Supplementary information for

Amino acids catalyze RNA formation under ambient alkaline conditions

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Materials and methods

Chemicals

Nucleoside 2',3'-cyclic phosphates (including cAMP, cUMP, cGMP from Biolog Life Science Institute and cCMP from Sigma Aldrich), amino acids (from Sigma Aldrich), glycogen, and ammonium acetate (from Sigma Aldrich), as well as KOH, HCl, and ethanol (from Carl Roth), were used in the experiment. Polymerization reactions were conducted using either glass slides with cavities (from Carl Roth) or in 24-well plates (from Greiner CELLSTAR®)

Dry-state polymerization of cyclic nucleotides

Stock solutions of amino acids and nucleotides (100-200 mM) were prepared in nuclease-free water (Invitrogen™). The reaction mix was prepared by mixing the corresponding stock solutions to attain the required concentration and pH (adjusted with KOH or HCl).

For a typical polymerization reaction, small volumes of the reaction mix (10-50 µL) were placed on the glass slide or multi-well plate and were allowed to dry under airflow (ca 0.5-2h). The dried samples were then incubated at room temperature for 20 hours. After the incubation, the samples were extracted at least twice the volume with nuclease-free water. In some experiments, the samples were further purified by ethanol precipitation to enrich the oligonucleotides as described in the following section.

For morphological characterization of the dried nucleotide and amino acids mixtures, microscopic images were acquired using an Olympus SZX10 stereomicroscope equipped with an Olympus DF PLAPO 1.25X objective and a Canon EOS 2000D camera.

Ethanol precipitation

The extracted samples were mixed with glycogen (20 µg) and ammonium acetate (at a final concentration of 500 mM). To this, 2-3x volume cold 100% Ethanol was added. After an overnight incubation at 4 °C, the sample was centrifuged at 4 °C for 30 minutes at 21000g. The supernatant was discarded, and the pellet was washed with 50 µl of cold 70% ethanol by mixing and centrifuging again at 4 °C for 30 minutes. The supernatant was removed, and the washed pellet was dissolved in nuclease-free water for the LC-MS analysis.

HPLC ESI-TOF analysis

Oligomer quantification was performed using high-performance liquid chromatography (Agilent 1260 Infinity II) coupled with an electrospray ionization time-of-flight mass spectrometer (Agilent 6230B with Dual AJS ESI). To ensure accuracy and determine the timing of the HPLC, pre-formed oligomers (polyG and polyC) ranging from lengths 2-10 with 3' phosphate (from Biomers) were used as standards. Reverse-phase ion-pairing HPLC was employed to separate oligomers of different lengths on an Agilent AdvanceBio Oligonucleotide C18 Column (4.6 x 150 mm, 2.7 µm, heated to 60°C), with a gradient elution at a flow rate of 1 mL/min. The eluent consisted of mixtures of water (Bottle A) and methanol (50% in water, Bottle B) containing 200 mM trimethylamine (TEA) and 8 mM

hexafluoroisopropanol (HFIP). The separation process began with a 5-minute flush with 1% B, followed by a gradual increase to 30% B over 22.5 minutes and then to 40% B over 15 minutes. Subsequently, the column was flushed with 100% B for 5 minutes before re-equilibration at 1% B for 6 minutes. Detection of eluted oligonucleotides was achieved using ESI-TOF in negative mode, employing specific source parameters: Gas temperature: 325 °C, Drying gas flow: 13 l/min, Sheath gas temperature: 400 °C, Sheath gas flow: 12 l/min, VCap: 3500V, Nozzle Voltage: 2000V.

MS data analysis by custom-written LabView program

The MS data acquired from HPLC ESI-TOF was converted to .mz5 format using MSConvert, a component of ProteoWizard[1]. Subsequently, it was imported into a custom LabView program (Spectral_browser_3.58) for detailed analysis (Figure M).

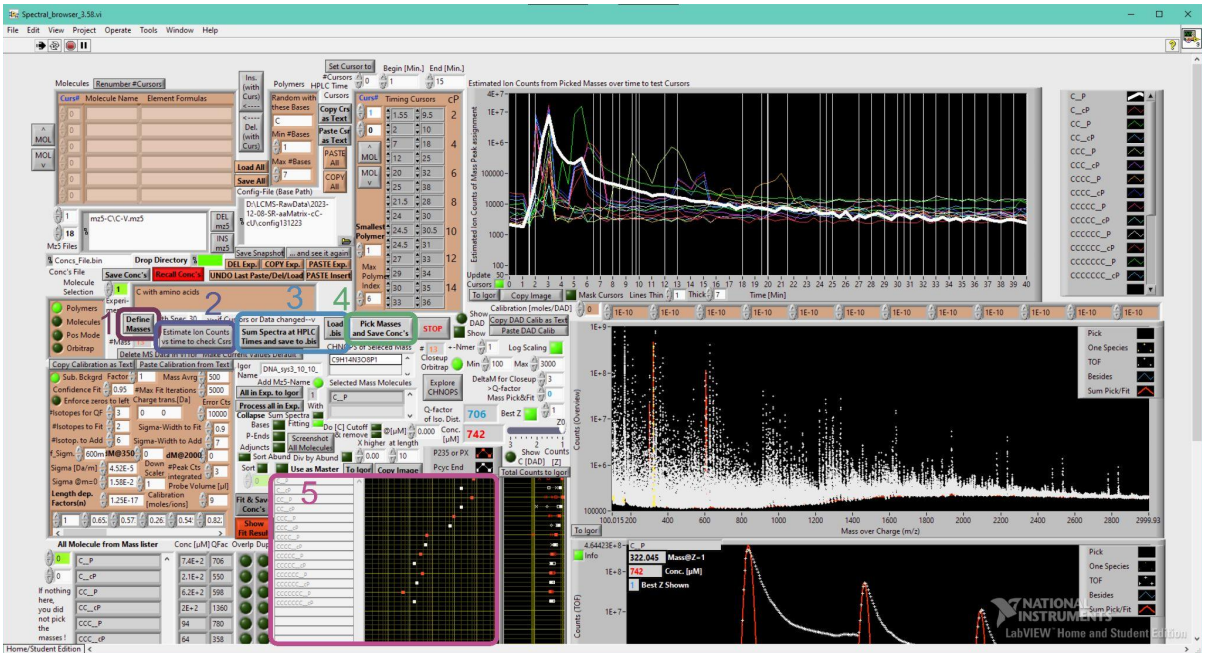


Figure M. User interface of SpectralBrowser v3.58. The MS data analysis pipeline involves a systematic process denoted by numbers (1-5), each step is color-coded and described below.

1. Mass List Generation

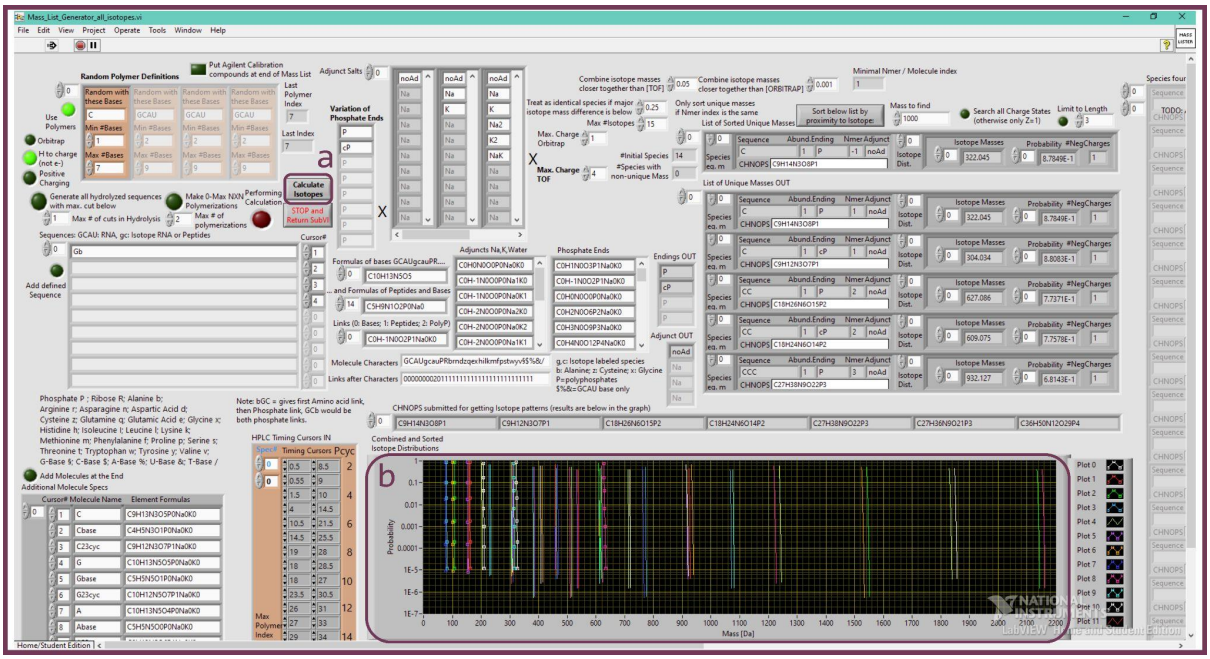
2. Data Loading and Mass Chromatogram Plotting

