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Title: Synthetic methodology for the preparation of nucleic acid containing peptides
Date: 2012-03-29
Chapter 6

Synthesis of Nucleotidylated Poliovirus VPg Proteins

Introduction

Nucleotidylation of proteins is a widespread post-translational modification.\textsuperscript{2-5} For example, adenylylation of tyrosine has been recognized as an important regulatory event, both in bacteria and in higher organisms.\textsuperscript{6,7} A number of RNA viruses use the uridylylated or guanylylated form of VPg (Viral Protein genome-linked) proteins to initiate the replication of the genome.\textsuperscript{8} Perhaps the most investigated protein nucleotidylation process takes place in Picornaviridae as described in Chapter 5.\textsuperscript{9} These viruses share the presence of a small VPg, that is covalently linked to the 5'-end of the viral genome.\textsuperscript{10} Although the mechanism of replication of picorna viruses has not been clarified completely, extensive research has shown that VPg and/or its uridylylated forms VPgpU or VPgpUpU can act as primers in both negative and positive strand RNA synthesis.\textsuperscript{11-15} VPgpU and VPgpUpU, in turn, are presumably formed by uridylylation at the O-4 of tyrosine-3 of the 22 amino acid long VPg protein of poliovirus under influence of a cis-acting replication element (CRE) within the viral RNA.\textsuperscript{16-20} It has been shown that the introduction of point mutations in this CRE motif led to 3D polymerase mediated reaction of CTP, ATP or GTP with O-4 tyrosine of VPg to produce VPgpC, VPgpA and VPgpG respectively.\textsuperscript{16} Although all four nucleosides can be incorporated, suggesting that the 3D polymerase is not specific for UTP as incoming nucleotide donor, VPgpG and VPgpA are not elongated towards the dinucleotide and VPgpC only in moderate amount when compared to VPgpU, resulting in a lower virulence.
As part of a program to develop synthetic routes towards nucleotidylated proteins and derivatives thereof a solid phase synthesis to obtain nucleotidylated VPg proteins, containing all four naturally occurring ribonucleotides was undertaken. The approach presented in Chapter 5 to synthesize a pre-uridylylated tyrosine building block is adapted here towards the preparation of adenylated, guanylylated and cytidylated Fmoc-tyrosine building blocks that were subsequently used in the synthesis of Poliovirus VPgpA, VPgpC and VPgpG (see Figure 1).

![Figure 1 Poliovirus VPgpC, VPgpA and VPgpG](image)

Results and Discussion

A solid phase synthesis using pre-nucleotidylated Fmoc-tyrosine building blocks, as was described for VPgpU in Chapter 5, is considered to be most convenient for the synthesis of poliovirus derived VPgpC, VPgpA and VPgpG. Key to the success of such a strategy is the choice of the protecting groups for the exocyclic amino functions of the nucleobases. Such protecting groups should be stable during the solid phase peptide synthesis and leave the peptide intact during deprotection at the end of the synthesis. Acyl protective groups are an obvious choice as these are well established in the field of nucleopeptide synthesis. Because the stability of acyl protecting groups depends on the nature of the nucleobase, the benzoyl group was selected to protect the exocyclic amino functions of both cytidine and adenosine while guanosine was equipped with the more labile phenoxyacetyl group.

The syntheses of protected nucleotidylated tyrosine building blocks 16-18 are shown in Scheme 1. Known N\(^4\)-benzoylcytidine 4, N\(^6\)-benzoyladenosine 5 and N\(^2\)-phenoxyacetylguanosine 6 were selectively silylated with TBDMSCl in pyridine, treated with isobutyric anhydride and subsequently desilylated with acid to give partially protected nucleosides 7, 8 and 9, respectively.

Treatment of 7, 8 and 9 with 2-cyanoethoxy-\(N,N\)-diisopropylaminochlorophosphine in the presence of triethylamine as base led to 5'-phosphoramidites 10, 11 and 12. Tetrazole mediated phosphitylation of the free hydroxyl function on the side chain of Fmoc-Tyr-OAll with amidites 10, 11 and 12 and oxidation of the intermediate phosphate triesters with \(m\)-CPBA was followed by liberation of the acid by palladium catalyzed removal of the allyl protective group to give the respective nucleotidylated amino acid building blocks 16, 17 and 18.
Scheme 1. Synthesis of Fmoc-tyrosine derivates

Reagents and conditions: i. TBDMSCl, pyr., ii. iBu2O, pyr., iii. pTsOH, iv. 2-cyanoethoxy-N,N-diisopropylaminochlorophosphine, TEA, DCM, 45 min, v. Fmoc-Tyr-OAll, tetrazole, MeCN/DCM, 45 min, r.t., vi. mCPBA, 0 °C, vii. Pd(PPh3)4, SnBu3H, THF/DCM, AcOH, r.t., 2h.

The solid phase synthesis of target compounds 1 - 3 started with the synthesis of the protected and immobilized 19-mer peptide 19, a common intermediate en route to VPgpC, VPgpA and VPgpG (Scheme 2). Tentagel S RAM resin was functionalized with the first amino acid by attachment of Fmoc-Glu-OtBu via the side chain acid moiety. Automated solid phase peptide synthesis based on Fmoc chemistry, using commercially available amino acid building blocks and HCTU as condensing agent proceeded uneventfully.

Immobilized 19-mer 19 was manually elongated by the sequential incorporation of the nucleotidylated tyrosine 16, Fmoc protected Ala and Gly using BOP/HOBt to give the fully protected VPgpC (20, Scheme 2). TFA mediated cleavage of the nucleopeptide from the resin and concomitant unmasking of the amino acid side chains, deprotection of exocyclic amine of cytosine with 25% aq. ammonia and final purification with RP-HPLC afforded homogeneous VPgpC 1 in 45% yield based on initial loading of the resin.
Scheme 2. Solid Phase Synthesis of VPgpC, VPgpA and VPgpG

Reagents and conditions: 

\( i. \) SPPS using a repetative cycle of a) 20% piperidine/DMF, b) Fmoc-AA-OH, HCTU, DiPEA, NMP, c) Ac₂O, DiPEA, HOBT, NMP, 

\( ii. \) SPPS using a repetitive cycle of a) 2% DBU/DMF, b) Fmoc-AA-OH, BOP/HOBt or HATU, DiPEA, NMP, c) Ac₂O, DiPEA, HOBT, NMP, 

\( iii. \) TFA/TIS/H₂O, 2.5 h, r.t., 

\( iv. \) 25% aq. ammonia/dioxane, 4 h, r.t.

A similar sequence of reactions as described for the formation of 20, using in this case adenylylated tyrosine 17 gave immobilized partially protected VPgpA 21. In the first instance, cleavage from the solid support, removal of all protecting groups and purification did not result in homogeneous VPgpA. It turned out that despite the relatively high acid stability of the glycosidic linkages of ribonucleosides, TFA treatment of fully protected VPgpA 21 was accompanied by depurination of the adenosine moiety. This was unexpected because the 5'-phosphorylated adenosine residue was reported to be stable to TFA treatment and 5'-adenyl O-tyrosine, specifically, is known to withstand a treatment with strong acid (0.3 N HCl, 37 °C, 3h). Homogeneous VPgpA 2 could be obtained in 10% yield based on initial loading of the resin by acidolysis, RP-HPLC purification of the intermediate nucleopeptide 21, ammonia treatment of the partially protected nucleopeptide and finally a second RP-HPLC purification.

Adoption of the procedure of VPgpC for the synthesis of VPgpG using guanylated tyrosine 18 and LCMS analysis of the crude product showed the presence of truncated sequences while depurination products were lacking. Guided by the work of Bae and Lakshman, who showed that BOP and PyBOP mediated condensations may lead to side reactions at the C-6 of the guanine base, the last three amino acids in the synthesis of VPgpG were incorporated using HATU as coupling reagent. Subsequently, acid mediated cleavage from the solid support and
deprotection using TFA followed by a 25% aq. ammonia treatment and RP-HPLC purification, afforded VPgpG in 29% based on initial loading of the resin.

