Chemical synthesis of picornaviral protein primers of RNA replication

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Received 15th June 2006, Accepted 10th August 2006
First published as an Advance Article on the web 30th August 2006
DOI: 10.1039/b608544j

Naturally occurring nucleopeptidic replication primers (VPg–pUpU) of poliovirus and coxsackie virus were chemically synthesized. The synthesis was accomplished *via* block-coupling of two minimally protected fragments of the target structures: a short RNA-nucleopeptide and a longer peptide segment containing diverse side-chain functionalities. The synthetic VPg–pUpU of coxsackie virus was characterized by NMR spectroscopy.

Introduction

Picornaviridae is a large family of plus-stranded RNA viruses that includes a number of important human and animal pathogens, the notable examples being poliovirus, rhinovirus and foot-and-mouth disease virus.[1] The genome of picornaviruses is characterized by the presence of a small (20–24 amino acids) protein (VPg) covalently attached to the 5′-end of the viral RNA.[2] This covalently attached peptide originates from the replication of the viral genome, which starts at a VPg–pUpU nucleopeptide primer containing the two uridines linked to a tyrosine side-chain present in the VPg sequence. The VPg–pUpU is assembled by the viral RNA polymerase on a two adenosine template of the *cis*-acting replication element (*cre*) located within the viral genome.[3,4] The replication mechanism involving the action of the *cre* has first been proposed for rhinovirus[5] and, as it appears now, is generally applicable for all members of *Picornaviridae*, including poliovirus[6,7] and coxsackie virus.[8] The functional *cre* is thought to comprise a stem-loop structure[9] (Fig. 1) where the first two conserved adenosines in a 5′-AAACA-3′ loop consensus sequence are crucial for VPg uridylylation as well as subsequent viral replication. A conspicuous structural feature of picornaviral *cre*s is the large size of the RNA loop, which can contain up to 14 nucleotides (Fig. 1). Limited structural information is available for RNA hairpins containing such large loops and no experimental data exist on the structure of VPg–pUpU bound to the *cre*. Only one NMR study on the *cre* of human rhinovirus 14 (HRV-14) free in solution has been reported, suggesting a structured loop without any hydrogen bond interactions. A theoretical model comprising VPg–pUpU bound to the RNA hairpin has been presented in the same study.[10]

As part of our effort to chemically synthesize RNA containing nucleopeptides[11,12] for functional and structural studies we embarked on the preparation of picornaviral VPg–pUpUs. These compounds are valuable assets in NMR studies aimed at refining the model of the viral replication process by unravelling the structure of VPg–pUpU in complex with *cre* and the viral proteins known to be involved in the replication process, such as $3D^{pol}$ (polymerase) and $3CD^{pro}$ (the precursor of the protease 3C and polymerase 3D). It can reasonably be expected that other spectroscopic and biological research directed at a better understanding of the viral replication will benefit from the availability of synthetic and well defined VPg–pUpU.

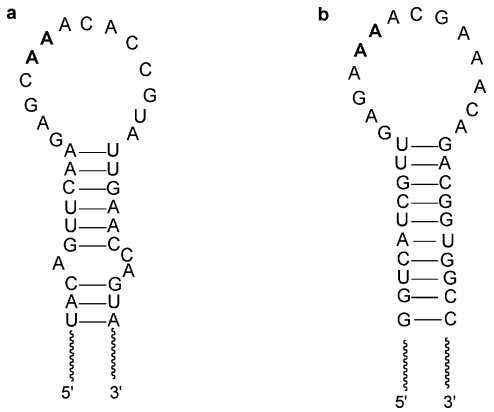

Fig. 1 Predicted partial structure of the *cre* of poliovirus (a) and human rhinovirus 14 (b), nucleotides essential for replication are shown in bold.

We report here a chemical synthesis of VPg–pUpUs **1** and **2** (Fig. 2) from poliovirus and a closely related coxsackie virus, respectively. The modular synthetic approach presented here is designed to be extensible to the synthesis of VPg–pUpUs from different picornaviruses because it takes advantage of the strong homology between the terminal pentapeptides of VPgs of the different members of *Picornaviridae*.

Results and discussion

We recently described a general solid phase approach to monouridylylated peptides employing pre-nucleotidylated Fmoc-amino acids.[11,12] However, such a method cannot be conveniently applied to the synthesis of VPg–pUpU. Not only because the required suitably protected tyrosine–pUpU building block is not readily accessible, but more importantly, the side-chain protection of the remaining amino acids in the VPg has to be fundamentally altered because of the occurrence of migration and/or cleavage

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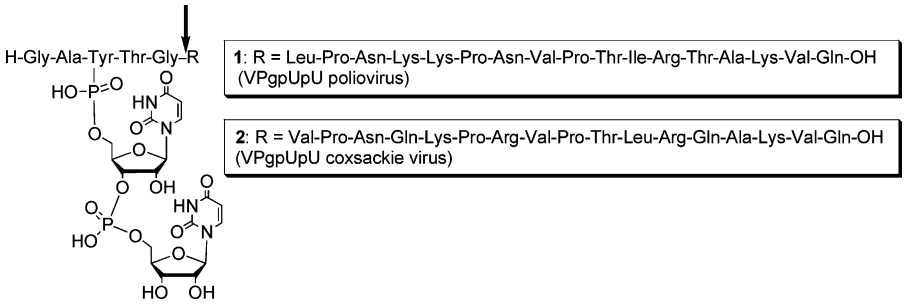


Fig. 2 Structures of the nucleopeptides from poliovirus (**1**) and coxsackie virus (**2**), the arrow indicates the retrosynthetic disconnection of the amide bond.

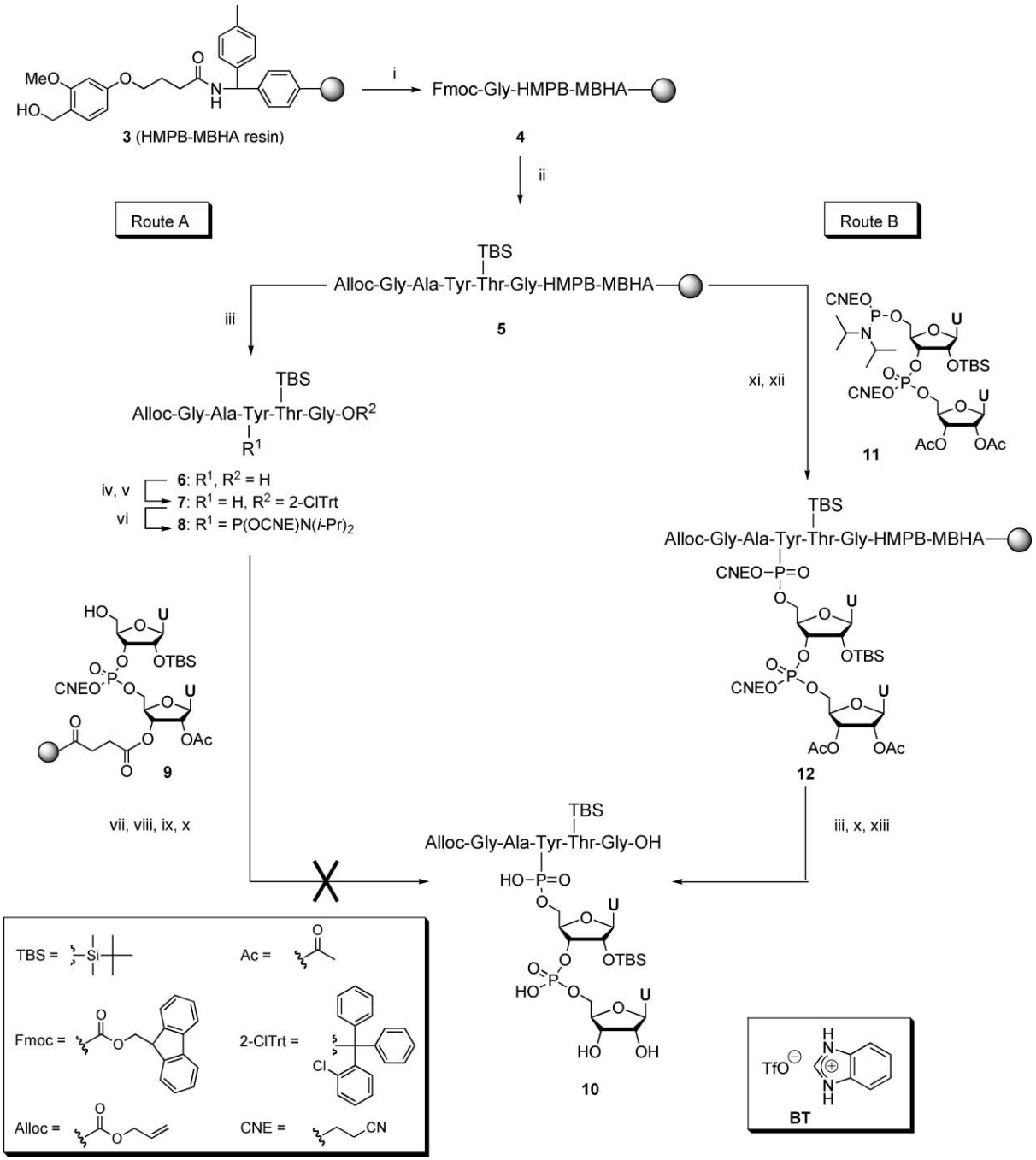
of the internucleosidic phosphodiester bond[13] in UpU during the acidic removal of the standard protecting groups.

