Electronic Supplementary Information

for manuscript entitled

Templates Direct the Sequence-Specific Anchoring of the C-Terminus of Peptido RNAs

by

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Contents

1. Materials and Methods

General. Starting materials: DMAP (4-(dimethylamino)pyridine), sodium perchlorate, sodium chloride, MOPS (3-(*N*-morpholino)propanesulfonic acid), glycine, dipeptide GlyGly and tripeptide GlyGlyGly were purchased from Sigma Aldrich (Taufkirchen, Germany). Other dipeptides are purchased from Bachem (Bubendorf, Switzerland). The condensation reagent EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) hydrochloride was from Roth (Karlsruhe, Germany). The phosphoramidite building blocks for the DNA synthesis were purchased form Sigma Aldrich (Taufkirchen, Germany). The controlled-pore glass (cpg) containing a 3'-terminal nucleoside was prepared in-house as previously described.[1] The 3'-aminoterminal oligodeoxynucleotide primers were then synthesized using this cpg, as reported previously.[2] The 2'-TBDMS-protected ribonucleotide phosphoramidites were from Sigma Aldrich. For the 5'-phosphorylation of the RNA dimer, 2-[2-(4,4'-dimethoxytrityloxy)ethylsulfonyl]ethyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)phosphoramidite[3] also known as chemical phosphorylation reagent from Glen Research (Sterling, VA, USA) was used in the final chain extension cycle.

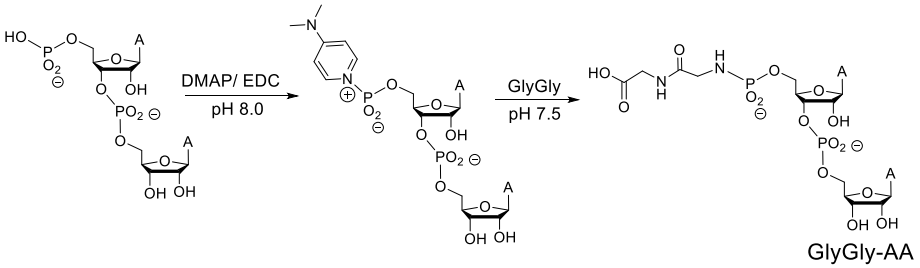
The RNA templates for assays were purchased from Biomers (Ulm, Germany). The HPLC purified samples were further desalted by using C18-catridge chromatography (details are given later). The HPLC grade water for assays was purchased from Sigma Aldrich, Switzerland.

The MALDI-TOF mass spectra were recorded on a Bruker Daltonik (Bremen, Germany) Microflex MALDI-TOF spectrometer with a N_2 laser (337 nm), in linear negative mode. A MSP BigAnchor 96 ground steel target was used to place samples for MALDI-TOF analysis and a mixture of 0.3 M trihydroxyacetophenone in EtOH and 0.1 M aqueous ammonium citrate (2/1, *v/v*) as matrix. An external calibration was done with a mixture of oligonucleotides with known masses and the analysis of the spectra was done with Flex Analysis, version 3.4 (Bruker). Analysis was performed on the $[M-H]^-$ ions. The spectral data were collected after applying 200 laser shots at 1.5 Hz frequency.

Solid-Phase Oligonucleotide Synthesis (General Protocol A). DNA and RNA syntheses were performed in the DMT-off mode on using an ABI 8909 Expedite synthesizer by following standard protocols and commercially available phosphoramidite building blocks. The oligonucleotides were cleaved from the solid support and deprotected by means of AMA reagent (20 min, 65° C; AMA = aq. Ammonia and methyl amine 1:1, *v/v*). The deprotected oligonucleotides were purified by water Sep-Pak Vac cartridge chromatography (500 mg;

Milford, MA, USA), using a gradient of ammonium carbonate in water. The pure compound was identified by MALDI-TOF spectroscopy. After that, the purified fractions were desalted by using via cartridge chromatography on Sep-Pak Vac C18 (500 mg; Milford, MA, USA), using a gradient of CH_3CN in water. Towards the end, the pure compounds were quantified by UV-Vis spectroscopy in Thermo Fischer NanoDrop spectrophotometers.

Representative protocol for the synthesis of peptido dimer (General Protocol B).



Scheme S1: Schematic representation for the synthesis of GlyGly-AA (**3gly$_2$**).

The 4-(dimethylamino)pyridine (DMAP, 19 mg, 0.15 mmol) was dissolved in water (0.9 mL), and the pH was adjusted to a value of 8.1 with 5 M HCl. To the resulting solution, EDC hydrochloride (157 mg, 0.82 mmol) was added. A sample of the resulting solution (120 µL) was added to the RNA dimer (AA, 270 nmol). The reaction mixture was kept at room temperature for 2.5 h. Afterwards, the solution was poured into 3 mL of a pre-chilled solution of 0.1 M $NaClO_4$ in acetone/diethyl ether (1:1, *v/v*). The mixture was vortexed, and kept at 0 °C for 15 min, followed by centrifugation for 5 min. The precipitate was separated and washed with diethyl ether (3 × 1 mL). After that, the precipitate was dried in air. A solution of GlyGly (0.3 mL, 0.3 M) was prepared and pH was adjusted to 7.4 by the addition of 5 M NaOH. From that, 110 µL solution was added to the dried precipitate and incubated at room temperature for 16 h. The crude mixture was analyzed by MALDI-TOF mass spectrometry, which indicated the formation of desired product in good amount.

After that, the crude mixture was purified by anion exchange chromatography. The pure fractions were obtained at 70-90 mM $(NH_4)_2CO_3$ in water. The pure fractions were lyophilized and subjected again to C18-chromatography for desalting and pure fractions were found only with water. At the end, the pure fractions were identified in combination with UV-Vis spectrometry and MALDI-TOF mass spectrometry. Prior to the lyophilization, the pH of the solution was adjusted to 7.5. The final yield was determined by UV-Vis spectrometry.

Yield: 34 %; *m/z* calculated for $C_{24}H_{30}N_{12}O_{15}P_2$, $[M-H]^-$: 788, found: 786.

2. Analytical Data

Primers

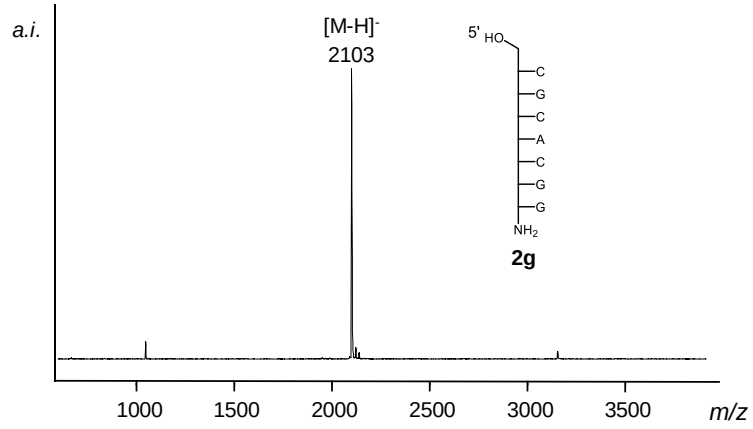


Figure S1. MALDI-TOF mass spectrum of primer **2g**.

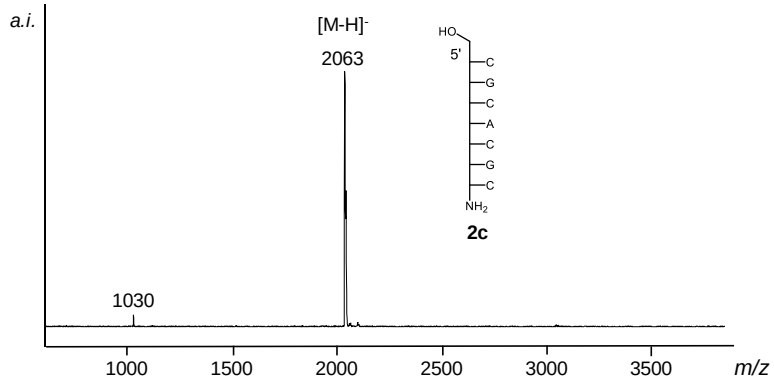


Figure S2. MALDI-TOF mass spectrum of primer **2c**.

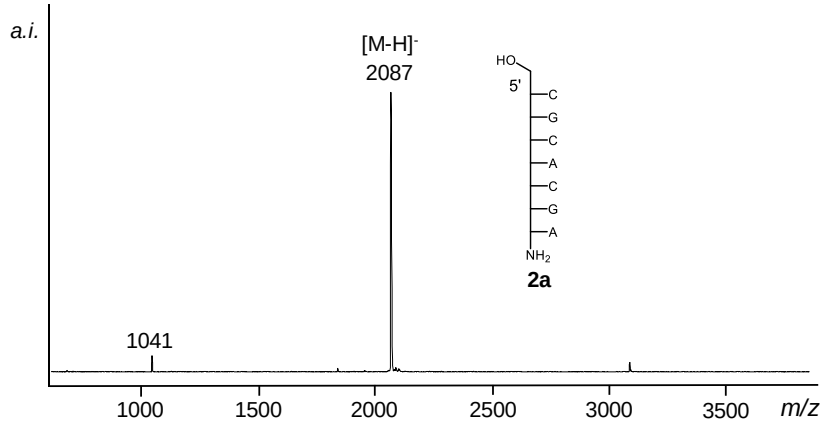


Figure S3. MALDI-TOF mass spectrum of primer **2a**.

Peptido Dinucleotides

Gly-AA (3gly). The glycyl dinucleotide was synthesized according to General Protocol B, with a coupling time of 14 h, using 0.5 M glycine and pH 7.7. The product was eluted from the anion-exchange column at 50-80 mM aqueous $(NH_4)_2CO_3$; a subsequent C18 cartridge chromatography was run using 0-1 % acetonitrile in water.

Yield: 36 %, *m/z*; MALDI-TOF MS, calculated for $C_{22}H_{28}N_{11}O_{14}P_2$, $[M-H]^-$: 732, found: 732.

GlyGlyGly-AA ($3gly_3$). The compound was synthesized according to General Protocol B, with a coupling time of 14 h, using GlyGlyGly (0.3 M) and pH 7.6. The product was eluted from the anion-exchange column at 30-70 mM aqueous $(NH_4)_2CO_3$; a subsequent C18 cartridge chromatography was run without a gradient, using pure water as eluant.

Yield: 28 %, *m/z*; MALDI-TOF MS, calculated for $C_{26}H_{33}N_{13}O_{16}P_2$, $[M-H]^-$: 846, found: 846.

LeuLeu-AA (4). The compound LeuLeu-AA was synthesized according to General Protocol B, with a coupling time of 18 h, using LeuLeu (0.3 M) and pH 8.3. The product was eluted from the anion-exchange column at 70-100 mM aqueous $(NH_4)_2CO_3$; a subsequent C18 cartridge chromatography was run using 1-3 % acetonitrile in water.

Yield: 63 %, *m/z*; MALDI-TOF MS, calculated for $C_{32}H_{46}N_{12}O_{15}P_2$, $[M-H]^-$: 900, found: 900.

PhePhe-AA (5). The compound PhePhe-AA was synthesized according to General Protocol B, with a coupling time of 36 h, using PhePhe (0.3 M) and pH 8.5. The product was eluted from the anion-exchange column at 70-90 mM aqueous $(NH_4)_2CO_3$; a subsequent C18 cartridge chromatography was run using 2-4 % acetonitrile in water.

Yield: 30 %, *m/z*; MALDI-TOF MS, calculated for $C_{38}H_{42}N_{12}O_{15}P_2$, $[M-H]^-$: 968, found: 967.

GlyPro-AA (6). The compound GlyPro-AA was synthesized according to General Protocol B, with a coupling time of 16 h, using ProGly (0.3 M) and pH 7.5. The product was eluted from the anion-exchange column at 70-80 mM aqueous $(NH_4)_2CO_3$; a subsequent C18 cartridge chromatography was run without a gradient, using pure water as eluant.

Yield: 29 %, *m/z*; MALDI-TOF MS, calculated for $C_{27}H_{34}N_{12}O_{15}P_2$, $[M-H]^-$: 828, found: 830.

GlyGly-CA (7). The compound GlyGly-CA was synthesized according to General Protocol B, with a coupling time of 16 h, using GlyGly (0.3 M) and pH 7.5. The product was eluted from the anion-exchange column at 60-80 mM aqueous $(NH_4)_2CO_3$; a subsequent C18 cartridge chromatography was run without a gradient, using pure water as eluant.

Yield: 30 %, *m/z*; MALDI-TOF MS, calculated for $C_{23}H_{30}N_{10}O_{16}P_2$, $[M-H]^-$: 764, found: 763.

LeuLeu-CA (8). The compound LeuLeu-CA was synthesized according to General Protocol B, with a coupling time of 16 h, using LeuLeu (0.3 M) and pH 8.1. The product was eluted from the anion-exchange column at 60-90 mM aqueous $(NH_4)_2CO_3$; a subsequent C18 cartridge chromatography was run using 1-3 % acetonitrile in water.

Yield: 58 %, *m/z*; MALDI-TOF MS, calculated for $C_{31}H_{46}N_{10}O_{16}P_2$, $[M-H]^-$: 876, found: 876.

PhePhe-CA (9). The compound PhePhe-CA was synthesized according to General Protocol B, with a coupling time of 36 h, using PhePhe (0.3 M) and pH 8.6. The product was eluted from the anion-exchange column at 60-90 mM aqueous $(NH_4)_2CO_3$; a subsequent C18 cartridge chromatography was run without a gradient, using pure water as eluant.

Yield: 23 %, *m/z*; MALDI-TOF MS, calculated for $C_{37}H_{42}N_{10}O_{16}P_2$, $[M-H]^-$: 944, found: 944.

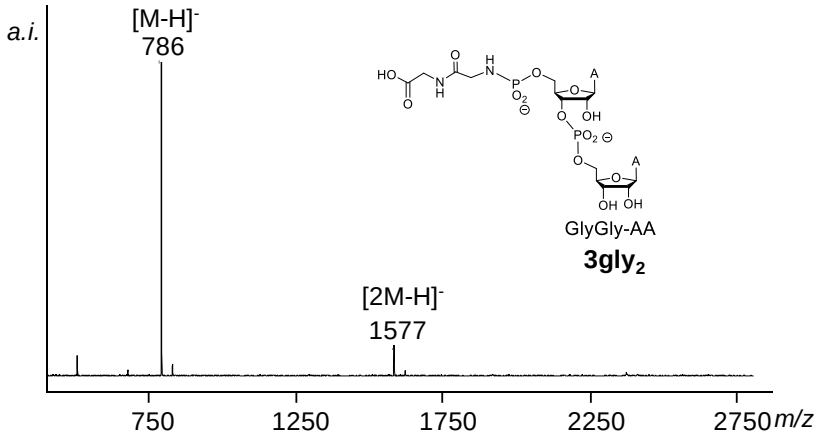


Figure S4. MALDI-TOF mass spectrum of GlyGly-AA (**3gly$_2$**).

