CH₃, 2'-O-Si-*t*Bu), 24.96, 24.89, 24.86, 24.84, 24.77 (4x CH₃, *i*Pr₂NR), 20.67, 20.59, 20.57, 20.50 (NC-CH₂-CH₂-OR), 19.19, 19.17, 18.98, 18.95 (2x CH₃, *i*Bu), 17.87, 17.86 (C_α, Si-*t*Bu), -5.11, -5.21, -5.40, -5.42 (2x CH₃, 2'-O-Si-Me₂).

³¹P NMR (162 MHz, CDCl₃) 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: [C₃₄H₅₆N₇O₉PSi+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:

$$L_{(\text{mmol/g})} = (A * \text{Volume}_{(\text{mL})}) / (C_{(\text{fmoc} = 7800)} * \text{Mass}_{(\text{g})})$$

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

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 a tube containing 5 mL of cold Et₂O, followed by 300 μL 3%TFA/DCM and 500 μL of Et₂O 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 H₂O. 200 μL was taken and diluted with 1mL of H₂O for LC-MS analysis. **LC-MS analysis:** (Alltima C₁₈ analytical column, linear gradient in 12.5 minutes; H₂O: 90 → 40% ; ACN: 0 → 50% ; aq. 0.5% TFA: 10%) **LC:**

R_t4.94 min **MS**: [C₁₈H₂₆N₄O₇+H]⁺: found 411.07, calculated 411.19. [2C₁₈H₂₆N₄O₇+H]⁺: found 821.13, calculated 821.37. [3C₁₈H₂₆N₄O₇+H]⁺: found 1230.93, calculated 1231.55.

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-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) 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 (4 mL / gram resin (~200 μmol)) before being added to the resin and shaken for 1 hour. **Capping:** 20 equivalents of Ac₂O (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 Et₂O, followed by 300 μL 3%TFA/DCM and 500 μL of Et₂O 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 H₂O. 200 μL was taken and diluted with 1mL of H₂O for LC-MS analysis. **LC-MS analysis:**(Alltima C₁₈ analytical column, linear gradient in 12.5 minutes; H₂O: 90 → 40% ; ACN: 0 → 50% ; aq. 0.5% TFA: 10%)**LC:**R_t4.41 min **MS**: [C₂₀H₂₉N₅O₈+H]⁺: found 468.13, calculated 468.21. [2C₂₀H₂₉N₅O₈+H]⁺: found 935.13, calculated 935.41. [3C₂₀H₂₉N₅O₈+H]⁺: found 1402.07, calculated 1402.61. [4C₂₀H₂₉N₅O₈+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) of Bpoc-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 $^\circ\text{C}$ 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 \rightarrow 40% ; ACN: 0 \rightarrow 50% ; aq. 0.5% TFA: 10%) **LC:** R_t 8.62 min **MS:** $[\text{C}_{43}\text{H}_{63}\text{N}_8\text{O}_{18}\text{PSi}+\text{H}]^+$: 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 $\mu\text{mol/g}$) 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 with 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) of Bpoc-Gly-Ala-Tyr(OH)-Thr(Otrt)-Gly-HMPB-HCP resin **38** (199 $\mu\text{mol/g}$) 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 with 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 $^\circ\text{C}$ 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 \rightarrow 40% ; ACN: 0 \rightarrow 50% ; aq. 0.5% TFA: 10%) **LC:** R_t 6.03 min

MS:[C₂₂H₃₁N₅O₉+H]⁺: found 510.20, calculated 510.22. [2C₂₂H₃₁N₅O₉+H]⁺: found 1019.20, calculated 1019.43. [3C₂₂H₃₁N₅O₉+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 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**, **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 with 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