3. Summation of Raw Mass Spectra

4. Pick and Fit of Summed Isotope Distributions

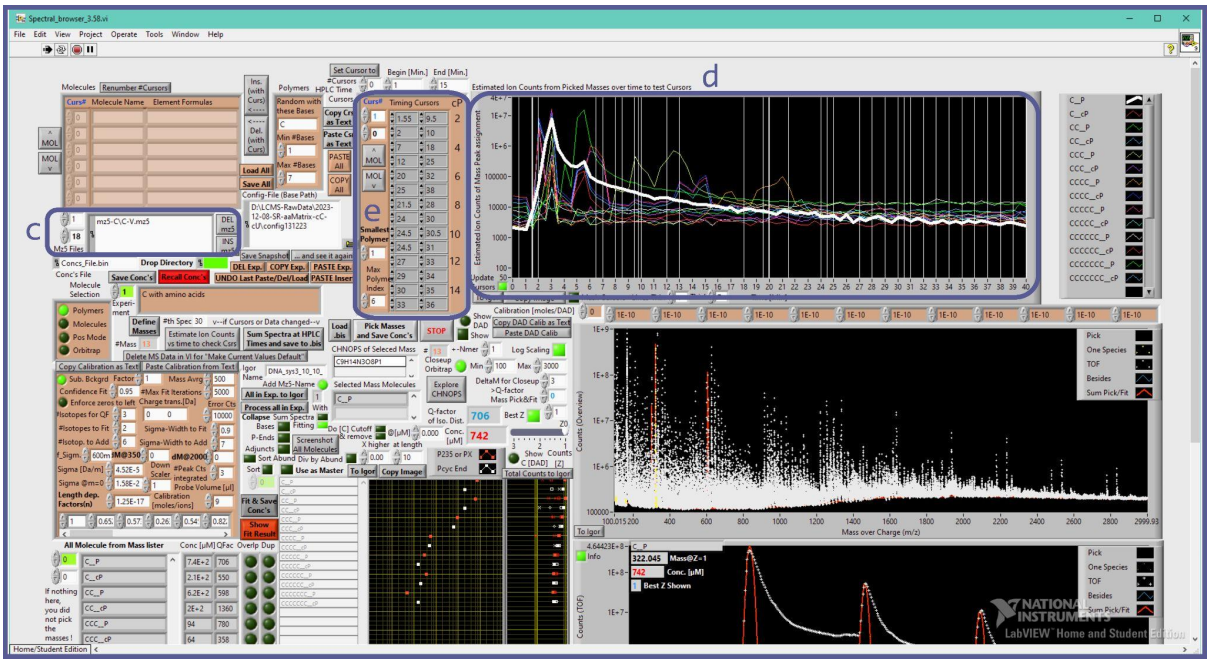
5. Concentration Determination

1. Mass List Generation



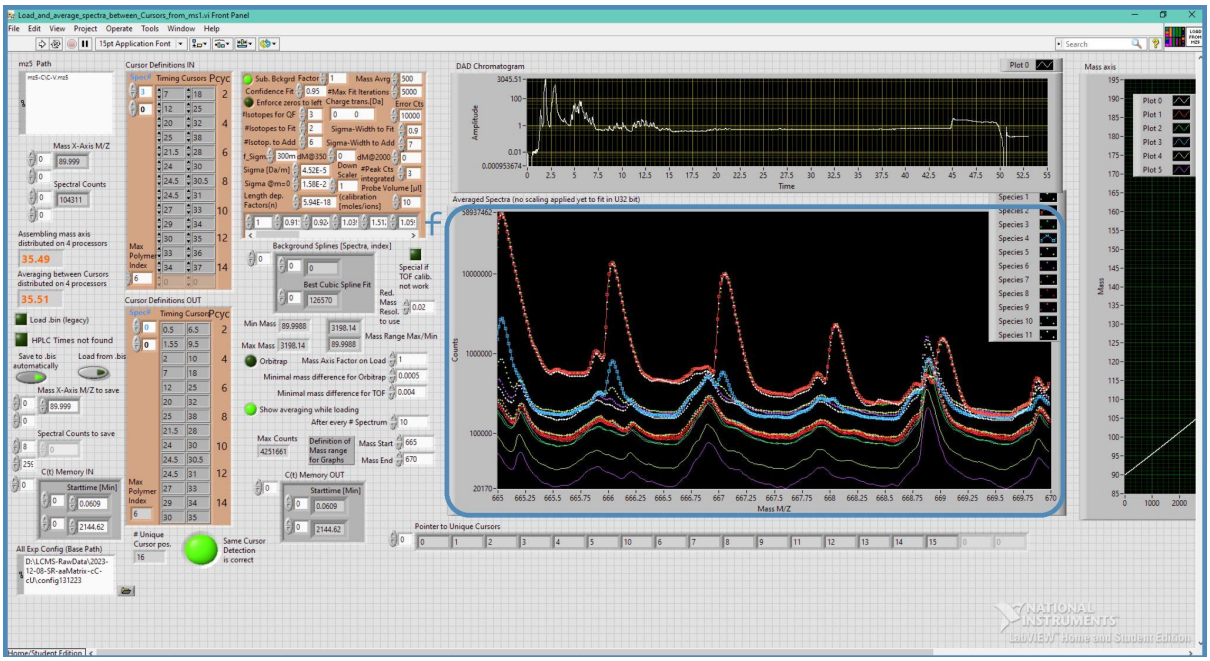
Define Masses: A sub-VI called "mass list generator" takes inputs such as nucleotide combinations, length range, charge states, and types of phosphate ends to generate a list of all chemical formulae **(Calculate Isotopes, a)**. This list is then passed via ZeroMQ message transfer protocol to Python, where IsoSpecPy [1] retrieves **theoretical isotope distributions (b)** for the corresponding chemical formulae.

2. Data Loading and Mass Chromatogram Plotting



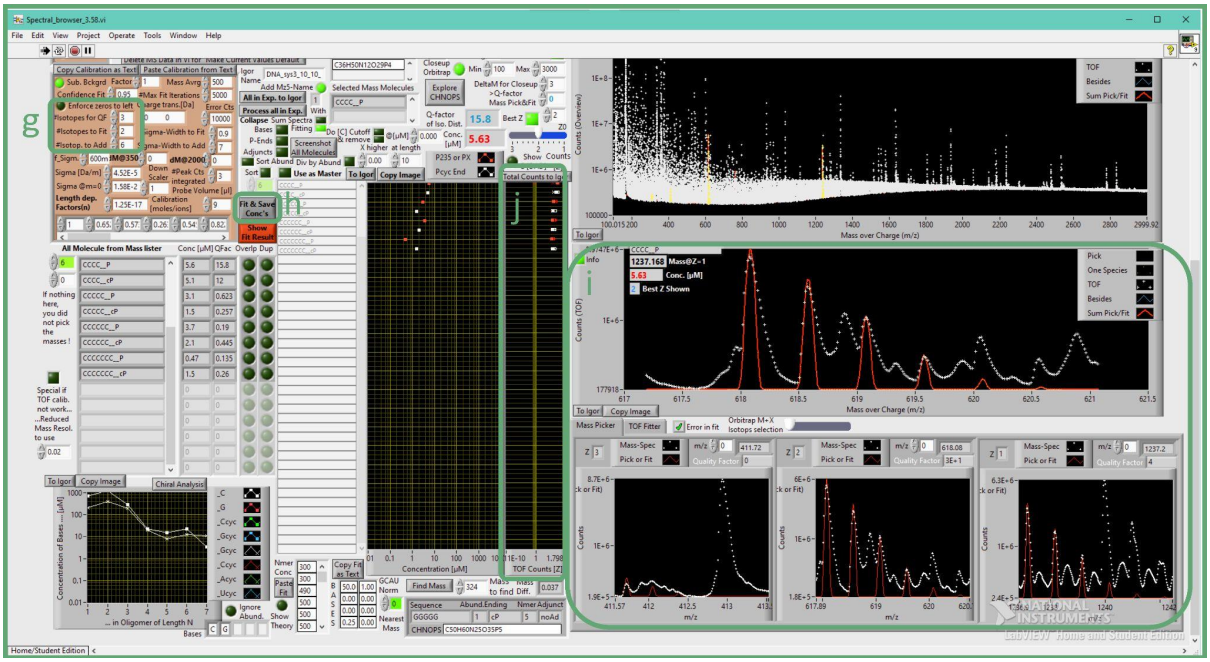
In the main VI, the **mz5 file is loaded (c)**, and mass chromatograms for the molecules are plotted **(Estimated Ion Counts, d)**. This step facilitates the determination of retention times for different oligonucleotide lengths. The retention times of oligonucleotide standards are utilized to set **cursor positions (e)** for different-length oligomers.

