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Synthesis of Nucleotidylated Poliovirus VPg Proteins

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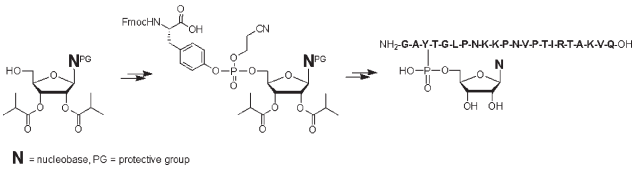

Phosphitylation of the side chain hydroxyl function of Fmoc protected tyrosine with 5′-phosphoramidites of suitably protected cytidine, adenosine, and guanosine, followed by oxidation gave three novel nucleotidylated amino acid building blocks. After protective group manipulation, these building blocks were used in a solid phase peptide synthesis to afford the nucleotidylated poliovirus proteins VPgpC, VPgpA, and VPgpG.

Nucleotidylation of proteins is a widespread posttranslational modification.[1−4] For example, adenylylation of tyrosine has been recognized as an important regulatory event both in bacteria and in higher organisms.[5,6] A number of RNA viruses use the uridinylylated or guanylylated form of so-called VPg proteins to initiate the replication of the genome (VPg = Viral Protein genome-linked).[7] Perhaps the most investigated protein nucleotidylation process takes place in *Picornaviridae*, a family of positive strand RNA viruses that causes illnesses such as poliomyelitis, common cold, hepatitis A, and foot and mouth disease.[8] All these viruses share the presence of a small VPg, which is covalently linked to the 5′-end of the viral genome.[9] Although the mechanism of replication of *Picornaviridae* has not been clarified completely extensive research on for instance poliovirus has shown that VPg and its uridinylylated forms, VPgpU and VPgpUpU, act as primers in both negative- and positive-strand RNA synthesis.[10−14] In poliovirus VPgpU the O^4 of tyrosine-3 of the 22 amino acid long VPg protein is modified with a 5′-uridinylyl moiety, whereas the elongated version VPgpUpU is modified with a 5′-(uridinylyl-(5′→3′))-uridinylyl moiety. These poliovirus nucleoproteins are formed under influence of a cis-acting replication element (CRE) within the viral RNA.[15−19] It has been shown that the introduction of point mutations in this CRE motif led to reaction of CTP, ATP, or GTP with O^4 tyrosine of VPg to produce VPgpC, VPgpA, and VPgpG, respectively.[15]

As part of a program[20] to develop synthetic routes to naturally occurring nucleotidylated proteins and derivatives thereof we set out to develop a solid phase synthesis of nucleotidylated poliovirus VPg's. We demonstrate the viability of a generic, solid-phase approach toward the nucleotidylated polypeptide containing all four naturally occurring ribonucleotides. In earlier reports VPgpU and VPgpUpU of certain picornaviruses were assembled either by block coupling or by solid-phase peptide synthesis.[21−23] These nucleopeptides prove to be valuable tools in the ongoing research on viral replication of picornaviruses.[24−26] For the synthesis of poliovirus derived VPgpC, VPgpA, and VPgpG a solid-phase synthesis with prenucleotidylated Fmoc-tyrosine building blocks appeared most convenient.[23] 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 their deprotection procedure at the end of the synthesis.

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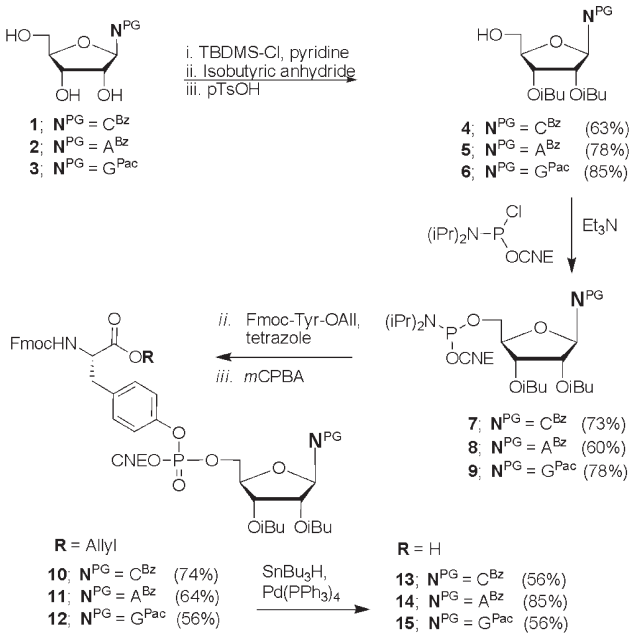
SCHEME 1. Synthesis of Fmoc-Tyrosine Derivatives