The expected difficulties of a stepwise synthesis of **1** guided us to explore a fragment condensation approach.[11] Retrosynthetic analysis indicated the possibility of disconnecting the marked bond (indicated by the arrow in Fig. 2) in nucleopeptide **1** to furnish a pentameric nucleopeptide fragment and a heptadecameric peptide fragment. The presence of a C-terminal glycine residue in the pentameric nucleopeptide fragment prevents the occurrence of racemization during the final condensation of the fragments. Moreover, a fragment condensation approach allows for a protective group strategy which is compatible with the presence of RNA.

The assembly of **1** commences with the development of an efficient route to a suitable protected pentameric nucleopeptide **10** (see Scheme 1). The hydroxyl function of threonine and the 2′-hydroxyl of the uridine moiety in **10** were protected with a *tert*-butyldimethylsilyl (TBS) group, while the terminal amine was protected with an allyloxycarbonyl (Alloc) group. Both these protective groups can be cleaved under mild conditions in the final stage of the synthesis. Two convergent approaches to pentameric nucleopeptide **10** (see Scheme 1) can be considered. Either an immobilized UpU is elongated with a pentapeptide-phosphoramidite (route A) or conversely, a resin-bound pentapeptide is phosphitylated with UpU-phosphoramidite (route B).

Route A (see Scheme 1) was first explored because of its potential usefulness for incorporation of longer oligonucleotides in the target structure. Esterification of Fmoc–Gly–OH with the acid-labile HMPB–MBHA resin **3** under the agency of DCC and a catalytic amount of DMAP afforded immobilized glycine derivative **4**. Deprotection of the Fmoc group in **4** was followed by sequential coupling of Fmoc–Thr(TBS)–OH,[14] Fmoc–Tyr–OH, Fmoc–Ala–OH and Alloc–Gly–OH[15] under the agency of BOP and HOBt. Cleavage of immobilized pentapeptide **5** from the resin with 1% TFA in CH_2Cl_2 gave C-terminal carboxylic acid **6**, which was converted into 2-chlorotrityl ester **7**. Phosphitylation of the phenolic hydroxyl in **7** with known[16] 2-cyanoethyl-(*N*,*N*-diisopropylamino)phosphochloridite yielded phosphoramidite **8**. Benzimidazolium triflate[17] (BT, Scheme 1) mediated condensation of **8** with the immobilized uridine dimer **9**, obtained by standard procedures,[18,19] was followed by oxidation of the intermediate phosphite triester. The immobilized nucleopeptide product was treated with 0.5% TFA to unmask the carboxylic acid function and subsequently with NH_3–MeOH to remove the cyanoethyl groups and to release the nucleopeptide from the solid support. Unfortunately, LC-MS analysis of the crude mixture only showed the presence of the uridine-dimer while the target nucleopeptide **10** could not be detected. The disappointing outcome of the final phosphitylation step agrees with reported precedents[20,21] on the inability to couple peptide-phosphoramidites. Therefore route B (Scheme 1) to nucleopeptide **10** was investigated. The immobilized pentapeptide **5** was condensed with UpU-phosphoramidite[22] **11** under the influence of BT. After 4 hours, gel-phase ^{31}P-NMR analysis[23] of the resin showed the presence of two broad phosphorus signals (δ 134.8; −1.5 ppm) indicating a phosphite and a phosphate triester, respectively. Oxidation of the intermediate phosphite triester with *t*-BuOOH furnished nucleopeptide-resin **12** (gel-phase ^{31}P-NMR analysis, δ −6.5; −1.6 ppm). Nucleopeptide **10** was obtained from **12** by acid-mediated release from the resin and subsequent ammonolysis to remove the acetyl and the cyanoethyl groups. Finally, RP HPLC purification afforded the desired nucleopeptide fragment **10** as the triethyl ammonium salt, the integrity of which was confirmed by mass spectroscopy, ^{1}H-NMR and ^{31}P-NMR analysis (D_2O: δ −0.28; −4.40 ppm).

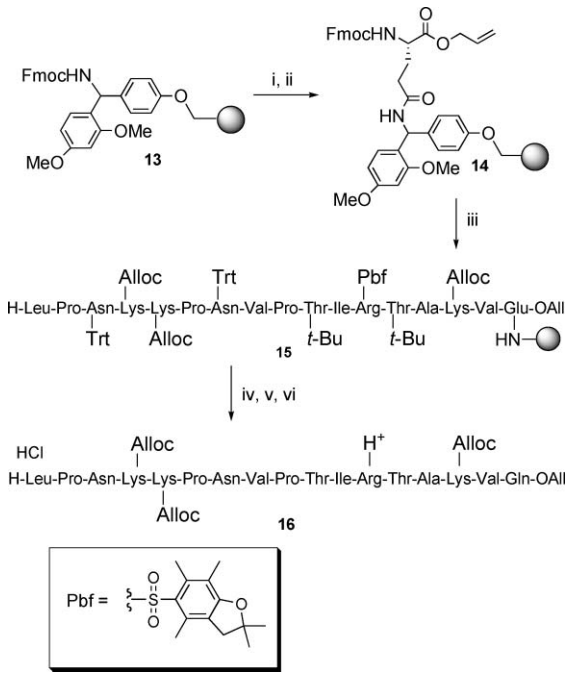
With the nucleopeptide fragment **10** in hand, the synthesis of the heptadecameric peptide fragment **16** (Scheme 2) originating from poliovirus was undertaken. An essential characteristic of **16** is the protection of the C-terminal carboxylic acid and the minimal protection of the side-chain functionalities of the amino acids. Only arginine and lysine are vulnerable to acylation and require protection in the final condensation step. Initially, the allyloxycarbonyl (Alloc) group was selected for protection of the amine function of lysine as well as the δ- and ω-positions of the guanidino function of arginine. Moreover, to minimize the number of deprotection steps in the final stage of the synthesis, the C-terminal carboxylic acid was protected as an allyl ester. Unfortunately, pilot experiments showed that the δ-Alloc group at the side-chain of arginine was not entirely stable to the conditions of solid phase peptide synthesis diminishing the yield of the target minimally protected peptide fragment. Therefore, we decided to examine whether protonation of the guanidino function would be sufficient to prevent acylation during the final condensation step. To this end we employed for the preparation of peptide fragment **16** a standard TFA labile Pbf-protection that is cleaved in the final stage of the solid phase synthesis leaving the arginine side-chain protonated. Following this approach to partially protected peptide fragment **16** (Scheme 2) Fmoc–Glu–OAll was attached to Rink Amide MBHA resin **13** *via* its side-chain acid functionality. In a standard protocol the Fmoc group in **14** was removed and commercially available amino acids were condensed under the