3. Summation of Raw Mass Spectra



Raw mass spectra within the cursor positions are summed **(Sum Spectra, f)**. Additionally, the program saves the summed spectra into a binary file format (.bis), which optimizes memory usage and enhances data loading time for future analysis. This feature allows for efficient retrieval and utilization of the summed spectra in subsequent analyses through the **"load bis"** functionality.

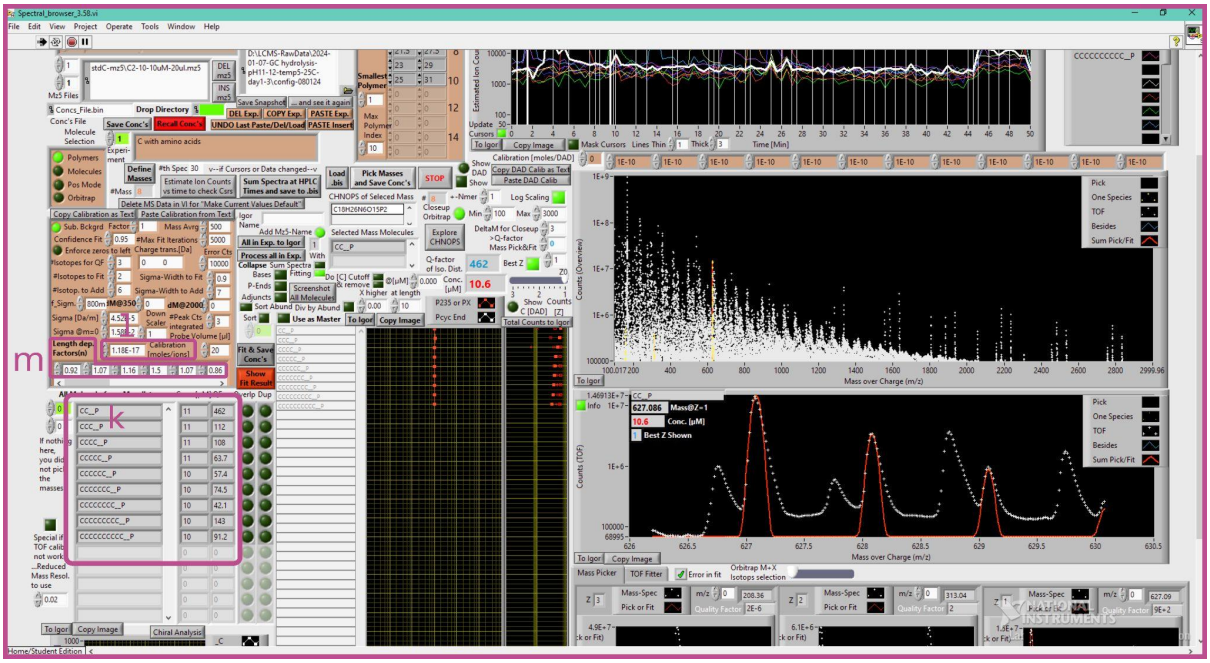
4. Pick and Fit of Summed Isotope Distributions



The theoretical isotope distributions for the molecules at the cursor positions are assigned to the corresponding m/z in the summed spectra using the most abundant isotope **(Pick Masses)**. Depending on the number of isotopes selected **(# isotope to fit, g)**, their theoretical distribution is fit to the

measured data **(Fit & Save Conc, h)** to generate the **TOF counts (i, j).** The sum of TOF ion counts at different charge states are calculated for each molecule based on the fitted distributions.

5. Concentration Determination



The concentration of the oligonucleotides in the sample is determined for the volume analyzed using the TOF counts for **oligonucleotide standards of 2-10 mer (k)**. Calibration curves (moles/ion counts) for each of the standard oligos are generated for known concentrations. The mean of the slopes **(Calibration, moles/ions, l)** is calculated, and the corresponding ratio to the mean for each length is used as **length dependent factors (m)**.

NMR measurements

The dry-state reactions of Nucleoside 2′, 3′-cyclic monophosphates were carried out in the presence of 5 equiv. valine under pH 10 conditions (adjusted with KOH) at room temperature for 20 h. The oligomers were solubilized in water and 8M urea with 5% D_2O and subjected to NMR measurements. The 1D- ^{31}P NMR spectra were acquired on a Bruker Avance III 300 MHz spectrometer. The data analysis was performed on Bruker TopSpin 4.3.0 to quantify the regio-isomers (3′-5′ and 2′-5′ linkages) in the RNA products.

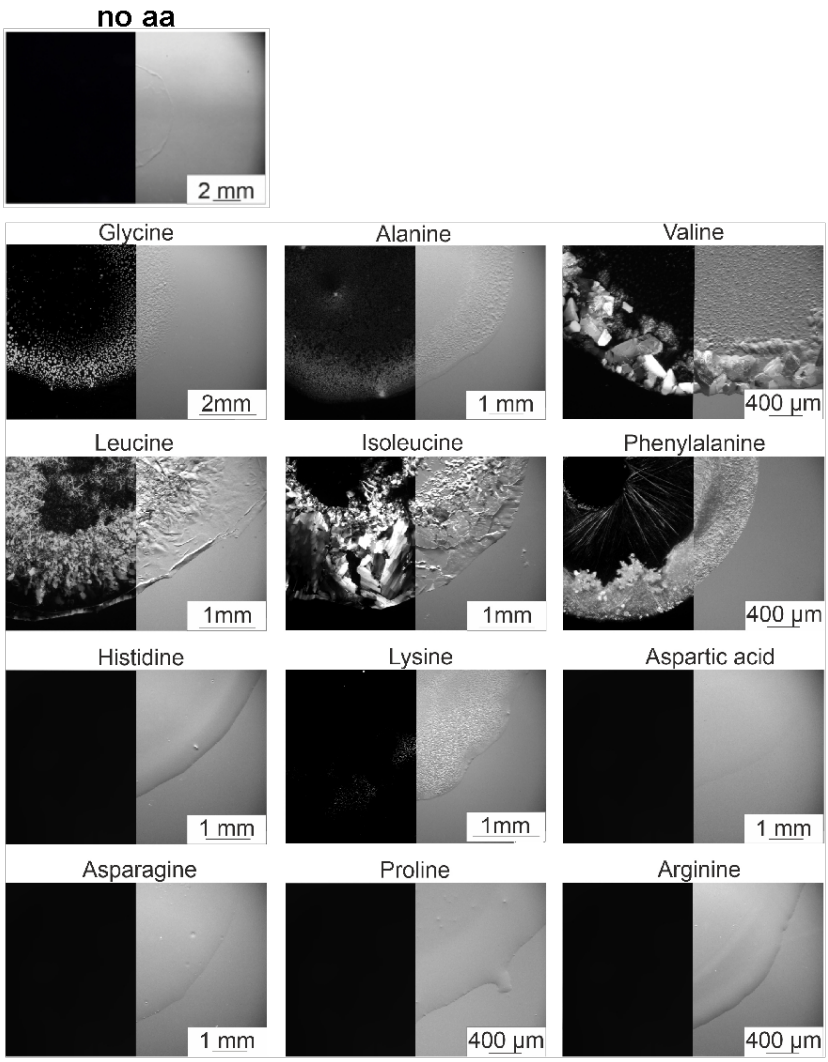


Figure S1. Morphological characterization of the dried mixtures of nucleotides and amino acids by optical microscopy. The solution containing cCMP and amino acids mixed in a 1:5 ratio was prepared at pH 10 and then incubated for 20 hours. The left half of the images show the internal structure of the samples viewed in cross-polarized light. In the experiment 100 nmol of nucleotides and 500 nmol of amino acids were used. The figures display only a zoomed-in section of the entire dried spot, revealing heterogeneity in its appearance.