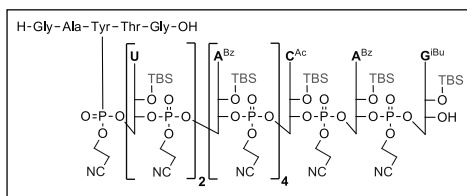
25 mg (≈ 5.0 μmol) of Bpoc-Gly-Ala-Tyr(OpUlev)-Thr(OTrt)-Gly-HMPB-HCP resin **41** was taken and put in a 2 mL filter syringe. ACN was added and the resin was allowed to swell while being shaken for 20 minutes. The solvent was drained and the resin was washed three times with ACN. Then 375 μmol of hydrazine (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 2 mL filter syringe. 300 μL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into a tube containing 5 mL of cold Et₂O, followed by 300 μL 3% TFA/DCM and 500 μL of Et₂O 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 H₂O. 200 μL was taken and diluted with 1 mL of H₂O for LC-MS analysis. The 800 μL of product containing solution and remaining LC-MS solution were combined and transferred to an eppendorf. The eppendorf was cooled using liquid nitrogen for 2 minutes and freeze-dried overnight resulting in 1.18 mg (1.25 μmol, 25%) of Compound **4** as a white fluffy solid. **LC-MS analysis:** (Alltima C₁₈ analytical column, linear gradient in 12.5 minutes; H₂O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:**R_t5.36 min **MS:**[C₃₈H₅₇N₈O₁₆PSi+H]⁺: found 941.33, calculated 941.35. [2C₃₈H₅₇N₈O₁₆PSi+H]⁺: found 1882.20, calculated 1881.69.

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

25 mg (≈ 5.0 μmol) of Bpoc-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 a 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 product containing solution and remaining LC-MS solution were combined and transferred to an eppendorf. The eppendorf was cooled using liquid nitrogen for 2 minutes and freeze-dried 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 \rightarrow 40%; ACN: 0 \rightarrow 50%; aq. 0.5% TFA: 10%) Compound **4**: **LC**:R:8.12 min **MS**: $[\text{C}_{38}\text{H}_{57}\text{N}_8\text{O}_{16}\text{PSi}+\text{H}]^+$: found 941.27, calculated 941.35. $[\text{C}_{238}\text{H}_{57}\text{N}_8\text{O}_{16}\text{PSi}+\text{H}]^+$: found 1882.07, calculated 1881.69. Compound **5**: **LC**:R:9.74 min **MS**: $[\text{C}_{56}\text{H}_{85}\text{N}_{11}\text{O}_{24}\text{P}_2\text{Si}_2+\text{H}]^+$: found 1414.40, calculated 1414.49.



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

50 mg ($\approx 10 \mu\text{mol}$) of Bpoc-Gly-Ala-Tyr(OpUlev)-Thr(OTrt)-Gly-HMPB-HCP resin **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*.

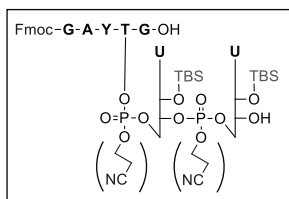
Nucleotides used: uridine **34**, adenosine **31**, cytosine **33** and guanosine **32**. After each step the resin washed multiple times with ACN. An extra levulinyl deprotection cycle was introduced at the end to remove the final Levulinyl protective group. As a final step the resin was washed multiple 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 ($\approx 4.0 \mu\text{mol}$) 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 a 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 NH₄OAc/ACN (1:1, v:v) (noted the residue is not soluble in either H₂O or ACN, only a mixture of both). 100 μL was taken and diluted with 1mL of H₂O for LC-MS analysis. The 1.9 mL solution and remaining LC-MS solution were combined and transferred to an eppendorf. The eppendorf was cooled using liquid nitrogen for 2 minutes and freeze-dried overnight yielding 7.5 mg (estimated 1.4 μmol, 35%) of a mixture of fragment intermediates and Compound **6** as a white fluffy solid. **LC-MS analysis:** (Gemini 3u C₁₈ 110A analytical column, linear gradient in 12.5 minutes; H₂O: 40 → 0% ; ACN: 50 → 90% ; aq. 0.1M NH₄OAc: 10%) **LC:** R_t 8.4 – 10.6 min. **MS:** [C₂₂₉H₃₁₆N₅₁O₇₅P₉Si₉+H]³⁺: m/z found 1839.3, calculated 1838.6

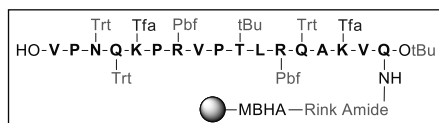


Compound 43: Fmoc-Gly-Ala-Tyr(OpUpU)-Thr-Gly-OH An eppendorf containing 2.8 μmol (3.2 mg) of Compound **5*** was taken and 5.5 μmol (1.9 mg) of Fmoc-OSu (250 μL of a freshly made 22 mM solution in DMF) followed by 11.0 μmol (1.42 mg, 1.82 μL) of DiPEA (250 μL of a 44 mM solution in DMF) and the mixture was left to react overnight at room temperature. 5 μL was taken and added to 50 μL of H₂O/ACN/tBuOH (1:1:1, v:v:v) for LC-MS analysis. The LC-MS showed successful protection of the N-terminus amine, resulting in full conversion of Compound **5** into