**Conclusion**

In conclusion, three unprecedented nucleotidylated amino acid building blocks were prepared and applied in the solid phase synthesis of poliovirus derived proteins VPgpC, VPgpA and VPgpG. This methodology opens the way to the synthesis of more elaborated nucleotidylated proteins such as VPgpG of Norovirus.\(^\text{35}\) The synthetic, well defined constructs of this type may prove to be invaluable in the structural and biological studies of viral replication.
Experimental

General: All chemicals were used as received. MeCN and THF were stored over activated 4Å molecular sieves for at least 24 hours prior to use. DCM was distilled over CaH₂ prior to use. Solvents were removed by rotary evaporation under reduced pressure at a temperature not exceeding 40°C. TLC analysis, silica gel column chromatography, NMR, LCMS, HPLC and HRMS techniques were used as described in Chapter 2.

Procedure for solid phase peptide synthesis: The protected peptide 19 was prepared via solid phase peptide synthesis on an ABI-433A (Applied Biosystems, division of Perkin-Elmer) automated peptide synthesizer using the Fmoc based peptide synthesis protocol starting from Tentagel S RAM resin (200 mg, 50 μmol) to which Fmoc-Glu-OtBu was attached via the side-chain carboxylic acid functionality. As repetitive cycle were used:
- Fmoc cleavage using 20% piperidine in NMP.
- Coupling of the appropriate amino acid applying a five-fold excess, activation by 5 eq. HCTU in NMP (0.25M) and 12.5 eq. DiPEA in NMP (1.25 M) for 1 hour.
- Capping using 0.5 M acetic anhydride, 0.125 M DiPEA and 0.015 M HOBt in NMP for 1 min.

The last three amino acids were introduced manually on 25 μmol 19 by a double coupling protocol:
- Fmoc cleavage using 2% DBU in DMF.
- Coupling of the appropriate amino acid applying a five-fold excess, activation by 5 eq. BOP/HOBt (in the preparation of 1 and 2) or HATU (in the preparation of 3) in NMP and 12.5 eq. DiPEA in NMP (1.25 M) for 1 hour.
- Capping using 0.5 M acetic anhydride, 0.125 M DiPEA and 0.015 M HOBt in NMP for 1 min.

N⁴-Benzoyl-2', 3'-di-O-isobutyryl-β-d-cytidine (7)

Cytidine 4 (1.0 g, 2.9 mmol) was dissolved in pyridine (5 mL) after coevaporation with pyridine. TBDMS-Cl (0.87 g, 5.8 mmol) was added and the reaction mixture was stirred until it became a clear solution. After this, isobutyric anhydride (11.6 mmol, 1.93 mL) was added and the reaction mixture was stirred at room temperature for 16 hours, upon which TLC analysis (10% MeOH/DCM) indicated complete conversion of the starting material. The reaction mixture was concentrated and taken up in EtOAc. After washing with sat. aq. NaHCO₃, 5% citric acid and H₂O the organic phase was dried (MgSO₄) and concentrated. The residue was taken up in 4:1 v/v MeCN/H₂O (30 mL), p-TsOH (0.55 g, 2.9 mmol) was added and the reaction mixture was stirred overnight at room temperature. The reaction mixture was concentrated and purified using silica gel column chromatography with a gradient of DCM/MeOH (100/0 – 98/2) to afford the title compound as a white solid (0.88 g, 1.8 mmol, 63 %). ^1H-NMR (400 MHz, CDCl₃) δ 8.36 (d, J = 7.5 Hz, 1H, H₆), 7.88 (d, J = 7.5 Hz, 2H, Arom. Bz), 7.59 - 7.36 (m, 5H, Arom Bz), 7.47 (t, J = 7.7 Hz, 2H, Arom Bz), 7.42 (t, J = 7.7 Hz, 2H, Arom Bz), 7.41 (d, J = 5.1 Hz, 1H, H1'), 5.62 (t, J = 5.3 Hz, 1H, H2'), 5.58 - 5.51 (m, 1H, H3'), 4.38 - 4.17 (m, 1H, H4'), 4.01 (dd, J = 12.4, 1.9 Hz, 1H, H5α'), 3.87 (dd, J = 12.4, 1.9 Hz, 1H, H5β'), 2.74 (t, J = 7.7 Hz, 2H, CH₂Bu), 1.21 - 1.08 (m, 3H, CH₃Bu). ^13C-NMR (100 MHz, CDCl₃); δ 175.6, 175.3 (CO iBu), 167.0 (C2), 162.94 (CO Bz), 155.8 (C6), 145.0 (C4), 132.7 (Cq Bz), 132.6, 128.3, 127.6 (Bz), 97.6 (C5), 88.0 (C1'), 83.6 (C4'), 74.0 (C3'), 70.0 (C2'), 60.6 (C5'), 33.4, 33.3 (CH iBu), 18.5, 18.3, 18.28, 18.2 (CH₃ iBu). IR: 1743, 1653, 1624, 1558, 1481, 1311, 1244, 1099. LCMS (10-90% B in 15 min), Rt = 6.90. ESI-MS: m/z 487.9 [M]+. HRMS [C₂₄H₂₉N₃O₈ + H]+; calcd. 488.2027, found 488.2026.
Adenosine 5 (1.8 g, 4.8 mmol) was dissolved in pyridine (25 mL) after coevaporation with pyridine. TBDMSCl (1.5 g, 10 mmol) was added and the reaction mixture was stirred for 1 hour at room temperature. After this, isobutyric anhydride (20 mmol, 3.7 mL) was added and the reaction mixture was stirred for 48 hours. The reaction mixture was concentrated and taken up in DCM. After washing with 5% citric acid and H₂O the organic phase was dried (MgSO₄) and concentrated. The residue was taken up in 4:1 v/v MeCN/H₂O (50 mL) and p-TsOH (0.8 g, 5 mmol) was added after which the reaction mixture was stirred at room temperature for 2 hours. The reaction mixture was concentrated and purified with silica gel column chromatography using a gradient of EtOAc/PE (0/100 – 45/55) to afford the title compound as a white foam (1.9 g, 3.9 mmol, 78%).

1H-NMR (400 MHz, CDCl₃) δ 9.82 (s, 1H, NH), 8.66 (s, 1H, H₈), 8.31 (s, 1H, H₂), 8.01 (d, J = 7.6 Hz, 2H, Arom. Bz), 7.53 (t, J = 7.3 Hz, 1H, Arom. Bz), 7.43 (t, J = 7.5 Hz, 2H, Arom. Bz), 6.21 (d, J = 7.1 Hz, 1H, H₁'), 6.04 - 5.92 (m, 1H, H₂'), 5.91 (s, 1H, 5'-OH), 5.69 (d, J = 5.2 Hz, 1H, H₃'), 4.31 (s, 1H, H₄'), 4.09 - 3.73 (m, 2H, H₅'), 2.75 - 2.38 (m, 1H, CH₃iBu), 2.56 - 2.38 (m, 1H, CH₂iBu), 1.21 – 1.07 (m, 12H, CH₃iBu).13C-NMR (100 MHz, CDCl₃); δ 175.6, 175.1 (COiBu), 164.6 (CO Bz), 152.3 (C₂), 150.9, 150.2 (C₄, C₆), 142.2 (C₈), 133.3 (C q Bz), 132.8 (Arom. Bz), 128.8 (Arom. Bz), 127.9 (Arom. Bz), 124.4 (C₅), 88.4 (C₁'), 86.3 (C₄'), 72.8 (C₂'), 72.2 (C₃'), 66.5 (CH₂ Pac), 61.9 (C₅'), 33.8, 33.5 (CH₃iBu), 18.8, 18.7, 18.6, 18.5 (CH₃iBu). IR: 3274, 2977, 1743, 1701, 1609, 1582, 1454, 1246, 1153, 1095. LCMS (10-90% B in 15 min), Rt = 6.57 . ESI-MS: m/z 512.2 [M + H]⁺. HRMS [C₂₅H₂₉N₅O₇ + H]⁺; calcd. 512.2135, found 512.2139.

