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Stepwise solid phase synthesis of uridylylated viral genome-linked peptides using uridylylated amino acid building blocks

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Abstract—The uridylylated amino acid building blocks 2-cyanoethyl-(N^{α}-9-fluorenylmethoxy-carbonyl-tyrosin-4-yl)-(2′,3′-di-*O*-acetyl-uridin-5′-yl) phosphate and 2-chlorophenyl-(N^{α}-fluorenyl-methoxycarbonyl-serin-3-yl)-(2′,3′-di-*O*-acetyluridin-5′-yl) phosphate have been used successfully in an on-line SPPS of the VPgpU from the polio, coxsackie and cowpea mosaic virus.

1. Introduction

Poliovirus is perhaps the best known member of the *Picornaviridae*, a family of plus-stranded RNA viruses including a large number of pathogens with widely different host range and disease symptoms.[1] An unusual but common feature of the picornaviruses genome is the presence of a small protein, VPg (viral protein genome-linked), covalently attached to the 5′-end of the plus-stranded viral genome which in turn is terminated with a poly(A)-tail.[2] Virus replication in the infected host is a two-step process, carried out primarily by the RNA polymerase in conjunction with other viral and possibly also cellular proteins. The incoming viral RNA is transcribed into complementary minus strands, which are then used as templates for the synthesis of the progeny plus strands. Although the basic steps are well known, very little is understood about the details of these processes and particularly about the exact function of the *cis*-acting RNA structures contained within picornaviral RNAs.[3] Thus, one of the important unanswered

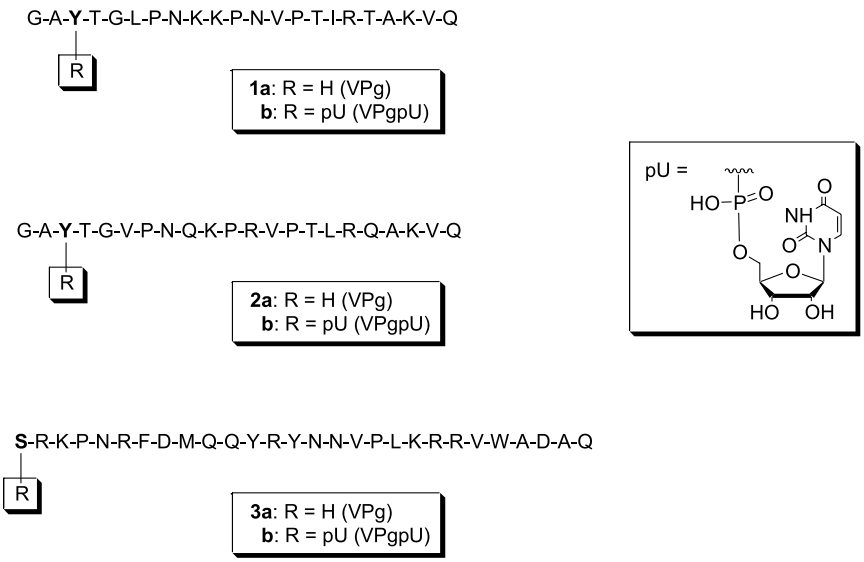

Figure 1. VPgpUs from poliovirus (**1b**), coxsackie virus (**2b**) and cowpea mosaic virus (**3b**).

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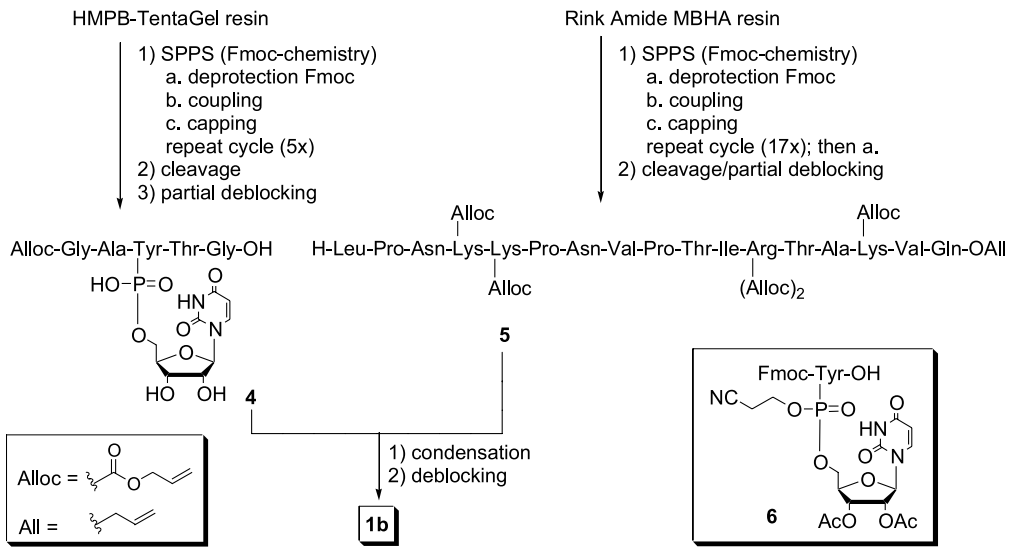


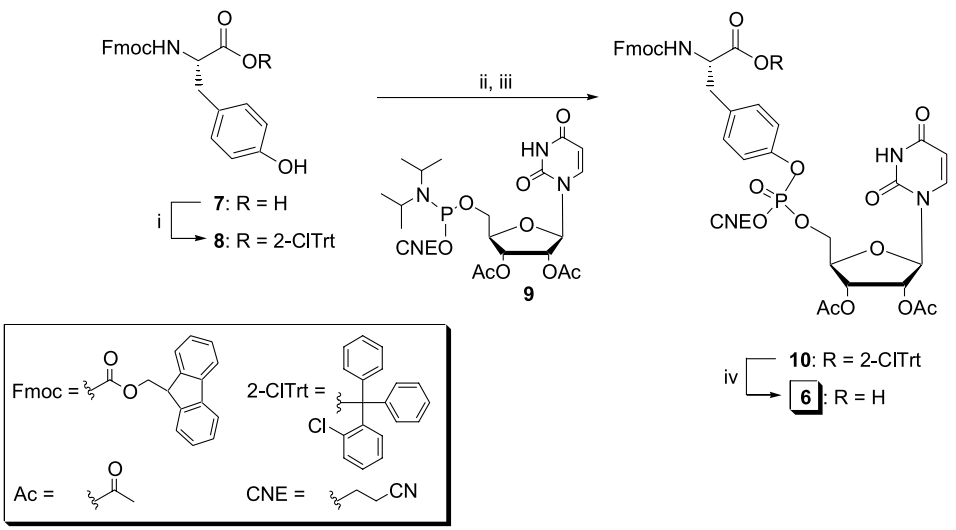
Chart 1. Synthesis of poliovirus VPgpU by fragment condensation.[5]

questions about minus-strand synthesis is how the viral RNA polymerase recognizes and selects only its own RNA as template. It has been shown[4] that the postulated first step in the replication of the plus-stranded poliovirus RNA could be initiated using both synthetic VPg (**1a**, Fig. 1) and VPgpU[5] (**1b**) as primers. For example, uridylylation of **1a** with uridine triphosphate, Mg^{2+} ions, purified poliovirus RNA polymerase $3D^{pol}$ and using poly(A) as a template led to the formation of VPg-poly(U), the 5′-end of the minus strand RNA.[4] It was envisaged that the poly(A)-dependent process would confer sufficient specificity to poliovirus RNA replication. However, recent studies[6] clearly indicated that a small RNA hairpin in the coding region of the protein 2C in poliovirus RNA serves as the primary template in the in vitro uridylylation of VPg (**1a**).