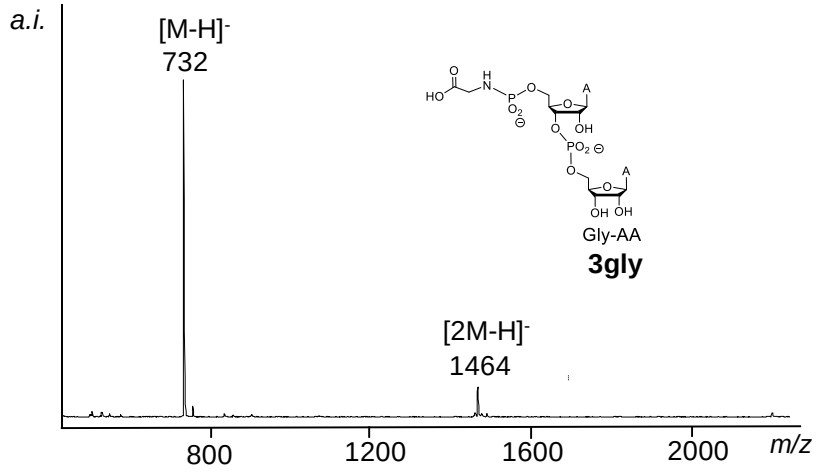


Figure S5. MALDI-TOF mass spectrum of Gly-AA (**3gly**).

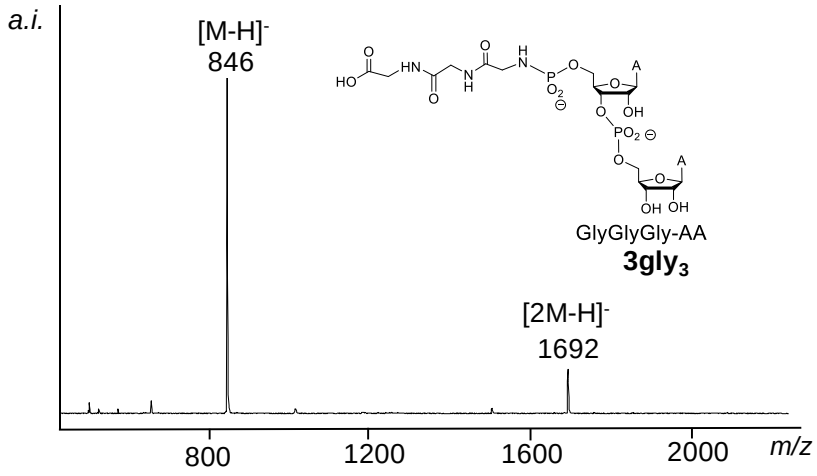


Figure S6. MALDI-TOF mass spectrum of GlyGlyGly-AA (**3gly$_3$**).

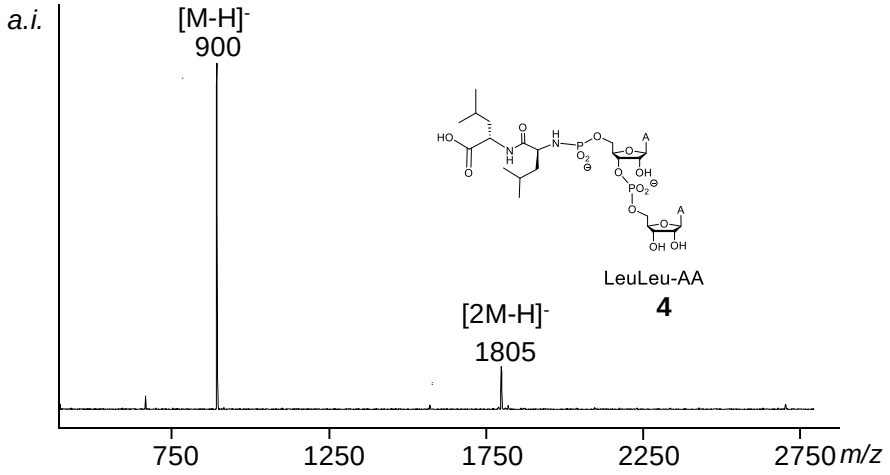


Figure S7. MALDI-TOF mass spectrum of LeuLeu-AA (**4**).

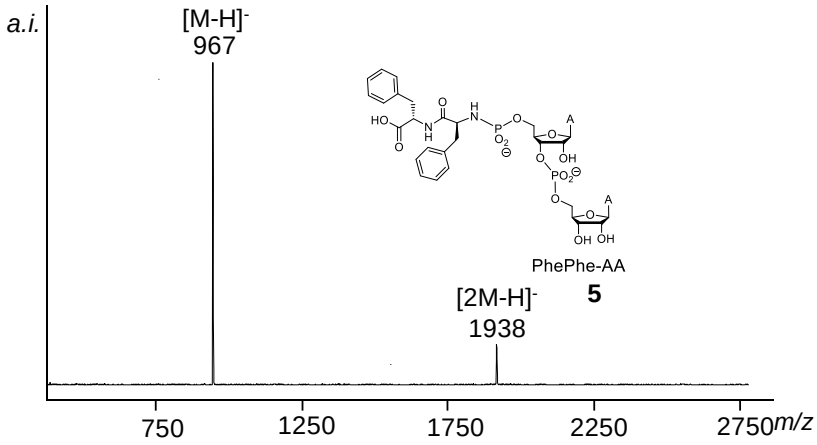


Figure S8. MALDI-TOF mass spectrum of PhePhe-AA (**5**).

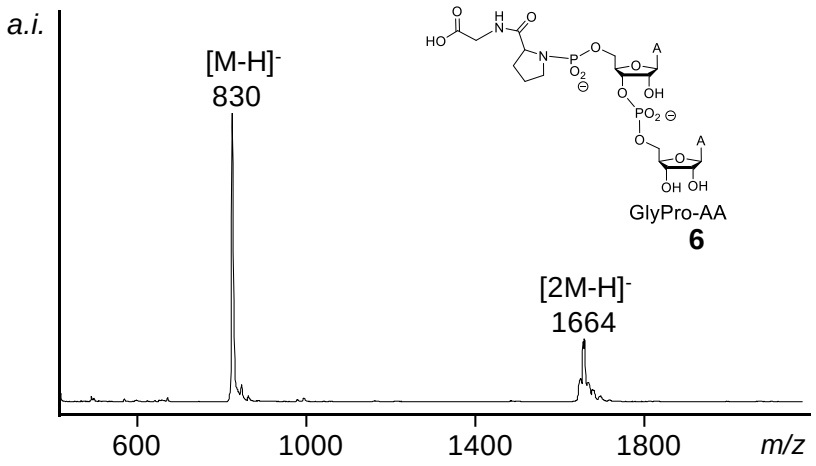


Figure S9. MALDI-TOF mass spectrum of GlyPro-AA (**6**).

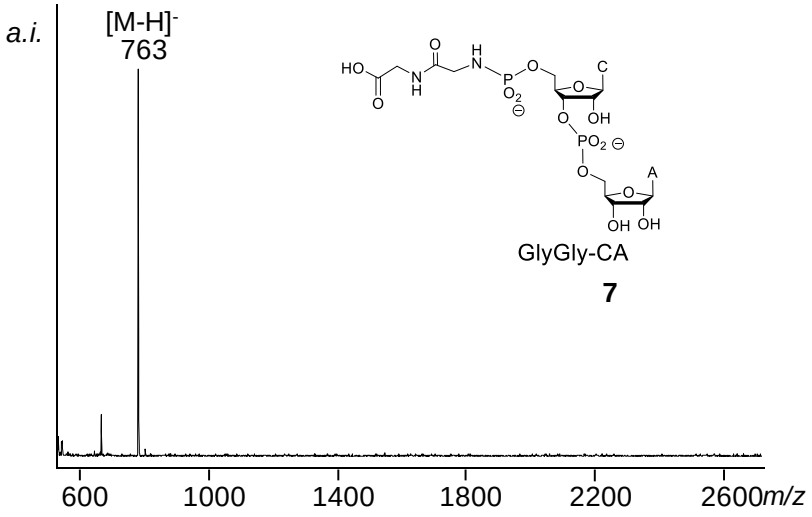


Figure S10. MALDI-TOF mass spectrum of GlyGly-CA (**7**).

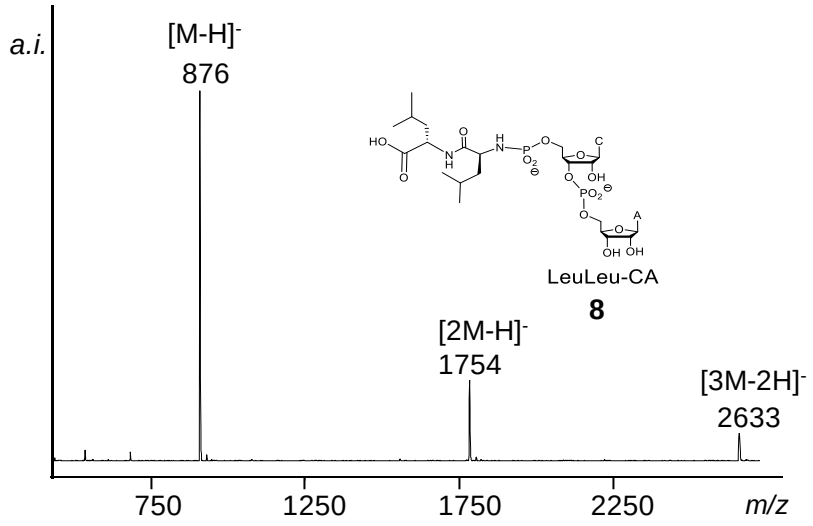


Figure S11. MALDI-TOF mass spectrum of LeuLeu-CA (**8**).

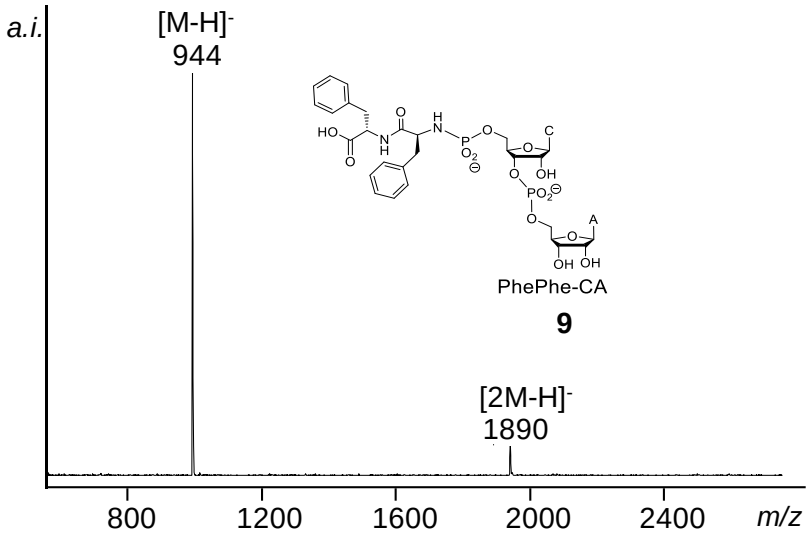
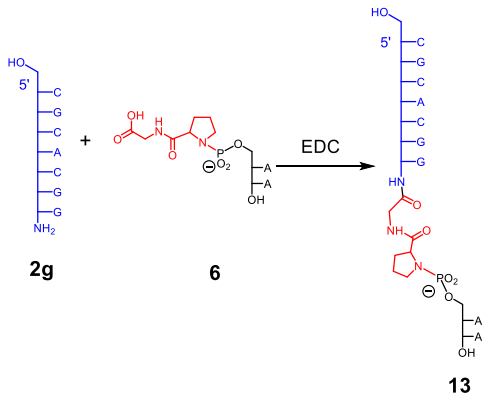


Figure S12. MALDI-TOF mass spectrum of PhePhe-CA (**9**).

3. Representative MALDI Calibration Data

Synthesis of the reference compound 13



Scheme S2. Synthesis of compound **13**.

Procedure: In a polypropylene vessel, primer **2g** (8 nmol) and GlyPro-AA (**6**, 60 nmol) were mixed together. To it, MOPS buffer (pH 7.4, end concentration: 20 mM) was added to maintain the pH of the solution during the reaction, followed by EDC hydrochloride (end concentration: 200 mM) as condensation reagent. Total volume of the reaction mixture was made up to 100 µL. The resultant mixture was incubated at room temperature for 4 d at 0 °C.

The crude mixture was analyzed by MALD-TOF mass spectroscopy, which indicated complete conversion of the primer **2g** to the product **13**. After that, the reaction mixture was subjected to anion exchange chromatography for purification, using 400 mM ammonium $(NH_4)_2CO_3$ in water.

Yield: 43 %, *m/z*; MALDI-TOF MS, calculated for **13**, $[M-H]^-$: 2915, found: 2915.

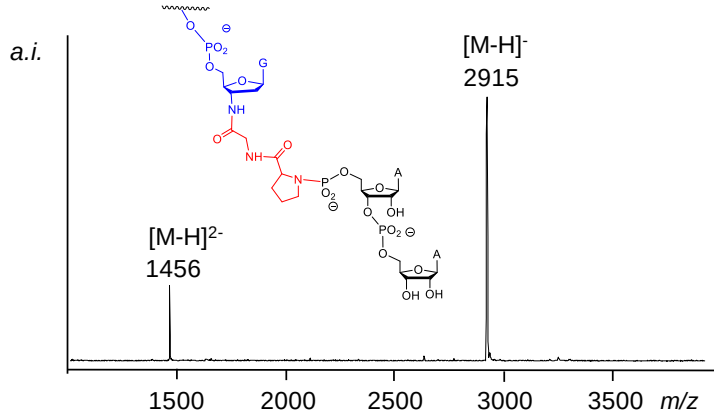


Figure S13. MALDI-TOF mass spectrum of compound **13**.