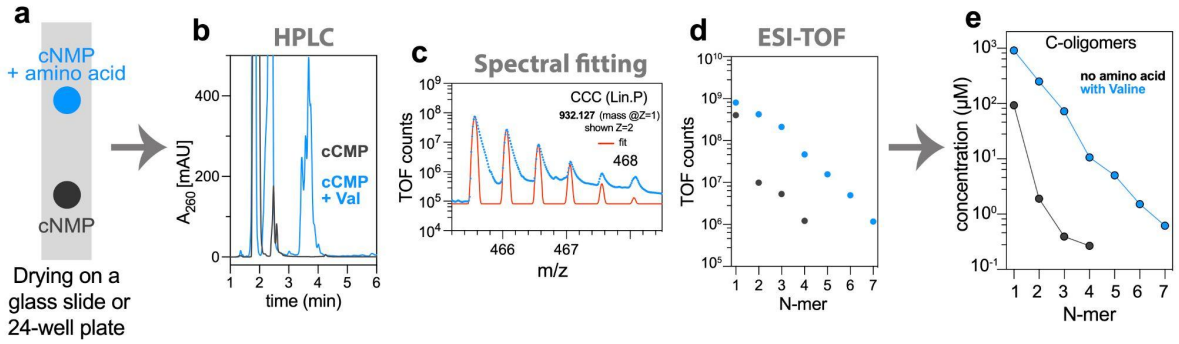


Figure S2. Summary of the experiment and analysis pipeline. a, Drying of the cyclic nucleotides (cCMP) in the absence and presence of amino acids (valine). **b**, Chromatogram at 260 nm is presented for cCMP oligomerization. **c**, Isotope fits for CCC (-2 charged state) in the Spectral_browser v3.58. **d,**

Total ion counts of each oligomer after spectral fitting. **e,** Concentration of each oligomer as determined by standard curve.

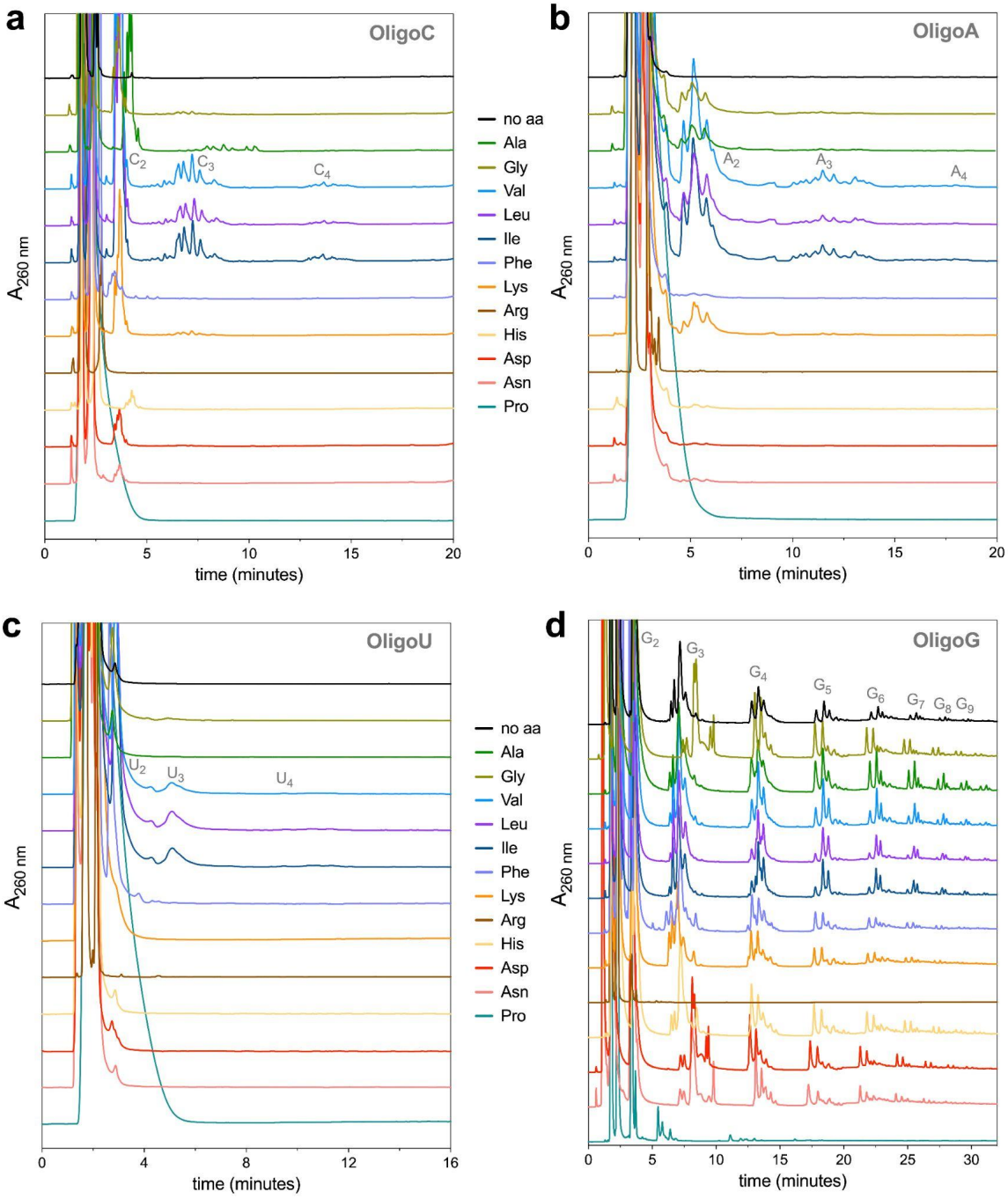


Figure S3. Analysis of the oligomeric RNA products by HPLC. a, The HPLC traces of oligoC at 260 nm in the absence and presence of amino acids are presented. In the experiments, 10 mM cyclic nucleotides and 50 mM amino acids at pH 10 were rapidly dried and incubated for 20 h at room temperature. **b-d**, Chromatograms for oligoA, oligoU and oligoG are plotted. The elution times of different oligomeric RNA are noted on the plot. All quantitative estimations were done by ESI-TOF and a custom-written LabView program.

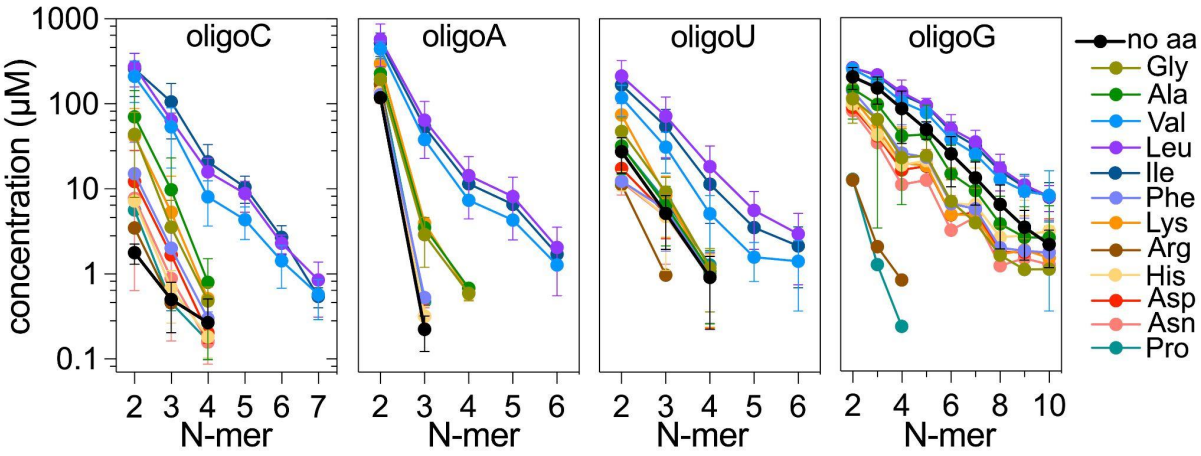


Figure S4. RNA oligomers with linear phosphate end from oligomerization of 2',3'-cyclic nucleotides catalysed by amino acids. Concentrations of the oligomers of different lengths with linear phosphate ends are plotted in a log scale for cCMP, cAMP, cUMP, and cGMP oligomerization. Controls without amino acids are shown in black (no aa), and amino acid-promoted reactions (corresponding three-lettered codes) are shown in other colours. All reactions were performed with 10 mM cNMP and 50 mM amino acid, at pH 10 for 20 h, and quantifications were done on a reverse phase HPLC column coupled to ESI-TOF and by a custom-written LabView program. Errors are given as S.D. of three independent experiments.