Guanosine 6 (4.17 g, 10 mmol) was dissolved in pyridine (50 mL) after coevaporation with pyridine. TBDMSCl (3.0 g, 20.0 mmol) was added and the reaction mixture was stirred at room temperature until TLC analysis indicated complete consumption of the starting material. Isobutyric anhydride (6.6 mL, 40.0 mmol) was added and the reaction mixture was stirred overnight. After concentration the residue was redissolved in EtOAc (100 mL) and washed with sat. aq. NaHCO₃, 5% citric acid and water. The organic layer was dried (MgSO₄) and directly desilylated without further purification. Towards this end the residue was dissolved in MeCN/water (100 mL, 4:1, v/v) and excess p-TsOH (2.4 g, 14.8 mmol) was added. After concentration the residue was taken up in EtOAc and washed with sat. aq. NaHCO₃ and water. Silica gel column chromatography purification using a gradient of EtOAc in toluene (0/100 – 70/30) yielded the title compound as an off-white powder (4.5 g, 8.5 mmol, 85%).

1H-NMR (400 MHz, CDCl₃) δ 7.78 (s, 1H, H₈), 7.37 (dd, J = 8.5, 7.6 Hz, 2H, Arom. Pac), 7.09 (t, J = 7.4 Hz, 1H, Arom. Pac), 7.05 (d, J = 7.9 Hz, 2H, Arom. Pac), 5.95 (d, J = 7.2 Hz, 1H, H₁'), 5.89 (dd, J = 7.1, 5.5 Hz, 1H, H₂'), 5.71 (dd, J = 5.3, 2.2 Hz, 1H, H₃'), 4.92 (s, 1H, NH), 4.72 (s, 2H, CH₂ Pac), 4.31 (d, J = 1.9 Hz, 1H, H₄'), 4.12 - 3.79 (m, 2H, H₅'), 2.76 - 2.58 (m, 1H, CH /Bu), 2.58 - 2.45 (m, 1H, CH₂ /Bu), 1.23 – 1.11 (m, 12H, CH₃iBu). 13C-NMR (100 MHz, CDCl₃); δ 175.7, 175.2 (CO /Bu), 169.9 (CO Pac), 156.4 (Cq Pac), 155.0 (C₆), 147.0, 146.5 (C₂, C₄), 138.9 (C₈), 129.7, 122.6, 114.7 (Arom. Pac), 87.5 (C₁'), 85.0 (C₄'), 72.6 (C₂'), 71.4 (C₃'), 66.5 (CH₂ Pac), 61.9 (C₅'), 33.7, 33.5 (CH /Bu), 18.8, 18.7, 18.6, 18.5 (CH₃iBu). IR: 2974, 1684, 1608, 1558, 1471, 1407, 1188, 1150, 1096. LCMS (10-90% B in 15 min), Rt = 7.94 . ESI-MS: m/z 558.0 [M+H]⁺. HRMS [C₂₆H₃₁N₅O₉ + H]⁺; calcd. 558.2194, found 558.2194.
2-cyanoethoxy-N,N'-di-isopropylamino-(N4-benzoyl-2', 3'-di-O-isobutyryl-β-D-cytidyl-5'-'yl) phosphine (10)

To a stirred solution of 7 (0.89 g, 1.8 mmol), coevaporated with MeCN, in DCM (5 mL), containing distilled TEA (0.84 mL, 5.9 mmol), 2-cyanoethoxy-N,N-di-isopropylaminochlorophosphine (0.50 mL, 2.25 mmol) was added under an argon atmosphere. The reaction mixture was stirred for 45 min at room temperature. After $^{31}$P-NMR showed complete consumption of the 2-cyanoethoxy-N,N-di-isopropylaminochlorophosphine (80 MHz, CDCl$_3$; $\delta$ 180.4) and formation of the phosphoramidite (80 MHz, CDCl$_3$; $\delta$ 150.3, 149.6), 30 mL DCM was added and the reaction mixture was washed with water and 5% NaHCO$_3$. The organic phase was dried (MgSO$_4$) and concentrated. The residue was applied on a pre-neutralized (1%TEA in PE) silica gel column and eluted with a gradient of EtOAc in PE (50/50 – 100/0) to yield the title compound as an off-white foam. 1H-NMR (400 MHz, CDCl$_3$); $\delta$ 8.18 (t, $J$ = 7.8 Hz 1H, H6), 7.94 – 7.76 (m, 2H, Bz), 7.44 - 7.32 (m, 3H, H5, Bz), 6.24 (d, $J$ = 5.7 Hz, 1H, H1'), 6.17 (d, $J$ = 4.5 Hz, 1H, H1'), 5.38 (dd, $J$ = 5.1, 3.7 Hz, 1H, H2'), 5.33 - 5.28 (m, 1H, H3'), 4.08 - 3.80 (m, 4H, H5', CH$_2$CNEO), 3.69 - 3.62 (m, 2H, CH$i$Pr), 2.76 - 2.69 (m, 2H, CH$_2$CNEO), 2.63 - 2.55 (m, 2H, CH$i$Bu), 1.27 - 1.14 (m, 24H, CH$_3$iBu, CH$_3$iPr). 13C-NMR (100 MHz, CDCl$_3$); $\delta$ 175.6, 175.5, 175.3, 175.2 (CO$i$Bu), 167.3 (C2), 162.7 (CO Bz), 154.6, 144.4, 144.3 (C6, C4), 133.6 (Cq Bz), 133.1, 128.7, 127.8 (Bz), 117.7 (CN), 97.4 (C5), 87.6, 87.1 (C1'), 82.8, 82.7, 82.4, 82.3 (C4'), 74.1, 74.0 (C2'), 70.8, 70.5 (C3'), 62.9, 62.7, 62.6, 62.4 (C5'), 58.8, 58.6, 58.4 (CH$_3$ CNEO), 43.2, 43.2, 43.1, 43.0 (CH$i$Pr), 33.69, 33.66, 33.55 (CH$i$Bu), 24.7, 24.6, 24.5, 24.5 (CH$_2$ CNEO), 20.4, 20.3, 20.2 (CH$_2$ CNEO), 19.0 (CH$i$Bu). $^{31}$P-NMR (161 MHz, CDCl$_3$); $\delta$ 150.3, 149.6. IR: 2980, 1746, 1666, 162 4, 1556, 1481, 1310, 1245, 1153. LCMS (10-90% B in 15 min), Rt = 6.80, ESI-MS: m/z 605.1 [M + H]$^+$ of the corresponding H-Phosphonate. TLCMS: 688.5 [M + H]$^+$.

2-cyanoethoxy-N,N'-di-isopropylamino-(N6-benzoyl-2',3'-di-O-isobutyryl-β-D-adenyl-5'-yl) phosphine (11)

To a stirred solution of 8 (1.3 g, 2.6 mmol), coevaporated with MeCN, in DCM (15 mL), containing distilled TEA (1.2 mL, 8.5 mmol), 2-cyanoethoxy-N,N-di-isopropylaminochlorophosphine (0.7 mL, 3.2 mmol) was added under an argon atmosphere. The reaction mixture was stirred for 45 min at room temperature. After $^{31}$P-NMR showed complete consumption of the 2-cyanoethoxy-N,N-di-isopropylaminochlorophosphine (161 MHz, CDCl$_3$; $\delta$ 180.4) and formation of the product (161 MHz, CDCl$_3$; $\delta$ 150.3, 149.6). 30 mL DCM was added and the reaction mixture was washed with water and 5% NaHCO$_3$. The organic phase was dried (MgSO$_4$) and concentrated. The residue was applied on a pre-neutralized (1%TEA in PE) silica gel column and eluted with a gradient of EtOAc in PE (50/50 – 100/0) to yield the title compound as an off-white foam. 1H NMR (400 MHz, CDCl$_3$); $\delta$ 9.64 (s, 1H, NH), 8.75 (d, $J$ = 4.1 Hz, 1H, H2), 8.53 (d, $J$ = 8.1 Hz, 1H, H8), 8.02 (d, $J$ = 7.3 Hz, 2H, Arom. Bz), 7.56 (t, $J$ = 7.2 Hz, 1H, Arom. Bz), 7.47 (t, $J$ = 7.3 Hz, 2H, Arom. Bz), 6.45, 6.40 (2x d, $J$ = 6.6 Hz, 1H, H1'), 6.00 - 5.79 (m, 1H, H2'), 5.76 - 5.50 (m, 1H, H3'), 4.42 (m, 1H, H4'), 4.09 - 3.77 (m, 4H, H5', CH$_2$ CNEO), 3.74 - 3.56 (m, 2H, CH$i$Pr), 2.79 - 2.43 (m, 4H, CH$_2$ CNEO, CH$i$Bu), 1.31 - 1.00 (m, 24H, CH$_3$iBu, CH$_3$iPr). $^{13}$C-NMR (100 MHz, CDCl$_3$); $\delta$ 175.91, 175.86, 175.4, 175.3 (CO$i$Bu), 164.6 (CO Bz), 154.9 (C2), 152.0, 151.9 (C6), 149.6 (C4), 141.4, 141.3 (C8), 133.7 (Cq Bz), 132.8, 128.9,

