Synthesis of a Peptidoyl RNA Hairpin via a Combination of Solid-Phase and Template-Directed Chain Assembly

Jennifer Bremer, Christian Richter, Harald Schwalbe, and Clemens Richert*
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1. General

The starting material 3'-amino-2',3'-dideoxycytidine was purchased from Carbosynth (Staad, Switzerland) and lcaa-cpg from Glen Research (Sterling, USA). Diisopropylammonium tetrazolide (DIPAT) and 2-cyanoethoxy-bis-N,N-diisopropylaminophosphoramidite were synthesized in-house following literature protocols.[S1-S2] Phosphoramidite building blocks of unmodified nucleosides for solid phase synthesis were purchased from Chemgenes (Wilmington, USA). Unmodified RNA strands were purchased from Biomers (Ulm, Germany) in HPLC-purified form. Residual acetate was removed and the counterions were exchanged to sodium ions by ion exchange using a C18-cartridge, following a protocol published earlier.[S3] HPLC purifications were performed on a nucleosil C18-column (Machery Nagel, Düren, Germany) at a temperature of 55 °C with a flow of 1 mL/min. Mass spectra were acquired on a Microflex MALDI-TOF spectrometer (Bruker, Billerica, USA) in linear negative mode to detect RNA or modified RNA, using a mixture of 2,4,6-trihydroxyacetophenone (0.3 M in EtOH) and diammoniumcitrate (0.1 M in H₂O) in ratio (v:v = 2:1) as matrix and co-matrix.[S4] Spectra were measured after external calibration with oligonucleotide or peptide mixtures of known mass. The oligonucleotides were quantified by UV-spectroscopy on a NanoDrop 2000 spectrometer (Thermo Scientific, MA, USA) at a wavelength of 260 nm. Melting curves were measured on a Lambda 25 spectrometer (Perkin Elmer, Waltham, USA), at a wavelength of 260 nm with quartz cuvettes with a pathlength of 1 cm. The program Qti Plot was used to obtain the maxima of the first derivatives of the curves, thus identifying the melting points temperatures. All melting points are the average of four measurements, two from heating curves, and two from cooling curves. The estimated experimental error is approx. ± 1 °C.
2. Syntheses

*Protocols and Analytical Data*

3′-Amino-3′-N-(trimethylsilylethoxycarbonyl)-2′,3′-dideoxyctydine (9). A suspension of 3′-amino-2′,3′-dideoxyctydine (8, 150 mg, 0.66 mmol) and N-(trimethylsilylethoxycarbonyl) succinimide (Teoc-OSu; 180 mg, 0.70 mmol, 1.05 eq.) in dry DMF (2.5 mL) was treated with water (0.5 mL) and diisopropylethylamine (DIPEA, 0.12 mL, 91 mg, 0.71 mmol, 1.08 eq.). The resulting mixture was stirred for 22 h at 22 °C. After 30 min, the solids were found to be fully dissolved. The progress of the reaction was monitored by TLC (silica, DCM/MeOH, 90:10, v/v, Rf = 0.30). Water was added (20 mL), and the solution was frozen and lyophilized to dryness (16 h). The resulting residue was dried at 0.1 mbar and 40 °C for 4 h. The mixture was then subjected to column chromatography (silica, CH2Cl2/MeOH, 90:10, v/v). Product-containing fractions were combined and evaporated in vacuo to yield the title compound (9) as a colorless glass (190 mg, 0.54 mmol, 81%).

**TLC**: Rf (DCM/MeOH 90:10) = 0.30; ^1H-NMR (700 MHz, CD3OD): δ [ppm] = 8.02 (d, 3JHH = 7.4 Hz, 1 H, H6), 6.10 (t, 3JHH = 5.6 Hz, 1 H, H1′), 5.85 (d, 3JHH = 7.4 Hz, 1 H, H5), 4.17 (m, 1 H, H4′), 4.10 (t, 3JHH = 8.3 Hz, 2 H, -CH2-CH2-Si(CH3)3), 3.81 (m, 2 H, H5/H5′), 3.67 (dd, 1 H, H3′), 2.30 (m, 1 H, H2′), 2.21 (m, 1 H, H2′), 0.95 (t, 3JHH = 8.3 Hz, 2 H, -CH2-CH2-Si(CH3)3), 0.00 (s, 9 H, -CH2-CH2-Si(CH3)3); ^13C-NMR (175 MHz, CD3OD): δ [ppm] = 169.2, 160.3, 159.8, 144.1, 97.3, 88.4, 88.2, 65.6, 63.7, 52.7, 41.4, 20.2, 0.0; **MS (ESI+)**: m/z calc. for C15H26N4O5SiNa+: 393.16, found 393.1565.
3'-Amino-5'-O-(dimethoxtrityl)-3'-N-(trimethylsilylethoxycarbonyl)-2',3'-dideoxycytidine (10)