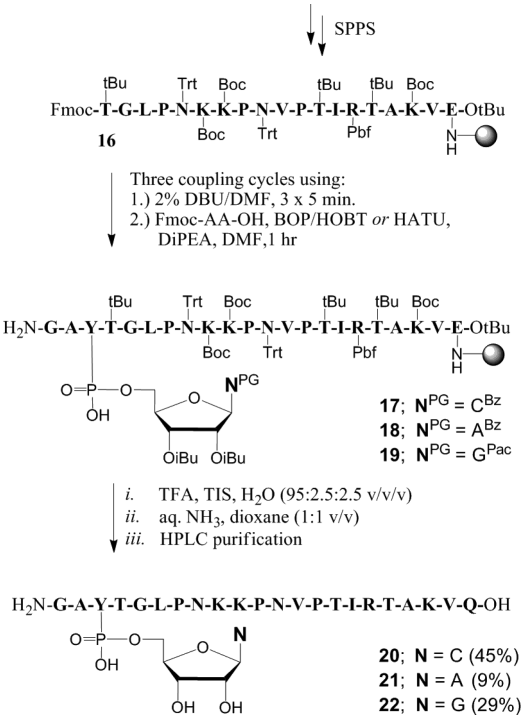
We selected the acyl-based protective groups as those are well established in the field of nucleopeptide synthesis.[27,28] Because the stability of acyl protecting groups depends on the nature of the nucleobase, the benzoyl group was selected to protect the exocyclic amino functions of both cytidine and adenosine while guanosine was equipped with the more labile phenoxyacetyl group.[29]

The solid-phase synthesis of the target nucleopeptides starts with the solution-phase synthesis of suitably protected nucleotidylated tyrosine building blocks **13**−**15** (Scheme 1). Known 4-*N*-benzoylcytidine[30] (**1**), 6-*N*-benzoyladenosine[31] (**2**), and 2-*N*-phenoxyacetylguanosine[32] (**3**) were silylated with TBDMSCl in pyridine, treated with isobutyric anhydride, and subsequently desilylated with acid to give partially protected nucleosides **4**, **5**, and **6**, respectively.

Treatment of **4**, **5**, and **6** with 2-cyanoethoxy-*N*,*N*-diisopropylaminochlorophosphine in the presence of triethylamine as base led to 5′-phosphoramidites **7**, **8**, and **9**. Tetrazole-mediated phosphitylation of the free hydroxyl function on the side chain of Fmoc-Tyr-OAll with amidites **7**, **8**, and **9** followed by oxidation of the intermediate phosphite triesters with *m*CPBA gave **10**, **11**, and **12**. Subsequent liberation of the acid by palladium-catalyzed removal of the allyl protective group gave the respective nucleotidylated amino acid building blocks **13**, **14**, and **15**.

Next attention was directed to the synthesis of the protected and immobilized 19-mer peptide **16**, a common intermediate en route to target VPgpC, VPgpA, and VPgpG (Scheme 2). Tentagel S Ram resin was functionalized with the first amino acid by attachment of Fmoc-Glu-OtBu via its side chain.[23]

SCHEME 2. Solid-Phase Synthesis of VPgpC, VPgpA, and VPgpG



Automated solid-phase peptide synthesis based on Fmoc chemistry with use of commercially available amino acid building blocks and HCTU as condensing agent proceeded uneventfully.

Immobilized 19-mer **16** was manually elongated by the sequential incorporation of citydinylated tyrosine **13**, using a BOP/HOBt based coupling protocol. Treatment with 2% DBU in DMF resulted in cleavage of the Fmoc and cyanoethyl protecting groups, after which the phosphodiester containing peptide was elongated with Fmoc-Ala-OH and Fmoc-Gly-OH to give the partially protected VPgpC (**17**, Scheme 2). TFA-mediated cleavage of the nucleopeptide from the resin and concomitant unmasking of the amino acid side chains, deprotection of all acyl protection groups with 25% aq ammonia, and final purification with RP-HPLC afforded homogeneous VPgpC **20** in 45% yield based on initial loading of the resin (Figure 1).