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128.8, 127.8 (Arom. Bz), 122.9 (C5), 117.7, 117.6 (CN), 85.4, 85.0 (C1'), 83.9, 83.8, 83.6, 83.5 (C4'), 74.0, 73.9 (C2'), 72.0, 71.7 (C3'), 63.2, 63.0, 62.8 (C5'), 58.8, 58.6, 58.4 (CH2 CNEO), 43.2, 43.1 (CH iPr), 33.9, 33.8, 33.59, 33.57 (CHi Bu), 24.7, 24.6 (CH3 iPr), 20.40, 20.36, 20.32, 20.29 (CH2 CNEO), 18.9, 18.8, 18.7, 18.6 (CH3 iBu). 31P-NMR (161 MHz, CDCl3); δ 150.0, 149.5. IR: 2968, 1746, 1702, 1610, 1582, 1508, 1458, 1245, 1184, 1153. LCMS (10-90% B in 15 min), Rt = 7.37. ESI-MS: m/z 629.1 [M + H]+ of the corresponding H-Phosphonate. TLCMS: 712.6 [M + H]+. HRMS: [C35H48N7O10P + H]+; 712.3218 calcd., 712.3229 found.

2-cyanoethoxy-N,N'-di-isopropylamino-(N2-phenoxyacetyl-2',3'-di-O-isobutyryl-β-D-guanyl-5'-yl) phosphine (12)

To a stirred solution of 9 (0.94 g, 1.77 mmol), coevaporated with MeCN in DCM (10 mL), containing freshly distilled TEA (0.8 mL, 5.8 mmol), 2-cyanoethoxy-N,N-di-isopropylchlorophosphine (0.5 mL, 2.2 mmol) was added under an argon atmosphere. The reaction mixture was subsequently stirred for 45 min at room temperature. After 31P-NMR showed complete consumption of the 2-cyanoethoxy-N,N-diisopropylchlorophosphine (80 MHz, CDCl3; δ 180.4) and formation of the phosphoramidite (31P-NMR, 80 MHz: δ 150.0, 149.1), 20 mL DCM was added and the reaction mixture was washed with water and 5% NaHCO3. The organic phase was dried (MgSO4) and concentrated. The residue was applied on a pre-neutralized (1%TEA in PE) silica gel column and eluted with a gradient of EtOAc in PE (50/50 – 100/0) to yield the title compound as an off-white foam (1.1 g, 1.4 mmol, 78%). 1H-NMR (400 MHz, CDCl3); δ 8.14 (s, 0.5 H, C2), 8.13 (s, 0.5 H, C2), 7.49 – 7.31 (m, 2H, Arom. Pac), 7.13 (t, J = 7.4 Hz, 1H, Arom. Pac), 7.08 – 6.94 (m, 2H, Arom. Pac), 6.11 (dd, J = 9.5, 6.1 Hz, 1H, H1'), 5.88 (dd, J = 10.5, 4.9 Hz, 1H, H2'), 5.72 (td, J = 5.6, 3.4 Hz, 1H, H3'), 4.73 (s, 2H, CH2 Pac), 4.39 - 3.38 (m, 3H, H4'), 3.99 - 3.80 (m, 4H, H5', CH2 CNEO), 3.67 - 2.60 (m, 2H, CHi Pr), 2.75 - 2.54 (m, 3H, CH2 Bu, CH2 CNEO), 1.24 - 1.05 (m, 24H, CH3 iBu, CH3 iPr). 13C-NMR (100 MHz, CDCl3); δ 175.82, 175.76, 175.2, 175.1 (COiBu), 169.6, 169.45 (CO Pac), 156.5, 156.4 (C6), 155.4 (Cq Pac), 148.0, 147.9 (C4), 146.4, 138.1, 137.7, 130.02 (Arom. Pac), 129.97 (Arom. Pac), 123.05 (Arom. Pac), 122.98 (Arom. Pac), 117.7, 114.9 (Arom. Pac), 85.7, 85.3 (C1'), 83.3, 83.2, 82.9, 82.8 (C4'), 73.44, 73.37 (C2'), 71.4, 71.3 (C3'), 67.0 (CH2 Pac), 62.92, 62.85, 62.75, 62.67 (C5'), 58.7, 58.5, 58.3 (CH2 CNEO), 43.46, 42.31, 43.13, 43.08 (CH iPr), 33.8, 33.6, (CH iBu), 24.71, 24.67, 24.64, 24.60 (CH3 iPr), 20.39, 20.35, 20.31 20.28 (CH2 CNEO), 18.91, 18.87, 18.8, 18.6 (CH3 iBu). 31P-NMR (161 MHz, CDCl3); δ 150.1, 149.7. IR: 2972, 1745, 1685, 1599, 1599, 1560, 1497, 1388, 1364, 1183, 1154. LCMS (10-90% B in 15 min), Rt = 7.93. ESI-MS: m/z 674.9 [M+H]+ of the corresponding H-Phosphonate. TLCMS: 758.5 [M+H]+. HRMS: [C16H16N7O10P + H]+; 758.3273 calcd., 758.3280 found.
2-cyanoethoxy-(N\textsuperscript{\textalpha}-Fmoc-tyrosin-4-yl allyl ester)-(N\textsuperscript{\textalpha}-benzoyl-2', 3'-di-O-isobutyryl-\textbeta-0-cytidyl- 5'-yl) phosphate (13)

\[
\text{FmocHN}^{-}\text{O}^{-}\text{All}^{+}
\]

\text{Phosphate (13)}

\(N\textsuperscript{\textalpha}-\text{Fmoc-tyrosine-allyl ester (0.33 g, 0.75 mmol) was added to compound 10 (0.50 g, 0.72 mmol) and the two compounds were coevaporated with MeCN and DCE and then dissolved in 1:1 v/v MeCN/DCM (5 mL). Tetrazole (200 mg, 2.88 mmol) was added and the reaction mixture was stirred for 45 min until }^{31}\text{P-}
\text{NMR (161 MHz: }\delta\text{ 135.36, 134.91) showed complete consumption of the phosphoramidite. The reaction mixture was cooled to 0 °C and }m\text{-CPBA was added in portions until }^{31}\text{P-}
\text{NMR showed complete consumption of the phosphite and formation of the phosphotriester (161 MHz: }\delta\text{ -6.17, -6.31). The reaction mixture was diluted in 20 mL DCM and washed with 5% Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} and water. The organic phase was dried (MgSO\textsubscript{4}) and concentrated. The residue was applied to a silica gel column and eluted with a gradient of EtOAc in PE (50/50 – 100/0) to yield the title compound as a colourless oil in 74 % (0.55 g, 0.53 mmol). 1H-NMR (400 MHz, CDCl\textsubscript{3}); \(\delta\text{ 8.18 - 7.06 (m, 18H, H6, Arom. Fmoc, Arom. Bz, Arom. Tyr.), 6.39 (d, }J\text{ = 8.2 Hz, 0.5H, H1'), 6.30 (d, }J\text{ = 8.2 Hz, 0.5H, H1'), 6.19 (d, }J\text{ = 6.5 Hz, 1H, H5), 5.73 (d, }J\text{ = 8.2 Hz, 0.5H, NH,), 5.41 - 5.18 (m, 4H, H3', H2', CH2 Allyl), 4.74 (m, 1H, CH }\text{α}), 4.40 - 4.26 (m, 10H, H4', H5', CH2 Fmoc, CH2 Allyl, CH2 CNEO, CH Fmoc), 3.16 - 3.06 (m, 2H, CH 2}, 2.59 - 2.55 (m, 2H, CH 2 CNEO), 2.61 - 2.53 (m, 2H, CH iBu), 1.21 - 1.14 (m, 12H, CH 3 iBu). 13C-NMR (100 MHz, CDCl\textsubscript{3}); \(\delta\text{ 175.62, 175.55, 175.4 (CO iBu), 171.2 (CO }\text{α}), 167.2 (C2), 163.1, 163.0 (CO Bz), 155.9, 155 .8 (CO Fmoc), 154.8 (C6), 149.1, 149.0 (Cq Arom. Tyr.), 144.6, 144.3 (C4), 143.8, 143.7, 141.2 (Cq Fmoc), 133.03, 132.99 (Bz), 132.7 (Arom. Tyr.), 132.09 (Bz), 131.5 (CH Allyl), 131.0 (Arom. Tyr.), 128.7 (Bz), 128.0 (Arom. Fmoc), 127.7 (Bz), 127.0, 125.1 (Arom. Fmoc, Arom. Tyr.), 118.9 (CH 2 Allyl), 116.7 (CN), 97.7 (C5), 89.5, 88.9 (C1'), 80.8, 80.7 (C4'), 73.5 (C2'), 69.63, 69.55 (C3'), 67.1, 66.9, 66.0 (CH 2 Fmoc, CH 2 Allyl, C5'), 63.3, 63.2 (CH 3 CNEO), 55.0 (CH }\text{α}), 47.0, 46.4 (CH Fmoc), 37.1 (CH }\text{β}), 33.7 (CH 2 iBu), 19.62, 19.55 (CH 2 CNEO), 18.80, 18.78, 18.75, 18.68 (CH 2 iBu). 31P-NMR (161 MHz, CDCl\textsubscript{3}); \(\delta\text{ -5.86, -5.89. IR: 2980, 1740, 1700, 1668, 1626, 1556, 1506, 1481, 1255, 1188, 1036. LCMS (50 – 90 % B in 15 min), Rt = 6.26. ESI-MS: }m/z 1046.5 [M+H]\textsuperscript{+}. HRMS: [C 54H56N5O15P + H]\textsuperscript{+}; calcd. 1046.3583, found 1046.3593.}