Scheme 1 Approaches in the synthesis of nucleopeptide fragment **10**. *Reagents and conditions*: (i) Fmoc–Gly–OH (3 eq), DCC (3.3 eq), DMAP (5 mol%); (ii) a. 20% piperidine–NMP, rt, 5 min; b. Fmoc–AA–OH (5 eq), BOP (5 eq), HOBt, (5 eq), DiPEA (10 eq), rt, 1 h; c. Ac_2O–DiPEA–HOBt–NMP, rt, 1 min; (iii) 1% TFA–CH_2Cl_2, rt, 10 min; (iv) Cs_2CO_3 (1.0 eq), DMF, rt, 10 min; (v) 2-chlorotrityl chloride (1.2 eq), DMF, rt, 1 h; (vi) Cl-P(OCNE)N(iPr)$_2$ (1.1 eq), DiPEA (5 eq), CH_2Cl_2, rt, 2 h; (vii) BT (4 eq), 1,4-dioxane–CH_3CN (3 : 1, v/v), rt, 4 h; (viii) 0.02 M I_2 in CH_3CN–*sym*-collidine–H_2O, rt, 1 min; (ix) 0.5% TFA–CH_2Cl_2, rt, 5 min; (x) NH_3–MeOH, rt, 1 h; (xi) BT (4 eq), CH_3CN–1,4-dioxane (1 : 5, v/v), rt, 4 h; (xii) *t*-BuOOH, CH_3CN–1,4-dioxane (1 : 5, v/v), rt, 20 min; (xiii) RP HPLC purification.

agency of BOP and HOBt to afford immobilized fully protected peptide **15**. Cleavage of **15** from the resin and removal of the acid-labile protecting groups *i.e.* Pbf, Trt and *t*-Bu was effected by treatment with TFA in the presence of triisopropylsilane (TIS) as a cation scavenger. Purification of crude **16** by RP HPLC furnished pure heptadecameric peptide **16** (TFA-salt). Fragment **16** was converted into the corresponding HCl-salt by anion exchange under the agency of Dowex resin (Cl^--form). The obtained peptide **16** was characterized by LC-MS analysis.

At this stage the palladium catalyzed removal of the Alloc groups and the allyl ester in **16** was examined. A borane–dimethylamine complex and thiosalicylic acid were selected as allyl cation scavengers.[24] LC-MS analysis of the crude deprotection mixtures showed in the case of $Me_2NH{\cdot}BH_3$ a fast and relatively



Scheme 2 Synthesis of the peptide fragment **16**. *Reagents and conditions*: (i) 20% piperidine–NMP, rt, 5 min; (ii) Fmoc–Glu–OAll (1.5 eq), BOP (2 eq), HOBt (3 eq), DiPEA (6 eq), rt, 24 h; (iii) a. 20% piperidine–NMP, rt, 5 min; b. Fmoc–AA–OH (5 eq), BOP (5 eq), HOBt (5 eq), DiPEA (10 eq), rt, 1 h; c. Ac_2O–DiPEA–HOBt–NMP, rt, 1 min; (iv) TFA–TIS–H_2O (95 : 2.5 : 2.5, v/v/v), rt, 2 h; (v) RP HPLC purification; (vi) DOWEX −2 (Cl-) anion exchange.

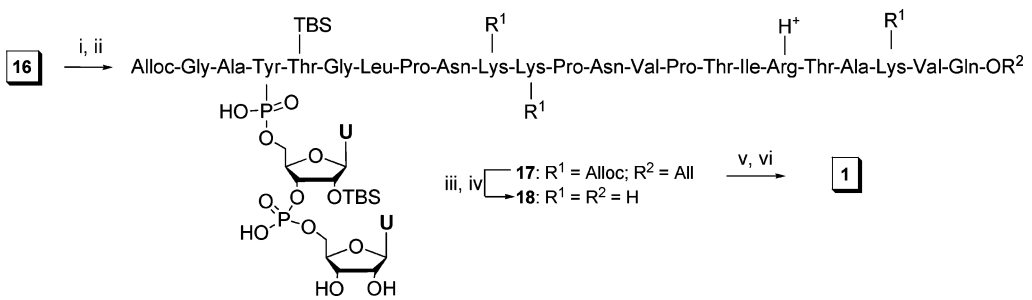
clean reaction. Furthermore, subjection of the nucleopeptide **10** to these conditions did not lead to degradation of the UpU moiety.

The key condensation reaction of nucleopeptide **10** with heptadecameric fragment **16** was executed as depicted in Scheme 3. Nucleopeptide fragment **10** (TEA-salt) was pre-activated with PyBOP in the presence of DiPEA and subsequently coupled with the minimally protected heptadecameric peptide **16** (HCl-salt). The progress of the reaction was monitored by LC-MS analysis, which showed after 22 h the formation of the partially protected nucleopeptide **17** that was isolated in 63% yield after gel filtration. MALDI-TOF-MS analysis showed partial loss of one of the TBS-protecting groups. The Alloc groups and the allyl ester in **17** were cleaved by means of Pd°-catalysed allyl transfer[24] in the presence of the borane–dimethylamine complex as the accepting nucleophile. The crude TBS-protected nucleopeptide **18** was purified by RP HPLC. The TBS groups in **18** were removed with a TEA·3HF complex in the presence of additional TEA[25] to furnish crude **1**. Purification by gel filtration gave pure VPg–pUpU **1** of poliovirus, which was analyzed by MALDI-TOF–MS (m/z = 2969.3 [M + H]$^+$) and ^{31}P-NMR (D_2O, δ −0.49; −4.34 ppm).

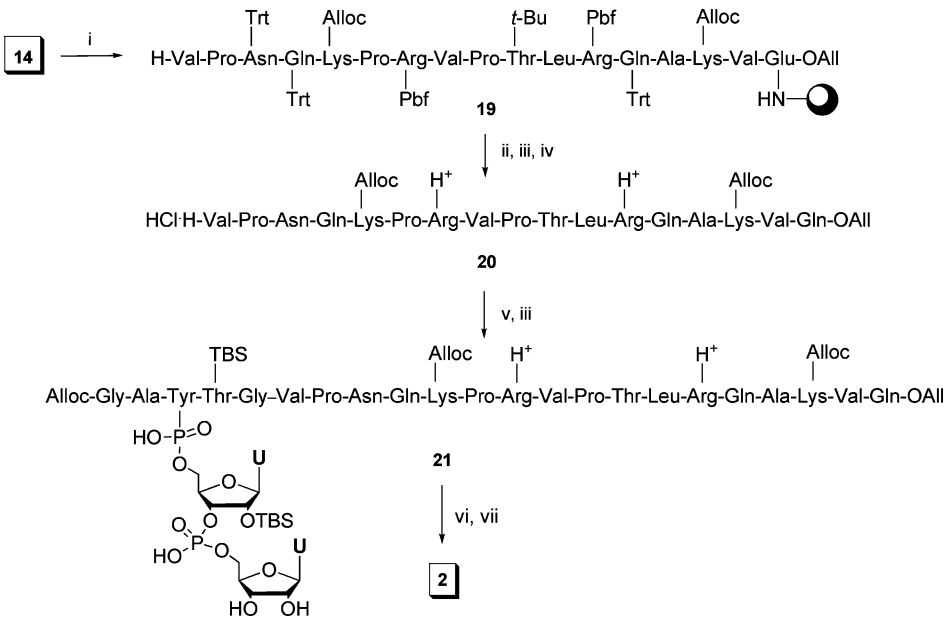
The successful outcome of the condensation of minimal protected fragments in the preparation of nucleopeptide **1** was an incentive to further explore this concept in the preparation of VPg–pUpU **2** of coxsackie virus. The route of synthesis followed to attain this goal is described in Scheme 4. The minimally protected heptadecameric peptide fragment **20** was assembled by a similar protocol as described for **16**. Acylation of thus obtained heptadecameric peptide fragment **20** with pre-activated pentameric nucleopeptide **10** afforded, after 27 h, the partially protected nucleopeptide **21** as was observed by LC-MS analysis in an estimated 90% yield. After RP HPLC purification under slightly acidic conditions followed by lyophilisation, MALDI-TOF-MS analysis (m/z = 3428.96 [M + H]$^+$) showed the almost complete loss of one of the TBS groups, nevertheless the partially protected target nucleopeptide was isolated in 50% yield. Subsequent treatment with $Pd(PPh_3)_4$ in the presence of the borane–dimethylamine complex effected simultaneous removal of the allyloxycarbonyl groups and the allyl ester. Surprisingly, LC-MS analysis of the crude mixture showed the fully deprotected target nucleopeptide **2** instead of its partially silylated derivative. Probably, the reaction mixture became slightly acidic during the latter deprotection step, resulting in cleavage of the remaining silyl-protecting groups. Purification of crude **2** by gel filtration afforded the VPg–pUpU **2** from coxsackie virus as was confirmed by RP HPLC, ^{31}P-NMR (D_2O, δ −0.28; −4.40 ppm) and MALDI-TOF-MS analysis (m/z = 3021.3 [M + H]$^+$).