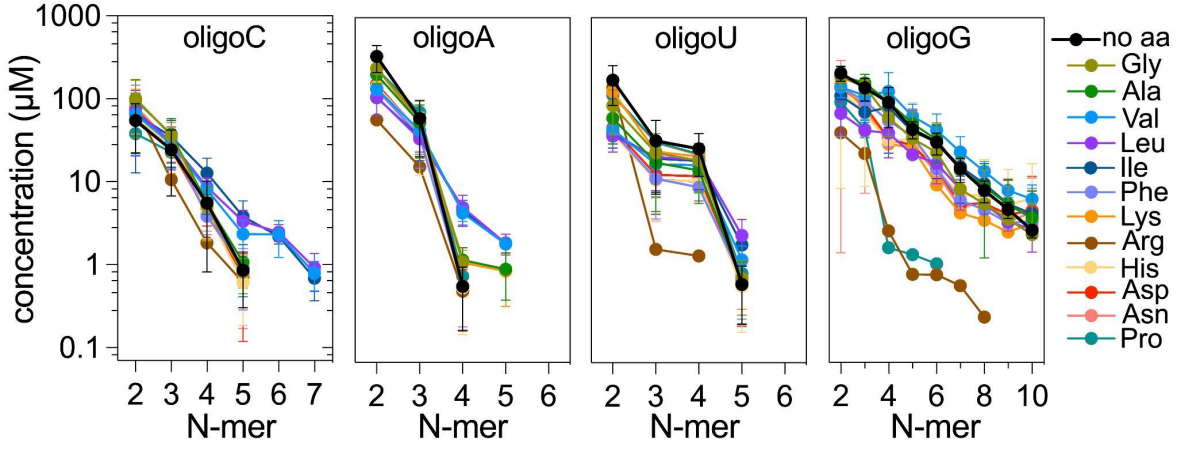


Figure S5. RNA oligomers with cyclic phosphate end from oligomerization of 2',3'-cyclic nucleotides catalysed by amino acids. Concentrations of the oligomers of different lengths with linear phosphate ends are plotted in a log scale for cCMP, cAMP, cUMP, and cGMP oligomerization. Controls without amino acids are shown in black (no aa), and amino acid-promoted reactions (corresponding three-lettered codes) are shown in other colours. All reactions were performed with 10 mM cNMP and 50 mM amino acid, at pH 10 for 20 h, and quantifications were done on a reverse phase HPLC column coupled to ESI-TOF and by a custom-written LabView program. Errors are given as S.D. of three independent experiments.

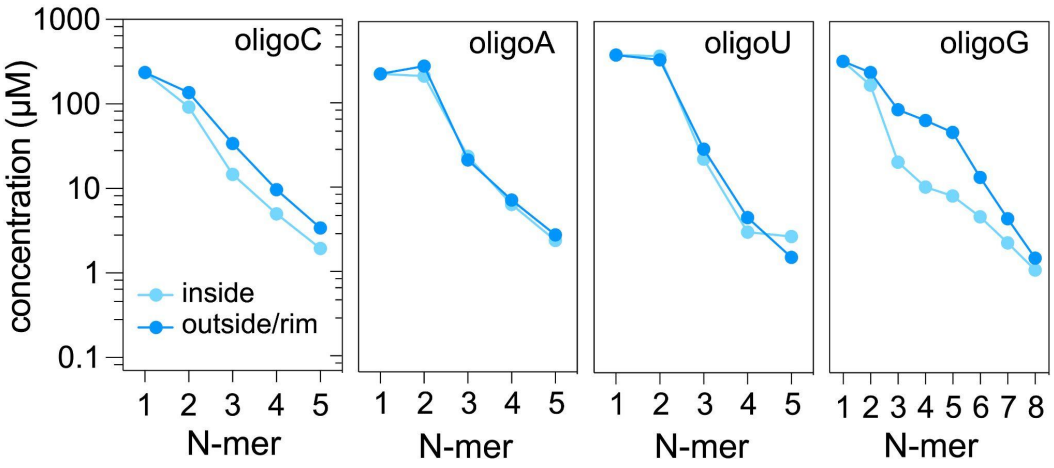


Figure S6. Estimation of oligomers from the outer and inner area of the dried nucleotide and valine mixture. Reactions were done using 10 mM nucleotides and 50 mM valine at pH 10 by drying for 20 h. The rim of the spot was carefully scraped and separated from the central area. Both samples were then dissolved in nuclease-free water for the LC-MS analysis. Despite some heterogeneity in appearance (see valine in fig. S1), the oligomerization process appears to be relatively consistent across the entire spot.

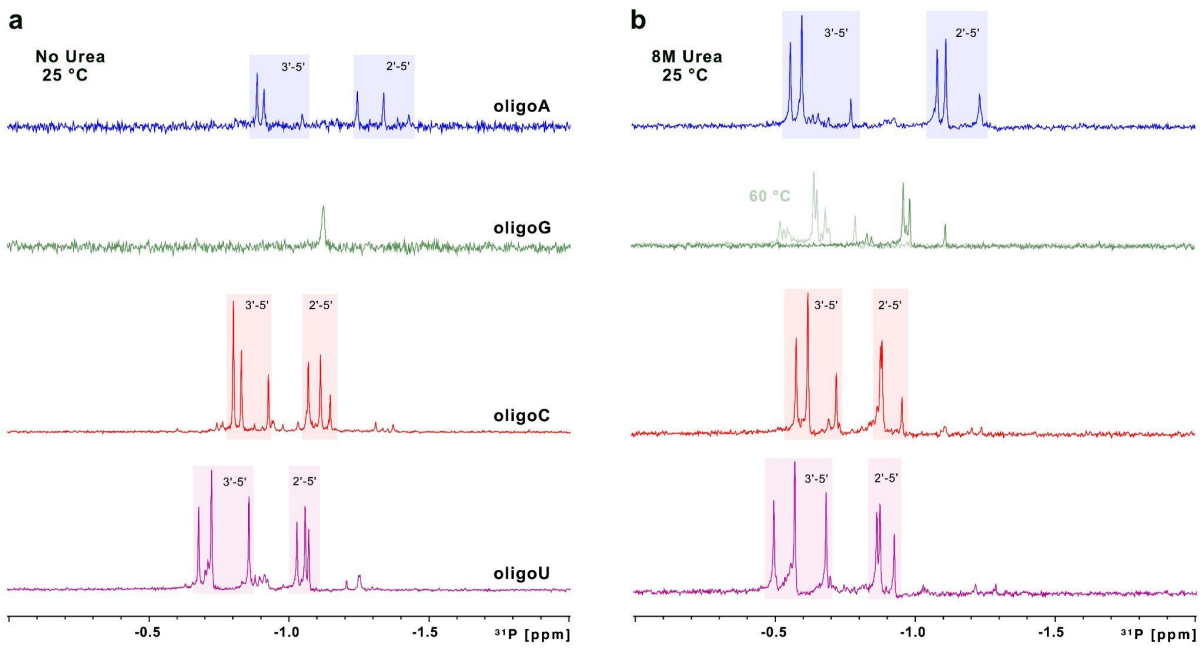


Figure S7. ^{31}P NMR spectra of RNA oligomers. NMR spectra (121.5 MHz, H_2O/D_2O 95:5, -1.5 – -0.5 ppm) to show the products of the reaction of cNMP (nucleoside 2', 3'-cyclic phosphate) in the presence of 5 equiv. valine at pH 10 following drying under airflow and incubation at room temperature for 20 h. The samples were dissolved in 5% D_2O/H_2O and spectra were acquired on a Bruker Avance III 300 MHz spectrometer.

The ratio of 3',5' to 2',5' linkages observed in the RNA products were as follows.

	Ratio of **3',5'** to **2',5'** linkages	
	(a) no Urea	**(b)** with Urea
oligoA	55:45	58:42
oligoG*	-	-
oligoC	55:45	58:42
oligoU	64:36	66:34

*oligoG forms aggregates that were not observed by NMR. The NMR signal was improved in 8 M urea and at 60 °C, however, these spectra were not used to measure the linkage ratio.