2-cyanoethoxy-(N\textsuperscript{\textalpha}-Fmoc-tyrosin-4-yl allyl ester)-(N\textsuperscript{\textalpha}-benzoyl-2', 3'-di-O-isobutyryl-\textbeta-0-adenylyl- 5'-yl) phosphate (14)

\[
\text{FmocHN}^{-}\text{O}^{-}\text{All}^{+}
\]

\text{Phosphate (14)}

\(N\textsuperscript{\textalpha}-\text{Fmoc-tyrosine-allyl ester (0.26 g, 0.58 mmol) was added to amidite 11 (0.40 g, 0.58 mmol) and the two compounds were coevaporated with MeCN and DCE and then dissolved in 1:1 v/v MeCN/DCM (10 mL). Tetrazole (0.12 g, 1.74 mmol) was added and the reaction mixture was stirred for 45 minutes until }^{31}\text{P-}
\text{NMR (161 MHz: }\delta\text{ 135.36, 134.91) showed complete consumption of the phosphoramidite (161 MHz, CDCl\textsubscript{3}; }\delta\text{ 150.31, 148.83). The reaction mixture was cooled to 0 °C and }m\text{-CPBA was added in portions until }^{31}\text{P-}
\text{NMR (161 MHz: }\delta\text{ -6.17, -6.31) showed complete consumption of the phosphate. The reaction...}
mixture was diluted in DCM and washed with 5% Na2S2O3 and water. The organic phase was dried (MgSO4) and concentrated. The residue was applied to a silica gel column and eluted with a gradient of EtOAc in PE (50/50 – 100/0) to yield the title compound as a colourless oil (0.39 g, 0.37 mmol, 64%) 1H-NMR (400 MHz, CDCl3); δ 9.30, 9.28 (m, 1H, H2), 8.78 (2x s, 1H, H8), 7.95 - 6.89 (m, 17H), 6.53 (d, J = 8.0 Hz, 1H, NH), 6.37 - 6.33 (m, 1H, H1'), 5.92 - 5.68 (m, 1H, CH Allyl, H3'), 5.54 - 5.51 (m, 1H, H2'), 5.28 - 5.15 (m, 2H, CH Allyl), 4.65 - 4.60 (m, 1H, CH α), 4.56 - 4.32 (m, 9H, C4', C5', CH2 Fmoc, CH2 Allyl, CH2 CO), 4.27 - 3.98 (m, 1H, CH Fmoc), 3.12 - 3.03 (m, 2H, CH β), 2.92 - 2.88 (m, 2H, CH2 CO), 2.82 - 2.47 (m, 2H, CH iBu), 1.26 - 1.04 (m, 12H, CH3 iBu). 13C-NMR (100 MHz, CDCl3); δ 175.7, 175.5, 175.2, 175.0 (CO iBu), 171.0, 170.8 (CO α), 165.0, 164.8 (CO Bz), 155.6 (CO Fmoc), 152.7 (C2), 151.3, 151.1 (C4, C6), 149.5, 148.8 (Cq Tyr.), 143.6, 143.2, 142.1, 141.15, 140.9, 140.5 (Cq Fmoc), 139.6 (C8), 134.0, 133.6, 133.4, 133.2 (Cq Arom. Tyr.), 122.7, 122.0 (C5), 119.9 (Arom.) 119.1 (CH2 Allyl), 116.1 (CN), 85.9, 85.5 (C1'), 81.7, 81.6, 81.44, 81.36 (C4'), 73.5, 73.1 (C2'), 70.3, 70.2 (C3'), 67.1, 66.0, 65.9, 65.3 (CH2 Fmoc, CH2 Allyl, CH2 CNEO), 62.9 (C5'), 54.4, 54.0 (CH α), 47.0, 46.9 (CH Fmoc), 36.6, 36.2 (CH2 β), 33.6, 33.4 (CH2 iBu), 19.5, 19.4 (CH2 CNEO), 18.8, 18.7, 18.5 (CH3 iBu). 31P-NMR (161 MHz, CDCl3); δ -6.97, 6.98. IR: 2976, 1738, 1699, 1608, 1583, 1505, 1456, 1247, 1189, 1155, 1029, 961. LCMS (50 – 90  % B in 15 min), Rt = 10.25. ESI-MS: m/z 1070.2 [M+H]+. HRMS: [C55H56N7O14P + H]+; 1070.3695 calcd., 1070.3706 found.

Nα-Fmoc-tyrosine-allyl ester (0.78 g, 1.77 mmol) was added to amidite 12 (1.0 eq., 1.77 mmol) and the two compounds were coevaporated with MeCN and DCE and then dissolved in 1:1 MeCN/DCM (10 mL, v/v). Tetrazole (295 mg, 4.2 mmol) was added and the reaction mixture was stirred for 45 minutes until 31P NMR showed complete consumption of the phosphoramidite (31P-NMR, 161 MHz: δ 134.18, 134.06). The reaction mixture was cooled to 0 oC and m-CPBA was added in portions until 31P NMR showed complete oxidation of the phosphite (31P-NMR, 161 MHz: δ -6.97, 6.98). The reaction mixture was diluted in 20 mL DCM and washed with 5% Na2S2O3 and water. The organic phase was dried (MgSO4) and concentrated. The residue was applied to a silica gel column using a gradient of EtOAc in PE (50/50 – 70/30) to yield the separate epimers at phosphorus as white foams, 233 mg (0.21 mmol, 12 %) (Rt = 0.5, EtOAc) and 476 mg (0.47 mmol, 27 %) (Rt = 0.6, EtOAc) of which analytical samples were repurified using silica gel column chromatography using a gradient of 2 % MeOH in DCM, and a mix of both epimers (0.43 g, 0.38 mmol, 24 %) respectively. A total of 1.14 g, 1.0 mmol, 56 %.