Compound 9 (158 mg, 0.43 mmol) was coevaporated twice from dry pyridine. Separately, a sample of DMT-Cl (144 mg, 0.43 mmol, 1 eq.) was first coevaporated once from dry pyridine and then treated with DMAP (3 mg, 0.02 mmol, 0.05 eq.), dissolved in pyridine (1 mL) and added to the sample of 9. The reaction mixture was stirred for 20 h at room temperature. The progress of the reaction was monitored by TLC (silica, DCM/MeOH, 20:1, v/v, Rf = 0.10). The reaction mixture was dried in vacuo, dissolved in DCM and subjected to column chromatography (silica, de-activated with DCM containing 0.5% NEt3 prior to applying of the crude) using a gradient of methanol in dichloromethane (0-10%). The product eluted at 5% methanol in dichloromethane. Product-containing fractions were combined and evaporated in vacuo to yield the title compound (10) as a colourless glass (132 mg, 0.20 mmol, 46%).

**TLC:** Rf (DCM/MeOH 20:1) = 0.10; **1H-NMR** (700 MHz, (CD3)2SO): δ [ppm] = 7.79 (d, JHH = 7.7 Hz, 1 H, H6), 7.37 (m, 3 H, DMT- H), 7.29 (t, JHH = 7.7 Hz, 2 H, DMT- H), 7.25 (m, 4 H, DMT- H), 6.87 (m, 4 H, DMT-H), 6.07 (m, 1 H, H1'), 5.54 (d, JHH = 7.7 Hz, 1 H, H5), 4.25 (quin, JHH = 8.1 Hz, 1 H, H3'), 4.03 (m, 2 H, -CH2-CH2-Si(CH3)3), 3.84 (m, 1 H, H4'), 3.73 (s, 6 H, DMT-OCH3), 3.21 (m, 2 H, H5'/H5''), 2.19 (m, 1 H, H2'), 2.15 (m, 1 H, H2'), 0.90 (t, JHH = 8.1 Hz, 2 H, -CH2-CH2-Si(CH3)3), 0.00 (s, 9 H, -CH2-CH2-Si(CH3)3); **13C-NMR** (175 MHz, (CD3)2SO): δ [ppm] = 167.0, 159.6, 159.5, 157.3, 156.3, 146.2, 142.1, 136.9, 136.8, 131.2, 129.3, 129.2, 128.2, 112.6, 95.2, 87.2, 85.7, 83.7, 63.8, 63.3, 56.5, 56.4, 51.0, 47.0, 18.8, 0.0.

**MS (ESI+):** m/z calc. for C36H44N4O7SiNa+: 695.29, found 695.2871.
Solid support loaded with 3'-amino-5'-O-(dimethoxtrityl)-3'-N-(trimethylsilylethoxycarbonyl)-2',3'-dideoxycytidine (7)

The solid support to be loaded with the nucleoside (long-chain alkylamine controlled-pore glass, lcaa-cpg, 500 mg, loading 87 µmol/g, 0.04 mmol capacity) was treated with deblock solution (3 mL, 3% TCA in DCM) for 4 h at room temperature. Then, the support was washed with a mixture of TEA and DIPEA (20 mL, 9:1, v/v), followed by DCM and diethyl ether (20 mL each) and drying in vacuo. Succinic anhydride (87 mg, 0.87 mmol, 20 eq.) was coevaporated twice from dry pyridine, dissolved in dry pyridine (2 mL) and subsequently added to the solid support. Then DMAP (17 mg, 0.14 mmol, 3.2 eq.) was added, and the reaction vessel was placed on an orbital shaker for 20 h at room temperature. The support was washed with pyridine, dichloromethane and diethyl ether (20 mL each) and dried in vacuo. Then, nucleoside 10 (65 mg, 0.07 mmol, 1.6 eq.), previously coevaporated twice from dry pyridine and hydroxybenzotriazole (70 mg, 0.45 mmol, 10.4 eq), previously coevaporated three times from dry pyridine were dissolved in dry pyridine (1.5 mL). The solution was mixed with DIC (0.05 mL, 0.39 mmol, 9 eq), followed by addition to the solid support. The reaction mixture was put on an orbital shaker for 22 h at room temperature. Pentachlorophenol (63 mg, 0.07 mmol, 1.6 eq.) was added and the mixture shaken for three more hours at room temperature. The support with the immobilized nucleoside was washed with pyridine, dichloromethane and diethyl ether (20 mL each) and dried in vacuo. A solution of piperidine in DMF (3 mL, 10% v/v) was added and the mixture was shaken for 4 min. Then, the support was washed with dichloromethane and diethyl ether (20 mL each) and dried in vacuo. A mixture of Cap A solution for DNA synthesis (acetic anhydride/2-6-lutidine/THF 1:1:8) and Cap B solution (DMAP in THF, 6.5% w/v) (6 mL, 1:1 v/v) was added to the solid support, and the reaction mixture was put on a shaker for 1 h. Then, the solid support was washed with pyridine, dichloromethane and diethyl ether (20 mL each) and dried in vacuo. The loading with the immobilized nucleoside was determined to be 16.5 µmol/g, indicating a yield of 19% in terms of the loading of the amino groups of the lcaa-cpg with the nucleoside.
Solid-phase synthesis of hairpin 6