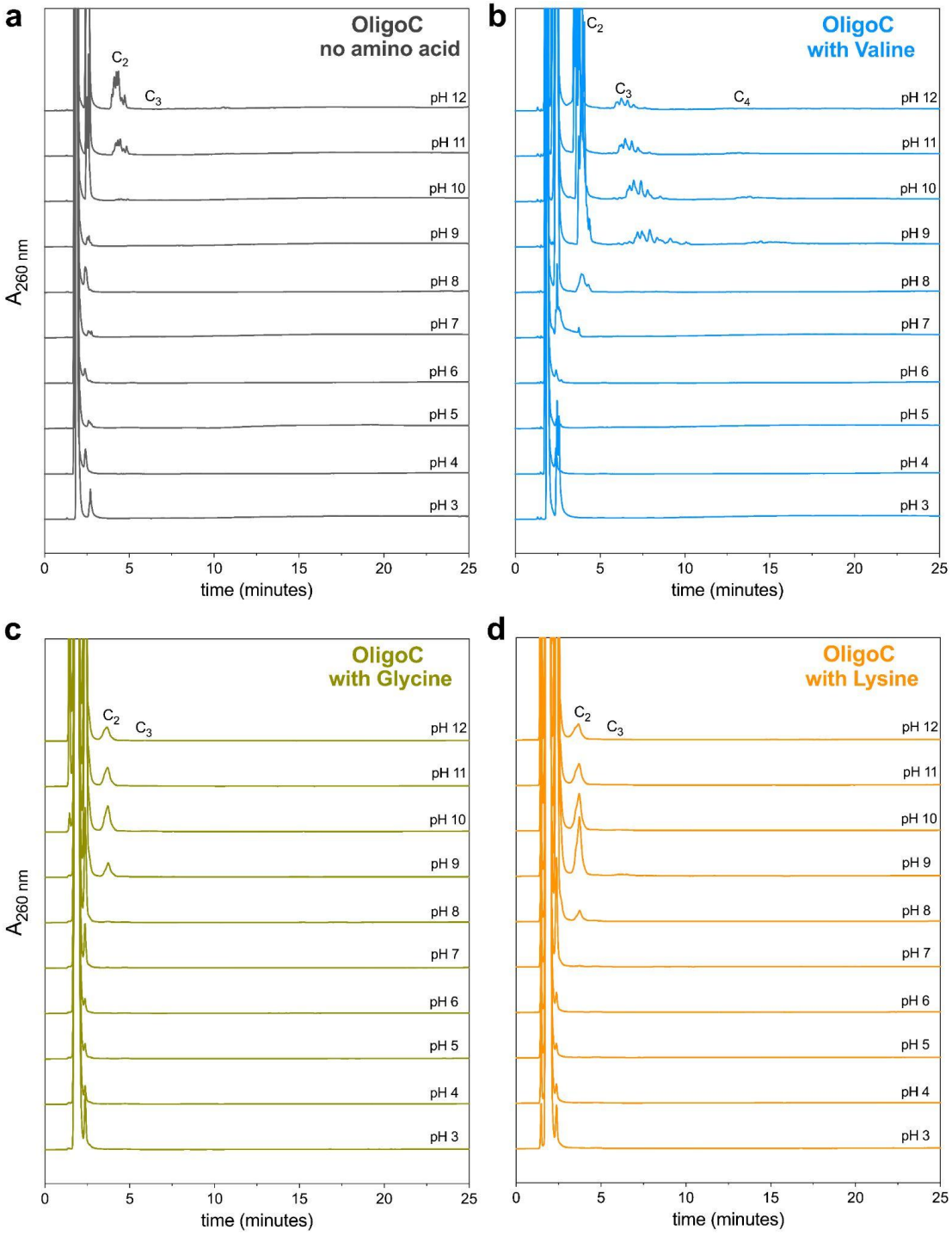


Figure S8. pH-dependent oligomerization of cCMP in the presence of amino acids investigated by HPLC. a, The HPLC traces of C-oligomerization across pH 3-12, recorded at 260 nm. **b-d**, Chromatograms for oligoC in the presence of valine, glycine and lysine are presented, similar to **a**. In the experiments, 10 mM cyclic nucleotides and 50 mM amino acids at pH 3-12 were rapidly dried and incubated for 20 h at room temperature. The elution times of different oligomeric RNA are indicated on the plot. All quantitative estimations were done by ESI-TOF and a custom written LabView program.

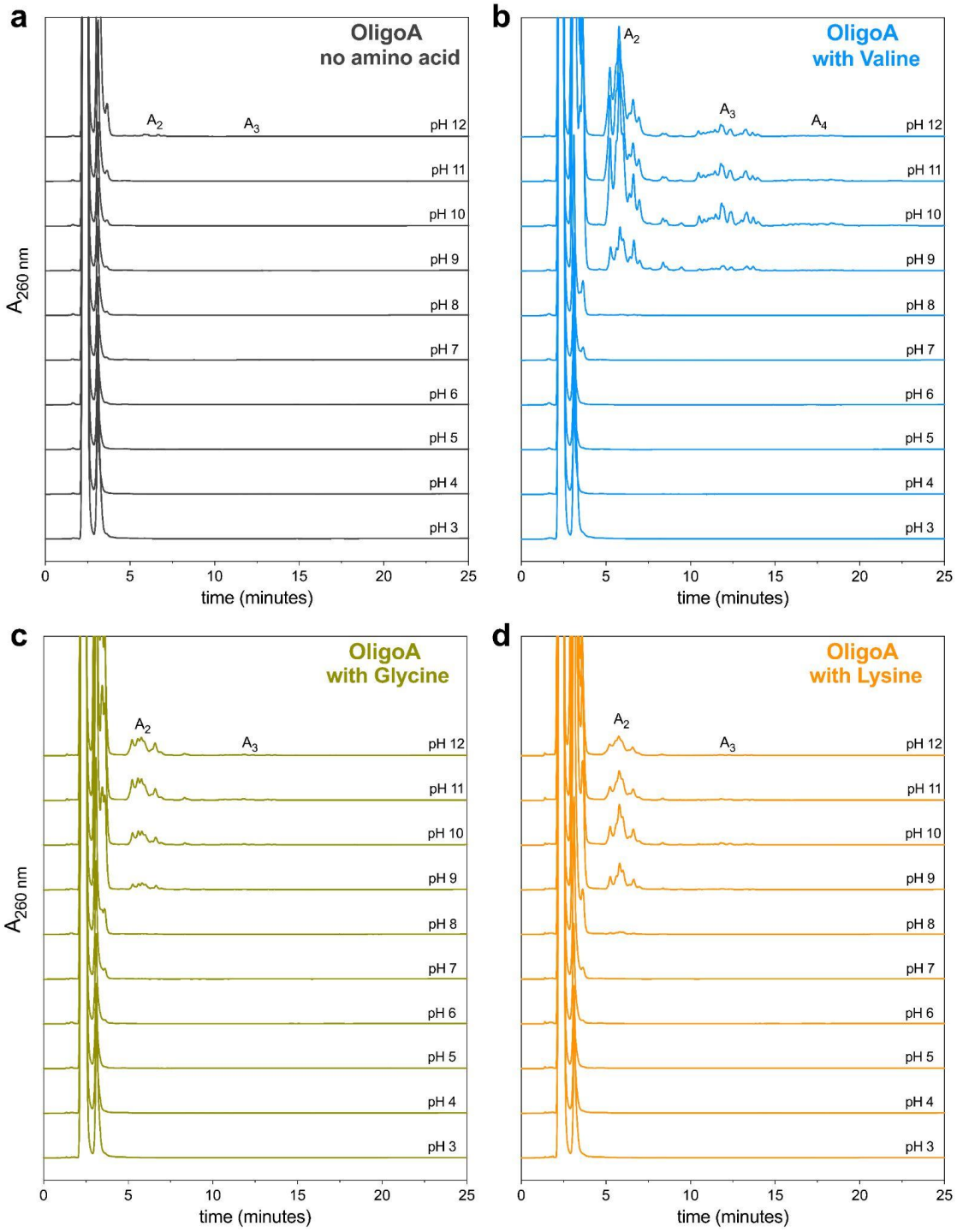


Figure S9. pH-dependent oligomerization of cAMP in the presence of amino acids investigated by HPLC. a, The HPLC traces of A-oligomerization across pH 3-12, recorded at 260 nm. **b-d**, Chromatograms for oligoA in the presence of valine, glycine and lysine are presented, similar to **a**. In the experiments, 10 mM cyclic nucleotides and 50 mM amino acids at pH 3-12 were rapidly dried and incubated for 20 h at room temperature. The elution times of different oligomeric RNA are indicated on the plot. All quantitative estimations were done by ESI-TOF and a custom-written LabView program.