2-cyanoethoxy-(Nα-Fmoc-tyrosin-4-yl-allyl ester)-(N2-phenoxyacetetyl-2',3'-di-O-isobutyryl-β-D-guanyl-5'-yl) phosphate (15)

Nα-Fmoc-tyrosine-allyl ester (0.78 g, 1.77 mmol) was added to amidite 12 (1.0 eq., 1.77 mmol) and the two compounds were coevaporated with MeCN and DCE and then dissolved in 1:1 MeCN/DCM (10 mL, v/v). Tetrazole (295 mg, 4.2 mmol) was added and the reaction mixture was stirred for 45 minutes until 31P NMR showed complete consumption of the phosphoramidite (31P-NMR, 161 MHz: δ 134.18, 134.06). The reaction mixture was cooled to 0 oC and m-CPBA was added in portions until 31P NMR showed complete oxidation of the phosphite (31P-NMR, 161 MHz: δ -6.56, -6.98). The reaction mixture was diluted in 20 mL DCM and washed with 5% Na2S2O3 and water. The organic phase was dried (MgSO4) and concentrated. The residue was applied to a silica gel using a gradient of EtOAc in PE (50/50 – 70/30) to yield the separate epimers at phosphorus as white foams, 233 mg (0.21 mmol, 12 %) (Rt = 0.5, EtOAc) and 476 mg (0.47 mmol, 27 %) (Rt = 0.6, EtOAc) of which analytical samples were repurified using silica gel column chromatography using a gradient of 2 % MeOH in DCM, and a mix of both epimers (0.43 g, 0.38 mmol, 24 %) respectively. A total of 1.14 g, 1.0 mmol, 56 %.

epimer A (Rt = 0.6), (93:7 epimeric ration). 1H-NMR (400 MHz, CDCl3); δ 11.74, 10.33 (2 x s, 1H, NH H1, NH H2), 7.77 (s, 1H, H8), 7.76 – 7.71 (m, 2H, Arom.), 7.56 – 7.53 (m, 2H, Arom.), 7.37 – 7.33 (m, 2H, Arom.), 7.31 - 7.24 (m, 4H, Arom.), 7.01 - 6.95 (m, 6H, Arom.), 6.12 - 6.09 (m, 1H, H2'), 5.97 - 5.95 (m, 2H, H1'), 5.89 - 5.78 (m, 2H, CH Allyl, H3'), 5.26 (m, 2H, CH2 Allyl), 4.60 - 4.52 (m, 7H, CH2 Allyl, CH2 Fmoc, Hδ', Hα), 4.43 (s, 1H, H4'), 4.36 - 4.23 (m, 2H, CH2 CNEO), 4.19 – 4.15 (m, 1H, CH Fmoc), 3.12 - 2.97 (m, 2H, CH β), 2.73 - 2.60 (m, 2H, CH2 CNEO), 2.56 - 2.49 (m, 2H, CH2 iBu), 1.22 – 1.10 (m, 12H, CH3 iBu). 13C-NMR (100 MHz, CDCl3); δ 175.6, 175.1 (CO iBu), 170.8, 170.1 (CO Pac, CO α), 156.9, 155.5 (Cq Pac, C6), 155.0 (CO Fmoc), 148.4, 148.3 (Cq Tyr.), 147.4, 146.5 (C4, C2), 143.6, 140.9 (Cq Fmoc), 134.0 (Cq Tyr), 131.2 (CH Allyl), 130.3 (Arom. Tyr.), 129.4 (Arom. Pac), 127.4, 126.8, 124.9 (Arom. Fmoc), 122.6, 121.9 (C5), 119.8 , 119.7, 119.6, (Arom. Fmoc, Arom. Tyr.), 118.8 (CH2 Allyl),
116.1 (CN), 114.6 (Arom. Pac), 87.0 (C1’), 81.0, 80.9 (C4’), 71.2 (C2’), 70.0 (C3’), 67.0, 66.63, 66.60 (CH2 Fmoc, CH2 Pac, C5’), 65.8 (CH2 Allyl), 62.8, 62.7 (CH2 CNEO), 54.6 (CH α), 46.8 (CH Fmoc), 36.8 (CH β), 33.5, 33.3 (CH iBu), 19.3, 19.2 (CH2 CNEO), 18.9, 18.6, 18.5, 18.4, 18.3 (CH3 iBu). 31P-NMR (161 MHz, CDCl3); δ - 6.94. IR: 2970, 1695, 1610, 1558, 1539, 1497, 1472, 1448, 1188, 1153, 1041. LCMS (50 - 90% B in 15 min), Rt = 7.56 ESI-MS: m/z 1116.6 [M+H]+. αD (CHCl3): -17.4. HRMS: C56H58N7O16P + H]+ calcd. 1116.3750, found 1116.3765.

epimer B (Rt = 0.5), (96:4 epimer ratio). 1H-NMR (400 MHz, CDCl3); δ 11.84, 10.42 (2 x s, 1H, NH α, NH H2), 7.92 - 7.87, 7.75 - 7.73, 7.54 - 7.47, 7.39 - 7.35, 7.29 - 7.25, 7.21 - 7.14 (m, 17H, H6, H5, Arom.), 6.07 (m, 1H, C1’), 5.72 (m, 1H, NH), 5.46 - 5.42 (m, 2H, C3’, C2’), 4.70 (m, 1H, CH α), 4.48 - 4.43 (m, 2H, CH2 Fmoc), 4.33 - 4.31 (m, 3H, C4’, C5’), 4.30 - 4.23 (m, 2H, CH2 CNEO), 4.13 (m, 1H, CH Fmoc), 3.18 - 3.11 (m, 2H, CH2 β), 2.75 - 2.63 (m, 2H, CH2 CNEO), 2.62 - 2.58 (m, 2H, CH iBu), 1.17 - 1.14 (m, 12H, CH3 iBu). 13C-NMR (100 MHz, CDCl3); δ 175.50, 175.46, 175.1 (CO iBu), 174.5 (CO α), 167.2 (C2), 163.1, 163.1 (CO Bz), 155.6 (CO Fmoc), 154.3 (C4), 148.8, 148.7 (Cq Arom. Tyr.), 145.0 (C6), 143.7, 143.6, 141.0 (Cq Arom. Fmoc), 134.0 (Cq Tyr.), 133.0 (Bz), 132.48 (Bz, 131.0 (Arom. Tyr.), 128.50 (Bz), 128.0 (Arom. Fmoc), 127.5 (Bz), 126.9, 125.0, 124.9 (Arom. Fmoc), 119.8, 119.6 (Arom. Tyr.), 116.3 (CN), 97.6 (C5), 89.8, 89.29 (C1’), 80.7, 80.6 (C4’), 73.4 (C2’), 69.4, 69.3 (C3’), 66.95, 66.86, 66.68 (CH2 Fmoc, C5’), 63.02, 62.97 (CH2 CNEO), 54.6 (Ch), 46.9 (CH Fmoc), 37.0 (Cq iBu), 19.43, 19.35 (CH2 CNEO), 18.64, 18.62 (CH3 iBu). 31P-NMR (161 MHz, CDCl3); δ -6.37, -6.77. IR: 1699,

2-cyanoethoxy-(Nα-Fmoc-tyrosin-4-yl)(N4-benzoyl-2’,3’-di-O-isobutyryl-β-D-cytidin-5’-yl) phosphate (16)