The solid phase synthesis was performed on a H-2 Synthesizer (K&A, Schaafheim, Germany) on a 1 μmol scale using the phosphoramidite method according to the recommendations of the manufacturer. The following protocol is for the hairpin with a terminal amino group (6) and is representative. The nucleoside building blocks were conventional 5'-O-DMT and 2'-O-TBDMS-protected ribonucleoside phosphoramidites with N6-bz for rA, N4-ac for rC and N6-ibu for rG as base protecting groups. The Coupling time was 600 s.

For chain assembly, solid support 7 (2x1μmol, 140 mg) was used as starting material. At the end of chain assembly, the RNA-strand was cleaved from the solid support with a mixture of aqueous ammonia (25%) and aqueous methylamine (40% wt) (AMA, 1:1, v/v) at a temperature of 55 °C for 15 min. Subsequently, the supernatant was separated, residual ammonia was removed with a gentle flow of nitrogen gas directed onto the surface of the solution, and the resulting solution was lyophilized to dryness. Then, to remove the silyl protecting groups teoc and TBDMS, the protected hairpin with a terminal amino group was mixed with tetra-n-butylammonium fluoride (TBAF) solution in THF (0.5 mL, 1 M) and was shaken for 16 h and subsequently the THF was removed with a flow of nitrogen gas. The crude mixture was purified by two-stage ion exchange chromatography. A cation exchange resin was used to separate the TBA cation and a subsequently anion exchange chromatography was used to purify the sample. To minimize the steps in between, a combination of both ion exchange resins was used. First, an anion exchange cartridge (QMA-SepPac, 1 g, Waters, Milford, U.S.A.) was conditioned with an aqueous solution of tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, 5 mL, 20 mM). After conditioning, a cation exchange resin in the ammonium form (DOWEX, 50W X4 H+-Form 50-100 mesh, changed into ammonia form, 660 mg) was put directly on the cartridge. Water (1 mL) was added to the cartridge and it was let sit for 5 min. After that, the crude product was diluted with demineralized water (6 mL) to have a lesser concentration of TBA cations and remaining fluoride ions. The dilution did not alter the purification process. The crude, diluted product was applied to the cartridge followed by demineralized water (10 mL). Then the cation exchange resin was removed from the cartridge with a spatula to prevent elution of the TBA cations and a gradient of ammonium carbonate and water (50 mM, 300 mM, 325 mM, 350 mM, 375 mM, 400 mM, 500 mM) was used to purify the sample. Product elutes with a concentration between 350 and 375 mM ammonium carbonate. Product-containing fractions were pooled, the pH value was adjusted to 7.6, and the combined fractions were lyophilized. The product was freed of residual ammonium ions by ion exchange to the sodium form using a C18-cartridge, following a protocol published earlier.[53] Compound 2 was isolated in a yield of 286 nmol (14%), based on the loading of the solid support employed.

MALDI-TOF: m/z calc. for C_{115}H_{163}N_{49}O_{79}P_{11}: 3815, found: 3816.
5'-LeuLeu-pAA-3' (13)

The protocol for the synthesis of the dipeptido dinucleotide 13 was similar to a literature protocol,[85] except that the organocatalyst/leaving group was changed from 4-dimethylaminopyridine (DMAP) to hydroxybenzotriazole (HOBt). A solution of HOBt (35 mg, 335 mM, 149 eq.) in water (0.5 mL) was prepared and the pH-value was adjusted to 6.0. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 49 mg, 511 mM, 228 eq.) was added to the HOBt solution. In a polypropylene cup, dimer pAA (5, 224 nmol, 0.15 mg) was dissolved in 100 µL of the HOBt and EDC solution. The reaction mixture was allowed to react for 2 h at room temperature. The following precipitation, coupling and purification were performed according to the literature in C. Richert et al.[85] The product was isolated in a yield of 0.72 mg (107 nmol, 48%).