At this stage we set out to conduct preliminary NMR assessments of VPg–pUpU **2** which we intend for future use in the structural studies of *cre*-VPg–pUpU complexes. A particular point of concern was a possible occurrence of the migration of the phosphodiester in the dinucleotide portion of the molecule from the natural 3′→5′ to an unnatural 2′→5′ position.

To distinguish the normal 3′→5′ from a possible 2′→5′ linkage in the structure of VPg–pUpU **2** the following NMR data were collected and analyzed. All proton and phosphorus resonances of the pUpU moiety were assigned by a combination of 2D DQF-COSY,[26] TOCSY,[27] ^{1}H-^{31}P HETCOR[28] and NOESY[29] experiments. The sugar spin systems were assigned using TOCSY



Scheme 3 Synthesis of VPg–pUpU **1** from poliovirus. *Reagents and conditions*: (i) **10** (TEA-salt, 1 eq), PyBOP (1.1 eq), HOBt (1.1 eq), DiPEA (2 eq), rt, 22 h; (ii) gel filtration, 63%; (iii) $Pd(PPh_3)_4$ (5 mol%), PPh_3 (cat. amount), $Me_2NH{\cdot}BH_3$ (2 eq), rt, 3 h; (iv) RP HPLC; (v) TEA·3HF, TEA, rt, 16 h; (vi) gel filtration.



Scheme 4 Synthesis of nucleopeptide **2** from coxsackie virus. *Reagents and conditions*: (i) a. 20% piperidine–NMP, rt, 5 min; b. Fmoc–AA–OH (5 eq), BOP (5 eq), HOBt (5 eq), DiPEA (10 eq), rt, 1 h; c. Ac_2O–DiPEA–HOBt–NMP, rt, 1 min; (ii) TFA–TIS–H_2O (95 : 2.5 : 2.5, v/v/v), rt, 2 h; (iii) RP HPLC; (iv) anion exchange; (v) **10** (TEA-salt, 1 eq), PyBOP (1.1 eq), HOBt (1.1 eq), DiPEA (2 eq), rt, 27 h; (vi) $Pd(PPh_3)_4$ (5 mol%), PPh_3 (cat. amount), $Me_2NH{\cdot}BH_3$ (2 eq), rt, 3 h; (vii) gel filtration.

and DQF-COSY experiments. The TOCSY experiment (Fig. 3) provided connectivities between the H1′ and the H3′ and H4′ resonances, and for one of the two uridines, U2, a full sequential walk from H1′ to H5″ could be easily traced out in the DQF-COSY (Fig. 4). Distinction between the U1 and U2 resonances was derived from the 2D-NOESY, which showed a clear sequential U1H2′–U2H6 NOE contact (data not shown). All H2′–H4′ resonances of U2 appear in a very small region between 4.1 and 4.3 ppm with all cross-peaks on or close to the diagonal. However, a constant time DQF-COSY, in which proton–proton *J*-couplings in the F1 dimension were refocused, provided additional resolution and by close inspection of the CT-DQF-COSY (Fig. 5) and TOCSY (Fig. 3) experiments the U2 sugar protons could be assigned as well. Finally, assignment of the phosphorus resonances and establishment of the type of phosphodiester bond between U1 and U2 could be deduced from the ^{31}P-^{1}H HETCOR (Fig. 6). One phosphorus, resonating at −0.66 ppm is connected to the H4′ and H5′/H5″ of U1, while the other phosphorus shows cross-peaks to

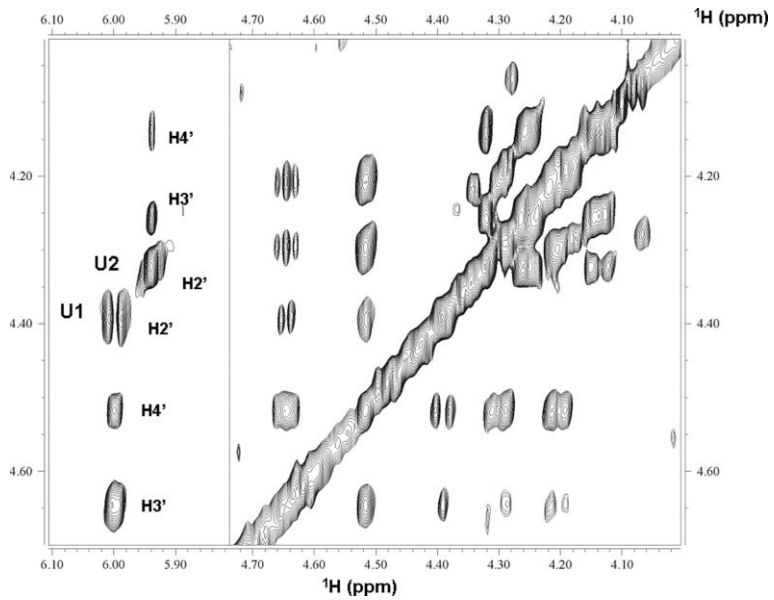


Fig. 3 Portions of the 2D-TOCSY experiment showing the aligned H1′ to other sugar proton region and the H2′–H5″ to H2′–H5″ region separated by a thin line. Some sugar proton assignments are given along the H1′ frequency line.

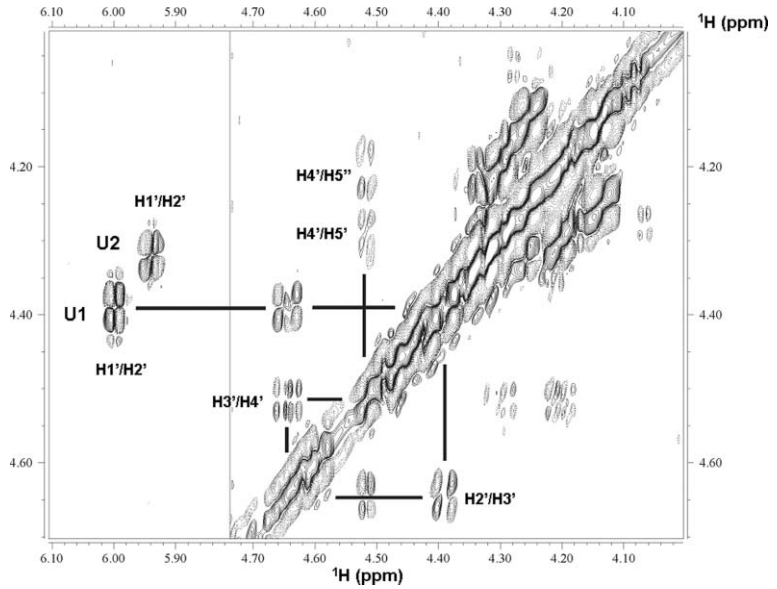


Fig. 4 Regions of the 2D-DQF-COSY spectrum identical to those in Fig. 3. Assignment of the U1 sugar protons is traced out. Assignments of the cross-peaks are quoted in F2, F1 dimensions.

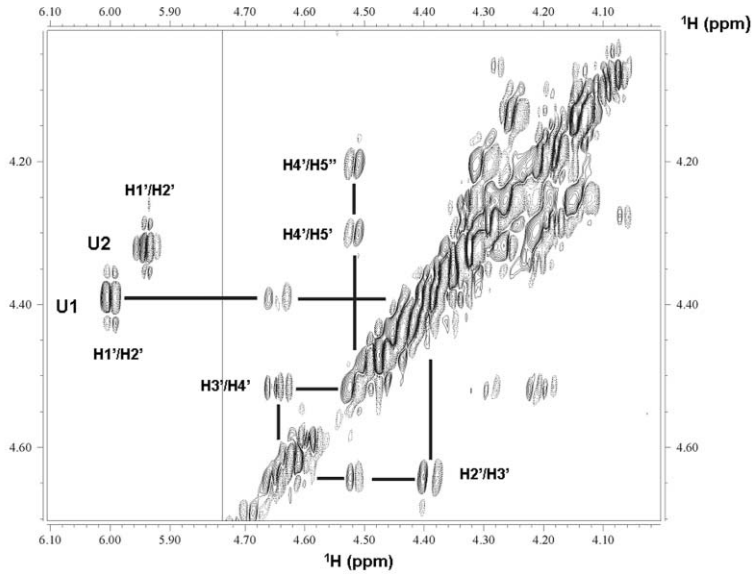


Fig. 5 Portions of the 2D-CT-DQF-COSY experiment, identical to those in Fig. 4. Resolution enhancement by refocusing the H1′–H1′ *J*-couplings in the F1 dimension is evident.

the U1H3′ and U2H5′/H5″. Hence, the phosphorus resonance at −0.66 ppm belongs to the phosphorus 5′ to U1, while the other one belongs to the phosphorus between U1 and U2. The latter shows a 3-bond *J*-coupling to the H3′ of the 5′-residue, which demonstrates a 3′–5′ linkage between the two uridines. The assignments are listed in Table 1.