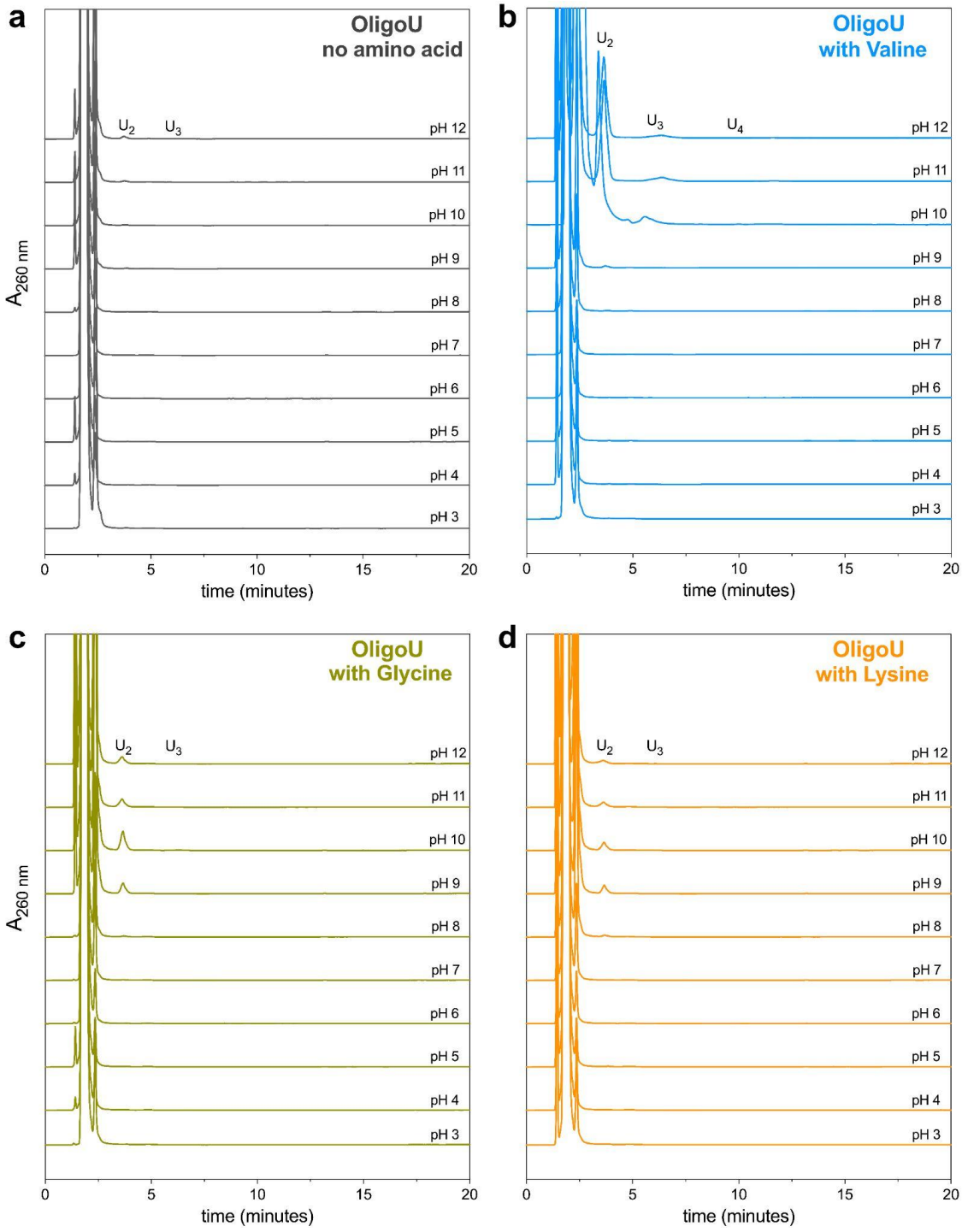


Figure S10. pH-dependent oligomerization of cUMP in the presence of amino acids investigated by HPLC. a, The HPLC traces of U-oligomerization across pH 3-12, recorded at 260 nm. **b-d**, Chromatograms for oligoU in the presence of valine, glycine and lysine are presented, similar to **a**. In the experiments, 10 mM cyclic nucleotides and 50 mM amino acids at pH 3-12 were rapidly dried and incubated for 20 h at room temperature. The elution times of different oligomeric RNA are indicated on the plot. All quantitative estimations were done by ESI-TOF and a custom-written LabView program.

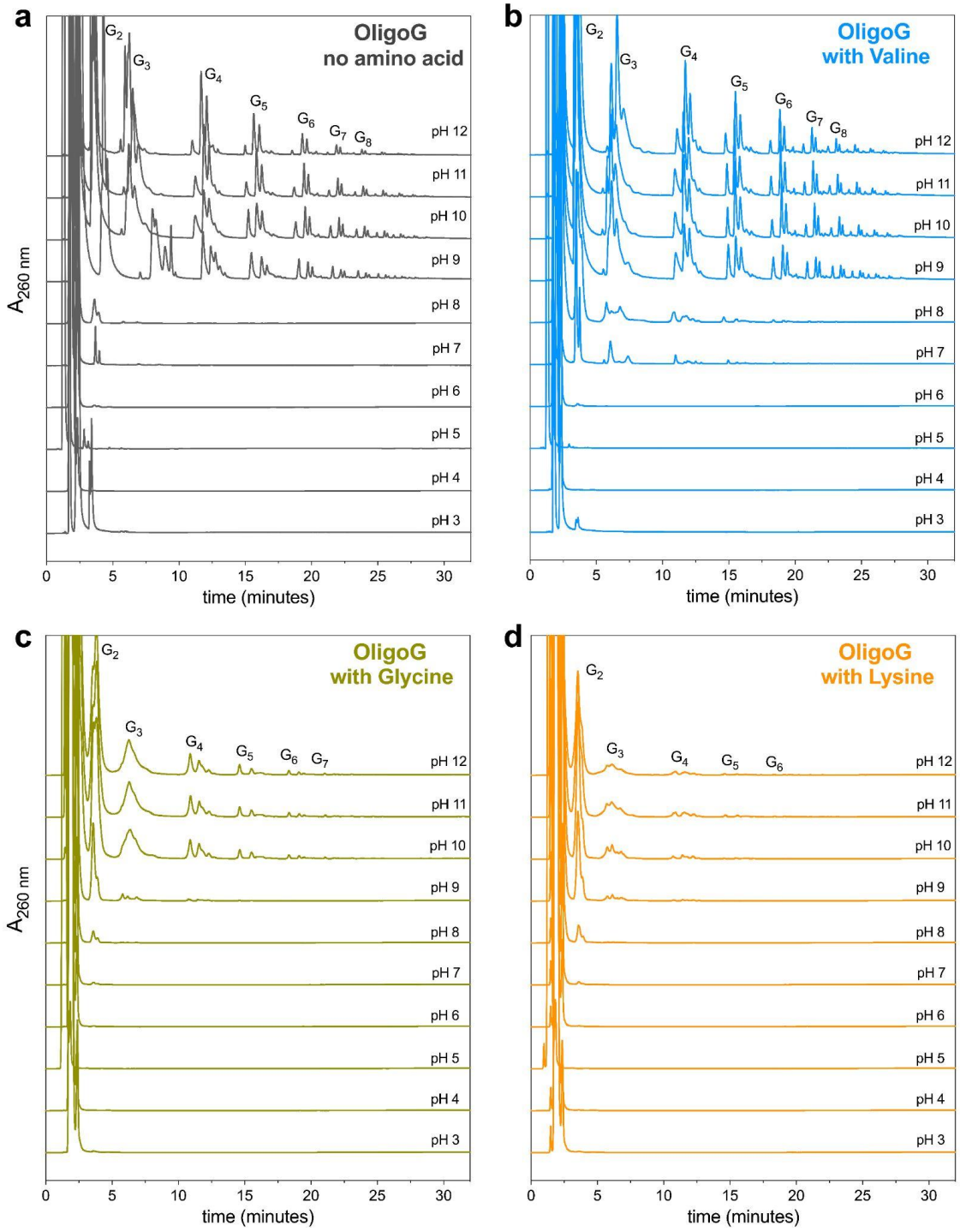


Figure S11. pH-dependent oligomerization of cGMP in the presence of amino acids investigated by HPLC. a, The HPLC traces of G-oligomerization across pH 3-12, recorded at 260 nm. **b-d**, Chromatograms for oligoG in the presence of valine, glycine and lysine are presented, similar to **a**. In the experiments, 10 mM cyclic nucleotides and 50 mM amino acids at pH 3-12 were rapidly dried and incubated for 20 h at room temperature. The elution times of different oligomeric RNA are indicated on the plot. All quantitative estimations were done by ESI-TOF and a custom written LabView program.