Correction factor for difference in desorption/ionization in MALDI-TOF MS

For the quantitative detection in anchoring assays, a correction factor for the desorption/ionization was determined for a representative case. A stock solution of compound **13** was prepared in distilled water with concentration of 52 mM. Similarly, a stock solution of primer **2g** was prepared at a concentration of 315 µM. The concentration of these components was determined by UV absorption spectroscopy. Then, primer **2g** and product **13** were mixed at different ratios (5:1 → 5:5) in an Eppendorf cap. In order to stick with the conditions of our assays, all the mixtures were prepared in solutions containing 50 mM MOPS buffer (pH 7.4) and 200 mM EDC hydrochloride. Prior to MALDI-analysis, the mixture was desalted with the ammonium form of cation exchange resin Dowex 50 WX8-200 in a volume of 10 µL for 45 min. An aliquot of the supernatant was aspirated and applied to the MALDI plate, followed by mixing with the matrix. The UV-based concentration of the analyte (**13**) was plotted against the MALDI-based relative signal intensity(to **2g**) to obtain the calibration curve (R^2 = 0.98). The slope of the linear fit to the calibration curve was found to be 1.12, which was used as correction factor.

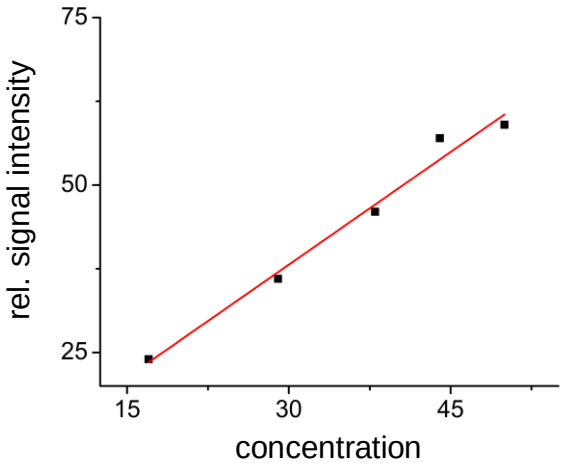


Figure S14. The linear fit of UV-determined concentration of the analyte against MALDI-based relative signal intensity.

4. Kinetics of Anchoring Reactions

Sample preparation. A mixture of primer, template and peptido dimer was prepared in a condensation buffer containing 50 mM MOPS (pH 7.4), 0.2 M NaCl and 0.2 M EDC hydrochloride. The final volume of the assay was 10 µL, and the reaction temperature was maintained at 0 °C. During the assays, 1 µL aliquots of the reaction mixture were subjected to cation exchange, using the ammonium form of cation exchange resin Dowex 50 WX8-200 for 1 h (10 µL total colume). From the supernatant, 2 µL were applied to the MALDI-plate and mixed with the matrix/comatrix. Mass spectrometric analysis used the $[M-H]^-$ ions. Ratios of peak heights were used for determining the conversion of the primer to the product (POR). The spectra were obtained by applying 200 laser shots at a frequency of 1.5 Hz .

Mathematical analysis. Apparent rate constants were calculated using the program Origin, version Pro 8.0. The rate constants were derived from fits using the following monexponential equation:

$$y = y_{max} (1- e^{-kt})$$

where y stands for the conversion of the primer to the POR, y_{max} stands for maximum yield of the reaction at infinite time, as calculated by the fit equation, k = rate constant, t = time in min; $t_{1/2}$ was determined using $t_{1/2} = 0.693/k$

Anchoring Assay with 1cuuc, 2g, and 3gly

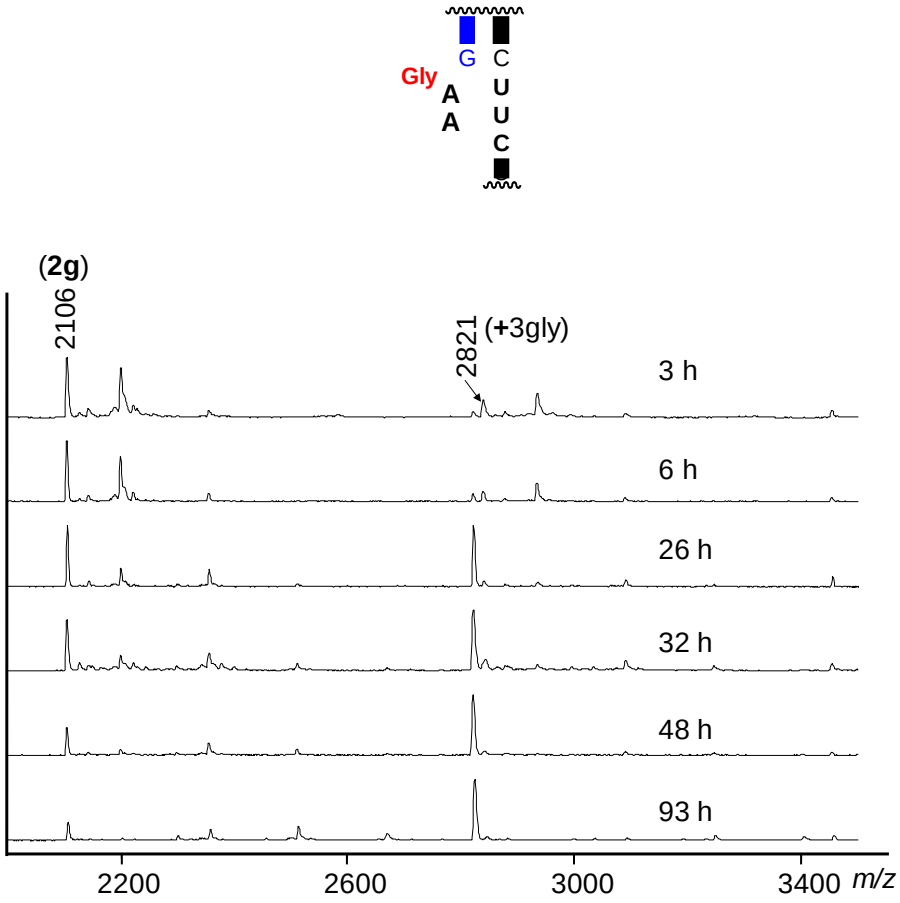


Figure S15. MALDI-TOF mass spectra from assay involving template **1cuuc**, primer **2g** and Gly-AA (**3gly**) in condensation buffer at different time points. Conditions: 20 µM primer, 20 µM template, 200 µM Gly-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

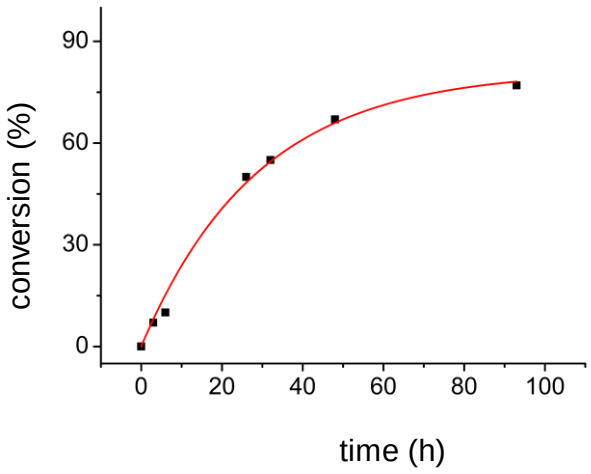


Figure S16. Data points and monoexponential fit for anchoring assay with **1cuuc**, **2g**, and **3gly** in condensation buffer at 0 °C.

Anchoring Assay with 1cuuc, 2g, and 3gly$_2$

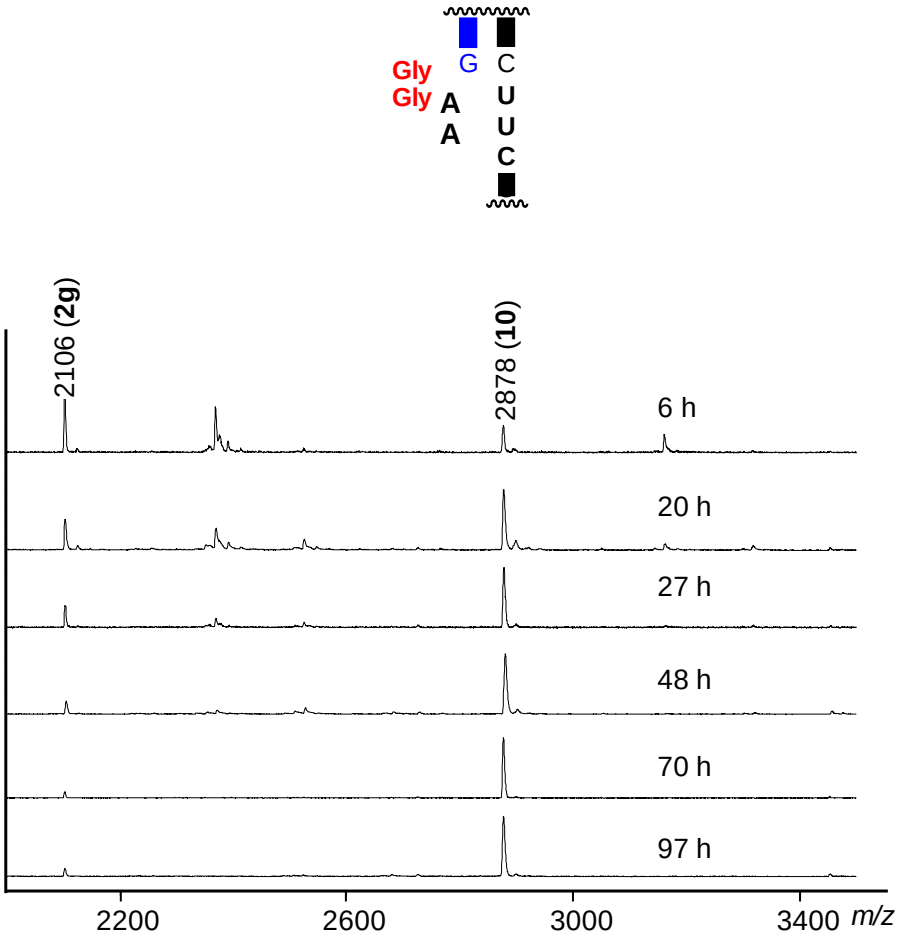


Figure S17. MALDI-TOF mass spectra from assays involving template **1cuuc**, primer **2g** and GlyGly-AA (**3gly$_2$**) in condensation buffer at different time points. Conditions: 20 µM primer, 20 µM template, 200 µM GlyGly-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

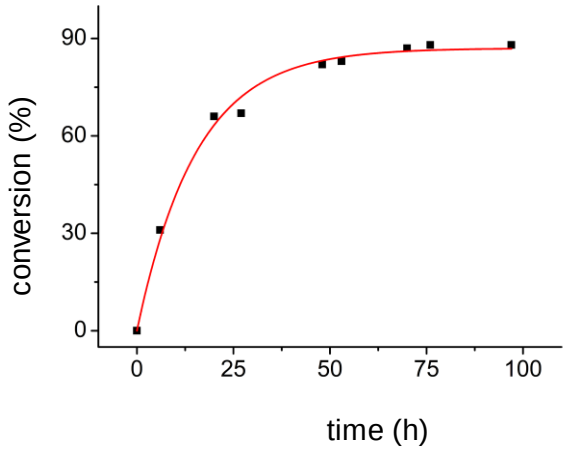


Figure S18. Data points and monoexponential fit for anchoring assay with **1cuuc**, **2g**, and **3gly$_2$** in condensation buffer at 0 °C.

Replicate 1 of Anchoring Assay with 1cuuc, 2g, and 3gly$_2$

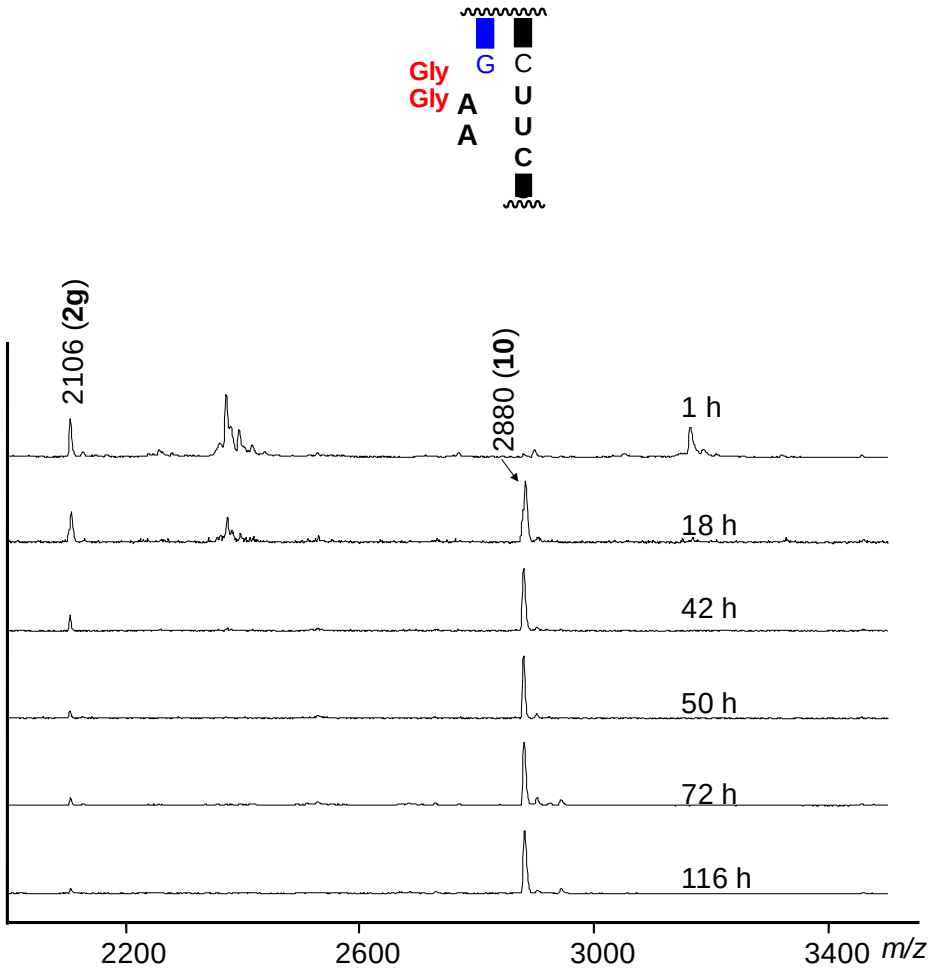


Figure S19. MALDI-TOF mass spectra from assays involving template **1cuuc**, primer **2g** and GlyGly-AA (**3gly$_2$**) in condensation buffer at different time points. Conditions: 20 µM primer, 20 µM template, 200 µM GlyGly-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

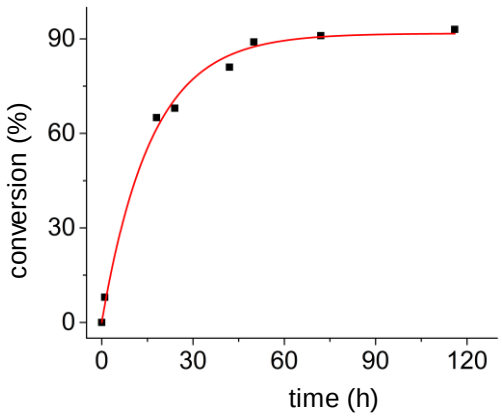


Figure S20. Data points and monoexponential fit for anchoring assay with **1cuuc**, **2g**, and **3gly$_2$** in condensation buffer at 0 °C.