Compound 13 (0.55 g, 0.53 mmol) was coevaporated with MeCN and dissolved in 1:1 THF/DCM (10 mL, v/v). After addition of AcOH (2.6 mmol, 0.15 mL), Bu3SnH (1.05 mmol, 0.32 mL) and Pd(PPh3)4 (0.02 mmol, 20 mg) the reaction was stirred for 2 hours. The reaction mixture was concentrated and the residue was applied to a silica gel column and eluted with a gradient of MeOH in DCM (0/100 – 5/95) to yield the title compound as a white foam (0.30 g, 0.29 mmol, 56 %). 1H-NMR (400 MHz, CDCl3); δ 7.92 - 7.87, 7.75 - 7.73, 7.54 - 7.47, 7.39 - 7.35, 7.29 - 7.25, 7.21 - 7.14 (m, 17H, H6, H5, Arom.), 6.07 (m, 1H, C1’), 5.72 (m, 1H, NH), 5.46 - 5.42 (m, 2H, C3’, C2’), 4.70 (m, 1H, CH α), 4.48 - 4.43 (m, 2H, CH2 Fmoc), 4.33 - 4.31 (m, 3H, C4’, C5’), 4.30 - 4.23 (m, 2H, CH2 CNEO), 4.13 (m, 1H, CH Fmoc), 3.18 - 3.11 (m, 2H, CH2 β), 2.75 - 2.63 (m, 2H, CH2 CNEO), 2.62 - 2.58 (m, 2H, CH iBu), 1.17 - 1.14 (m, 12H, CH3 iBu). 13C-NMR (100 MHz, CDCl3); δ 175.50, 175.46, 175.1 (CO iBu), 174.5 (CO α), 167.2 (C2), 163.1, 163.1 (CO Bz), 155.6 (CO Fmoc), 154.3 (C4), 148.8, 148.7 (Cq Arom. Tyr.), 145.0 (C6), 143.7, 143.6, 141.0 (Cq Arom. Fmoc), 134.0 (Cq Tyr.), 133.0 (Bz), 132.48 (Bz, 131.0 (Arom. Tyr.), 128.50 (Bz), 128.0 (Arom. Fmoc), 127.5 (Bz), 126.9, 125.0, 124.9 (Arom. Fmoc), 119.8, 119.6 (Arom. Fmoc, Arom. Tyr.), 116.3 (CN), 97.6 (C5), 89.8, 89.29 (C1’), 80.7, 80.6 (C4’), 73.4 (C2’), 69.4, 69.3 (C3’), 66.95, 66.86, 66.68 (CH2 Fmoc, C5’), 63.02, 62.97 (CH2 CNEO), 54.6 (Ch), 46.9 (CH Fmoc), 37.0 (Cq iBu), 19.43, 19.35 (CH2 CNEO), 18.64, 18.62 (CH3 iBu). 31P-NMR (161 MHz, CDCl3); δ -6.37, -6.77. IR: 1699,
1668, 1624, 1558, 1481, 1450, 1249. LCMS (10 – 90 % B in 15 min) Rt = 9.06. ESI-MS: m/z 1006.7 [M+H]+. HRMS: [C_{51}H_{52}N_{5}O_{15}P + H]+; calcd. 1006.3270, found 1006.3281.

2-cyanoethoxy-(N^α-Fmoc-tyrosin-4-yl)-(N^6-benzoyl-2',3'-di-O-isobutyryl-β-d-adenyl-5'-yl) phosphate (17)

![Diagram of compound 17]

Compound 14 (0.39 g, 0.37 mmol) was coevaporated with MeCN and dissolved in 1:1 THF/DCM (10 mL, v/v). After addition of AcOH (1.7 mmol, 0.10 mL), Bu_{3}SnH (0.74 mmol, 0.21 mL) and Pd(PPh_{3})_{4} (0.015 mmol, 18 mg) the reaction was stirred for 2 hours. The reaction mixture was concentrated and the residue was applied to a silica gel column and eluted with a gradient of MeOH in DCM (0/100 – 5/95) to yield the title compound as a white foam (0.32 g, 0.31 mmol, 85 %). 1H-NMR (400 MHz, CDCl_{3}); δ 8.78 (2x s, 1H, H8), 8.12, 7.86 (2x s, 1H, H2), 7.98 - 6.89 (m, 17H), 6.43, 6.38 (2x d, J = 7.2 Hz, 1H, H1'), 5.97, 5.95 (d, J = 7.6 Hz, 1H, NH), 5.75 - 5.59 (m, 2H, C3', C2'), 4.52 - 4.36 (m, 7H, CH_{2}Fmoc, CH_α, C4', CH_2CNEO, CH Fmoc), 4.17 - 4.15 (m, 2H, H5'), 3.27 - 3.21 (m, 2H, CH_β), 2.98 - 2.92 (m, 2H, CH CNEO), 2.72 - 2.48 (2x m, 2H, CH_iBu), 1.29 - 1.06 (m, 12H, CH_3iBu). 13C-NMR (100 MHz, CDCl_{3}); δ 175.5, 175.3 (CO_iBu), 174.4, 174.0 (CO_α), 165.7 (CO Bz), 155.4 (CO Fmoc), 153.1 (C2), 151.6 (Cq Arom. Tyr.), 149.8 (C6), 149.0 (C4), 143.75, 143.68, 143.61, 141.2 (Cq Arom. Fmoc), 140.4 (Cq Tyr.), 133.5 (Cq Bz), 132.7 (Arom. Bz), 131.2, 131.0 (Arom. Tyr.), 128.5, 128.5 (Arom. Bz), 128.2 (Arom. Fmoc), 127.7, 127.6 (Arom. Bz), 127.0, 125.0, 125.0, 124.9, 124.7 (Arom. Fmoc), 122.0, 121.8 (C5), 119.9, 119.9, 119.7 (Arom. Fmoc, Arom. Tyr.), 116.2, 116.1 (CN), 85.2, 84.8 (C1'), 82.0, 81.9 (C4'), 73.6, 73.3 (C2'), 70.4, 70.0 (C3'), 67.6, 67.1, 66.6, 66.4 (CH_2 Fmoc, CH_2 CNEO), 63.0, 63.0, 62.8, 62.8 (C5'), 55.0, 54.8 (CH_α), 47.2 (CH Fmoc), 38.3, 37.9 (CH_β), 33.79, 33.77, 33.56, 33.53 (CH_iBu), 19.7, 19.6 (CH_3 CNEO), 18.9, 18.8, 18.7, 18.6, 18.60, 18.57 (CH_iBu). 31P-NMR (161 MHz, CDCl_{3}); δ -6.8, -7.1. IR: 2976, 2360, 1738, 1714, 1700, 1584, 1505, 1455, 1247, 1155, 1032. LCMS (10 – 90 % B in 15 min) Rt = 9.39. ESI-MS: m/z 1030.1 [M+H]^+. HRMS: [C_{52}H_{52}N_{7}O_{14}P + H]^+; calcd., 1030.3394 found.

2-cyanoethoxy-(N^α-Fmoc-tyrosin-4-yl)-(N^3-phenoxyacetetyl-2',3'-di-O-isobutyryl-β-d-guanyl-5'-yl) phosphate (18)

![Diagram of compound 18]

A mixture epimers of 15 (0.43 g, 0.38 mmol) was coevaporated with MeCN and dissolved in 1:1 v/v THF/DCM (10 mL). After addition of AcOH (2.0 mmol, 0.11 mL), Bu_{3}SnH (0.36 mL, 0.76 mmol) and Pd(PPh_{3})_{4} (0.02 mmol, 20 mg) the reaction was stirred for 2 hours. The reaction mixture was concentrated and the residue was applied to a silica gel column and eluted with a gradient of MeOH in DCM (0/100 – 4/96) containing 1% of AcOH to yield the title compound as a white foam (0.23 g, 0.21 mmol, 56 %).
NMR (400 MHz, CDCl₃); δ 7.88 (s, 1H, H8), 7.75 - 6.99 (m, 2H, Arom.), 7.59 - 7.57 (m, 2H, Arom), 7.39 - 7.35 (m, 2H, Arom), 7.31 - 7.27 (m, 4H, Arom), 7.13 (m, 2H, Arom), 7.04 - 6.99 (m, 5H, Arom), 6.00 - 5.92 (m, 2H, H1′, H3′), 5.88 - 5.85 (m, 1H, H2′), 4.68 (m, 2H, CH₂ Pac), 4.48 - 4.17 (m, 5H, CH₂), 4.17 - 4.08 (m, 5H, CH₂), 4.02 - 3.78 (m, 1H), 3.70 (m, 1H), 3.51 - 3.39 (m, 2H), 2.30 - 2.25 (m, 4H), 2.17 - 1.93 (m, 4H), 1.93 - 1.40 (m, 4H), 1.22 - 1.18 (m, 9H), 1.16 (m, 9H). 

VPgPC, (H-Gly-Ala-Tyr(pC)-Thr-Leu-Pro-Asn-Lys-Lys-Pro-Val-Pro-Thr-Ile-Arg-Thr-Ala-Lys-Val-Glu(OH)) (1)

The crude protected nucleopeptide was cleaved off the resin using TFA/TIS/H₂O (95/5/5, v/v/v, 5 mL) by shaking for 2.5 hours. After filtration into cold Et₂O the precipitate was coevaporated with toluene (2 x 5 mL) and then treated with 25%aq. ammonia (2.5 mL) in 1,4-dioxane (2.5 mL). After stirring for 4 hours, concentration of the reaction mixture afforded the crude title compound. RP-HPLC purification was conducted on an automated HPLC system supplied with a semi-preparative C₁₈ column (250 x 10.00 mm, 5 μM, flow: 4 mL/min). The applied eluent system was: A: H₂O, B: MeCN, C: 0.5% TFA and detection at 280 nm. Gradient: 10 - 25% B in 3 CV. The collected fractions were lyophilized, yielding 14.89 mg, 5.6 μmol (45% based on original loading of the resin) of the nucleopeptide. 31P-NMR (161 MHz, D₂O); δ - 4.24. LCMS (00 - 90% B in 15 min.), Rt = 4.18 min. ESI-MS: m/z 2659.2 [M]⁺, 1330.4 [M]²⁺, 887.6 [M]³⁺, HRMS: [C₁₁₄H₁₈₉N₃₄O₃₇P + H]⁺ calc. 1329.6918, found 1329.6937, [M + H] 3+ calcd. 886.7981, found 886.7981, [M + H] 4+ calcd. 1076.3437, found 1076.3449.