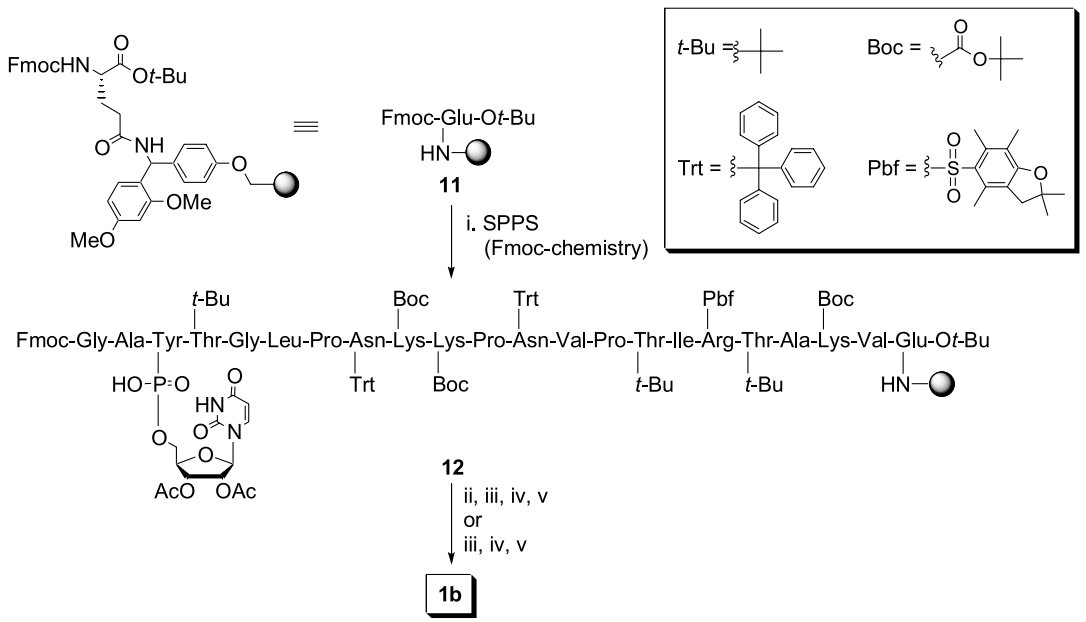
With the objective of studying the latter process in more detail, we here report an online SPPS of the initially formed transcripts VPgpU (see Figure 1) from polio (**1b**), coxsackie[7] (**2b**) as well as cowpea mosaic[8] (**3b**) virus.

2. Results and discussion

The synthetic route to VPgpU (**1b**) devised earlier in our laboratory[5] comprised, as denoted in Chart 1, a fragment condensation of the *N*-terminal allyloxycarbonyl (Alloc) protected peptide **4** with the Alloc side-chain protected *C*-terminal allyl (All) ester peptide **5**, and subsequent removal under neutral conditions of the protecting groups. Both fragments were readily accessible by a stepwise solid phase peptide synthesis (SPPS) based on Fmoc-chemistry. Thus, sequential elongation of the acid-labile 4-hydroxymethyl-3-methoxyphenoxybutyric acid (HMPB)-Tentagel resin with the appropriately protected amino acids and the uridylylated tyrosine unit **6** gave, after cleavage and



Scheme 1. Synthesis of VPgpU **1b** from poliovirus. *Reagents and conditions*: (i) (a) Cs_2CO_3 (0.98 equiv.), DMF, 0°C→room temperature, 20 min; (b) 2-chlorotrityl chloride (1.1 equiv.), DMF, room temperature, 30 min. (ii) **9** (1 equiv.), *o*-NPT (2 equiv.), CH_2Cl_2, room temperature, 10 min. (iii) *t*-BuOOH, CH_2Cl_2, room temperature, 30 min. (iv) TFA/CH_2Cl_2 (2/98, v/v), TES, room temperature, 1 h, 38% yield based on **7**.



Scheme 2. Synthesis of VPgpU **1b** from poliovirus. *Reagents and conditions*: (i) repeat (21×) the following three-step cycle: (a) 2% DBU in DMF, room temperature, 5 min; (b) Fmoc-AA-OH (5 equiv.), BOP (5 equiv.), HOBt (5 equiv.), DiPEA (10 equiv.), NMP, room temperature, 1 h; (c) Ac_2O/DiPEA/NMP/HOBt, room temperature, 1 min. (ii) 2% DBU in DMF, room temperature, 5 min. (iii) TFA/TIS/H_2O (95/2.5/2.5, v/v/v), room temperature, 2 h. (iv) 25% conc. $NH_3{\cdot}H_2O$/1,4-dioxane (1/1, v/v), room temperature, 3 h. (iv) RP-HPLC purification.

partial unmasking, the uridylylated fragment **4**. Similarly, the partially protected sequence **5** was obtained by elongation of the 4-(2′,4′-dimethoxyphenyl-aminomethyl)-phenoxyacetamido-L-norleucyl-MBHA resin (Rink Amide MBHA resin) followed by cleavage and concomitant removal of the acid-labile side-chain protecting groups. This rather elaborate and time-consuming approach was an incentive to explore the feasibility of constructing VPgpU **1b** and **2b** via a continuous solid phase synthesis. In this respect, it was essential to find out whether both target compounds would be unscathed by the harsh acidic conditions required for the deprotection of the acid-labile side-chain protecting groups (cf. Chart 1) of the amino acids introduced in the on-line SPPS of **1b** and **2b** based on Fmoc-chemistry. To this end, the uridylylated tyrosine building unit **6** (see Chart 1) was subjected to neat trifluoroacetic acid (TFA). Work-up of the mixture, after 24 h at 20°C, led to a near quantitative recovery of **6**, indicating that the phosphodiester in both target peptides will be compatible with the unblocking of the side-chain protecting groups in the final stage of the synthesis of VPgpU **1b** and **2b**. It also turned out that the number of steps in the synthesis of the earlier reported[5] uridylylated tyrosine building block **6** could be reduced following the procedure depicted in Scheme 1. Thus, tritylation of the cesium-salt of Fmoc-Tyr (**7**) with 2-chlorotrityl chloride led, after work-up, to the isolation of **8**. Phosphitylation of the phenolic hydroxyl in crude **8** with known[5] 2-cyanoethyl-(2′,3′-di-*O*-acetyl-uridin-5′-yl)-phosphoramidite (**9**) in the presence of 5-(*ortho*-nitrophenyl)tetrazole (*o*-NPT),[9] followed by in situ oxidation with *tert*-butylhydroperoxide, resulted in the isolation of **10**. Acidolysis of the 2-chlorotrityl ester in **10** using triethylsilane (TES) as a trityl cation scavenger gave after purification the homogeneous tyrosine building block **6** in 38% overall yield.