Replicate 2 of Anchoring Assay with 1cuuc, 2g, and 3gly$_2$

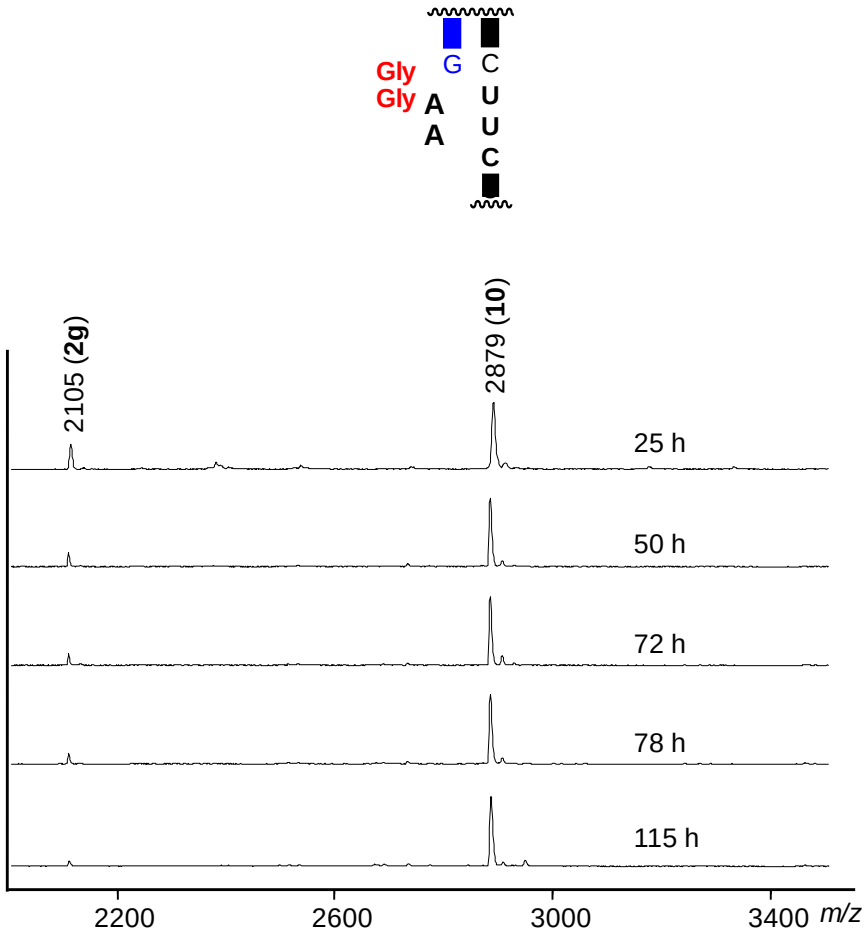


Figure S21. MALDI-TOF mass spectra from assays involving template **1cuuc**, primer **2g** and GlyGly-AA (**3gly$_2$**) in condensation buffer at different time points. Conditions: 20 μM primer, 20 μM template, 200 μM GlyGly-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

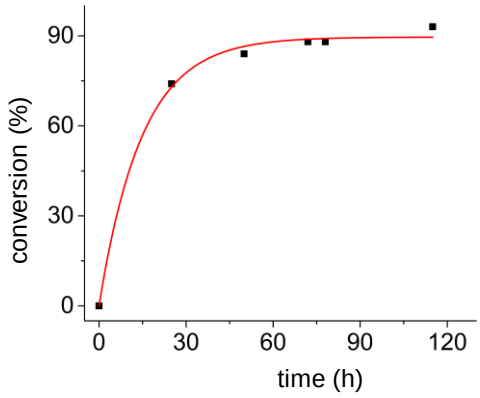


Figure S22. Data points and monoexponential fit for anchoring assay with **1cuuc**, **2g**, and **3gly$_2$** in condensation buffer at 0 °C.

Anchoring Assay with 1cuuc, 2g, and 3gly$_3$

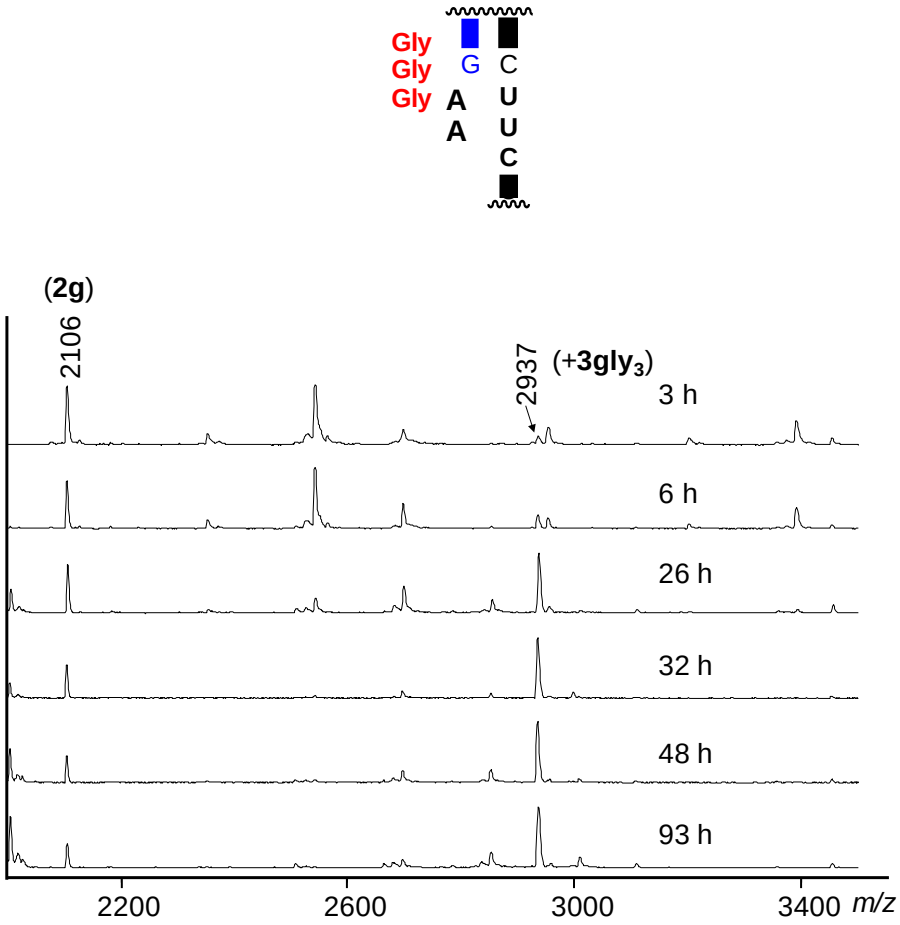


Figure S23. MALDI-TOF mass spectra from assays involving template **1cuuc**, primer **2g** and GlyGlyGly-AA (**3gly$_3$**) in condensation buffer at different time points. Conditions: 20 µM primer, 20 µM template, 200 µM GlyGlyGly-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

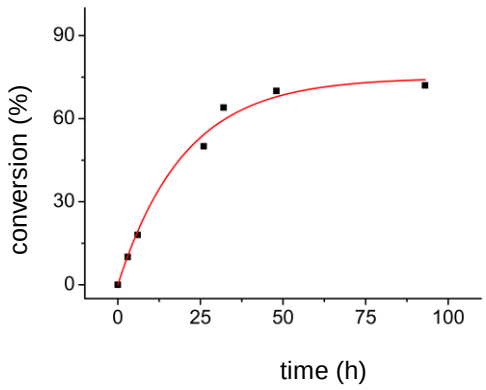


Figure S24. Data points and monoexponential fit for anchoring assay with **1cuuc**, **2g**, and **3gly$_3$** in condensation buffer at 0 °C.

Numerical values for data shown in Figure 2c and additional details

Table S1. Results obtained from monoexponential fits to data points from anchoring reactions involving **3gly**, **3gly$_2$**, **3gly$_3$**, primer **2g**, and RNA template **1cuuc**.

Dinucleotide derivative	**k (h^{-1})** [a]	**$t_{1/2}$ (h)**	**y_{max} (%)**[a]	**R^2**
3gly	0.035 ± 0.003	19.8	81 ± 3	0.99
3gly$_2$	0.065 ± 0.002 [b]	10.7 ± 0.5 [b]	90 ± 2 [b]	0.99 [c]
3gly$_3$	0.050 ± 0.006	13.9	75 ± 3	0.99

[a] Errors are standard errors, as calculated by OriginPro, version 8 for monoexponential fits to the experimental data, except where replicates were performed. [b] Mean ± one standard deviation, calculated from the results of three independent experiments. [c] For each of the three individual fits.

Anchoring Assay with 1cuc, 2g, and 4

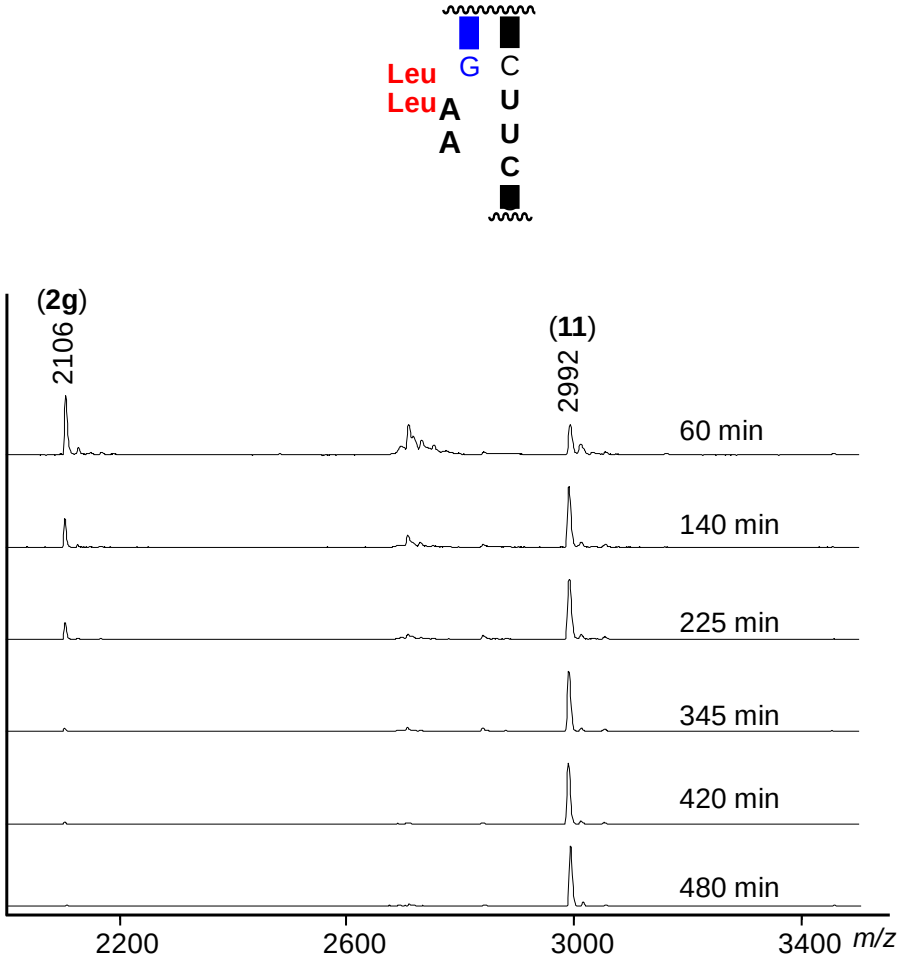


Figure S25. MALDI-TOF mass spectra from assays involving template **1cuuc**, primer **2g** and LeuLeu-AA (**4**) in condensation buffer at different time points. Conditions: 20 µM primer, 20 µM template, 200 µM LeuLeu-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

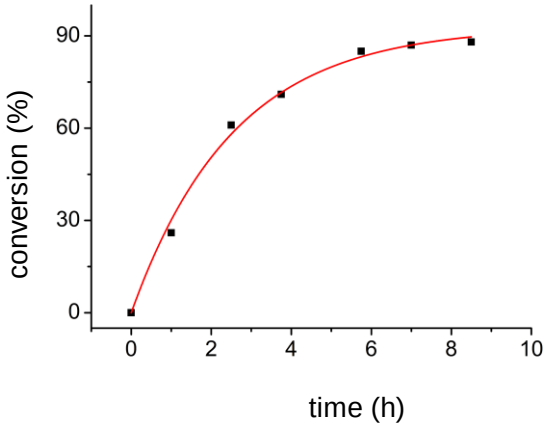


Figure S26. Data points and monoexponential fit for anchoring assay with **1cuuc**, **2g**, and **4** in condensation buffer at 0 °C.

