Fully Automated Sequential Solid Phase Approach Towards Viral RNA-Nucleopeptides

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Supporting Information

General: 3'-O-DMT-2'-O-TBDMS-N°-benzoyladenosine-5'-(N,N-di-isopropylamino-2-cyanoethoxy) phosphoramidite, 3'-O-DMT-2'-O-TBDMS-uridine-5'-(N,N-di-isopropylamino-2-cyanoethoxy) phosphoramidite, -O-DMT-2'-O-TBDMS – N°-acetylcytidine-5'-(N,N-di-isopropylamino-2-cyanoethoxy) phosphoramidite and 3'-O-DMT-2'-O-TBDMS – N°-isobutyrylguanosine-5'-(N,N-di-isopropylamino-2-cyanoethoxy) phosphoramidite were purchased from ChemGenes Corporation, Wilmington, USA and used as received. HCP Custom Primer Support™ Amino 200 resin was purchased from GE Healthcare.

LC/MS analysis was performed on A) Jasco HPLC system (UV detection simultaneously at 214 and 254 nm) coupled to a PE/SCIEX API 165 single quadruple mass spectrometer (Perkin-Elmer) using an analytical Gemini C18 column (Phenomex, 50 x 4.60 mm, 3 micron) in combination with eluents A: H2O; B: MeCN and C: 0.1 M aq. NH4OAc B) Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer with an electrospray ion source coupled to Surveyor HPLC system (Thermo Finnegan) using an analytical Gemini C18 column (Phenomex, 50 x 4.60 mm, 3 micron) in combination with eluents A: H2O; B: MeCN and C: 1% aq. TFA as the solvent system.

High resolution mass spectra spectra were recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000) and dioctylphalate (m/z = 391.2842) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).

HCP-HMBA resin

Amino HCP resin (1 g, 200 μmol) was treated with 4-hydroxymethylbenzoic acid (HMBA) (92 mg, 600 μmol), BOP (266 mg, 600 μmol), HOBt (82 mg, 600 μmol) and DiPEA (200 μl, 1.2 mmol) in 10 ml DMF for 5 hours at room temperature. After washing with DCM three times, the unreacted amines were capped using 230 μl acetic anhydride, 270 μl DiPEA, 10 mg HOBt in 5 ml NMP for 5 minutes. The resin was then washed three times with DMF, DCM and subsequently air dried.

Fmoc-Gly-Ala-Tyr-Thr-Gly-HMBA-HCP resin (3)

Starting from HCP-HMBA resin (500 mg, 100 μmol) Fmoc-Gly-OH (150 mg, 500 μmol) was coupled using DIC (101 μL, 500 μmol) and catalytic DMAP in DCM (5 mL) for 4 hours at room temperature. After washing with DCM the unreacted resin was capped using the procedure used for oligonucleotide capping as described below. The resin was transferred to an automated peptide synthesizer (ABI-433A, Applied Biosystems, Perkin-Elmer) and the peptide was elongated with Fmoc-Thr(OTBDMS)-OH, Fmoc-Tyr-OH, Fmoc-Ala-OH and Fmoc-Gly-OH using the following repetitive steps:

- Fmoc cleavage using 20% piperidine in DMF.
- Coupling of the appropriate amino acid applying a five-fold excess, activation by 5 eq. HCTU in NMP (0.25M) and 12.5 eq. DiPEA in NMP (1.25 M) for 1 hour.

An analytical sample of resin 3 (ca. 5 μmol) was treated with a saturated solution of NH3 in trifluoroethanol (1 mL) for 16 hours at room temperature after which the reaction mixture was filtrated and concentrated. LCMS (0 – 90 % MeCN, conditions B, 15
Procedure for solid phase oligonucleotide synthesis:

The oligoribonucleotides were prepared on a 10 µmol scale using an Akta Oligo Pilot (GE) automated nucleic acid synthesizer starting from peptidyl-HMBA-HCP resin 3. Couplings were performed using 5 eq. of commercially available amidites at 0.1 M concentration. 5-benzylmercaptotetrazole (BMT, 0.3 M) was used as the activating agent in 15 minute coupling cycles. Oxidation was performed by means of standard procedure using I₂ (50 mM) in pyridine/H₂O (90:10, v/v). Capping was performed using a standard protocol employing cap A (40% N-methylimidazole in MeCN) and cap B (20% Ac₂O, 20% lutidine in MeCN) 1:1 (v/v).

Cleavage of the DMT group was accomplished using 5% DCA in toluene.

H-Gly-Ala-Tyr(pUpUpApA)-Thr-Gly-NH₂ (1)

The RNA-fragment was assembled on pentapeptide resinate 3 using the automated oligonucleotide synthesis protocol as described above on 10 µmol scale. The fully protected product was treated with sat. NH₄Cl in trifluoroethanol (10 mM) at room temperature for 16 hours to cleave the peptidylated oligonucleotide off the resin as well as removed all base labile protective groups. The resin was washed with 1 mL H₂O/ EtOH, 1:1, v/v and the filtrate was concentrated. The crude peptidylated oligonucleotide was dissolved in DMSO (500 µL) and treated with TEA.HF (3 mL) for 16 hours at room temperature followed by precipitation in cold nBuOH, centrifugation and collection of the precipitate. The crude nucleopeptide was purified by ion-exchange chromatography using a Source-S column (16 x 100 mm, GE-Healthcare) and a gradient of 0 – 50 % 20 mM NaOAc/I M NaCl in 20 mM NaOAc/ 20 mM NaCl with a flow of 5 mL/min. Subsequent desalting on a Sephadex G25 column (26 x 200 mm, GE-Healthcare) using 0.15 M NH₄HCO₃ with a flow of 5 mL/min resulted in the title compound 1 as ammonium salt (4.2 mg, 2.3 µmol, 23 %). LC-MS (0 - 20 % MeCN, conditions A, 15 min run), Rt = 620. ESI-MS m/z: 1737.8 [M + H]+, 896.9 [M + 2H]+.