Table 1 Proton (relative to TMA) and phosphorus chemical shifts (relative to TSP) of the pUpU moiety in the VPg–pUpU nucleopeptide **2**

	H6	H5	H1′	H2′	H3′	H4′	H5′	H5″	P[a]
U1	7.90	5.89	5.60	4.39	4.64	4.52	4.30	4.20	−0.66
U2	7.72	5.72	5.94	4.32	4.26	4.14	4.25	4.14	3.44

[a] Phosphorus resonances are denoted 5′ to each residue.

Conclusions

In conclusion, the VPg–pUpUs of poliovirus and coxsackie virus have been synthesized by fragment condensation of a small nucleopeptide and the corresponding minimally protected heptadecameric peptides. The applied protection strategy *i.e.* Alloc group for lysine and protonation for arginine was shown to be compatible with the presence of the dinucleotide.

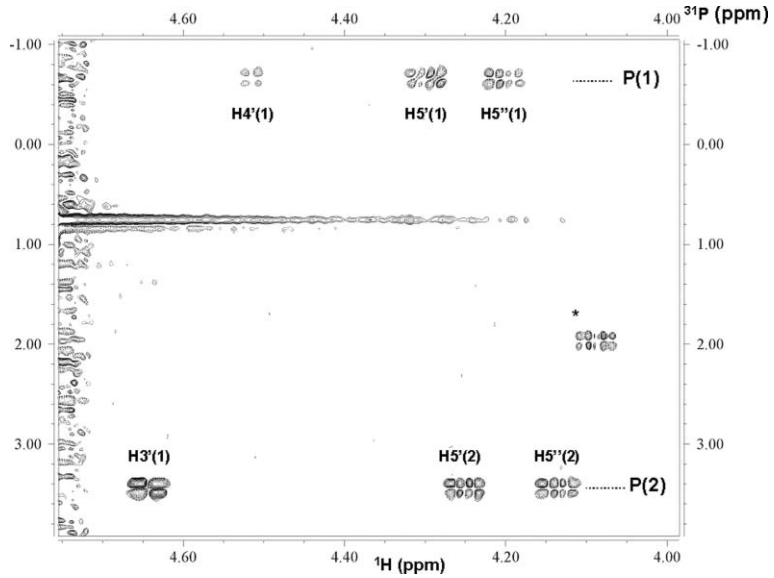


Fig. 6 Plot of the ^{31}P-^{1}H HETCOR experiment. Assignments of the phosphates are given by P(Ni), which indicates the phosphate 5′ to residue number Ni. The asterisk denotes a cross-peak from an unidentified impurity.

Experimental

Pyridine (Acros Organics), *N*,*N*-dimethylformamide (Baker, p.a.), 1,4-dioxane (Baker, p.a.) and 1,2-dichloroethane (Baker, p.a.) were stored over molecular sieves (4 Å). Acetonitrile (extra dry, DNA synthesis grade) was purchased from Biosolve. Methanol (HPLC grade) and triethylammonium acetate buffer (TEAA, pH 7) were purchased from Biosolve. Sephadex G-25 was obtained from Pharmacia. All reagents were obtained from Acros Chemicals, unless otherwise stated, and used as received. $Pd(PPh_3)_2Cl_2$ was bought at Aldrich.

Solvents used in the automated peptide synthesis, DiPEA and TFA were all of peptide synthesis grade (Biosolve) and used as received. BOP reagent, anhydrous HOBt and 2-chlorotrityl chloride were obtained from Senn Chemicals, 4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid (HMPB) and 4-methylbenzhydrylamine (MBHA) resin were obtained from NovaBiochem. PyBOP was obtained from Senn Chemicals and the protected amino acids were obtained at NovaBiochem. Alloc–Gly–OH was synthesized as described[15] and *tert*-butyldimethylsilyl ether of N^{α}-(9-fluorenylmethoxycarbonyl) threonine [Fmoc–Thr(TBS)–OH] was synthesized as described.[14]

The UV absorption in the determination of the loading of the resin was measured with a Varian DMS 200 UV–VIS spectrophotometer. TLC analysis was performed on Merck 25DC Plastikfolien Kieselgel 60 F_{254}. Detection by UV absorption (254 nm) and spraying with one of the following solutions: (a) 20% H_2SO_4 in EtOH followed by charring; (b) ammonium molybdate (25 g L^{-1})–ceric ammonium sulfate (10 g L^{-1}) in 10% aq. H_2SO_4 followed by charring; (c) $KMnO_4$ (10 g L^{-1} in 2% aq. Na_2CO_3). Fluka silica gel (230–400 mesh) was used for column chromatography. The solvents for chromatography were of technical grade and distilled before use.

^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR spectra were recorded with a Bruker AC200 instrument at 200, 50.1 and 80.7 MHz, respectively. Chemical shifts (δ) are given in ppm, relative to tetramethylsilane as an internal standard for ^{1}H-NMR and ^{13}C-NMR and 85% H_3PO_4 as an external standard for ^{31}P-NMR. LC-MS analysis was performed on a Jacso HPLC system (detection simultaneously at 214 and 254 nm) coupled to a Perkin Elmer Sciex API 165 mass instrument equipped with a custom-made Electrospray Interface (ESI). An analytical Alltima C_{18} column (Alltech, 4.6 mmD × 250 mmL, 5 µ particle size) was used. Buffers: A: H_2O; B: CH_3CN and C: 0.5% aq TFA.

For RP HPLC purification, a BioCAD "Vision" automated HPLC system (PerSeptive Biosystems, inc.), supplied with a semi-preparative Alltima C_{18} column (Alltech, 10.0 mmD × 250 mmL, 5 µ particle size, running at 4 mL min^{-1}) was used. An appropriate gradient of buffer B (CH_3CN) in buffer A (H_2O) was applied while buffer C (as specified) was kept at 10% throughout the purification run. Detection was performed by UV, simultaneous at 214 nm and 254 nm. MALDI-TOF-MS spectra were recorded on a Voyager-DE PRO mass spectrometer (PerSeptive Biosystems, Inc.).

Solid phase peptide synthesis

The peptides were prepared on an ABI 433A (Applied Biosystems, division of Perkin-Elmer) automatic peptide synthesizer, using the peptide synthesis protocol supplied with the synthesizer. The peptides were synthesized on a 50 µmol, 250 µmol or 1 mmol scale. The consecutive steps performed in each cycle were: 1) Deprotection of the Fmoc-group with 20% piperidine for 5 × 2 min, unless stated otherwise. 2) Coupling of the appropriate amino acid applying an excess of an appropriate Fmoc-amino acid (5-fold for 50 µmol scale, 4-fold for 250 µmol scale and 3-fold for 1 mmol scale). Thus for 50 µmol synthesis, the Fmoc-amino acid (0.25 mmol) was dissolved in NMP (0.5 mL) and subsequently 0.25 mmol of BOP–HOBt (0.5 M BOP–0.5 M HOBt in DMF–NMP 1 : 1, v/v) and 0.63 mmol of DiPEA (1.25 M in NMP) were added. The resulting solution was transferred to the reaction vessel, which was then shaken for 1 hour. 3) The remaining amino functions were capped by addition of a

solution of 0.5 M acetic anhydride, 0.125 M DiPEA and 0.015 M HOBt in NMP. The resulting suspension was shaken for 1 min. The side-chain protections were: Arg(Pbf), Asn(Trt), Asp(O*t*-Bu), Gln(Trt), Lys(Boc), Thr(*t*-Bu), unless stated otherwise. Double couplings were performed for Val, Thr, Ile, Arg and Asn.