A similar sequence of reactions as described for the formation of **17**, using in this case adenylylated tyrosine **14**, gave immobilized VPgpA **18**. In the first instance, cleavage from the solid support, removal of the remaining protecting groups, and purification did not result in homogeneous VPgpA. It turned out that despite the relative acid stability of the glycosidic linkages of ribonucleosides, TFA treatment of partially protected VPgpA **18** was accompanied by depurination of the adenosine moiety, which was not separable from nucleoprotein **21**.[33] This was unexpected because the 5′-phosphorylated adenosine residue was reported to be stable to TFA treatment[34] and 5′-adenylyl *O*-tyrosine, specifically, is known to withstand a treatment with strong acid (0.3 N HCl, 37 °C, 3 h).[35] Homogeneous VPgpA

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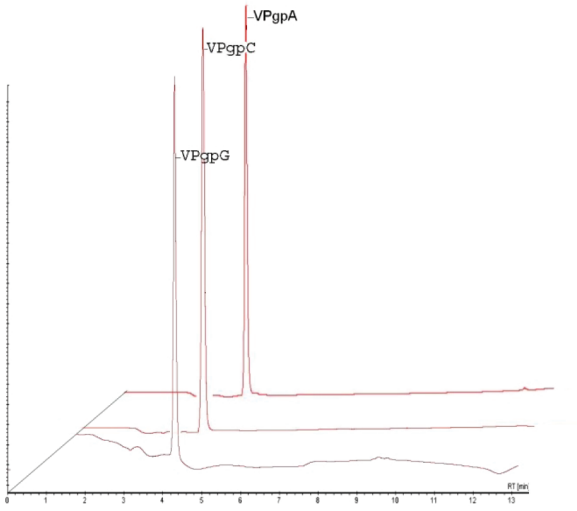

FIGURE 1. LCMS traces of purified VPgpG, VPgpC, and VPgpA.

21, however, could be obtained in 9% yield based on initial loading of the resin by acidolysis, RP-HPLC purification of the intermediate nucleopeptide, ammonia treatment, and finally a second RP-HPLC purification (Figure 1).

Adoption of the procedure of VPgpC for the synthesis of VPgpG 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[36,37] reporting that BOP and PyBOP mediated condensations may lead to side reactions at the C-6 of guanosine we introduced the last three amino acids in the synthesis of VPgpG using the HATU coupling reagent.[38] Treatment with TFA, 25% aq ammonia, and RP-HPLC purification afforded VPgpG in 29% yield based on initial loading of the resin (Figure 1).

In conclusion, three unprecedented nucleotidylated amino acid building blocks were prepared and applied in a concise solid-phase procedure to the 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. The synthetic, well-defined constructs of this type may prove to be invaluable in the structural and biological studies of viral replication.

Experimental Section

The experimental data for compounds **4**, **7**, **10**, **13**, and **20** are given as typical representatives of VPgpN synthesis.

4-*N*-Benzoyl-2′,3′-di-*O*-isobutyryl-*β*-D-cytidine (4). 4-*N*-benzoyl-*β*-D-cytidine (**1**) (1.0 g, 2.9 mmol) was dissolved in pyridine (5 mL) after coevaporation with pyridine. TBDMS-Cl (5.8 mmol, 0.87 g) was added and the reaction mixture was stirred until it became a clear solution. After this isobutyric anhydride (1.6 mmol, 1.93 mL) was added and the reaction mixture was stirred at room temperature for 16 h, 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 $NaHCO_3$, 5% citric acid, and H_2O the organic phase was dried ($MgSO_4$) and concentrated. The residue was taken up in 4:1 MeCN/H_2O (30 mL, v/v) and *p*-TsOH (1 equiv, 0.55 g) was added after which the reaction mixture was stirred overnight at room temperature. The reaction mixture was concentrated and purified with silica gel column chromatography DCM/MeOH (100/0 to 98/2) to afford the title compound as a white foam (0.88 g, 1.8 mmol, 63%). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.1, 175.6, 162.8, 155.4, 145.7 (C6 Cyt.), 133.1, 133.0 (Arom. Bz), 128.8 (Arom. Bz), 127.8 (Arom. Bz), 97.7 (C5 Cyt.), 88.9 (C1′), 84.1 (C4′), 74.2 (C2′), 71.0 (C3′), 61.3 (C5′), 33.8, 33.7 (CH *i*Bu), 18.8, 18.7 (CH_3 *i*Bu). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42, 8.40 (d, 1H, H6 Cyt.), 7.83, 7.81 (d, 2H, Bz), 7.52−7.49 (m, 2H, Arom. Bz, H5 Cyt.), 7.41−7.37 (m, 2H, Arom. Bz), 6.19, 6.18 (d, 1H, H1′), 5.58−5.53 (m, 2H, H2′, H3′), 4.40 (br s, 1H), 4.22−4.21 (m, 1H, H4′), 3.98−3.83 (dd, 2H, H5′), 2.58−2.49 (m, 2H, CH *i*Bu), 1.13−1.08 (m, 12H, CH_3 *i*Bu). IR 1743, 1653, 1624, 1558, 1481, 1311, 1244, 1099. LCMS (10−90% MeCN in 15 min), Rt = 6.90. ESI-MS *m*/*z* 487.9 $[M]^+$. HRMS $[C_{24}H_{29}N_3O_8 + H]^+$ calcd 488.2027, found 488.2026.