The sequence of events in the stepwise SPPS of poliovirus VPgpU (**1b**) is depicted in Scheme 2. Accordingly, the Fmoc-group in the commercially available glutamic acid derivative **11**, attached via the side-chain to Rink Amide MBHA resin, was removed with 2% 1,8-diazabicyclo-[5,4,0]-undec-7-ene (DBU) in *N*,*N*-dimethylformamide (DMF).[10] Subsequently, the free amine was condensed with the suitably protected amino acids under the agency of benzotriazole-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) and 1-hydroxybenzotriazole (HOBt) in the presence of *N*,*N*-diisopropylethylamine (DiPEA). The β-branched amino acids, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH as well as the key uridylylated tyrosine building block **6** were incorporated by executing a double condensation step. Although it is known[5] that removal of the Fmoc group at this stage of the synthesis is accompanied by β-elimination of the cyanoethyl group, the resulting phosphodiester is not expected[11] to give rise to the formation of unwanted side-products in the ensuing two elongation steps. The synthesis of the fully protected immobilized sequence **12** was completed by double coupling of the last two amino acids i.e. Fmoc-Ala-OH and Fmoc-Gly-OH. The nucleopeptide **12** was deprotected and cleaved from the resin by the following three-step procedure. First, the *N*-terminal Fmoc-group was removed by treatment with 2% DBU in DMF. Secondly, the peptide was cleaved from the resin with concomitant removal of the side-chain protecting groups by acidolysis with 95% aq. TFA in the presence of triisopropylsilane (TIS) as cation scavenger. Deblocking of the acetyl groups in the uridine moiety with concentrated aqueous ammonia in 1,4-dioxane led to the isolation of the crude nucleopeptide **1b**. RP-HPLC purification afforded homogeneous poliovirus VPgpU **1b** (see Figure 2a), the identity of which was ascertained by ^{31}P NMR (D_2O, δ −4.1 ppm) and MALDI-TOF-MS (m/z=2659.6 $[M+H]^+$) analysis. The steps involved in the transformation of **12** into target VPgpU **1b** could be reduced by taking into consideration that the Fmoc group can be

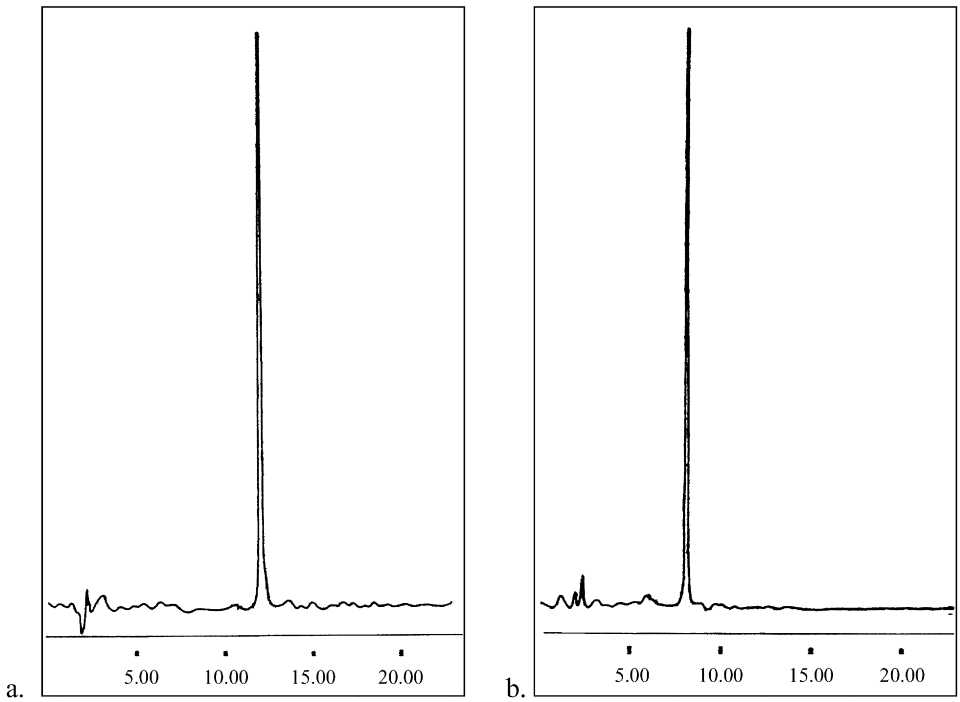
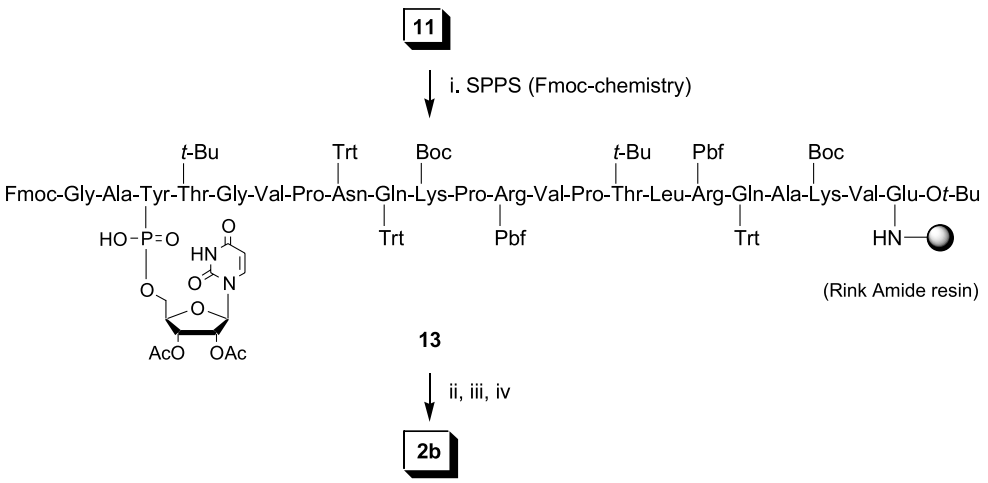


Figure 2. RP-HPLC trace of (a) poliovirus VPgpU **1b** and (b) coxsackie virus VPgpU **2b**.