HMPB–MBHA resin (3). Under a blanket of argon, 4-methylbenzhydrylamine resin (2.4 g, 1.5 mmol, 0.62 mmol g^{-1}) was suspended in a mixture of NMP and dichloromethane (1 : 1, v/v, 60 mL). BOP (1.99 g, 4.5 mmol), HOBt (0.6 g, 4.5 mmol), HMPB (1.1 g, 4.5 mmol) and DiPEA (1.53 mL, 9.0 mmol) were added. The suspension was shaken overnight after which the solvents and reagents were removed by filtration. The resin was washed with CH_2Cl_2 (2 × 20 mL), MeOH (20 mL) and CH_2Cl_2 (2 × 20 mL). The resin was dried followed by subjecting a sample of the resin to the Kaiser test,[30] which showed the coupling of the HMPB linker to be complete.

Fmoc–Gly–HMPB–MBHA resin (4). HMPB–MBHA resin (1.5 mmol) was dried by co-evaporation with 1,4-dioxane (2 × 20 mL) and suspended in dichloromethane (60 mL). Subsequently, Fmoc–Gly–OH (1.3 g, 4.5 mmol), DCC (1.0 g, 4.9 mmol) and DMAP (27 mg, 0.23 mmol) were added. The reaction mixture was shaken for 2 hours after which the resin was filtered and washed with CH_2Cl_2 (2 × 10 mL), MeOH (10 mL), CH_2Cl_2 (2 × 10 mL) and dried in a high vacuum. Finally, of an analytical sample of resin **4**, the loading (0.39 mmol g^{-1}) was determined as described.[31]

5′-*O*-[2-Cyanoethyl-(*N*,*N*-diisopropyl)phosphitamido]-2′-*tert*-butyldimethylsilyluridyl-[3′-*O*^{P}-2-cyanoethyl] → 5′]-2′,3′-di-*O*-acetyluridine (11). Known[12] 2′,3′-di-*O*-acetyluridine (1.9 g, 5.8 mmol) was treated with commercially available 3′-*O*-[5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-*tert*-butyldimethylsilyl-uridinyl] 2-cyanoethyl-(*N*,*N*-diisopropyl)phosphoramidite (5 g, 5.8 mmol) and 5-(*ortho*-nitrophenyl)tetrazole (2.2 g, 11.6 mmol) in 75 mL of dry CH_3CN for 15 min. Next *t*-BuOOH was added as a solution in di-*t*-butyl peroxide (80%, 5 mL) and the mixture was stirred for 15 min, pyridine (100 mL) was added followed by acetic anhydride (4 mL). After 5 min the reaction mixture was quenched with 5 mL ethanol, diluted with EtOAc (250 mL), washed with aq. $NaHCO_3$ and H_2O, dried with $MgSO_4$ and concentrated to dryness. Next the DMT group was cleaved by treatment of the resulting oil with 50 mL of 3% TCA in DCM for 10 min followed by slow addition of TES (1.9 mL, 12 mmol). The reaction mixture was diluted with DCM (150 mL) washed with aq. $NaHCO_3$ and H_2O, dried with $MgSO_4$ and upon concentration to near dryness, purified by silica gel column chromatography (DCM–MeOH from 100 : 0 to 95 : 5 v/v) to give 2.7 g (3.4 mmol, 58%) of the partially protected dinucleotide. The latter material was co-evaporated with pyridine, dissolved in dry dioxane (40 mL, freshly distilled from K/Na-benzophenone ketyl) and treated with TEA (23.5 mmol, 3.3 mL) and next with Cl-P(OCNE)N(iPr)$_2$ (4 mmol, 0.860 mL in 10 mL DCM). After stirring for 30 min the reaction mixture was diluted with DCM (150 mL) washed with aq. $NaHCO_3$ and H_2O, dried with $MgSO_4$ and upon concentration to near dryness purified by precipitation from pentane to give **11** (3.4 g, quant., off-white powder) as a mixture of 4 diastereomers at the phosphorus centres. The latter material was used in the next step without further purification. ^{31}P-NMR (200 MHz, CH_3CN, acetone-d_6, external lock): δ 150.1, 149.0, 148.8 (phosphoramidite); −0.8, −1.0, −1.3, −1.5 (phosphotriester).

Alloc–Gly–Ala–Tyr[pU(TBS)pU]–Thr(TBS)–Gly–OH (10)

Route A. Immobilized dinucleotide **9** (1 μmol) was prepared as described[18,19] using controlled pore glass (0.32 μmol g^{-1}, 500 Å) as a solid support. Dinucleotide resin **9** was subsequently treated with a mixture of peptide phosphoramidite **8** (0.13 M in dioxane, 115 μL, 15 μmol) and benzimidazolium triflate[17] (BT) (0.2 M in dioxane–CH_3CN 1 : 1, 300 μL, 60 μmol) for 4 h. Compound **8** was rendered dry by co-evaporation with dioxane, BT was repeteadly co-evaporated with dry CH_3CN. The resulting support was oxidized with 0.02 M I_2 in CH_3CN–*sym*-collidine–H_2O (11 : 1 : 5), rinsed with dry CH_3CN, treated with 0.5% TFA in CH_2Cl_2, rinsed with CH_2Cl_2 and the product was cleaved with NH_3–MeOH for 1 h. LC-MS analysis revealed the presence of UpU as the only component while no trace of desired product **10** was detected.

Route B. The synthesis of the pentapeptide was performed as described above (**Solid phase peptide synthesis**). The Fmoc-group of immobilized glycine derivative **4** was cleaved with 20% piperidine and subsequently the appropriate building blocks were coupled to obtain peptide resin **5**. An analytical sample of **5** was treated with 1% TFA in CH_2Cl_2 for LC-MS analysis (R_t = 18.93 min, Alltima C_{18}, analytical column, buffers A: H_2O; B: CH_3CN and C: 0.5% aq. TFA, linear gradient 5 → 50% B in 20 min; ESI-MS: m/z = 666.4 $[M + H]^+$). For coupling of the phosphoramidite of the uridine dimer, the peptide resin **5** (0.89 g, 0.25 mmol) was dried by co-evaporation with 1,4-dioxane (2 × 20 mL). The amidite **11** (1.0 g, 1.0 mmol) was added, co-evaporation was repeated twice using pyridine–1,4-dioxane (1 : 1, v/v, 2 × 20 mL) and the solids were dried in a high vacuum (15 min). Subsequently, BT (1.1 g, 4.0 mmol, dried by co-evaporation with CH_3CN) was added and the mixture was dried in a high vacuum for 15 more minutes. Under a blanket of argon, the resin was suspended in 1,4-dioxane (8 mL) and CH_3CN (1.6 mL). The mixture was shaken for 4 hours, filtered, and the resin was washed with CH_2Cl_2 (2 × 10 mL) and CH_3CN (2 × 20 mL). A sample of the resin was taken for gel-phase ^{31}P-NMR analysis (($CDCl_3$): δ 134.8 (phosphite triester); −1.5 (phosphate triester), both broad signals). Next, *t*-BuOOH (2 mL) was added, the reaction mixture was shaken for 20 more minutes after which NMR analysis[23] (gel-phase ^{31}P-NMR ($CDCl_3$): δ −1.6; −6.5) showed the complete conversion of the phosphite triester into phosphate triester. The resin containing the immobilized nucleopeptide (**12**) was filtered, washed with CH_2Cl_2 (2 × 10 mL), MeOH (10 mL), CH_2Cl_2 (2 × 10 mL) and dried in a high vacuum to give **12** (1.3 g). A small batch of the resin **12** (300 mg, ± 58 μmol) was transferred onto a glass filter equipped with an adapter and flask filled with dry toluene (50 mL). Under an argon atmosphere, the resin was washed with 1% TFA in CH_2Cl_2 (25 mL). The volume of the filtrate was reduced to 20 mL (temperature of the waterbath 35 °C), diluted with toluene (2 × 25 mL) and concentrated to dryness. The residue was dissolved in NH_3–MeOH (10 mL) and stirred for 2 hours. After evaporation, the residue was redissolved in water and purified by RP HPLC (Alltima C_{18}, semi-preparative column, buffers A: H_2O; B: CH_3CN; C: TEAA 0.2 M, pH 7; 27 → 37% B for 5 CV) to give title compound **10** (41.8 mg, 43%, based on the loading of resin **5**). LC-MS analysis: R_t 15.81 min