Compound **43****. **LC-MS analysis:** (Alltima C₁₈ analytical column, linear gradient in 12.5 minutes; H₂O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) Compound **44'**: **LC:** R_t 6.83 min **MS:** [C₅₀H₆₄N₇O₁₈PSi+H]⁺: found 1110.33, calculated 1110.39. Compound **44**: **LC:** R_t 7.21 min **MS:** [C₅₃H₆₇N₈O₁₈PSi+H]⁺: found 1163.33, calculated 1163.42. Compound **45'**: **LC:** R_t 7.76 min **MS:** [C₅₆H₈₅N₁₁O₂₆P₂Si₂+H]⁺: found 1583.27, calculated 1583.53. Compound **45**: **LC:** R_t 7.88 min **MS:** [C₇₁H₉₅N₁₁O₂₆P₂Si₂+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 H₂N-Rink Amide-MBHA-PS (780 μmol/g) elongated using Solid Phase Peptide Synthesis (SPPS) yielding 89.3 μmol (425

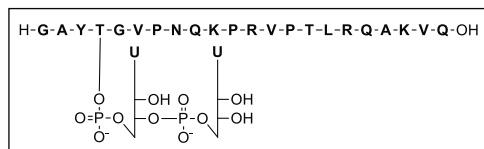
mg, 89.5%) of H-Val-Pro-Asn(Trt)-Gln(Trt)-Lys(Tfa)-Pro-Arg(Pbf)-Val-Pro-Thr(tBu)-Leu-Arg(Pbf)-Gln(Trt)-Ala-Lys(Tfa)-Gln(NH-Rink Amide-MBHA-PS)-OtBu (210 μmol/g).

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 was 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 3.2 mL of a solution of 20% Piperidine in NMP per 100 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 Ac₂O (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/H₂O (95/2.5/2.5, v:v:v) was added and left for 30 minutes. The solvent was dropped into a tube containing 10 mL of cold Et₂O, followed by 500 μL TFA/TIS/H₂O (95:2.5:2.5, v:v:v) and 500 μL of Et₂O 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 H₂O. 200 μL was taken and diluted with 1 mL of H₂O for LC-MS analysis. **LC-MS analysis:** (Alltima C₁₈ analytical column, linear gradient in 12.5 minutes; H₂O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:** R_t 4.48 min **MS:** [C₉₀H₁₄₉F₆N₂₉O₂₅+2H]²⁺: found 1076.60, calculated 1076.57.

full conversion of Compound **45** and **45'** into Compound **48**. **LC-MS analysis:** (Alltima C₁₈ analytical column, linear gradient in 12.5 minutes; H₂O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) Compound **48**: **LC:** R_t 5.09 min **MS:** [C₁₃₆H₂₂₈N₃₈O₄₆P₂Si₂+2H]²⁺: found 1625.73, calculated 1625.29. [C₁₃₆H₂₂₈N₃₈O₄₆P₂Si₂+H+F₃CCOOH]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 NH₄⁺HCO₃⁻ (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. NH₄HCO₃ eluent. After LC-MS analysis the obtained fractions were concentrated, redissolved in 1 mL of H₂O/ACN/tBuOH (1:1:1, v:v:v) and freeze-dried to remove NH₄HCO₃ salt. **LC-MS analysis:** (Alltima C₁₈ analytical column, linear gradient in 12.5 minutes; H₂O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) Compound **49**: **LC:** R_t 5.10 min **MS:** [C₁₂₄H₂₀₀N₃₈O₄₆P₂+2H]²⁺: found 1511.53, calculated 1511.20 [C₁₂₄H₂₀₀N₃₈O₄₆P₂+H+F₃CCOOH]²⁺: found 1566.87, calculated 1568.20 [C₁₂₄H₂₀₀N₃₈O₄₆P₂+3H]³⁺: found 1007.67, calculated 1007.81.

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