VPgPA, (H-Gly-Ala-Tyr(pA)-Thr-Leu-Pro-Asn-Lys-Lys-Pro-Val-Pro-Thr-Ile-Arg-Thr-Ala-Lys-Val-Glu(OH)) (2)

The crude partially protected nucleopeptide was cleaved off the resin using TFA/TIS/H₂O (95/5/5, v/v/v, 5 mL) by shaking for 4 hours. After filtration into cold Et₂O the precipitate was concentrated and RP-HPLC purification was conducted on an automated HPLC system supplied with a semi-preparative C₁₈ column (250 x 10.00 mm, 5 μM, flow 4 mL/min). The applied eluent system was: A: 0.5% TFA in H₂O, B: MeCN, Gradient: 20 - 50% B in 3 CV and detection at 280 nm. to give partially protected VPgA, (H-Gly-Ala-Tyr(pA)-Thr-Leu-Pro-Asn-Lys-Lys-Pro-Val-Pro-Thr-Ile-Arg-Thr-Ala-Lys-Val-Glu(OH)) (2)

The crude partially protected nucleopeptide was cleaved off the resin using TFA/TIS/H₂O (95/5/5, v/v/v, 5 mL) by shaking for 4 hours. After filtration into cold Et₂O the precipitate was concentrated and RP-HPLC purification was conducted on an automated HPLC system supplied with a semi-preparative C₁₈ column (250 x 10.00 mm, 5 μM, flow: 4 mL/min). The applied eluent system was: A: 0.5% TFA in H₂O, B: MeCN, Gradient: 20- 50% B in 3 CV and detection at 280 nm. The collected fractions were lyophilized, yielding the target nucleopeptide 21; 6.1 mg, 2.3 μmol (9% based on initial loading of the resin). 31P-NMR (161 MHz, D₂O); δ - 4.21. LCMS (00 - 90% B in 15 min.), Rt = 4.18 min. ESI-MS: m/z 1345.0 [M]⁺, 896.6 [M]²⁺, 672.7 [M]³⁺. HRMS: [C₁₁₄H₁₈₉N₃₄O₃₇P + H]⁺ calc. 1341.6974, found 1341.6949, [M + H] 3+ calc. 894.8007, found 894.8008, [M + H] 4+ calc. 671.3524, found 671.3532, [M + H] 5+ calc. 537.2833, found 537.2839. ¹H NMR (400 MHz, D₂O) δ 8.36 (s, 1H), 8.33 (s, 1H), 6.96 (q, J = 8.8 Hz, 4H), 6.13 (d, J = 5.6 Hz, 1H), 4.70 - 4.56 (m, 1H), 4.56 - 4.39 (m, 4H), 4.39 - 4.27 (m, 6H), 4.27 - 4.08 (m, 5H), 4.02 - 3.78 (m, 4H).
VPgpG, \((H\text{-Gly-Ala-Tyr(pG)}\text{-Thr-Gly-Leu-Pro-Asn-Lys-Pro-Asn-Val-Pro-Thr-Ile-Arg-Thr-Ala-Lys-Val-Glu-OH}) (3)\)

The crude partially protected nucleopeptide was cleaved of the resin using TFA/TIS/H\textsubscript{2}O (95/5/5, v/v/v, 5 mL) by shaking for 2.5 hours. After filtration into cold Et\textsubscript{2}O the precipitate was coevaporated with toluene (2 x 5 mL) and then treated with 25% aq. ammonia (1.25 mL) in 1,4-dioxane (1.25 mL). After stirring for 5 hours careful concentration of the reaction mixture afforded the crude title compound. RP-HPLC purification was conducted on an automated HPLC system supplied with a semi-preparative C\textsubscript{18} column (250 x 10.00 mm, 5 \(\mu\)M, flow: 4 mL/min). The applied eluent system was; A: H\textsubscript{2}O, B: MeCN, C: 0.5 % TFA in H\textsubscript{2}O, in a gradient of 10-30 % B (10% C as stationary phase) in 3 CV and detection at 280 nm. The collected fractions were lyophilized, yielding the target nucleopeptide 22; 9.8 mg, 3.6 \(\mu\)mol (29% based on initial loading of the resin). \(^{31}\text{P}-\text{NMR} \ (161 \text{ MHz, D}_2\text{O}); \delta - 4.21. \text{LCMS} \ (00 - 90\% \text{ B in 5 min.}, \ \text{Rt} = 4.21 \text{ min.}, \ \text{ESI-MS} \ : \text{m/z} 
2700.2 \ [\text{M + H}]^+, \ 1350.2 [\text{M+H}]^{2+}, \ 900.6 [\text{M+H}]^{3+}. \ \text{HRMS}: [\text{C}_{115}\text{H}_{189}\text{N}_{36}\text{O}_{37}\text{P} + \text{H}]^{2+} \text{calcd. 1349.6948, found 1349.6952, [M + H]}^{3+} \text{calcd. 900.1323, found 900.1327, [M + H]}^{4+} \text{calcd. 675.3511, found 675.3508, [M + H]}^{5+} \text{calcd. 540.4823, found 540.4816.} \ ^{1}\text{H-NMR} \ (600 \text{ MHz, D}_2\text{O}) \ \delta \ 7.85 \ (s, 1\text{H}), \ 7.08 - 6.84 \ (m, 4\text{H}), \ 5.86 \ (d, J = 5.9 \text{ Hz}, 1\text{H}), \ 4.90 \ (ap. t, J = 5.6 \text{ Hz}, 1\text{H}), \ 4.66 \ (ap. t, J = 7.1 \text{ Hz}, 1\text{H}), \ 4.63 - 4.59 \ (m, 2\text{H}), \ 4.57 \ (ap. d, J = 11.2 \text{ Hz}, 1\text{H}), \ 4.50 - 4.37 \ (m, 9\text{H}), \ 4.37 - 4.26 \ (m, 13\text{H}), \ 4.26 - 4.08 \ (m, 15\text{H}), \ 3.91 \ (ap. d, J = 13.7 \text{ Hz}, 3\text{H}), \ 3.89 - 3.79 \ (m, 5\text{H}), \ 3.77 \ (ap. d, J = 3.6 \text{ Hz}, 3\text{H}), \ 3.70 \ (ap. d, J = 7.7 \text{ Hz}, 2\text{H}), \ 3.64 \ (s, 4\text{H}), \ 3.19 \ (ap. t, J = 6.0 \text{ Hz}, 5\text{H}), \ 3.09 - 2.92 \ (m, 15\text{H}), \ 2.89 \ (ap. dd, J = 13.8, 8.9 \text{ Hz}, 2\text{H}), \ 2.83 - 2.62 \ (m, 8\text{H}), \ 2.38 - 2.20 \ (m, 11\text{H}), \ 2.13 - 1.39 \ (m, 92\text{H}), \ 1.37 \ (ap. d, J = 7.2 \text{ Hz}, 10\text{H}), \ 1.27 \ (ap. d, J = 7.2 \text{ Hz}, 7\text{H}), \ 1.21 - 1.11 \ (m, 23\text{H}), \ 0.95 \ (ap. dd, J = 13.3, 6.8 \text{ Hz}, 23\text{H}), \ 0.90 \ (ap. dd, J = 13.5, 5.4 \text{ Hz}, 25\text{H}), \ 0.85 \ (ap. t, J = 7.3 \text{ Hz}, 10\text{H}).\)
Notes and References

Synthesis of Nucleotidylated Poliovirus VPg Proteins