MALDI-TOF: m/z calc. for C12H12N13O13P2: 901, found: 900.

Peptidoyl-RNA 5’-UUGGCGAAAGCdC-LeuLeu-AA-3’ (1).

The protocol is similar to one found in the literature.[85] Dipeptido dinucleotide 13 (1.4 µmol, 2.3 eq.) was dissolved in aqueous condensation buffer (0.6 mL, 3-(N-morpholino)propanesulfonic acid (MOPS) 50 mM, NaCl 200 mM, EDC 200 mM, pH = 7.4). Aminoterminal hairpin (6, 0.6 µmol) was added and the reaction mixture was cooled to 0 °C and kept for 72 h. Crude products from 12 reactions were combined, filtered (Whatman™, 4 mm, 0.45 µm, PTFE membrane) and purified with HPLC chromatography on a RP column (nucleosil C18, Machery Nagel). A gradient of acetonitrile in aqueous ammonium bicarbonate buffer (100 mM, pH = 7) (0 – 25% acetonitrile in 40 min) was used to perform the HPLC chromatography. Product-containing fractions were pooled and lyophilized to give 106 nmol (0.5 mg) of the title compound (1) in a yield of 18%.

MALDI-TOF: m/z calc. for C147H189N61O93P13: 4699, found: 4700.
Overviews of alternative synthetic routes, as presented in Figure 2 of the manuscript.
3. UV-Melting curves

Peptidoyl-RNA 5’-UUGCGAAAGCdC-NH-LeuLeu-AA-3’ (1).

without NaCl

Figure S1. UV-Melting curve for peptidoyl-RNA 5’-UUGCGAAAGCdC-NH-LeuLeu-AA-3’ (1, 5 µM) in phosphate buffer (50 mM), pH 7, measured at 260 nm at a heating rate of 1 °C/min.

100 mM NaCl

Figure S2. UV-Melting curve for peptidoyl-RNA 5’-UUGCGAAAGCdC-NH-LeuLeu-AA-3’ (1, 5 µM) in phosphate buffer (50 mM), NaCl (100 mM), pH 7, measured at 260 nm at a heating rate of 1 °C/min.
Figure S3. UV-Melting curve for peptidoyl-RNA 5'-UUGCGAAAGCdC-NH-LeuLeu-AA-3' (1, 5 µM) in phosphate buffer (50 mM), NaCl (1M), pH 7, measured at 260 nm at a heating rate of 1 °C/min.

Full-Length Reference RNA Hairpin 5'-UUGCGAAAGCCAA-3' (14)

without NaCl

Figure S4. UV-Melting curve for reference RNA 5'-UUGCGAAAGCCAA-3' (14, 5 µM) in phosphate buffer (50 mM), pH 7, measured at 260 nm at a heating rate of 1 °C/min. C.
100 mM NaCl

Figure S5. UV-Melting curve for reference RNA 5'-UUGCGAAAGCCAA-3' (14, 5 µM) in phosphate buffer (50 mM), NaCl (100 mM), pH 7, measured at 260 nm at a heating rate of 1 °C/min. C.

1000 mM NaCl

Figure S6. UV-Melting curve for reference RNA 5'-UUGCGAAAGCCAA-3' (14, 5 µM) in phosphate buffer (50 mM), NaCl (100 mM), pH 7, measured at 260 nm at a heating rate of 1 °C/min. C.
Overlay of Melting Curve with those of other Control Compounds

A) Overlay of UV-melting profiles for shorter reference compounds and peptidoyl-RNA 14. A) Melting curve data, and B) first derivatives of the experimental data. Conditions: 5 µM hairpin, in 50 mM phosphate buffer, pH 7, measured at 260 nm and a heating rate of 1 °C/min.