Anchoring Assay with 1cuuc, 2g, and 5

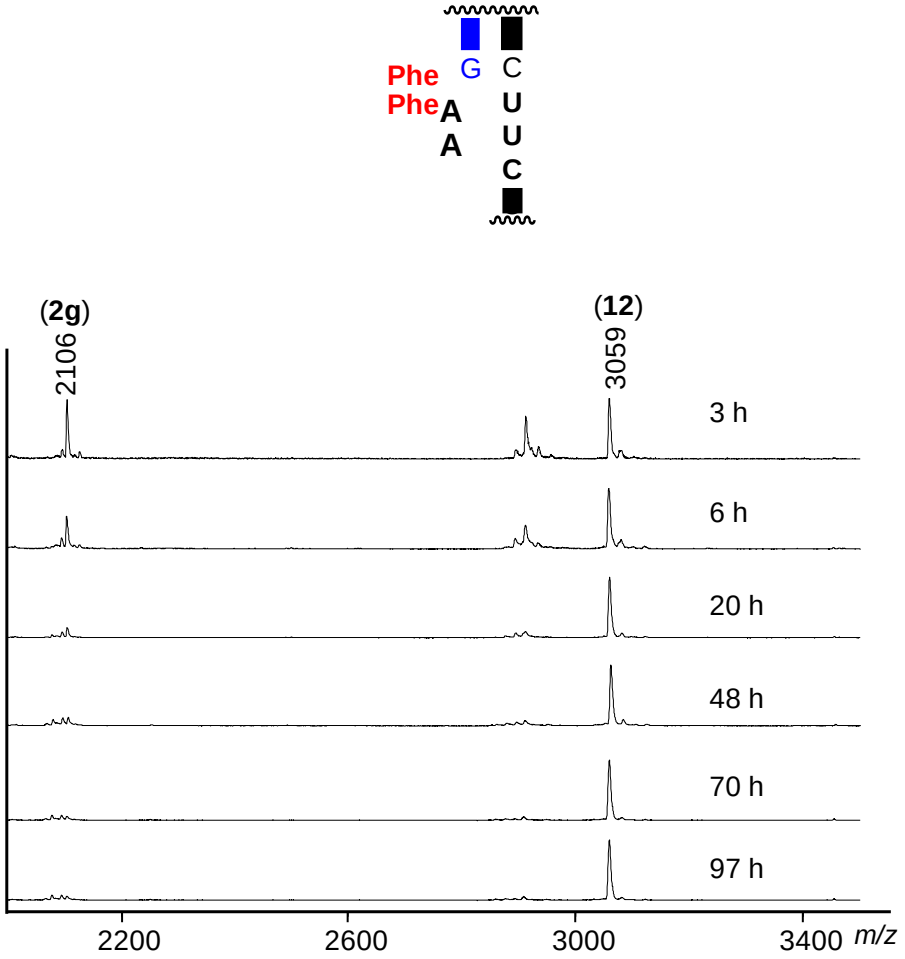


Figure S27. MALDI-TOF mass spectra from assays involving template **1cuuc**, primer **2g** and PhePhe-AA (**5**) in condensation buffer at different time points. Conditions: 20 µM primer, 20 µM template, 200 µM PhePhe-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

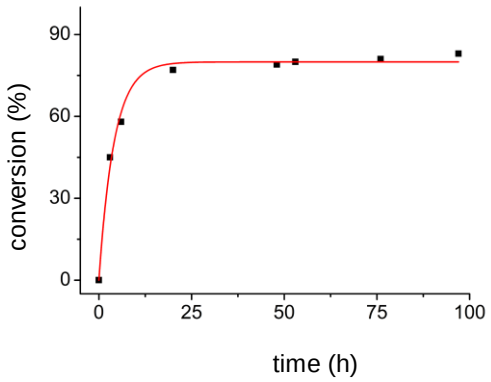


Figure S28. Data points and monoexponential fit for anchoring assay with **1cuuc**, **2g**, and **5** in condensation buffer at 0 °C.

Anchoring Assay with 1cuuc, 2g, and 6

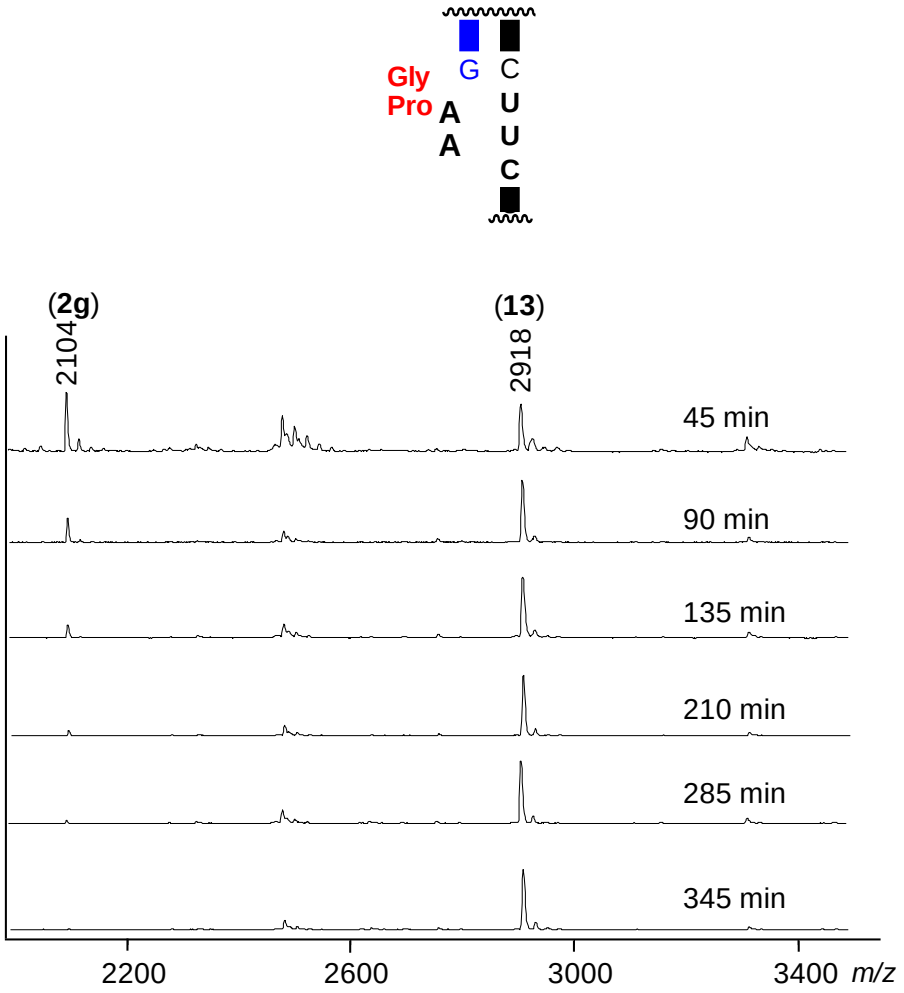


Figure S29. MALDI-TOF mass spectra from assays involving template **1cuuc**, primer **2g** and GlyPro-AA (**6**) in condensation buffer at different time points. Conditions: 20 µM primer, 20 µM template, 200 µM GlyPro-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

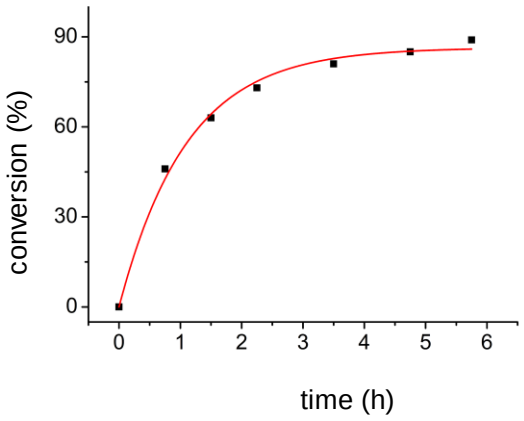


Figure S30. Data points and monoexponential fit for anchoring assay with **1cuuc**, **2g**, and **6** in condensation buffer at 0 °C.

Anchoring Assay with 1cguu, 2g, and 7

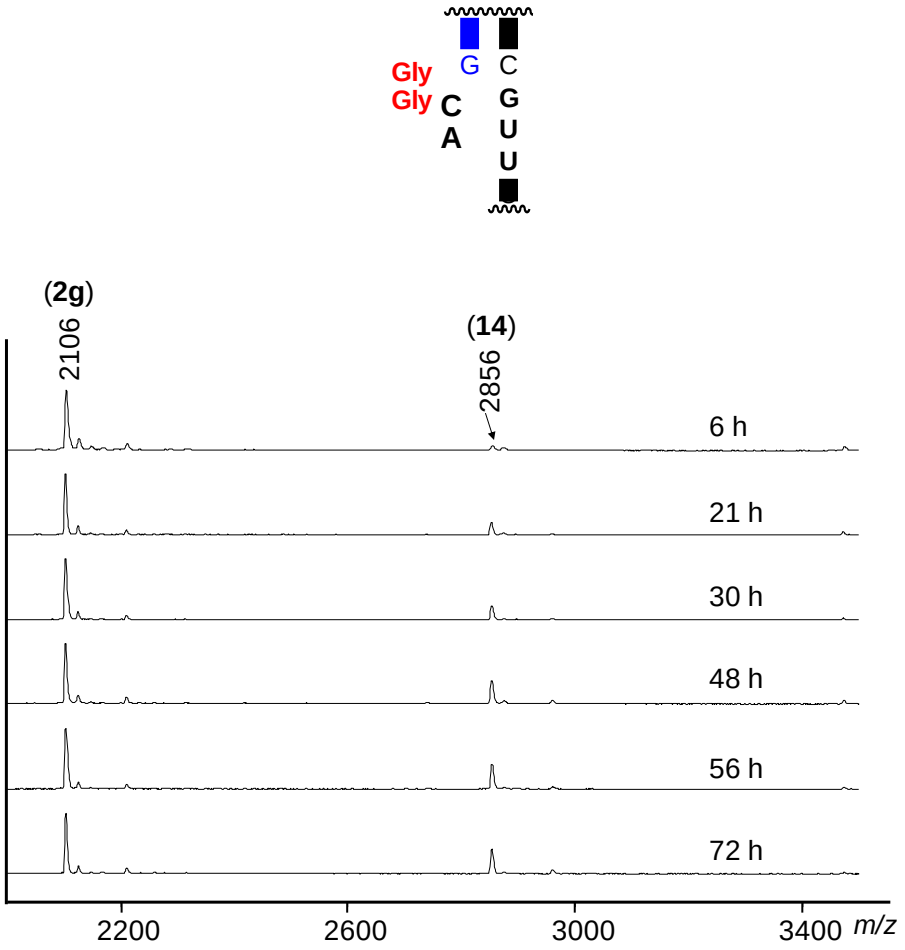


Figure S31. MALDI-TOF mass spectra from assays involving template **1cguu**, primer **2g** and GlyGly-CA (**7**) in condensation buffer at different time points. Conditions: 20 µM primer, 20 µM template, 200 µM GlyGly-CA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

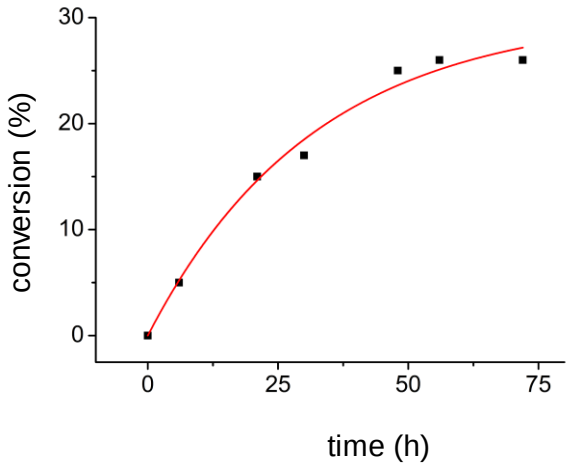


Figure S32. Data points and monoexponential fit for anchoring assay with **1cguu**, **2g**, and **7** in condensation buffer at 0 °C.

Anchoring Assay with 1cguu, 2g, and 8

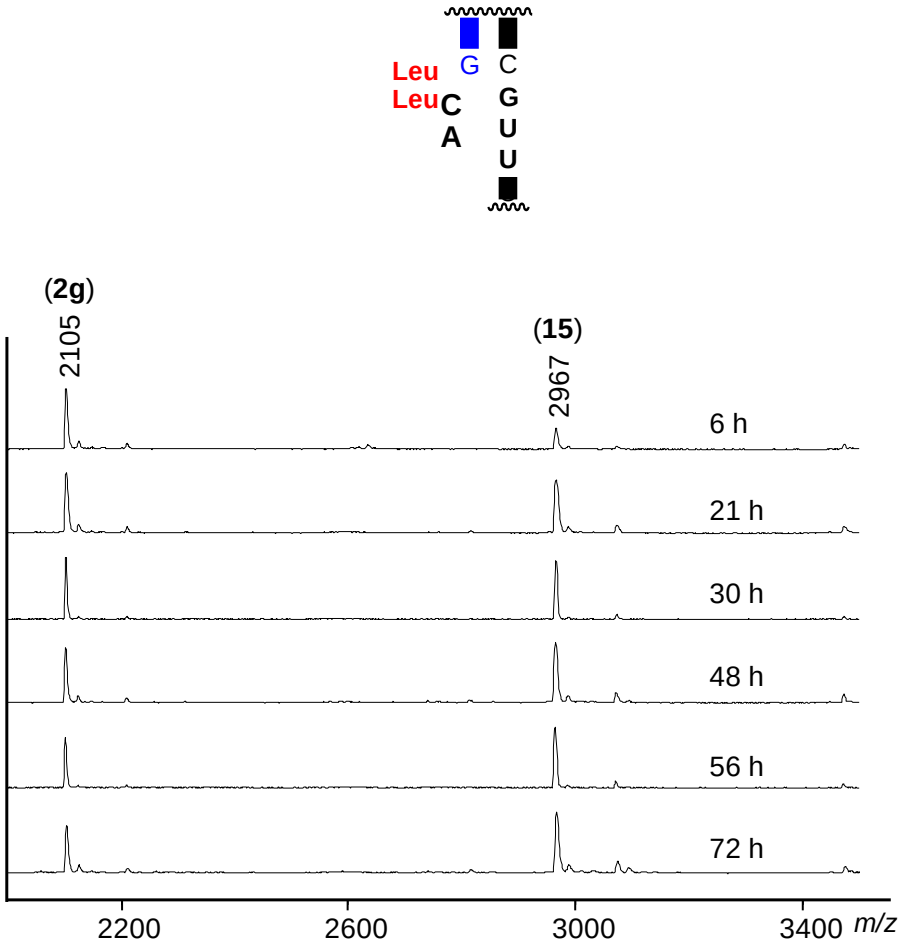


Figure S33. MALDI-TOF mass spectra from assays involving template **1cguu**, primer **2g** and LeuLeu-CA (**8**) in condensation buffer at different time points. Conditions: 20 µM primer, 20 µM template, 200 µM LeuLeu-CA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

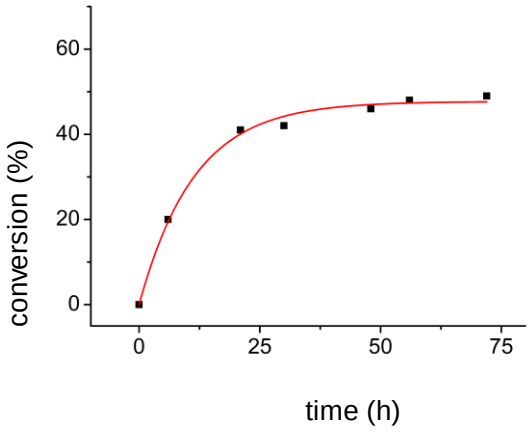


Figure S34. Data points and monoexponential fit for anchoring assay with **1cguu**, **2g**, and **8** in condensation buffer at 0 °C.