(Alltima C_{18} analytical column, buffers A: H_2O; B: CH_3CN; C: aq. 0.5% TFA; linear gradient 10 → 90% B in 20 min); ESI-MS: $m/z = 1392.7$ $[M + H]^+$; 697.1 $[M + 2H]^{2+}$; calc. 1391.45 $[M + H]^+$. ^{31}P-NMR (PH COSY, 600 MHz, D_2O): δ −0.4 (Tyr–pU**p**U); −4.1 (Tyr–**p**UpU). ^{1}H-NMR (HHCOSY, 600 MHz, D_2O): δ 7.86 (d, 1H, $J = 8.1$ Hz, H-6 U_1); 7.77 (d, 1H, $J = 8.04$ Hz, H-6 U_2); 7.14 (d, 2H, $J = 8.04$ Hz, H-2, H-6 Tyr); 7.05 (d, 2H, $J = 8.12$ Hz, H-3, H-5 Tyr); 6.03 (d, 1H, $J = 7.01$ Hz, H-1′); 5.94–5.82 (m, 3H, H-1′ U_1, = CH Alloc, H-5 U_1); 5.77 (d, 1H, $J = 7.58$ Hz, H-5 U_2); 5.29 (d, 1H, $J = 17.1$ Hz, CH=C**H**H Alloc); 5.21 (d, 1H, $J = 10.1$ Hz, CH=CH**H** Alloc); 4.61 (m, 2H, H-4′ U_1, H-4′ U_2); 4.55 (br d, 2H, $J = 5.0$ Hz, OCH_2 Alloc); 4.34 (br m, 12H, H-2′ U_1, H-2′ U_2, H-3′ U_1, H-3′ U_2, H-5a′, H-5b′ U_1, H-5a′, H-5b′ U_2, αCH Ala, βCH Thr, βCH_2 Tyr); 3.77 (m, 4H, 2 × αCH_2 Gly); 0.88 (s, 9H, $(CH_3)_3C$ TBS); 0.82 (s, 9H, $(CH_3)_3C$ TBS); 0.07(s, 6H, $(CH_3)_2Si$ TBS); 0.06 (s, 6H, $(CH_3)_2Si$ TBS).

Fmoc–Gln(Rink Amide MBHA resin)–OAll (14). Rink amide MBHA resin **13** (1.0 g, 0.5 mmol) was suspended in piperidine–DMF (1 : 4, v/v, 10 mL) and shaken for 5 min. The resin was filtered and the piperidine treatment was repeated twice after which the resin was washed with CH_2Cl_2 (2 × 20 mL), MeOH (20 mL) and CH_2Cl_2 (2 × 20 mL) and dried in a high vacuum. The free amine containing resin was suspended in DMF (25 mL) and subsequently were added Fmoc–Glu–OAll (320 mg, 0.75 mmol), BOP (442 mg, 1.0 mmol), HOBt (200 mg, 1.5 mmol) and DiPEA (0.52 mL, 3.0 mmol). The mixture was shaken for 24 hours after which the solvent and reagents were removed by filtration. The resin **14** was washed with DMF (3 × 20 mL), CH_2Cl_2 (2 × 20 mL), MeOH (2 × 20 mL) and CH_2Cl_2 (2 × 20 mL). The resin was dried in a high vacuum and of an analytical sample, the loading of **14** (0.44 mmol g^{-1}) was determined as described.[31]

HCl·H–Leu–Pro–Asn–Lys(Alloc)–Lys(Alloc)–Pro–Asn–Val–Pro–Thr–Ile–Arg–Thr–Ala–Lys(Alloc)–Val–Gln–OAll (16). The partially protected peptide fragment **16** was assembled on the solid support as described above (**Solid phase peptide synthesis**) starting from resin **14** (50 μmol). After completion of the synthesis, the fully protected peptide resin **15** was removed from the synthesizer and placed into a round-bottom flask. A mixture of TFA–TIS–H_2O (95 : 2.5 : 2.5, v/v/v, 10 mL) was added and the obtained suspension was gently shaken for 2 hours. The resin was filtered and washed with TFA. The filtrate was diluted with toluene (15 mL) and the solvents were removed under reduced pressure. The crude peptide **16** was dissolved in TFA (0.75 mL) and precipitated by dropwise addition to ether (10 mL). The suspension was centrifuged and the solution was decanted. The precipitate was dissolved in a mixture of H_2O–CH_3CN (3 : 1, v/v) and purified by RP HPLC (Alltima C_{18} semi-preparative column; buffers A: H_2O; B: CH_3CN and C: 1% aq. TFA; linear gradient in B 25 → 35%, 17 min, 3.5 CV). The purified peptide **16** (15 mg, TFA-salt) was converted into the HCl-salt by passing through a DOWEX-2 Cl^- column. After lyophilization pure **16** (10.7 mg, 9% overall based on the loading of **14**) was obtained. LC-MS analysis: R_t 18.85 min (Alltima C_{18} analytical column, buffers A: H_2O; B: CH_3CN; C: 0.5% aq. TFA, linear gradient in B: 5 → 50% in 20 min); ESI-MS: $m/z = 2197.8$ $[M + H]^+$; 1099.6 $[M + 2H]^{2+}$.

Alloc–Gly–Ala–Tyr[pU(TBS)pU]–Thr(TBS)–Gly–Leu–Pro–Asn–Lys(Alloc)–Lys(Alloc)–Pro–Asn–Val–Pro–Thr–Ile–Arg–Thr–Ala–Lys–Val–Gln–OAll (17). In a 5 mL flask, nucleopeptide fragment **10** (8.0 mg, 4.7 μmol, TEA-salt) was co-evaporated with DMF (2 × 1 mL). Solutions of PyBOP (2.7 mg, 5.2 μmol) and HOBt (0.7 mg, 5.2 μmol) in DMF (100 μL) and DiPEA (1.6 μL, 9.4 μmol) in DMF (100 μL) were added and the mixture was stirred for 10 minutes (pre-activation). Next, a solution of **16** (11 mg, 4.7 μmol, HCl-salt, dried by co-evaporation with DMF (2 × 1 mL)) in DMF (100 μL) was added and stirring was continued. The progress of the condensation was followed by LC-MS analysis which showed the reaction to be complete after 22 hours (R_t 13.3 min, Alltima C_{18} analytical column, buffers A: H_2O; B: CH_3CN and C: 0.5% aq. TFA; linear gradient of B: 5 → 50% in 20 min; ESI-MS: $m/z = 1787.0$ $[M + 2H]^{2+}$; 1191.6 $[M + 3H]^{3+}$). The crude nucleopeptide was subjected to gel filtration (LH-20, DMF–H_2O, 2 : 1, v/v) and the obtained fractions were analyzed by mass spectrometry and RP HPLC (BioCAD, Alltima C_{18} analytical column, buffers A: H_2O; B: CH_3CN; C: 0.2 M TEAA pH 7, linear gradient of 35 → 90% B in 12.5 min, 5.0 CV). Lyophilization of the nucleopeptide containing fractions afforded **17** (11 mg, 63%). MALDI-TOF-MS: $m/z = 3575.2$ $[M + H]^+$; 3460 $[M + H]^+$ (product-TBS). ^{31}P-NMR (DMF–H_2O, 9 : 1, v/v; acetone-d_6, external lock): δ −0.52; −4.34.