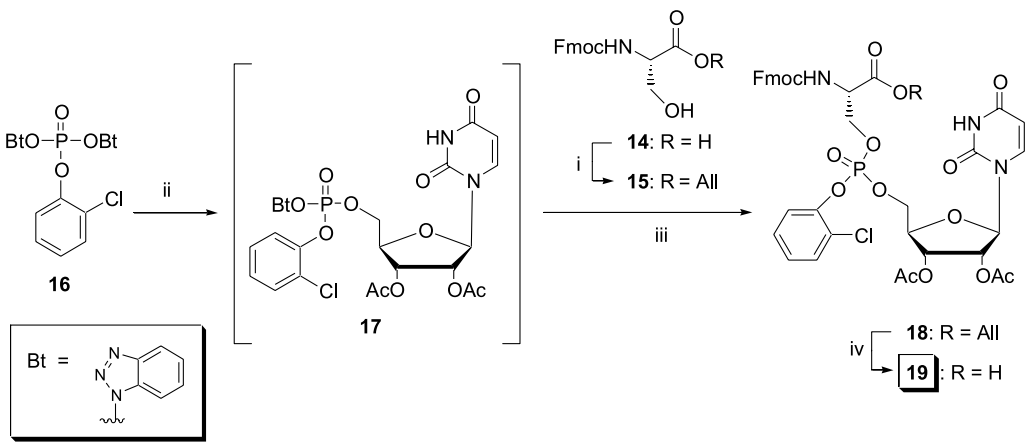
readily removed[12] under the same basic conditions as used in the deacetylation step. Indeed, acidolysis of **12** followed by treatment with concentrated ammonia led to the isolation of **1b**, which was in every aspect identical to the same compound obtained via deblocking of **12** by the three-step protocol.

The successful outcome of the on-line SPPS of VPgpU (**1b**) of poliovirus was an incentive for constructing VPgpU (**2b**) from coxsackie virus by the same methodology. Accordingly, elongation of the immobilised glutamic acid derivative **11**, as outlined in Scheme 3, with suitably protected amino acids proceeded uneventfully to give the immobilized and fully protected uridylylated peptide **13**. Deprotection and cleavage of **13** from the resin following the two-step protocol, as described for the unmasking of **12**, gave crude **2b**. Purification by RP-HPLC yielded homogeneous coxsackie virus VPgpU **2b** (see Figure 2b), the structure of which was ascertained by ^{31}P NMR (D_2O, δ −4.2 ppm) and MALDI-TOF-MS (m/z=2715.9 $[M+H]^+$).

The uneventful and smooth on-line SPPS of both VPgpU's **1b** and **2b** urged us to adopt the same approach to the preparation of the VPgpU **3b** of cowpea mosaic virus containing an *N*-terminal serine linked via a phosphodiester bond to the 5′-hydroxyl function of uridine. It is well established[13] that the serine uridylylated moiety in the target VPgpU **3b** will be rather prone, especially so under the influence of a strong base, to β-elimination leading to the unwanted dehydroalanine derivative of VPgpU **3b** and

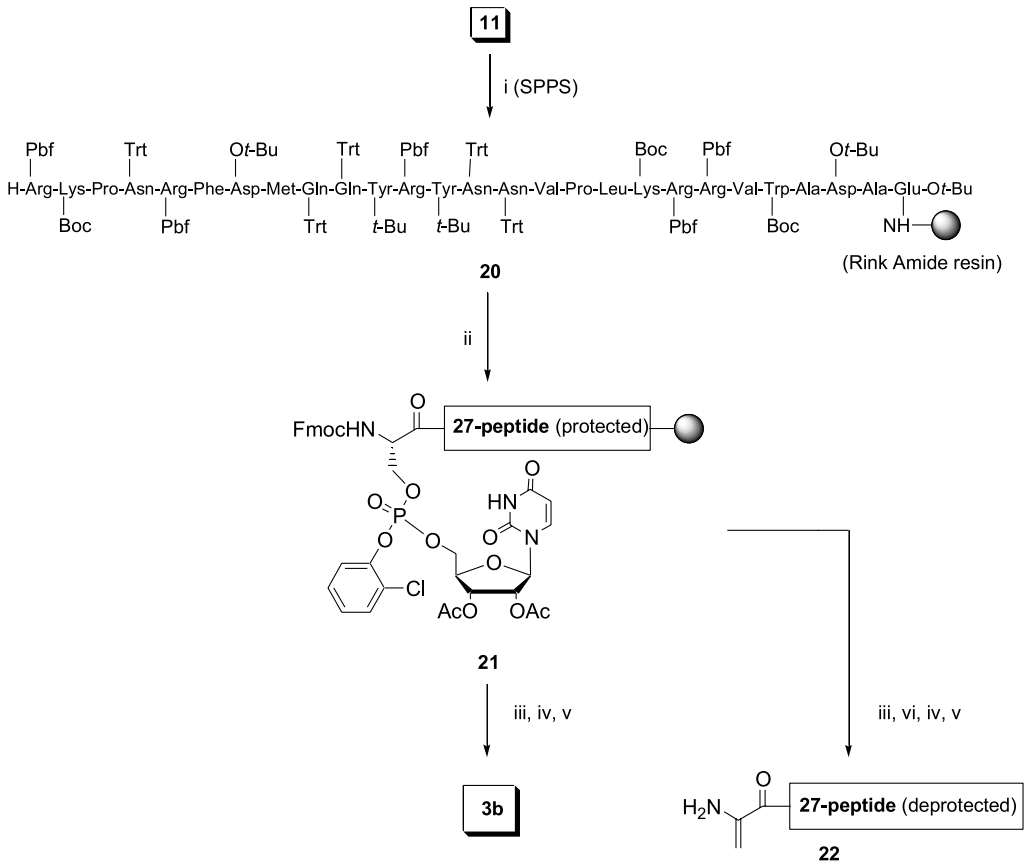


Scheme 3. Synthesis of VPgpU **2b** from coxsackie virus. *Reagents and conditions*: (i) repeat (21×) the following three-step cycle: (a) 2% DBU in DMF, room temperature, 5 min; (b) Fmoc-AA-OH (5 equiv.), BOP (5 equiv.), HOBt (5 equiv.), DiPEA (10 equiv.), NMP, room temperature, 1 h; (c) Ac_2O/DiPEA/NMP/HOBt, room temperature, 1 min. (ii) TFA/TIS/H_2O (95/2.5/2.5, v/v/v), room temperature, 2 h. (iii) 25% conc. $NH_3{\cdot}H_2O$/1,4-dioxane (1/1, v/v), room temperature, 3 h. (iv) RP-HPLC purification.



Scheme 4. Synthesis of the uridylylated serine derivative **19**. *Reagents and conditions*: (i) Ag_2CO_3 (1.3 equiv.), AllBr (4 equiv.), DMF, 0°C→room temperature, 3 h, 89%. (ii) 2′,3′-Di-*O*-acetyluridine (1 equiv.), 1,4-dioxane. (iii) **15**, pyridine/1,4-dioxane, room temperature, 2 h, 87%. (iv) AcOH (4.5 equiv.), Bu_3SnH (2 equiv.), $Pd(PPh_3)_4$ (4 mol%), CH_2Cl_2/THF (1/1, v/v), room temperature, 1 h, 91%.

uridine-5′-phosphate. The latter undesired scission can, however, be decreased substantially using a less strong base. For example, the integrity of the phosphodiester linkage can be preserved for a relatively long period of time in concentrated ammonia.[14] Moreover, the same basic conditions can also be used successfully, as mentioned before, in the unmasking of an Fmoc protecting group.[12] It may therefore be expected that the unblocking of the *N*-terminal Fmoc group from the serine uridylylated moiety, introduced in the final stage of the SPPS, will be compatible with the presence of the phosphodiester linkage in the target compound **3b**. Previous studies from our laboratory on the synthesis of serine uridylylated containing peptides revealed[15] that a 2-chlorophenyl protecting group could be removed under mild conditions[16] with fluoride ions (i.e. TBAF/pyridine/DMF/H_2O). The latter operation generates