Anchoring Assay with 1cguu, 2g, and 9

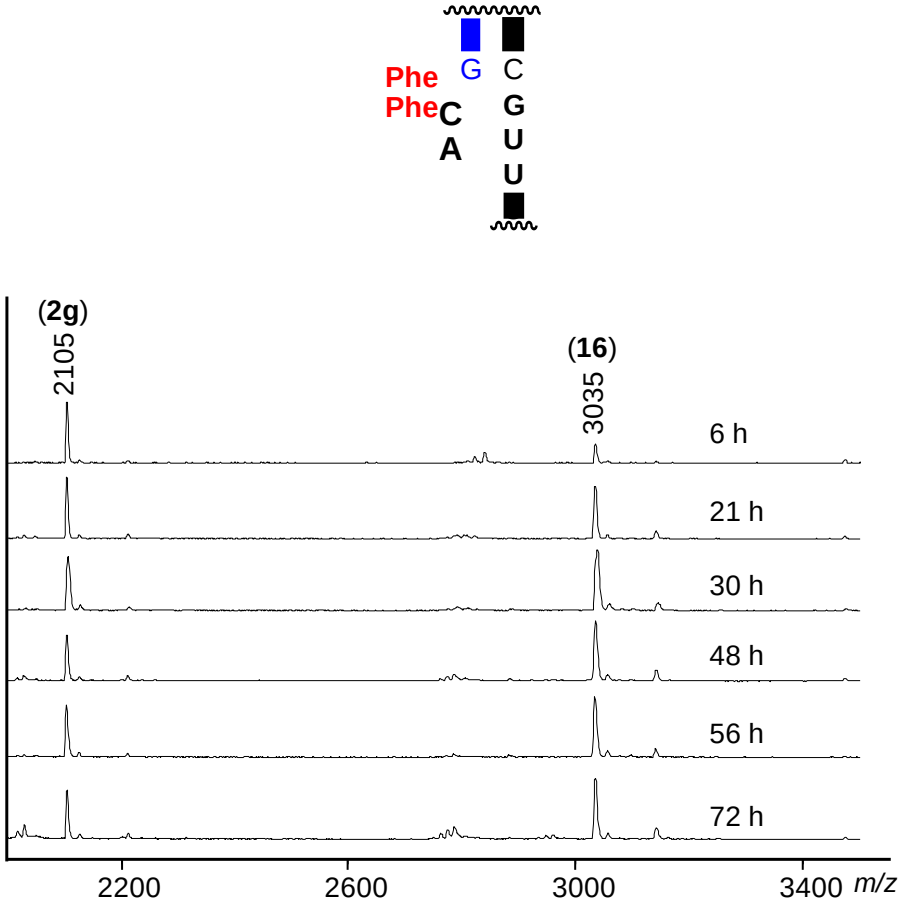


Figure S35. MALDI-TOF mass spectra from assays involving template **1cguu**, primer **2g** and LeuLeu-CA (**8**) in condensation buffer at different time points. Conditions: 20 µM primer, 20 µM template, 200 µM LeuLeu-CA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

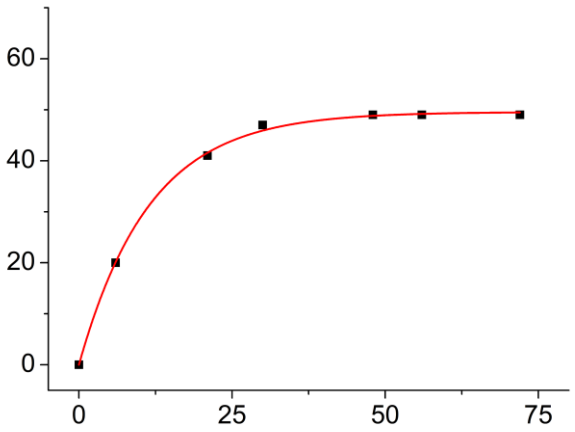


Figure S36. Data points and monoexponential fit for anchoring assay with **1cguu**, **2g**, and **9** in condensation buffer at 0 °C.

5. Additional Data on the Template-Dependent Yield of Anchoring Reactions

Anchoring assays with 2g and 3gly

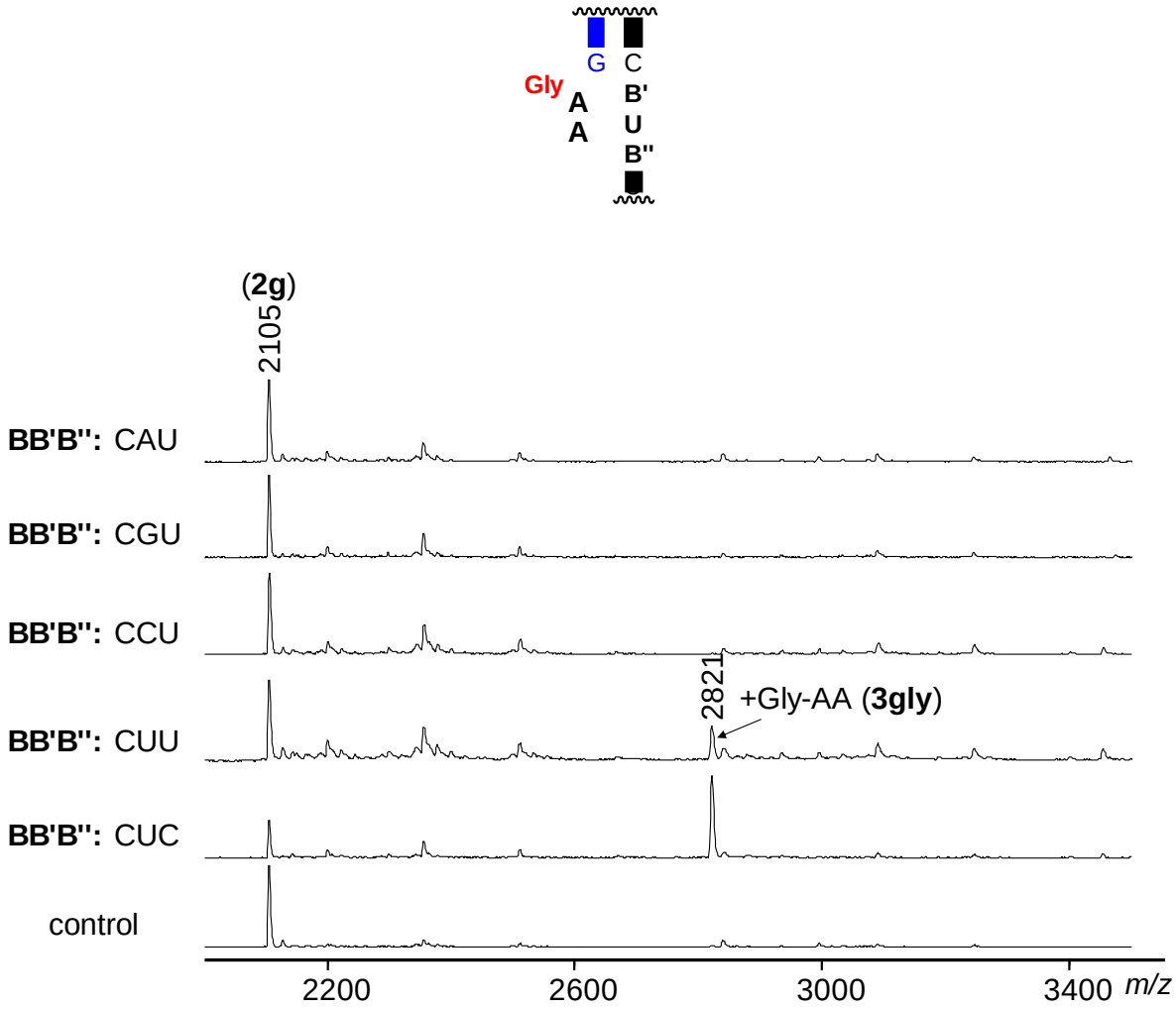


Figure S37. MALDI-TOF mass spectra from assays involving primer **2g** Gly-AA (**3gly**) and templates **1cauu**-**1cuuc** in condensation buffer after 48 h. Conditions: 20 µM primer, 20 µM template, 200 µM Gly-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

Table S2. Conversions (in %) of primer in assays involving RNA template, primer **2g**, and Gly-AA (**3gly**) in condensation buffer after 48 h at 0 °C.

	1cauu	**1cguu**	**1ccuu**	**1cuuu**	**1cuuc**	**control**
Gly-AA	3	<1	<1	23	67	2

Anchoring assays with 2g and 3gly$_2$

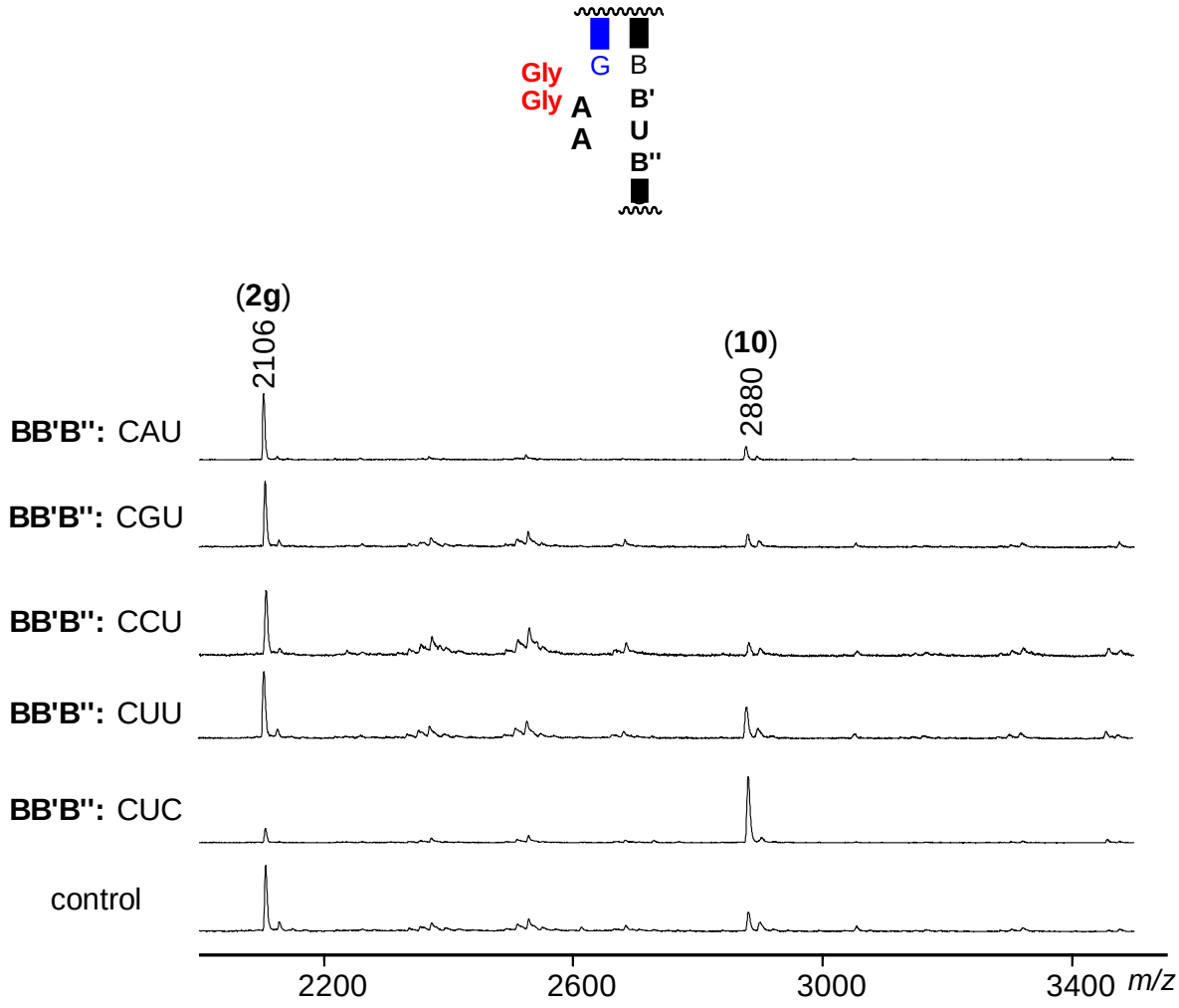


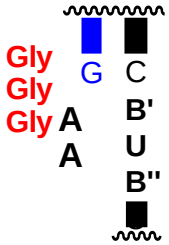
Figure S38. MALDI-TOF mass spectra from assays involving primer **2g**, **3gly$_2$** and templates **1cauu-1cuuc** in condensation buffer after 48 h. Conditions: 20 μM primer, 20 μM template, 200 μM GlyGly-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl 0 °C.

Table S3. Conversions of primer* (in %) in assays involving RNA template, primer **2g**, and **3gly$_2$** in condensation buffer after 48 h at 0 °C.