2-Cyanoethoxy-*N*,*N*′-diisopropylamino(4-*N*-benzoy-2′,3′-di-*O*-isobutyrylcytidinyl-5′-yl)phosphine (7). To a stirred solution of 4-*N*-benzoyl-2′,3′-di-*O*-isobutyryl-*β*-D-cytidine (**4**) (0.89 g, 1.8 mmol), coevaporated with MeCN, in DCM (5 mL), containing TEA (0.84 mL, 5.9 mmol) was added 2-cyanoethoxy-*N*,*N*-diisopropylaminochlorophosphine (0.50 mL, 2.25 mmol) 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*-diisopropylaminochlorophosphine (200 MHz, $CDCl_3$: δ 180.4) and formation of the product (200 MHz, $CDCl_3$: δ 150.3, 149.6), 30 mL of 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 silica gel column and eluted with a gradient of EtOAc in PE (50/50 to 100/0) to yield the title compound as an off-white foam (0.9 g, 1.3 mmol, 73%). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.2, 175.0, 174.8, 174.7, 162.2, 154.2, 144.0 (C6 Cyt.), 143.9 (C6 Cyt.), 132.7, 132.5 (Arom. Bz), 128.3 (Arom. Bz), 127.4 (Arom. Bz), 117.3, 117.2, 97.0, 96.9 (C5 Cyt.), 87.1, 86.7 (C1′), 82.4, 82.3, 82.0, 81.9 (C4′), 73.6, 73.5 (C2′), 70.3, 70.1 (C3′), 62.4, 62.3, 62.2, 62.0 (C5′), 58.4, 58.1, 57.9 (CH_2 CNEO), 42.79, 42.76, 42.7, 42.6 (CH *i*Pr), 33.3, 33.2, 33.1 (CH *i*Bu), 24.22, 24.15, 24.10, 24.08 (CH_3 *i*Pr), 19.96, 19.88, 19.86, 19.79 (CH_2 CNEO), 18.31, 18.29, 18.27, 18.13 (CH_3 *i*Bu). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20−8.16 (m, 1H, H6 Cyt.), 7.84−7.82 (m, 2H, Bz), 7.46−7.42 (m, 1H, Bz), 7.36−7.32 (m, 3H, Bz, H5 Cyt.), 6.25, 6.24, 6.18, 6.17 (2 × d, 1H, H1′), 5.39−5.28 (m, 1H, H2′), 4.23 (m, 1H, H3′), 4.08−3.80 (m, 5H, H4′, 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 *i*Bu, CH_3 *i*Pr). ^{31}P NMR (161 MHz, $CDCl_3$) δ 150.3, 149.6. IR 2980, 1746, 1666, 1624, 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 $[M + H]^+$. HRMS $[C_{33}H_{46}N_5O_9P + H]^+$ calcd 688.3106, found 688.3118.