Scheme 5. Synthesis of VPgpU **3b** of cowpea mosaic virus. *Reagents and conditions*: (i) repeat (26×) the following three-step cycle: (a) 20% piperidine in DMF, room temperature, 5 min; (b) Fmoc-AA-OH (5 equiv.), BOP (5 equiv.), HOBt (5 equiv.), DiPEA (10 equiv.), NMP, room temperature, 1 h; (c) Ac_2O/DiPEA/NMP/HOBt, room temperature, 1 min; then 20% piperidine in DMF, room temperature, 5 min. (ii) **19** (10 equiv.), DIC (10 equiv.), HOBt (10 equiv.), CH_2Cl_2/DMF, (95/5, v/v), room temperature, 1 h. (iii) TBAF (0.25 M) in pyridine/DMF/H_2O (3/4/1, v/v/v), room temperature, 2 h. (iv) TFA/TIS/H_2O/PhOH/EDT (90/0.12/0.8/0.12/0.8, v/v/v/w/v), room temperature, 4 h. (v) aq. conc. $NH_3 \cdot H_2O$ (25%)/1,4-dioxane (1/1, v/v), room temperature, 3 h. (vi) 20% piperidine in DMF, room temperature, 5 min.

a phosphodiester function which will survive, as required, ensuing deblocking steps involving base and acid treatments. On the basis of the foregoing information it was decided[17] to use the serine uridylylated derivative **19** (see Scheme 4), the phosphodiester function of which is temporarily protected with the 2-chlorophenyl group, as the building unit in the on-line SPPS of target VPgpU **3b**.

The synthesis of **19** is depicted in Scheme 4 and commences with the selective allylation of the silver-salt of Fmoc-serine **14** to give allyl ester **15**. The uridine phosphate moiety was then introduced by the following one-pot-two-step procedure. Thus, phosphorylation of 2′,3′-di-*O*-acetyluridine with the known[18] bifunctional phosphorylating agent **16**, and in situ condensation of intermediate **17** with the protected serine derivative **15**, gave the 2-chlorophenyl phosphotriester derivative **18**. Pd(0)-catalyzed hydrostannolysis[19] of the allyl ester in **18** furnished the serine building block **19** in 70% overall yield.

The synthesis of **3b** started, as outlined in Scheme 5, with the assembly of the immobilized peptide **20**. Initially, a stepwise SPPS of **20** was performed under the same conditions as previously described for the assembly of nucleopeptide **1b**. However, deprotection and cleavage from the resin followed by extensive LC/MS analysis showed the presence of truncated fragments due to incomplete couplings of the amino acids: [Gln^{10}], [Gln^{11}], [Tyr^{12}], [Tyr^{14}], [Pro^{18}], [Leu^{19}] and [Lys^{20}]. The latter could be circumvented by executing double couplings for these amino acids. The resulting immobilized peptide **20** was then elongated by condensation with 2-chlorophenyl-N^{α}-(9-fluorenylmethoxycarbonyl)-serin-3-yl-(2′,3′-di-*O*-acetyluridin-5′-yl) phosphate **19**. To prevent the possible occurrence of β-elimination, the introduction of building block **19** was executed under slightly acidic conditions using *N*,*N*′-diisopropylcarbodiimide (DIC) and HOBt to furnish the immobilised and fully protected nucleopeptide **21**. Transformation of **21** into VPgpU **3b** was performed in a three-step sequence starting with the removal of the 2-chlorophenyl group by treatment with aq. TBAF. The peptide was then subjected to TFA-mediated cleavage from the resin with concomitant removal of all acid-labile protecting groups. Unmasking of both the *N*-terminal amine function as well as the hydroxyl groups with concentrated aqueous ammonia in 1,4-dioxane yielded crude VPgpU **3b** not contaminated, as evidenced by LC/MS analysis, with the undesired dehydroalanine derivative **22**. Purification by cation exchange followed by desalting, gave homogeneous (see Figure 3) **3b**, the identity of which was corroborated by ESI-MS (m/z=1923.4 $[M+2H]^{2+}$) and ^{31}P NMR (D_2O, δ −0.1 ppm).

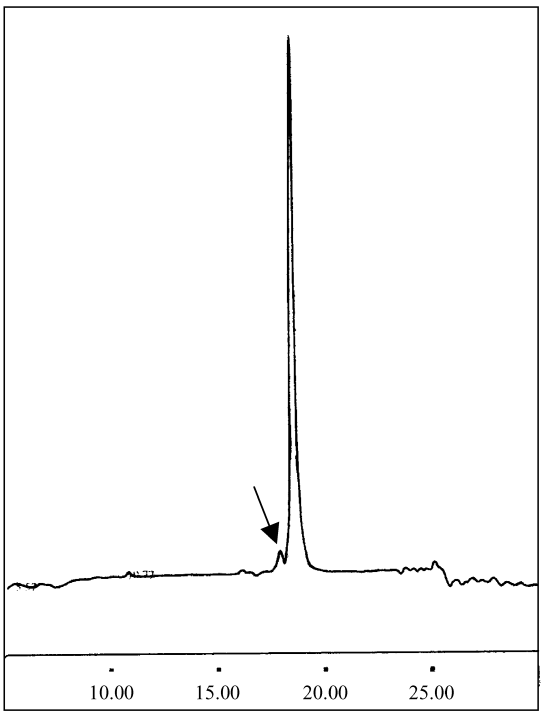


Figure 3. RP-HPLC trace of cowpea mosaic virus VPgpU **2**. The purified product is contaminated with a small quantity (see arrow) of a product having a mass of 16 m.u. higher than the target VPgpU **3b**. The latter finding may be ascribed to oxidation of the methionine unit in **3b**.

On the other hand, subjection of **21** to the following sequential four-step deblocking process (i.e. fluoride ions, 20% piperidine, TFA/TIS/H_2O/PhOH/EDT and then ammonolysis) revealed, as gauged by LC/MS analysis, the presence of the dehydroalanine derivative **22** of VPgpU **3b** as the major product. The latter observation also nicely confirms the incompatibility of the serine phosphodiester function, resulting from the fluoride treatment of **21**, with the removal of the Fmoc-group using piperidine as the base.

In conclusion, the results presented in this paper clearly show that biologically important uridylylated VPg's can be readily prepared via an on-line SPPS approach.