	1cauu	**1cguu**	**1ccuu**	**1cuuu**	**1cuuc**	**control**
GlyGly-AA	16 (14)*	19 (17)*	19 (17)*	32 (28)*	82 (73)*	23 (20)*

** A correction factor of 1.1 was applied to obtain the values reported in the parentheses.*

Anchoring assays with 2g and 3gly$_3$



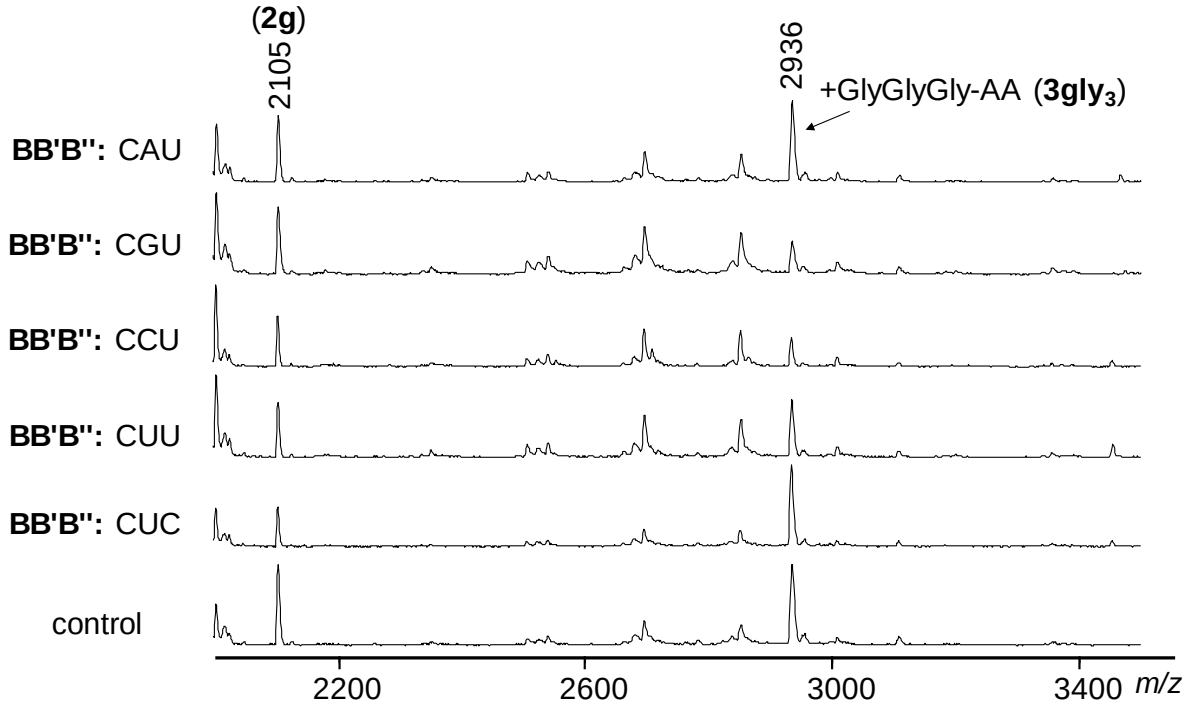


Figure S39. MALDI-TOF mass spectra from assays involving primer **2g**, **3gly$_3$** and templates **1cauu-1cuuc** in condensation buffer after 48 h. Conditions: 20 μM primer, 20 μM template, 200 μM GlyGlyGly-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl 0 °C.

Table S4. Conversions (in %) of primer in assays involving RNA template, primer **2g**, and GlyGlyGly-AA (**3gly$_3$**) in condensation buffer after 48 h at 0 °C.

	1cauu	**1cguu**	**1ccuu**	**1cuuu**	**1cuuc**	**control**
GlyGlyGly-AA	56	29	36	54	70	46

Anchoring assays with 2g and 4

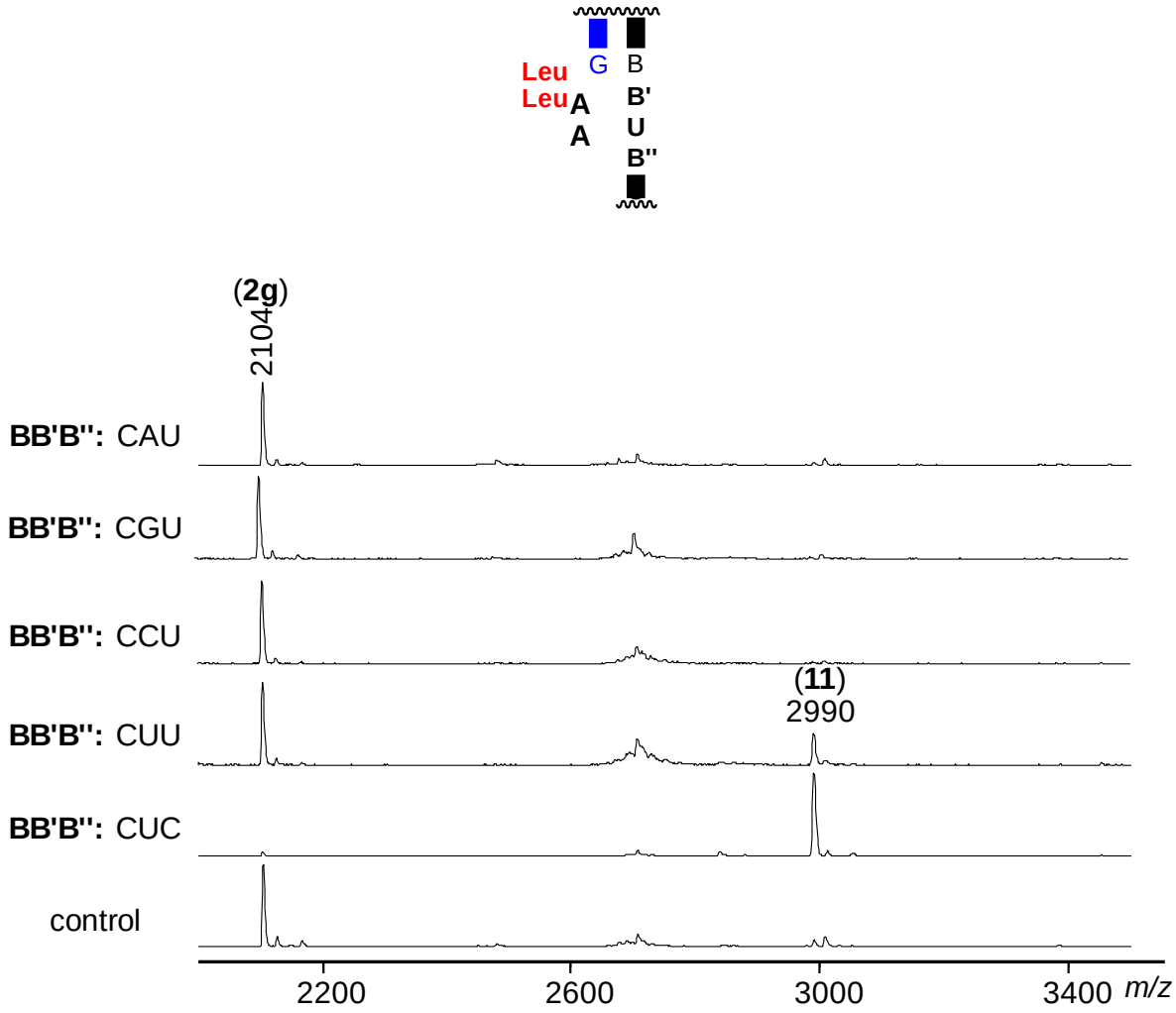


Figure S40. MALDI-TOF mass spectra from assays involving primer **2g**, **4** and templates **1cauu-1cuuc** in condensation buffer after 6 h. Conditions: 20 µM primer, 20 µM template, 200 µM LeuLeu-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

Table S5. Conversions of primer* (in %) in assays involving RNA template, primer **2g**, and **4** in condensation buffer after 6 h at 0 °C.

	1cauu	**1cguu**	**1ccuu**	**1cuuu**	**1cuuc**	**control**
LeuLeu-AA	3	<1	<1	20	85	4

* A correction factor of 1.1 was applied.

Anchoring assays with 2g and 5

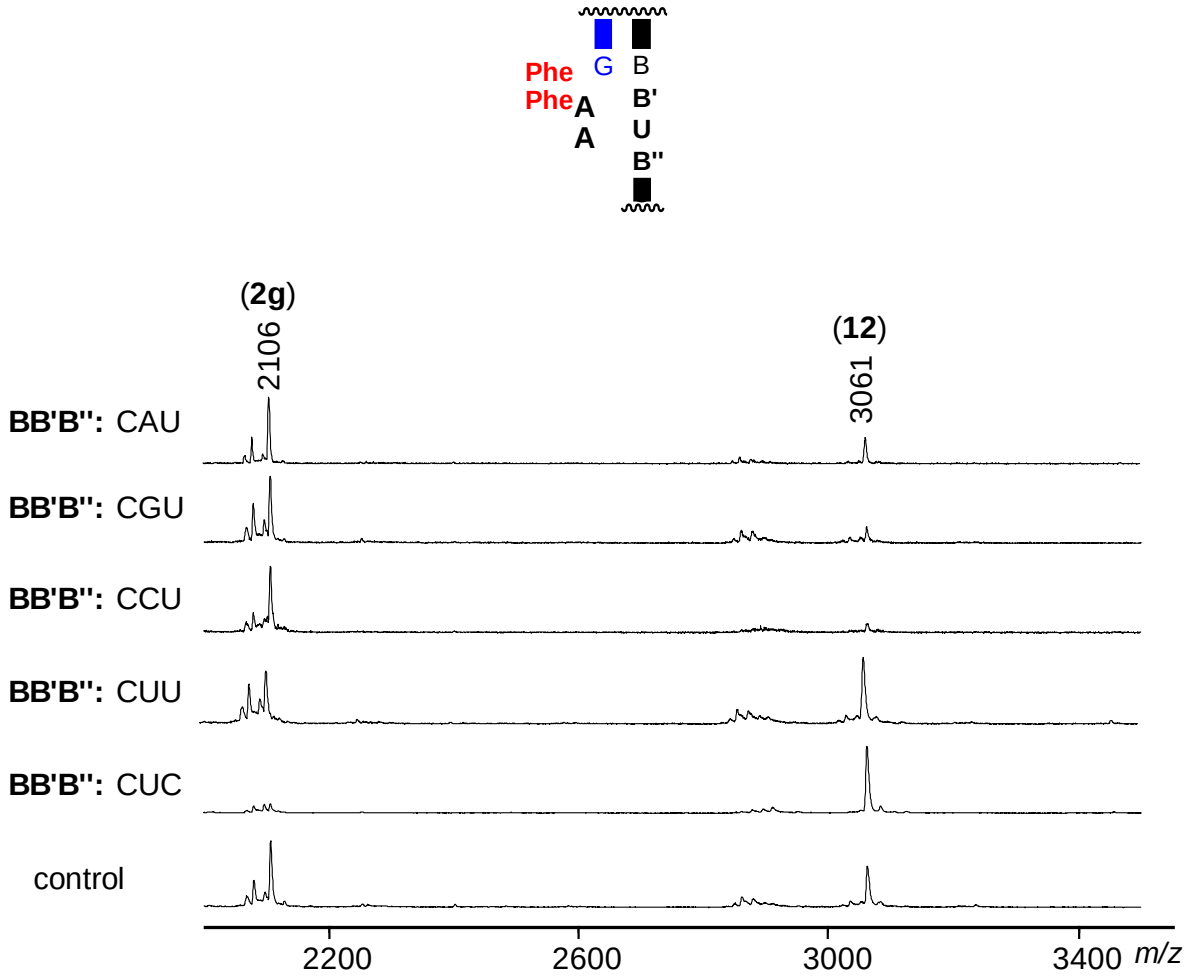


Figure S41. MALDI-TOF mass spectra from assays involving primer **2g**, **5** and templates **1cauu-1cuuc** in condensation buffer after 48 h. Conditions: 20 µM primer, 20 µM template, 200 µM PhePhe-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

Table S6. Conversions of primer* (in %) in assays involving RNA template, primer **2g**, and **5** in condensation buffer after 48 h at 0 °C.

	1cauu	**1cguu**	**1ccuu**	**1cuuu**	**1cuuc**	**control**
PhePhe-AA	26	19	11	51	79	34

* A correction factor of 1.1 was applied.

Anchoring assays with 2g and 6

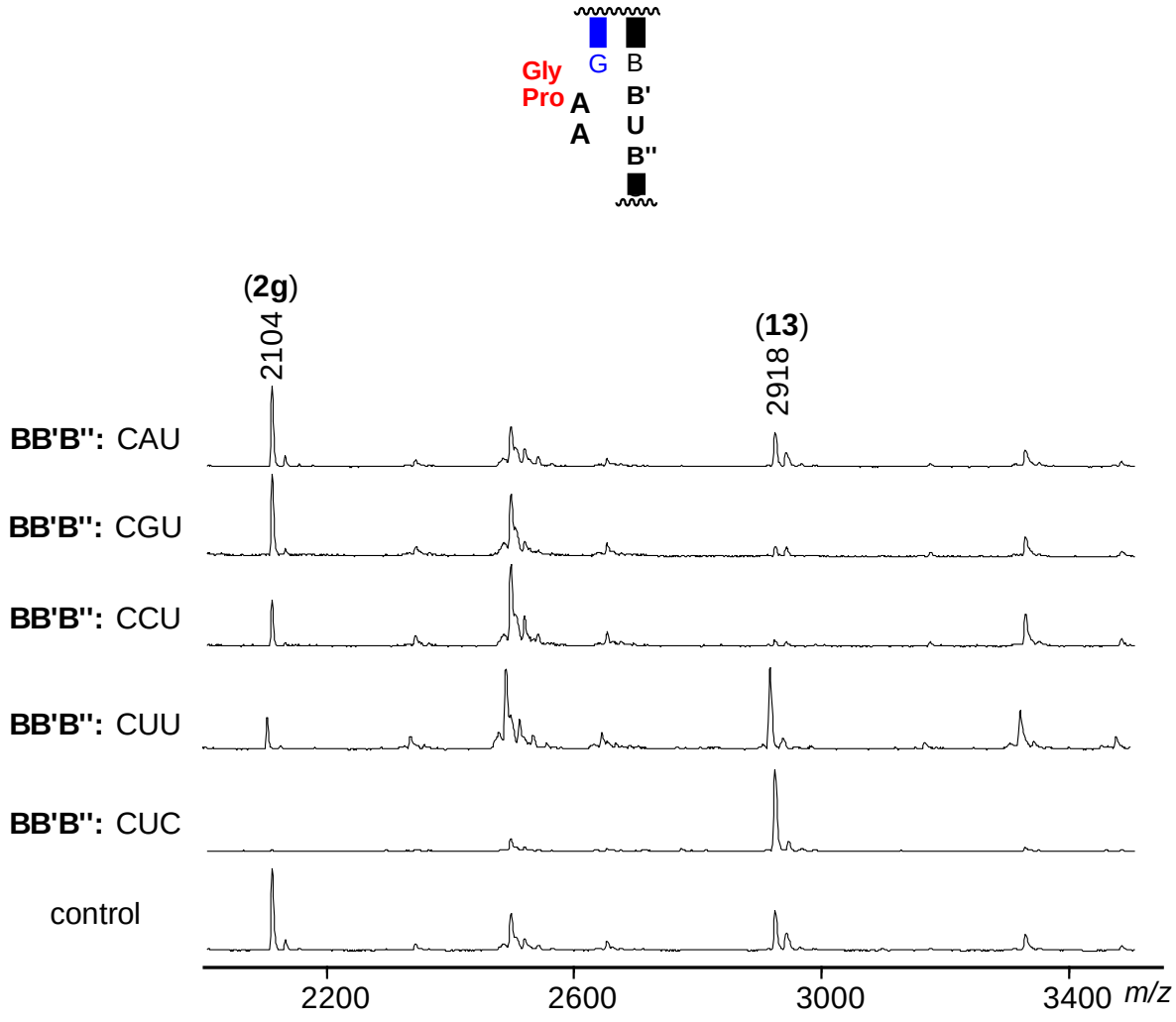


Figure S42. MALDI-TOF mass spectra from assays involving primer **2g**, GlyPro-AA (**6**) and templates **1cauu**-**1cuuc** in condensation buffer after 6 h. Conditions: 20 µM primer, 20 µM RNA template, 200 µM LeuLeu-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

Table S7. Conversions of primer* (in %) in assays involving RNA template, primer **2g**, and **6** in condensation buffer after 6 h at 0 °C.