2-Cyanoethoxy(N^{α}- Fmoc-tyrosin-4-yl allyl ester)(4-*N*-benzoyl- 2′,3′-di-*O*-isobutyrylcytidinyl-5′-yl) Phosphate (10). N^{α}-Fmoc-tyrosine allyl ester [Fmoc-Tyr-OAll] (0.33 g, 0.75 mmol) was added to compound **7** (0.50 g, 0.72 mmol) and the two compounds were coevaporated with MeCN (5 mL) and DCE (5 mL) and then dissolved in 1:1 MeCN/DCM (5 mL, v/v). Tetrazole (200 mg, 2.88 mmol) was added and the reaction mixture was stirred for 45 min until ^{31}P NMR (200 MHz: δ 135.4, 134.9) showed complete consumption of the phosphoramidite. The reaction mixture was cooled to 0 °C and *m*-CPBA (5 equiv) was added in portions until ^{31}P NMR (200 MHz: δ −6.2, −6.3) showed complete consumption of the phosphite. The reaction mixture was diluted in 20 mL of DCM and washed with 5% $Na_2S_2O_3$ and water. The organic phase was dried

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($MgSO_4$) and concentrated. The residue was applied to a silica gel column and eluted with a gradient of EtOAc in PE (50/50 to 100/0) to yield the title compound as a colorless gel in 74% over two steps (0.55 g, 0.53 mmol). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.7, 175.6, 175.5, 171.0, 170.9, 162.5, 155.7, 155.6, 149.03, 148.96, 143.8 (C6 Cyt.), 143.7 (C6 Cyt.), 141.2, 133.8, 133.7, 133.2, 132.8, 131.3, 131.04, 131.00, 128.9, 127.7, 127.6, 127.01, 126.98, 125.0, 119.9, 119.2, 119.1 (CH_2 Allyl), 116.1 (CN), 89.0, 88.4 (C1′), 81.01, 80.96, 80.9 (C4′), 73.5 (C2′), 69.7, 69.6 (C3′), 67.1 (C5′), 66.9, 66.8, 66.11, 66.08 (CH_2 Fmoc, CH_2 Allyl), 63.02, 62.97 (CH_2 CNEO), 54.7 (C_α), 47.1 (CH Fmoc), 37.3, 37.2 (C_β), 33.70, 33.68, 33.64 (CH *i*Bu), 19.63, 19.55 (CH_2 CNEO), 18.75, 18.72, 18.6 (CH_3 *i*Bu). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89−7.76, 7.74−7.73, 7.61−7.45, 7.41−7.36, 7.31−7.24, 7.19−7.12 (6 × m, 19H), 6.25, 6.24, 6.18, 6.17 (2 × d, 1H, H1′), 5.91−5.82 (m, 1H, CH Allyl), 5.65, 5.63 (d, 1H, NH), 5.49−5.44 (m, 1H, H3′), 5.39−5.22 (m, 4H, CH_2 Allyl), 5.22−5.18 (m, 1H, H2′), 4.75−4.73 (m, 2H, CH_2 β), 4.65−4.62 (m, 5H, CH α, CH_2 CNEO, CH_2 Fmoc), 4.40−4.33 (m, 2H, H5′), 4.27−4.26 (m, 1H, H4′), 4.20−4.14 (m, 1H, CH Fmoc), 3.20−3.08 (m, 2H, CH_2 β), 2.81−2.70 (m, 2H, CH *i*Bu), 2.63−2.55 (m, 2H, CH_2 CNEO), 1.21−1.14 (m, 12H, CH_3 *i*Bu). ^{31}P NMR (161 MHz, $CDCl_3$) δ −5.7, −5.9. IR 2980, 1740, 1700, 1668, 1626, 1556, 1506, 1481, 1250, 1188, 1036. LCMS (50−90% B in 15 min), Rt = 6.26. ESI-MS *m*/*z* 1046.5 $[M + H]^+$. HRMS $[C_{54}H_{56}N_5O_{15}P + H]^+$ calcd 1046.3583, found 1046.3593.