H–Gly–Ala–Tyr(pUpU)–Thr–Gly–Leu–Pro–Asn–Lys–Lys–Pro–Asn–Val–Pro–Thr–Ile–Arg–Thr–Ala–Lys–Val–Gln–OH (VPg–pUpU, 1). Partially protected nucleopeptide **17** (4.1 mg, 1.1 μmol) was dissolved in DMF (300 μL through which argon had been passed for 10 min). Under a blanket of argon were added $Me_2NH{\cdot}BH_3$ (0.63 mg, 11 μmol), PPh_3 (cat. amount) and $Pd(PPh_3)_4$ (0.3 mg, 0.3 μmol). After stirring for 3 hours with the exclusion of light, the reaction mixture was diluted with RNase-free water (3 mL) and extracted with diethyl ether (1 mL). The water layer was evaporated and analyzed with LC-MS and the deprotection was found to be complete (R_t 10.70 min, Alltima C_{18} analytical column, buffers: A: H_2O; B: CH_3CN; C: 0.5% aq. TFA, linear gradient in B, 10 → 90% over 20 min; ESI-MS: $m/z =$ 1598.2 $[M + 2H]^{2+}$ product; 1541.6 $[M + 2H]^{2+}$ product-TBS; 1484.0 $[M + 2H]^{2+}$ product −2 × TBS). Several procedures *i.e.* cation exchange and RP HPLC at pH 7.4 and 6.2 using a TEAA buffer were explored to purify crude **18**, however these methods were not suitable for the obtained product. A good separation was obtained by RP HPLC applying buffers A: 10% CH_3CN in 0.1% aq. AcOH and B: 90% CH_3CN in 0.1% aq. AcOH (Jasco, Alltima C_{18} analytical column, linear gradient of 10 → 26% B in 15 min, detection at 254 nm). R_t 13.3 min. MALDI-TOF-MS: $m/z = 3198.6$ $[M + H]^+$. Partially protected nucleopeptide **18** was dissolved in DMF (0.75 mL) and TEA (375 μl) and TEA·3HF (500 μL) were added. The reaction mixture was gently stirred for 16 hours after which the reaction was quenched by addition of NH_4^+ HCO_3^- (0.15 M, 5 mL). The crude nucleopeptide **1** was purified by gel filtration (Sephadex G-25, buffer 0.15 M aq. $NH_4^+HCO_3^-$, running at 300 mL $hour^{-1}$, detection at 254 nm). The appropriate fractions were concentrated and the residue was dissolved in MeOH–H_2O (1 : 1, v/v, 3 × 10 mL) and evaporated to dryness. Lyophilization from H_2O afforded pure VPg–pUpU **1** (0.66 mg) from poliovirus. ^{31}P-NMR (D_2O)

δ −0.49; −4.34, MALDI-TOF-MS: m/z = 2969.3 $[M + H]^+$; calc. 2964.38

HCl·H–Val–Pro–Asn–Gln–Lys(Alloc)–Pro–Arg–Val–Pro–Thr–Leu–Arg–Gln–Ala–Lys(Alloc)–Val–Gln–OAll (20). The partially protected peptide fragment **19** was assembled on solid support **14** (50 μmol) as described above (**Solid phase peptide synthesis**). A five-fold excess of amino acid building blocks was used in BOP–HOBt–DiPEA-mediated couplings. Lysine was incorporated as Fmoc–Lys(Alloc)–OH. Double couplings were performed for β-branched amino acids, Fmoc–Arg(Pbf)–OH and Fmoc–Asn(Trt)–OH. After the assembly of the peptide was complete, the resin was removed from the vessel and placed in a round-bottom flask. A cleavage mixture of TFA, TIS and H_2O (95 : 2.5 : 2.5, v/v/v, 10 mL) was added and the suspension was shaken for 2 hours at room temperature. Subsequently the cleavage mixture was filtered, the filtrate was diluted with toluene (30 mL) and concentrated. The residue was redissolved in TFA (0.5 mL) and precipitated by addition to diethyl ether (5 mL). The crude fragment **20** (TFA-salt) was purified by RP HPLC (Alltima C_{18} semi-preparative column, buffer A: H_2O; B: CH_3CN; C: 1% aq. TFA, linear gradient of B 25 → 30.5% in 15 min, 3 CV). The purified fragment **20** was converted into the HCl-salt by passing through a DOWEX-2 Cl^- column (17.2 mg, 15%, overall based on the loading of **14**). ESI-MS: m/z = 2168.6 $[M + H]^+$; 1085.2 $[M + 2H]^{2+}$; 723.4 $[M + 3H]^{3+}$. RP HPLC R_t 16.29 min (Alltima C_{18}, analytical column, buffer A: H_2O; B: CH_3CN; C: 0.5% aq. TFA, linear gradient of B, 5 → 50% in 20 min).

Alloc–Gly–Ala–Tyr(pUpU)–Thr–Gly–Val–Pro–Asn–Gln–Lys-(Alloc)–Pro–Arg–Val–Pro–Thr–Leu–Arg–Gln–Ala–Lys(Alloc)–Val–Gln–OAll (21). In a 5 mL flask, nucleopeptide fragment **10** (12.8 mg, 7.6 μmol, TEA-salt) was co-evaporated with DMF (2 × 1 mL) and dissolved in DMF (100 μL). Fragment **10** was pre-activated by addition of PyBOP (4.3 mg, 8.3 μmol) and HOBt (1.1 mg, 8.3 μmol) in DMF (100 μL) and DiPEA (2.6 μL, 15 μmol) in DMF (100 μL). After stirring this mixture for 10 minutes, a solution of **20** (17 mg, 7.6 μmol, HCl-salt), dried by co-evaporation with DMF (2 × 1 mL), in DMF (100 μL) was added and stirring was continued. The progress of the condensation was followed by LC-MS analysis (Alltima C_{18} analytical column, buffer A: H_2O; B: CH_3CN; C: 0.5% aq. TFA; linear gradient of B: 10 → 90% in 20 min) which showed the reaction to be complete after 27 hours. The reaction mixture was diluted with H_2O (3 mL) and purified by RP HPLC (Alltima, C_{18}, semi-preparative column, buffer A: H_2O; B: CH_3CN; C: 1% aq. AcOH; linear gradient of B, 10 → 48%, 24 min, in 5 CV) to give pure title compound **21** (12.6 mg, 3.8 μmol, 50%). ESI-MS: m/z = 1714.8 $[M + 2H]^{2+}$. RP HPLC R_t 16.56 min (Alltima analytical column C_{18}, buffer A: H_2O; B: CH_3CN; C: 0.5% aq. TFA) linear gradient in B (10 → 50%) in 20 min. MALDI-TOF-MS: m/z = 3428.96 $[M + H]^+$ (product-TBS); ^{31}P-NMR (D_2O) δ 0.44; −3.73.

VPg–pUpU 2. Partially protected nucleopeptide **21** (12.6 mg, 3.8 μmol) was dissolved in DMF (1 mL). Under a blanket of argon were added $Me_2NH{\cdot}BH_3$ (2.3 mg, 40 μmol), PPh_3 (cat. amount) and $Pd(PPh_3)_4$ (1.1 mg, 1 μmol). After stirring for 3 hours with the exclusion of light, the reaction mixture was diluted with RNase-free water (3 mL) and extracted with diethyl ether (1 mL). The water layer was evaporated and analyzed with LC-MS. The deprotection was found to be incomplete and the procedure described above was repeated once more. The obtained crude **2** was diluted with aq. $NH_4^+HCO_3^-$ (0.15 M, 5 mL) and applied onto a Sephadex G-25 column. Elution with aqueous ammonium bicarbonate (0.15 M) gave pure VPg–pUpU **2** (1.5 mg, 0.5 μmol, 13%), which was lyophilized twice from H_2O. MALDI-TOF-MS: m/z = 3021.4 $[M + H]^+$; 3043.5 $[M + Na]^+$; 3060.3 $[M + K]^+$; calc. 3019.40 $[M + H]^+$. RP HPLC R_t 14.1 min (Jasco, Alltima C_{18} analytical column, buffer A: 5% CH_3CN in 0.1% aq. AcOH and; B: 75% CH_3CN in 0.1% aq. AcOH, linear gradient in B, 0 → 50%, in 25 min). ^{31}P-NMR (D_2O): δ −0.28; −4.40.

NMR experiments with coxsackie VPg–pUpU (2). Roughly 0.8 mg of the VPg–pUpU nucleopeptide **2** was dissolved in 240 μl D_2O, yielding a ∼1.2 mM sample. The pH of the sample was adjusted to 5.5 (meter reading) and transferred to a Shigemi NMR tube. All spectra were recorded at 25 °C on a Varian Inova 500 MHz spectrometer. Resonances were assigned using standard 2D DQF-COSY,[26] TOCSY,[27] ^{1}H-^{31}P HETCOR,[28] and NOESY[29] experiments. For the CT-DQF-COSY, a 67 ms constant time delay was used in the t_1 dimension.

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