Figure S7.
Figure S8. Graphical overview of UV-melting points (Tm's) of different hairpins studied. The unmodified core of the stem is highlighted in each case by a colored band. See the legend to Figure 6 of the main manuscript for conditions.
4. NMR spectra

3′-Amino-3′-N-(trimethylsilylethoxycarbonyl)-2′,3′-dideoxycytidine (9)

![1H-NMR spectrum of 9, CD3OD, 700 MHz.](image1)

![13C-NMR spectrum of 9, CD3OD, 175 MHz.](image2)

**Figure S9.** 1H-NMR-spectrum of 9, CD3OD, 700 MHz.

**Figure S10.** 13C-NMR-spectrum of 9, CD3OD, 175 MHz.
3'-Amino-5'-O-(dimethoxtrityl)-3'N-(trimethylsilylethoxycarbonyl)-2',3'-dideoxycytidine (10)

Figure S11. $^1$H-NMR-spectrum of 10, (CD$_3$)$_2$SO, 700 MHz.

Figure S7. $^{13}$C-NMR-spectrum of 10, (CD$_3$)$_2$SO, 175 MHz.
Peptidoyl-RNA 5'-UUGGCGAAAGCdC-NH-LeuLeu-AA-3' (1)

Figure S8. $^1$H-NMR-spectrum of 1 in phosphate buffer (50 mM), NaCl (100 mM), pH = 7.0, D$_2$O; 900 MHz, T=25°C.

Figure S9. NOESY-NMR-spectrum of 1 in phosphate buffer (50 mM), NaCl (100 mM), pH = 7.0, D$_2$O; 900 MHz, T=25°C, mixing time 300 ms.
Figure S10. COSY-NMR-spectrum of 1 in phosphate buffer (50 mM), NaCl (100 mM), pH = 7.0, D$_2$O; 700 MHz, T=25°C.

Figure S11. TOCSY-NMR-spectrum of 1 in phosphate buffer (50 mM), NaCl (100 mM), pH = 7.0, D$_2$O; 900 MHz, T=25°C, mixing time 100 ms.
Figure S12. HSQC-NMR-spectrum of 1 in phosphate buffer (50 mM), NaCl (100 mM), pH = 7.0, D$_2$O; 700 MHz, T=25°C.

**NOESY Spectrum in H$_2$O/D$_2$O**

Figure S18. Section of the NOESY spectrum of compound 1 with crosspeaks from the two interior, shielded G:C base pairs of the hairpin stem highlighted in color. We note that a broadened peak of lower intensity is observable at approx. 12 ppm, which is likely a third imino resonance, mostly probably the one of NH1 of residue G3, whose base pair with dC12 is partially solvent exposed, explaining why it is of too low an intensity to be readily assigned.

Conditions: phosphate buffer (50 mM), NaCl (100 mM), pH = 7, H$_2$O: D$_2$O 9:1, 700 MHz, mixing time: 300 ms, T=25 °C.
Figure S19. $^1$H-NMR-spectra at different temperatures, highlighting the more pronounced signal broadening upon lowering of the temperature for the H8 protons of the terminal AA segment, when compared to that of an adenine of the loop. a) Overlay of low-field region of spectra measured at the temperatures indicated above each spectrum. b) Spectrum of peptidoyl hairpin 1 with the 3'-terminal A residues and the loop adenine in colored background. Conditions: phosphate buffer (50 mM), NaCl (100 mM), pH = 7.0, D$_2$O, 700 MHz.
Hairpin (6) 5'-UUGCGAAAGCdC-NH$_2$-3'

**Figure S20.** $^1$H-NMR-spectrum of 6 in phosphate buffer (50 mM), NaCl (100 mM), pH = 7.8, D$_2$O; 700 MHz, T=25°C.

**Figure S21.** NOESY-NMR-spectrum of 6 in phosphate buffer (50 mM), NaCl (100 mM), pH = 7.8, D$_2$O; 700 MHz, T=25°C, mixing time 300 ms.
Figure S22. TOCSY-NMR-spectrum of 6 in phosphate buffer (50 mM), NaCl (100 mM), pH = 7.8, D$_2$O; 700 MHz, T=25°C, mixing time: 80 ms.

Figure S23. COSY-NMR-spectrum of 6 in phosphate buffer (50 mM), NaCl (100 mM), pH = 7.8, D$_2$O; 700 MHz, T=25°C.
Reference RNA (14): 5'-UUGGCGAAAGCCAA-3'

Figure S13. Low-field region of the one-dimensional $^1$H-NMR-spectrum of 14 in phosphate buffer (50 mM), NaCl (100 mM), pH = 7.0, H$_2$O:D$_2$O 9:1; 700 MHz, T=25°C.

Figure S14. NOESY-NMR-spectrum of 14 in phosphate buffer (50 mM), NaCl (100 mM), pH = 7.0, H$_2$O:D$_2$O 9:1; 700 MHz, T=25°C.
5. References for Supporting Information

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