2-Cyanoethoxy(*N*$^\alpha$-Fmoc-tyrosin-4-yl)(4-*N*-benzoyl-2′,3′-di-*O*-isobutyrylcytidinyl-5′-yl) Phosphate (13). Compound **10** (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), Bu_3SnH (0.32 mL), and $Pd(PPh_3)_4$ (20 mg) the reaction was stirred for 2 h. 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 to 5/95) to yield the title compound as a white foam (0.30 g, 0.29 mmol, 56%). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.50, 175.46, 175.4, 174.3, 167.2, 163.2, 163.1, 155.6, 154.3, 148.8, 148.7, 145.0 (C6 Cyt.), 143.7, 143.6, 141.1, 134.0, 133.0, 132.5, 131.0, 128.5, 128.0, 127.5, 126.9, 125.0, 124.9, 119.8, 119.6, 116.3, 97.7 (C5 Cyt.), 89.8, 89.3 (C1′), 80.7, 80.6 (C4′), 73.4 (C2′), 69.4, 69.3 (C3′), 66.9, 66.9, 66.7 (CH_2 Fmoc, C5′), 63.0, 63.0 (CH_2 CNEO), 54.6 (C_α), 46.9 (CH Fmoc), 37.0 (C_β), 33.5 (CH *i*Bu), 19.4, 19.3 (CH_2 CNEO), 18.64, 18.62 (CH_3 *i*Bu). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92−7.87 (m, 3H), 7.75, 7.73 (m, 2H), 7.54−7.47 (m, 4H), 7.39−7.35 (m, 5H), 7.29−7.25 (m, 2H), 7.21−7.14 (m, 4H), 6.10, 6.06 (d, 1H, C1′), 5.76, 5.74, 5.70, 5.68 (dd, 1H, NH), 5.46−5.42 (m, 2H, C3′, C2′), 4.70 (m, 1H, CH α), 4.48−4.43 (m, 2H, CH_2 Fmoc), 4.33−4.31 (m, 3H, C4′, C5′), 4.30−4.23 (m, 2H, CH_2 CNEO), 4.13 (m, 1H, CH Fmoc), 3.18−3.11 (m, 2H, CH_2 β), 2.75−2.63 (m, 2H, CH_2 CNEO), 2.62−2.58 (m, 2H, CH *i*Bu), 1.16 (m, 12H, CH_3 *i*Bu). ^{31}P NMR (161 MHz, $CDCl_3$) δ −6.4, −6.8. 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 $[C_{51}H_{52}N_5O_{15}P + H]^+$ calcd 1006.3270, found 1006.3281.

VPgpC (20). The protected peptide **16** was prepared via solid-phase peptide synthesis starting from Tentagel S RAM resin (200 mg, 50 μmol) to which Fmoc-Glu-OtBu was attached via the side chain carboxylic acid functionality by using an automated peptide synthesizer. As repetitive cycle was used: (1) Fmoc cleavage with 20% piperidine in NMP, (2) coupling of the appropriate amino acid applying a 5-fold excess, activation by 5 equiv of HCTU in NMP (0.25M) and 12.5 equiv of DiPEA in NMP (1.25 M) for 1 h, and (3) capping with 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 12.5 μmol of **16** by a double coupling protocol: (1) Fmoc cleavage with 2% DBU in DMF, (2) coupling of the appropriate amino acid applying a 5-fold excess, activation by 5 equiv of BOP/HOBt in NMP (0.25M) and 12.5 equiv of DiPEA in NMP (1.25 M) for 1 h, and (3) capping with 0.5 M acetic anhydride, 0.125 M DiPEA, and 0.015 M HOBt in NMP for 1 min. The crude partially protected nucleopeptide was cleaved off the resin with TFA/TIS/H_2O (95/5/5, v/v/v, 5 mL) by shaking for 2.5 h. After filtration into cold Et_2O the precipitate was coevaporated with toluene (2 × 5 mL) and then treated with 25% aq ammonia (2.5 mL) in 1,4-dioxane (2.5 mL). After stirring for 4 h, concentration of the reaction mixture afforded the crude title compound. RP-HPLC purification was conducted on an automated HPLC system supplied with a semipreparative C_{18} column (250 × 10.00 mm, 5 μ, flow: 4 mL/min). The applied eluent system was (A) H_2O, (B) MeCN, and (C) 0.5% TFA in H_2O with detection at 280 nm. Gradient: 10−25% B (10% C as stationary phase) in 3 CV. The collected fractions were lyophilized, yielding 14.89 mg, 5.6 μmol (45% based on original loading of resin) of the nucleopeptide. ^{31}P NMR (161 MHz, D_2O) δ − 4.24. LCMS (10−90% B in 15 min.), Rt = 4.18 min. ESI-MS *m*/*z* 2659.2 $[M]^+$, 1330.4 $[M]^{2+}$, 887.6 $[M]^{3+}$. HRMS $[C_{114}H_{189}N_{34}O_{37}P + H]^{2+}$ calcd 1329.6918, found 1329.6934.

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Supporting Information Available: Experimental procedures and spectroscopic data for all compounds, as well as copies of ^{1}H, ^{13}C, and ^{31}P NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.