3. Experimental

3.1. General methods and materials

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. All reagents were obtained from Acros Chemicals and used as received unless otherwise stated. $Pd(PPh_3)_2Cl_2$ was bought from Aldrich. Solvents used in the automated peptide synthesis, DiPEA and TFA were all of peptide synthesis grade (Biosolve) and used as received. BOP was obtained from Senn Chemicals and anhydrous HOBt from Neosystem. Fmoc-Glu (Rink Amide MBHA resin)-O*t*-Bu, 2-chlorotrityl chloride and the protected amino acids were bought at NovaBiochem. All the amino acids applied in the synthesis were the naturally occurring L-amino acids. 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 μm particle size) was used. Buffers: A: H_2O, B: CH_3CN and C: 0.5% aq. TFA. For RP-HPLC purification of the uridylylated peptides 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 μm particle size, running at 4 mL min^{-1}) was used. The applied buffers were A: H_2O, B: CH_3CN and C: 1% aq. TFA. Detection was performed by UV, simultaneous at 214 and 254 nm. Cation exchange chromatography was executed on a Poros® 10S column running at 5 mL min^{-1} (PerSeptive Biosystems, inc., 4.6 mm×100 mm) using A: H_2O, B: CH_3CN, C: NaCl (3 M) and D: Hepes (0.05 M) in NaOAc (0.05 M, pH 7.5) as buffer-solutions and detection at 280 nm. Subsequent desalting was achieved on a Poros® R2 column (4.60 mm×100 mm, 5 mL min^{-1}, PerSeptive Biosystems, inc.) applying buffers: A: H_2O, B: CH_3CN and C: 1% aq. TFA. Detection was performed at 280 nm. MALDI-TOF-MS spectra were recorded on a Voyager-DE PRO mass spectrometer (PerSeptive Biosystems, inc.).

General procedure for solid phase peptide synthesis (*SPPS*). The nucleopeptides **1b**, **2b** and **3b** were prepared on an ABI 433A (Applied Biosystems, division of Perkin–Elmer) automatic peptide synthesiser using the FastMoc® peptide synthesis. The peptides were prepared on a 50 μmol scale starting from 4-(2′,4′-dimethoxyphenyl-aminomethyl)-phenoxyacetamido-L-norleucyl-MBHA resin (Rink Amide MBHA resin) to which Fmoc-Glu-O*t*-Bu is attached via the side-chain carboxylic acid functionality (initial loading: 0.34 mmol g^{-1}).

The consecutive steps performed in each cycle were:

1. Deprotection of the Fmoc-group with 2% DBU in DMF for 5×1 min, unless stated otherwise.
2. Coupling of the appropriate amino acid applying a five-fold excess. Generally, the 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 h.
3. The unreacted amino functions were capped by acetylation with 0.5 M acetic anhydride, 0.125 M DiPEA and 0.015 M HOBt in NMP. The resulting suspension was shaken for 1 min.

3.1.1. N^{α}-(9-Fluorenylmethoxycarbonyl)-tyrosine 2-chlorotrityl ester (8). To a cooled solution (0°C) of N^{α}-Fmoc-tyrosine **7** (1.6 g, 4.0 mmol) in DMF (50 mL) was added a solution of Cs_2CO_3 (0.64 g, 1.9 mmol) in water (1 mL). The reaction mixture was stirred at room temperature for 20 min and concentrated in high vacuum. The residue was suspended in DMF (50 mL) and 2-chlorotrityl chloride (1.3 g, 4.4 mmol) was added resulting in a clear solution followed by rapid formation of a white precipitate. After stirring for 2 h the reaction was quenched by addition of methanol (5 mL) and the solvents were evaporated. The residue was dissolved in EtOAc (100 mL), washed with water (50 mL) and aqueous $NaHCO_3$ (5%, 50 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated in vacuo. Obtained crude title compound **8** was used without further purification in the phosphitylation reaction.

3.1.2. 2′,3′-Di-*O*-acetyl-5′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylaminophosphoramidite (9). 2′,3′-Di-*O*-acetyluridine[20] (1.3 g, 4.0 mmol) was dried by coevaporation with 1,4-dioxane (2×15 mL) and dissolved in CH_2Cl_2 (20 mL). Under a blanket of nitrogen triethylamine (2.7 mL, 19 mmol) was added followed by slow addition of a solution of 2-cyanoethoxy-*N*,*N*-diispropylamino chlorophosphine[21] (1.1 mL, 4.8 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was stirred for 30 min, diluted with CH_2Cl_2 (50 mL) and washed with water (40 mL) and aq. $NaHCO_3$ (5%, 20 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated. The residue was dissolved in CH_2Cl_2 (10 mL) and precipitated by addition to light petroleum (75 mL). After centrifugation, the supernatant was carefully decanted and **9** was obtained as a slightly yellow coloured oil, which was used in the next step without further purification. ^{31}P NMR (CH_2Cl_2, acetone capillary): δ 150.2, 149.5.

3.1.3. 2-Cyanoethyl-N^{α}-(9-fluorenylmethoxycarbonyl)-tyrosin-4-yl-(2′,3′-di-*O*-acetyluridin-5′-yl)phosphate (6). N^{α}-Fmoc-tyrosine 2-chlorotrityl ester **8** (crude, 4 mmol) and 2′,3′-di-*O*-acetyluridine phosphoramidite **9** (crude, 4 mmol) were coevaporated with CH_3CN (2×20 mL) and dissolved in CH_3CN (20 mL). Under nitrogen atmosphere dry *o*-NPT (1.5 g, 8 mmol) was added and the reaction mixture was stirred for 1 h after which ^{31}P NMR analysis (acetone capillary, δ 135.3 ppm) showed complete consumption of the amidite. Subsequently, *t*-BuOOH (1.9 mL, 16 mmol) was added and stirring was continued for 30 min. The mixture was poured into EtOAc/H_2O (2/1, v/v, 60 mL) and the layers were separated. The organic layer was washed with aq. $NaHCO_3$ (5%, 25 mL), dried ($MgSO_4$), filtered and concentrated to give crude **10**. ^{31}P NMR ($CDCl_3$): δ −6.1; −6.2. Crude **10** was dissolved in TFA/CH_2Cl_2 (2/98, v/v, 10 mL) and stirred for 10 min followed by addition of TES (0.5 mL). After 10 min the addition of TFA/CH_2Cl_2 and TES with intermediates of 10 and 5 min respectively, was repeated twice. The reaction mixture was concentrated and purified (silica gel, light petroleum/EtOAc/MeOH, 1/1/0→0/95/5, v/v/v). Concentration of the appropriate fractions furnished title compound **6** (1.3 g, 1.5 mmol, 38% based on Fmoc-tyrosine) as an oil. ^{13}C NMR ($CDCl_3$/MeOD/D_2O, 45/50/5, v/v/v): δ 173.4 (C=O Tyr), 169.8 (2×C=O Ac), 163.9 (C-4 U), 155.8 (C=O Fmoc), 150.4 (C-2 U), 144.9 (C_q Tyr), 143.4, 140.9 (C_q Fmoc), 140.1 (C-6 U), 134.2 (C_q Tyr), 130.7, 127.3, 126.7, 124.7, 119.5 (C_{arom} Fmoc, Tyr), 116.4 (CN), 102.7 (C-5 U), 87.6 (C-1′), 72.2, 69.3, 66.4 (C-2′, C-3′, C-4′), 66.4 (CH_2 Fmoc), 63.0 (C-5′), 54.7 (αCH Tyr), 46.7 (CH Fmoc), 36.7 (βCH_2 Tyr), 19.7, 19.1 (2×CH_3 Ac). ESI-MS: *m*/*z*= 847.5 $[M+H]^+$, 869.4 $[M+Na]^+$.