3¹P-NMR (161 MHz, D₂O); δ -0.32, -0.39, -0.42, -4.40. ¹H-NMR (600 MHz, D₂O); δ 8.27 (s, 1H, H-8 A), 8.21 (s, 1H, H-8 A), 8.09 (s, H-2 A), 7.88 (s, 1H, H-2 A), 7.75 (d, 1H, H-5 U), 7.55 (d, 1H, H-5 U), 7.07 (d, 1H, H-4 a aminoadic), 3.85 – 3.83 (m, 2H, H a aminoadic), 3.60 – 3.59 (m, 1H, H a aminoadic), 3.04 – 3.01 (m, 1H, β Tyr), 2.92 – 2.88 (m, 1H, β Tyr), 1.23 – 1.19, 1.14 – 1.11 (2 m, 2 x 3H, 2 x CH₃, Ala + Thr). HRMS [C₈H₇N₅O₅P₄ + H]+: found 1737.8384, calc. 1737.8350, [C₈H₇N₅O₆P₄ + Na]+: found 1759.3611, calc. 1759.3624


The RNA-fragment was assembled on pentapeptide resinate 3 using the automated oligonucleotide synthesis protocol as described above on 10 µmol scale. The fully protected product was treated with sat. NH₄Cl in trifluoroethanol (10 mM) at a temperature of 40 °C for 16 hours to cleave the peptidylated oligonucleotide off the resin as well as removed all the base labile protective groups. The resin was washed with 1 mL H₂O/EtOH, 1:1, v/v and the filtrate was concentrated. The crude peptidylated oligonucleotide was dissolved in DMSO (500 µL) and treated with TEA.HF (3 mL) for 16 hours at room temperature followed by precipitation in cold nBuOH, centrifugation and collection of the precipitate. The crude nucleopeptide was purified by ion-exchange chromatography using a Source-Q column (16 x 100 mm, GE-Healthcare) and a gradient of 0 – 50 % 20 mM NaOAc/I M NaCl in 20 mM NaOAc/ 20 mM NaCl with a flow of 5 mL/min. Subsequent desalting on a Sephadex G25 column (26 x 200 mm, GE-Healthcare) using 0.15 M NH₄HCO₃ with a flow of 5 mL/min resulted in the title compound 2 as ammonium salt (5.1 mg, 1.4 µmol, 14 %). LC-MS (0 - 25 % MeCN, conditions A, 15 min run), Rt = 570. ESI-MS m/z: 1126.1 [M + H]+. ³¹P-NMR (161 MHz, D₂O); δ -0.46, -0.51, -0.65, -0.69, -0.85, -4.42. ¹H-NMR (600 MHz, D₂O); δ 7.98 – 7.45 (m, 12 H, 5x H-2 A, 5x H-8 A, H-8 G, H-5 C), 7.24 (d, 1H, H-5 U), 7.10 (d, 1H, H-5 U), 6.83 (d, 1H, H-4 a aminoadic), 6.70 (d, 1H, H-4 a aminoadic), 5.78 – 4.25 (m, 10H, 7x H-1' furanose, 2x H-6 U, H-6 C), 4.97 – 4.70 (m, 2H, 2x H-1' furanose), 4.53 – 3.73 (m, 49H, 45x CH furanose, 3x CH α, Ala, Thr, Tyr, 1 x β Thr) 2.79 – 2.76 (m, 1H, β Tyr), 2.65 – 2.62 (m, 1H, β Tyr), 1.02 – 0.97, 0.89 – 0.87 (2 m, 2 x 3H, 2 x CH₃, Ala + Thr). HRMS [C₁₀₆H₁₉₂N₉₀O₃₉P₉ + H]+: found 3374.6152, calc. 3374.6267, [C₁₀₇H₁₉₃N₉₀O₃₉P₉ + Na]+: found 3396.6006, calc. 3396.6087, [C₁₀₈H₁ₙ₂N₉₀O₃₉P₉ + 2H]+: found 1687.8194, calc. 1687.8174, [C₁₀₉H₁ₙ₃N₉₀O₃₉P₉ + Na + H]+: found 1698.0879, calc. 1698.0880, [C₁₀₁₀H₁ₙ₄N₉₀O₃₉P₉ + 3H]+: found 1125.5483, calc. 1125.5471, [C₁₀₁₁H₁ₙ₅N₉₀O₃₉P₉ + Na + 2H]+: found 1132.8757, calc. 1125.5471.
LC-MS analysis of crude 3.
$^{31}P$-NMR (161 MHz), D2O, compound 1
LC-MS analysis of purified compound 2

H-Gly-Ala-Tyr-Thr-Gly-NH₂

![Chemical structure of H-Gly-Ala-Tyr-Thr-Gly-NH₂]

LCMS conditions A

![LC-MS chromatogram and mass spectrum]
31P-NMR (161 MHz), D2O, compound 2
$^1$H-NMR (600 MHz), D$^2$O, compound 2
1H-NMR (600 MHz), D2O, compound 2