	1cauu	**1cguu**	**1ccuu**	**1cuuu**	**1cuuc**	**control**
GlyPro-AA	21	8	10	66	quant.	23

** A correction factor of 1.1 was applied.*

6. Additional Data from Competitive Anchoring Assays

Competitive Anchoring Assays with 2g and 3, 4, 5, 6

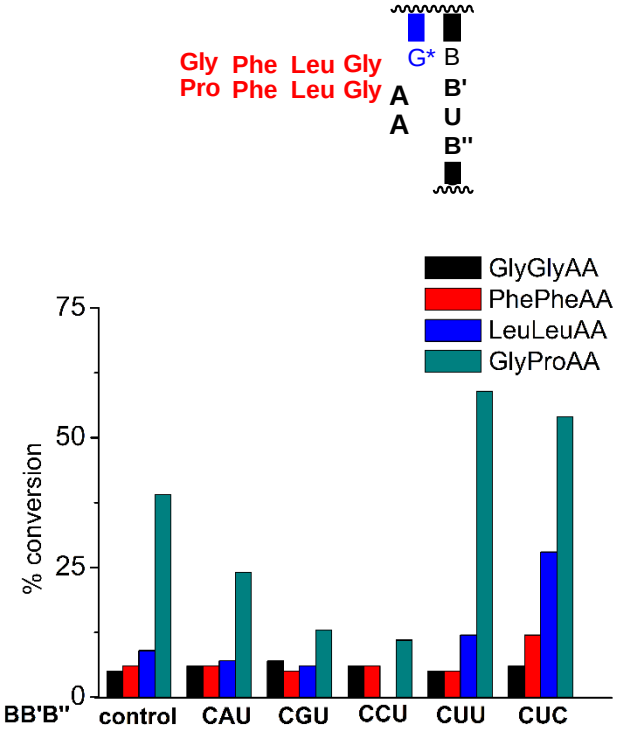


Figure S43. Extent of anchoring of **3gly$_2$**, **4**, **5**, **6** on the primer **2g** specified by the bases listed below each bar graph after 2 d reaction time. Conditions: 50 µM each of GlyGly-AA, PhePhe-AA, and LeuLeu-AA, GlyPro-AA, 20 µM primer, 20 µM template **1cauu-1cuuc**; 50 mM MOPS buffer, pH 7.4 with 0.2 M EDC and 0.2 M NaCl; 0 °C.

Table S8. Conversion of primer (in %) for molecular competition assays with primer **2g**, GlyGly-AA (**3gly$_2$**), LeuLeu-AA (**4**), PhePhe-AA (**5**), GlyPro-AA (**6**) and templates **1cauu-1cuuc** in condensation buffer after 2 d at 0 °C.

	control	**1cauu**	**1cguu**	**1ccuu**	**1cuuu**	**1cuuc**
GlyGly-AA	5	6	7	6	5	6
PhePhe-AA	6	6	5	6	5	12
LeuLeu-AA	9	7	6	<1	12	28
GlyPro-AA	39	24	13	11	59	54

Competitive Anchoring assays with 2g and 3, 4, 5

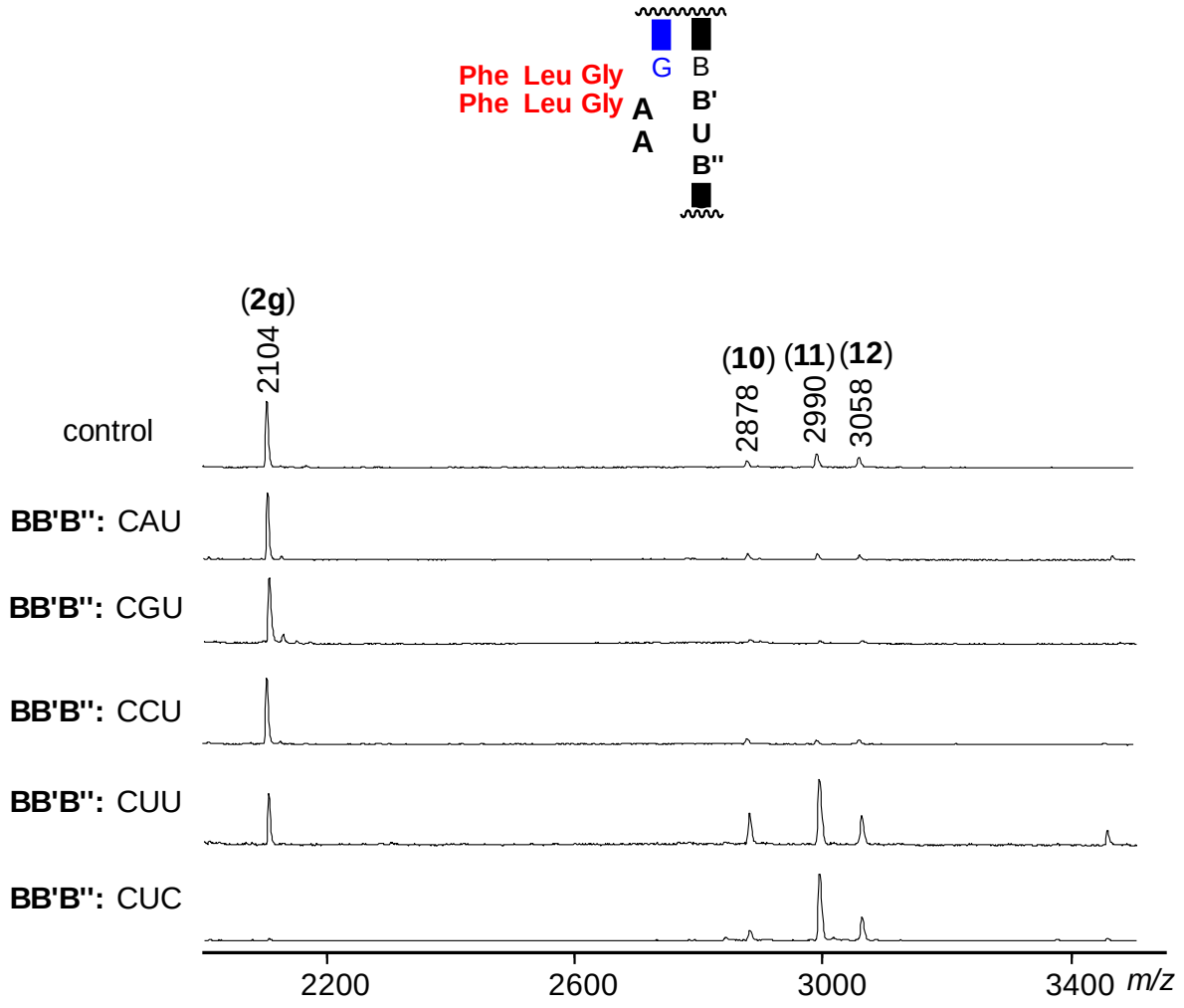


Figure S44. MALDI-TOF mass spectra from competitive assays involving primer **2g**, GlyGly-AA (**3gly$_2$**), LeuLeu-AA (**4**), PhePhe-AA (**5**) and templates **1cauu-1cuuc** in condensation buffer after 2 d. Conditions: 20 µM primer, 20 µM template, 67 µM of each GlyGly-AA, PhePhe-AA, LeuLeu-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M EDC hydrochloride and 0.2 M NaCl at 0 °C.

Table S9. Conversion of primer (in %) for molecular competition assays with primer **2g**, GlyGly-AA (**3gly$_2$**), LeuLeu-AA (**4**), PhePhe-AA (**5**), and templates **1cauu-1cuuc** in condensation buffer after 2 d at 0 °C.

	control	**1cauu**	**1cguu**	**1ccuu**	**1cuuu**	**1cuuc**
GlyGly-AA	8	7	7	8	18	11
PhePhe-AA	10	6	6	7	16	22
LeuLeu-AA	13	6	6	6	36	65

Competitive Anchoring assays with primers 2g, 2c, 2a and dipeptido deadenylates 3, 4, 5

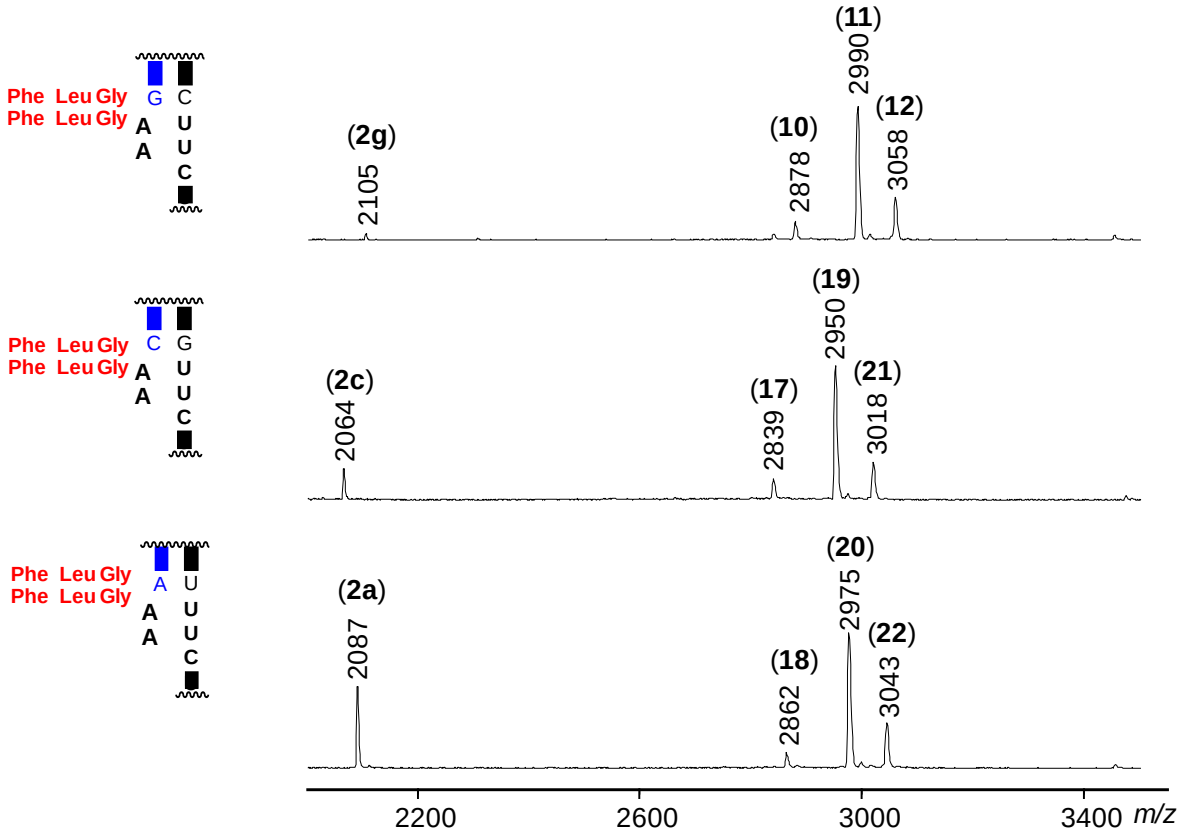


Figure S45. MALDI-TOF mass spectra from competitive assays involving primer **2g, 2c, 2a**, GlyGly-AA (**3gly$_2$**), LeuLeu-AA (**4**), PhePhe-AA (**5**) and templates **1cuuc, 1guuc, 1uuuc** in condensation buffer after 1 d. Conditions: 20 µM primer, 20 µM template, 67 µM of each GlyGly-AA, PhePhe-AA, LeuLeu-AA in 50 mM MOPS buffer (pH 7.4) containing 0.2 M NaCl, and 0.2 M EDC hydrochloride at 0 °C.

Product distribution at an earlier time point

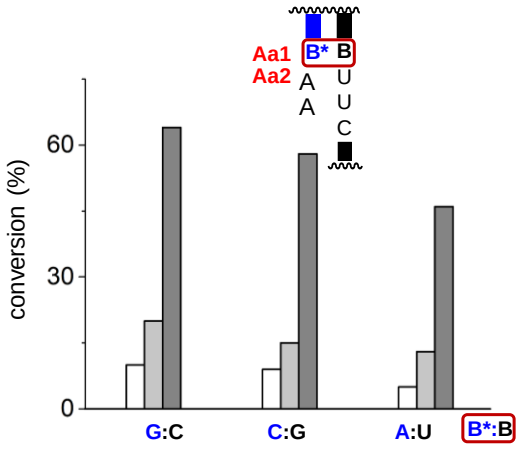


Figure S46. Results from read-out of the competition assays with dipeptido dinucleotides **3gly$_2$**/**4**/**5** (67 µM each) reacting with different primers (20 µM), directed by primer:template duplex. This data was collected at an earlier time point (after 18 h). Conditions: 50 mM MOPS buffer, pH 7.4, 0.2 M EDC, 0.2 M NaCl, 0 °C.

Table S10. Conversion of primer (in %) for molecular competition assays with primer **2g**, **2c**, **2a**, GlyGly-AA (**3gly$_2$**), LeuLeu-AA (**4**), PhePhe-AA (**5**) and template (**1cuuc**, **1guuc**, **1uuuc)** in condensation buffer after 1 d at 0 °C.

template	Primer	GlyGly-AA	PhePhe-AA	LeuLeu-AA
1cuuc	**B* = 2g**	9	20	66
1guuc	**B* = 2c**	9	17	61
1uuuc	**B* = 2a**	5	16	50

7. References for Supplementary Information

1. R. Eisenhuth, C. Richert, Convenient syntheses of 3'-amino-2',3'-dideoxynucleosides, their 5'-monophosphates, and 3'-aminoterminal oligodeoxynucleotide primers. *J. Org. Chem.* **2009**, *74*, 26-37.
2. E. Kervio, B. Claasen, U. E. Steiner, C. Richert, The strength of the template effect attracting nucleotides to naked DNA. *Nucleic Acids Res*. **2014**, *42*, 7409-7420.
3. A. Guzaev, H. Salo, A. Azhayev, H. Lönnberg, A new approach for chemical phosphorylation of oligonucleotides at the 5'-terminus. *Tetrahedron* **1995**, *51*, 9375-9384.