3.1.4. Poliovirus VPgpU (1b). Protected peptide **12** was synthesised as described in the general procedure for solid phase peptide synthesis starting from Fmoc-Glu (Rink Amide MBHA resin)-O*t*-Bu **11** (147 mg, 50 μmol, 0.34 mmol g^{-1}). The following amino acid derivatives were applied: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Thr (*t*-Bu)-OH and Fmoc-Val-OH. Amino acid derivative **6** was coupled twice by a standard coupling cycle. A small portion of the fully protected immobilised peptide **12** (100 mg, approximately 12 μmol) was transferred into a round bottom flask, a solution of 2% DBU in DMF (5 mL) was added and the suspension was shaken for 5 min. The resin was placed on a glass filter and washed with DMF (5×5 mL). Next, the resin was suspended in a mixture of TFA/TIS/H_2O (95/2.5/2.5, v/v/v, 10 mL) and shaken for 4 h. The resin was filtered, washed with TFA and the filtrate was concentrated to dryness. The residue was coevaporated with toluene (2×10 mL) and subsequently dissolved in a mixture of 25% conc. aq. ammonia and 1,4-dioxane (1/1, v/v, 10 mL). After 5 h, careful evaporation of the solvents yielded crude **1b**. RP-HPLC purification (linear gradient of 15→20% B in 17 min; 3.5 CV) followed by lyophilisation furnished nucleopeptide **1b** (6.8 mg, 19%, TFA-salt) as a fluffy solid. ^{31}P NMR (D_2O): δ −4.14. MALDI-TOF-MS: *m*/*z*=2659.56 $[M+H]^+$, 2681.38 $[M+Na]^+$, 2697.64 $[M+K]^+$, calculated mass 2659.4. LC/MS analysis: R_t 8.35 min (linear gradient of 5→50% B in 20 min), ESI-MS: *m*/*z*=2660.0 $[M+H]^+$, 1330.8 $[M+2H]^{2+}$, 887.4 $[M+3H]^{3+}$, 665.6 $[M+4H]^{4+}$.

3.1.5. Coxsackie virus VPgpU (2b). The nucleopeptide **13** was synthesised starting from **11** and deprotected as described for **1b**. The crude nucleopeptide **2b** (50 μmol) was purified by RP-HPLC (linear gradient of 15→23% B in 12.3 min, 2.5 CV). Lyophilisation yielded 22.5 mg (14%, TFA-salt) of pure coxsackie virus VPgpU **2b** as a fluffy solid. ^{31}P NMR (D_2O): δ −4.15. MALDI-TOF-MS: *m*/*z*=2715.85 $[M+H]^+$, 2738.01 $[M+Na]^+$, calculated mass 2715.7. LC/MS analysis: R_t 8.01 min (linear gradient of 5→50% B in 20 min), ESI-MS: *m*/*z*=2716.0 $[M+H]^+$, 1358.4 $[M+2H]^{2+}$, 906.0 $[M+3H]^{3+}$, 679.8 $[M+4H]^{4+}$.

3.1.6. N^{α}-(9-Fluorenylmethoxycarbonyl)-serine allyl ester (15). To a cooled solution (0°C) of Fmoc-Ser-OH **14** (3.7 g, 10 mmol) in DMF (40 mL) silver carbonate (3.6 g, 13 mmol) was added and the mixture was stirred for 15 min at room temperature. After addition of allylbromide (4.0 mL, 46 mmol), stirring was continued for 2.5 h. The mixture was filtered, diluted with ethyl acetate (200 mL) and washed with 10% aq. $KHSO_4$ (2×50 mL) and water (2×75 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by silica gel column chromatography (light petroleum/EtOAc, 100/0→1/3, v/v) afforded compound **15** as a white solid (3.7 g, 8.9 mmol, 89%). ^{13}C NMR ($CDCl_3$): δ 169.5 (C=O ester), 155.5 (C=O Fmoc), 143.0, 142.8, 140.1 (C_q Fmoc), 130.9 (=CH All), 126.7, 126.1, 124.3, 118.9 (C_{arom} Fmoc), 116.9 (=CH_2 All), 65.9, 64.6, 61.5 (OCH_2 All, Fmoc; βCH_2 Ser), 55.7 (αCH Ser), 46.0 (CH Fmoc). ^{1}H NMR ($CDCl_3$): δ 7.77 (m, 2H, H_{arom} Fmoc), 7.61 (m, 2H, H_{arom} Fmoc), 7.37 (m, 4H, H_{arom} Fmoc), 5.92 (br m, 1H, =CH All), 5.70 (d, 1H, *J*=6.58 Hz, NH), 5.31 (t, 2H, *J*=17.55 Hz, *J*=11.69 Hz, =CH_2 All), 4.70 (d, 2H, *J*=5.12 Hz, OCH_2 All), 4.44 (d, 2H, *J*=6.58 Hz, OCH_2 Fmoc), 4.24 (t, 1H, *J*= 6.58 Hz, *J*=7.31 Hz, CH Fmoc), 4.02 (m, 3H, αCH, βCH_2 Ser). ESI-MS: *m*/*z*=368.2 $[M+H]^+$, 390.3 $[M+Na]^+$.

3.1.7. *O*-(2-Chlorophenyl)-N^{α}-(9-fluorenylmethoxycarbonyl-serin-3-yl allyl ester)-(2′,3′-di-*O*-acetyl-uridin-5′-yl) phosphate (18). To a stirred solution of 2′,3′-di-*O*-acetyluridine (1.6 g, 5.0 mmol, coevaporated with pyridine (2×20 mL) in 1,4-dioxane (20 mL) was added *O*-(2-chlorophenyl)-*O*,*O*-bis(1-benzotriazolyl) phosphate **17**[15] (0.2 M in 1,4-dioxane, 25 mL, 5 mmol). After 1 h, TLC analysis (MeOH/CH_2Cl_2, 1/9, v/v) indicated complete conversion of the starting material into the lower running compound **18**. Then a solution of N^{α}-Fmoc-serine allyl ester **15** (1.8 g, 5.0 mmol) in pyridine (15 mL) was added and stirring was continued for 2.5 h. The reaction mixture was diluted with CH_2Cl_2 (100 mL), washed with aq. $NaHCO_3$ (2%, 2×50 mL), water (2×75 mL) and aq. AcOH (2%, 2×50 mL), dried ($MgSO_4$), filtered and concentrated. Purification by silica gel column chromatography (EtOAc/light petroleum, 1/1→100/0, v/v) furnished **18** as a colourless oil (3.8 g, 4.4 mmol, 87%, based on 2′,3′-di-*O*-acetyluridine). ^{31}P NMR ($CDCl_3$): δ −6.4, −6.9. ^{13}C NMR ($CDCl_3$): δ 169.4, 169.5 (2×C=O Ac), 168.1 (C=O ester), 163.0 (C-4 U), 155.7 (C=O Fmoc), 150.3 (C-2 U), 143.4, 141.0 (C_q Fmoc), 139.6 (C-6 U), 130.5 (=CH All), 130.9, 127.9, 127.4, 126.8, 126.3, 124.9, 121.2, 119.7 (C_{arom} Fmoc, Ph), 118.8 (=CH_2 All), 103.2 (C-5 U), 86.9. 86.5 (C-1′ U), 80.2, 72.2, 69.4 (C-2′, C-3′, C-4′ U), 68.5, 67.1, 66.4, 60.1 (OCH_2 All, Fmoc; C-5′ U; βCH_2 Ser), 54.2 (αCH Ser), 46.7 (CH Fmoc), 20.2 (2×CH_3 Ac). ^{1}H NMR ($CDCl_3$): δ 9.77 (br d, 1H, NH), 7.76 (m, 2H, H_{arom} Fmoc), 7.61 (m, 2H, H_{arom} Fmoc), 7.43-7.10 (m, 9H, H_{arom} Fmoc, Ph; H-6 U), 6.08 (m, 2H, H-1 U, NH Ser), 5.89 (m, 1H, =CH All), 5.69 (d, 1H, *J*=8.04 Hz, H-5 U), 5.40 (m, 2H, H-2′, H-3′), 5.26 (m, 2H, =CH_2 All), 4.70-4.22 (m, 11H, OCH_2 All, Fmoc; CH Fmoc; H-4′, H-5′a, H-5′b U; αCH, βCH_2 Ser), 2.11 (s, 6H, 2×CH_3 Ac). ESI-MS: *m*/*z*=868.3 $[M+H]^+$, 890.2 $[M+Na]^+$.

3.1.8. *O*-(2-Chlorophenyl)-N^{α}-(9-fluorenylmethoxycarbonyl)-serin-3-yl-(2′,3′-di-*O*-acetyluridin-5′-yl) phosphate (19). Allyl ester **18** (3.68 g, 4.2 mmol) was dissolved in a mixture of THF/CH_2Cl_2 (1/1, v/v, 20 mL). Acetic acid (1.1 mL, 19 mmol), Bu_3SnH (2.2 mL, 8.4 mmol) and $Pd(PPh_3)_2Cl_2$ (0.12 g, 0.17 mmol) were added, resulting in a yellow reaction mixture that immediately turned dark red. After stirring at room temperature for 30 min, the solvents were evaporated. The remaining oil was redissolved in CH_2Cl_2 and applied onto a silica gel column. Elution with CH_2Cl_2/MeOH (100/0→95/5, v/v) yielded uridylylated building block **19** as slightly yellow coloured oil (3.2 g, 3.8 mmol, 90%). ^{31}P NMR ($CDCl_3$): δ −6.52, −6.62. ^{13}C NMR ($CDCl_3$/MeOD, 3/1, v/v): δ 171.0 (C=O acid), 169.6 (2×C=O Ac), 165.1 (C-4 U), 157.2 (C=O Fmoc), 151.3 (C-2 U), 144.4, 141.9 (C_q Fmoc), 141.4 (C-6 U), 131.5, 129.0, 128.4, 127.7, 127.5, 125.8, 122.1, 120.5 (C_{arom} Fmoc, Ph), 103.6 (C-5 U), 88.7 (C-1′ U), 80.8, 73.4, 70.3 (C-2′, C-3′, C-4′ U), 69.6, 67.9, 62.1 (OCH_2 Fmoc, βCH_2 Ser, C-5′ U), 46.9 (CH Fmoc), 20.4 (2×CH_3 Ac). ^{1}H NMR ($CDCl_3$): δ

7.75 (m, 2H, H_{arom} Fmoc), 7.61 (m, 2H, H_{arom} Fmoc), 7.48-7.07 (br m, 9H, H_{arom} Fmoc, Ph; H-6 U), 6.11 (m, 2H, H-1′ U, NH Ser), 5.73 (d, 1H, J=8.04 Hz, H-5 U), 5.40, 5.29 (2×m, 2H, H-2′, H-3′ U), 4.65-3.88 (br m, 9H, CH, OCH_2 Fmoc; H-4′, H-5′a, H-5′b U; αCH, βCH_2 Ser), 2.08 (s, 6H, 2×CH_3 Ac). ESI-MS: m/z=828.3 $[M+H]^+$, 850.1 $[M+Na]^+$.

3.1.9. Cowpea mosaic virus VPgpU (3b). Immobilised peptide **20** was synthesised according to the general procedure for solid phase peptide synthesis. In every Fmoc deprotection cycle piperidine/DMF (1/4, v/v) was used. Additional double couplings were introduced for [Gln^{10}], [Gln^{11}], [Tyr^{12}], [Tyr^{14}], [Pro^{18}], [Leu^{19}] and [Lys^{20}]. A small amount of peptide-resin **20** (50 mg, ±13 μmol) was removed from the synthesiser, transferred into a round bottom flask and suspended in CH_2Cl_2 (2 mL). Subsequently, uridylylated serine building block **19** (107 mg, 0.13 mmol) and HOBt (18 mg, 0.13 mmol) were added, and the reaction mixture was gently stirred for 15 min. Then DIC (20 μL, 0.13 mmol) was added and stirring was continued for 1 h. The solvent and reagents were removed by filtration and the resin was repeatedly washed with CH_2Cl_2 (2×5 mL), MeOH (5 mL) and CH_2Cl_2 (2×5 mL). After the coupling-procedure was repeated once more, a negative Kaiser-test showed complete coupling of the uridylylated building block. The fully protected peptide-resin **21** was suspended in a solution of 0.2 M TBAF (1 M in THF, 1.25 mL) in DMF/H_2O/pyridine (4/1/3, v/v/v, 4 mL) and stirred for 2 h. The resin was filtered and washed with CH_2Cl_2 (2×5 mL), MeOH (5 mL) and CH_2Cl_2 (2×5 mL). Next, the resin was suspended in a mixture of TFA/TIS/H_2O/PhOH/EDT (90/1.2/0.8/0.8/1.2, v/v/v/w/v, 10 mL) and gently stirred under a blanket of argon for 4 h. After filtration, the resin was washed with TFA (3×5 mL) and the filtrate was concentrated. The remaining oil was dried in high vacuum (0.5 mm Hg, 15 min), redissolved in TFA (1 mL) and precipitated by addition to diethyl ether (15 mL). After washing with ether (3×5 mL), the white solid was taken up in $NH_3{\cdot}H_2O$ (25%)/1,4-dioxane (1/1, v/v, 10 mL) and stirred for 5 h at room temperature. Careful evaporation of the solvents afforded crude **3b**. The obtained product **3b** was purified by cation exchange chromatography (linear gradient (A/B/C/D, 60/20/0/20→52.5/20/7.5/20, v/v/v/v in 12 CV). Next, the obtained product was desalted (linear gradient of (10→50% B in 10 CV). After lyophilisation nucleopeptide **3b** was obtained as a white fluffy solid (2.7 mg, 5%, TFA-salt). ^{31}P NMR (D_2O): δ −0.10. LC/MS analysis: R_t 18.54 min (linear gradient of 10→50% B in 20 min). ESI-MS: m/z= 1923.0 $[M+2H]^{2+}$, 1282.1 $[M+3H]^{3+}$, 962.0 $[M+4H]^{4+}$, 770.1 $[M+5H]^{5+}$, calculated mass 3842.9.

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