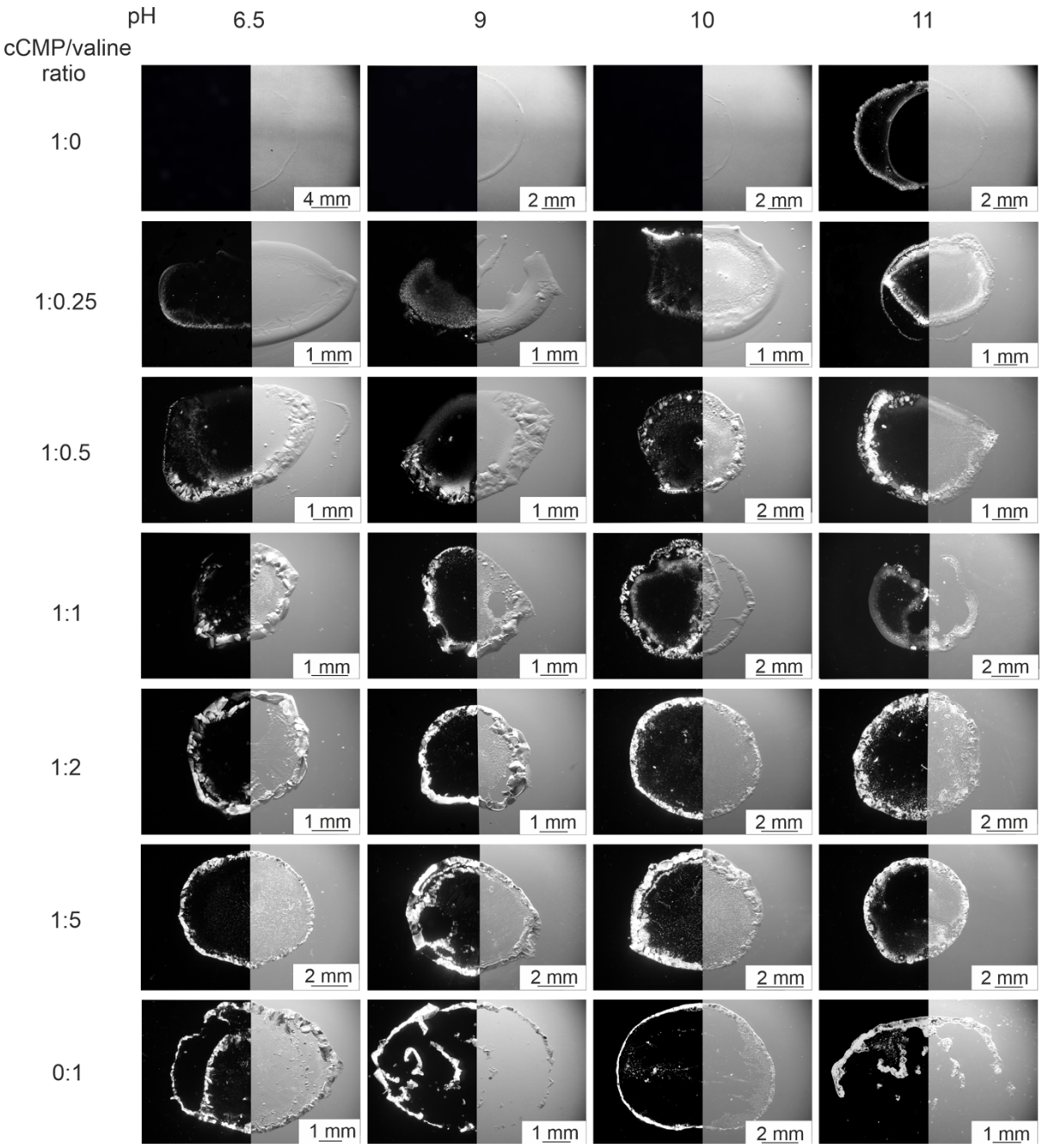


Figure S12. Morphological characterization of cCMP and valine mixtures with varying ratios across different pH ranges by optical microscopy. The solutions containing cCMP and valine mixed in various ratios were prepared within pH 6.5-11 and subsequently incubated for 20 hours at room temperature. The left half of the images displays the internal structure of the samples viewed in cross-polarized light. In the experiment, 100 nmol of nucleotides and 25-500 nmol of valine were used. With increased pH and valine concentrations, a noticeable outer rim and the heterogeneity within the sample become more pronounced.

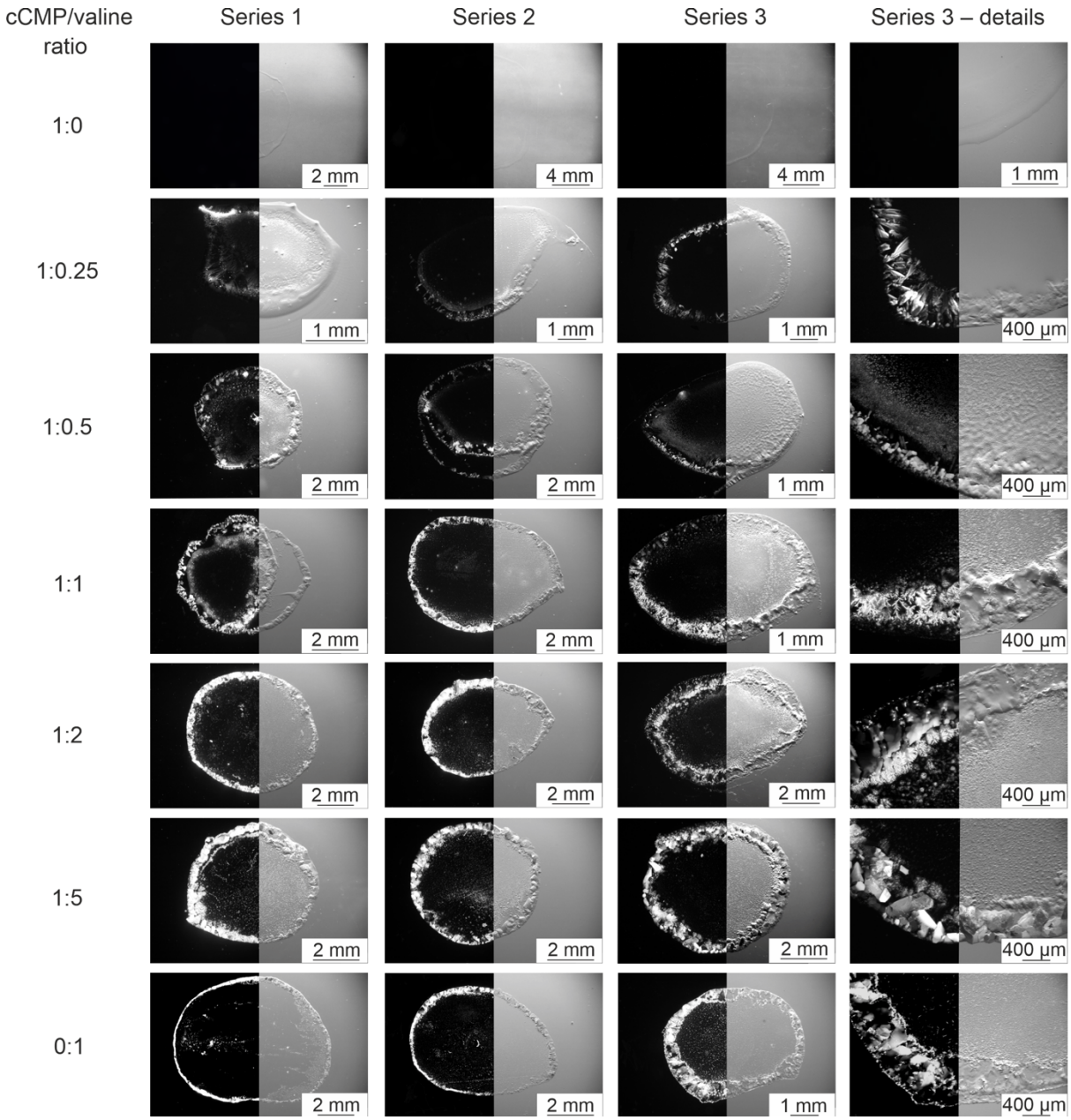


Figure S13. **Morphological characterization of cCMP and valine mixtures with varying ratios at pH 10 by optical microscopy.** The solutions containing cCMP (100 nmol) and valine (25-500 nmol) were prepared at pH 10 and subjected to drying for 20 hours at room temperature. The left half of the images displays the internal structure of the samples viewed in cross-polarized light. Images were collected from three sets of experiments. Additionally, a detailed zoom-in image is also provided. As a result of the drying process, the distinct outer rim and the heterogeneity within the sample are clearly discernible, although the macroscopic feature of the dried spot does not influence the oligomerization chemistry (Fig. S6).

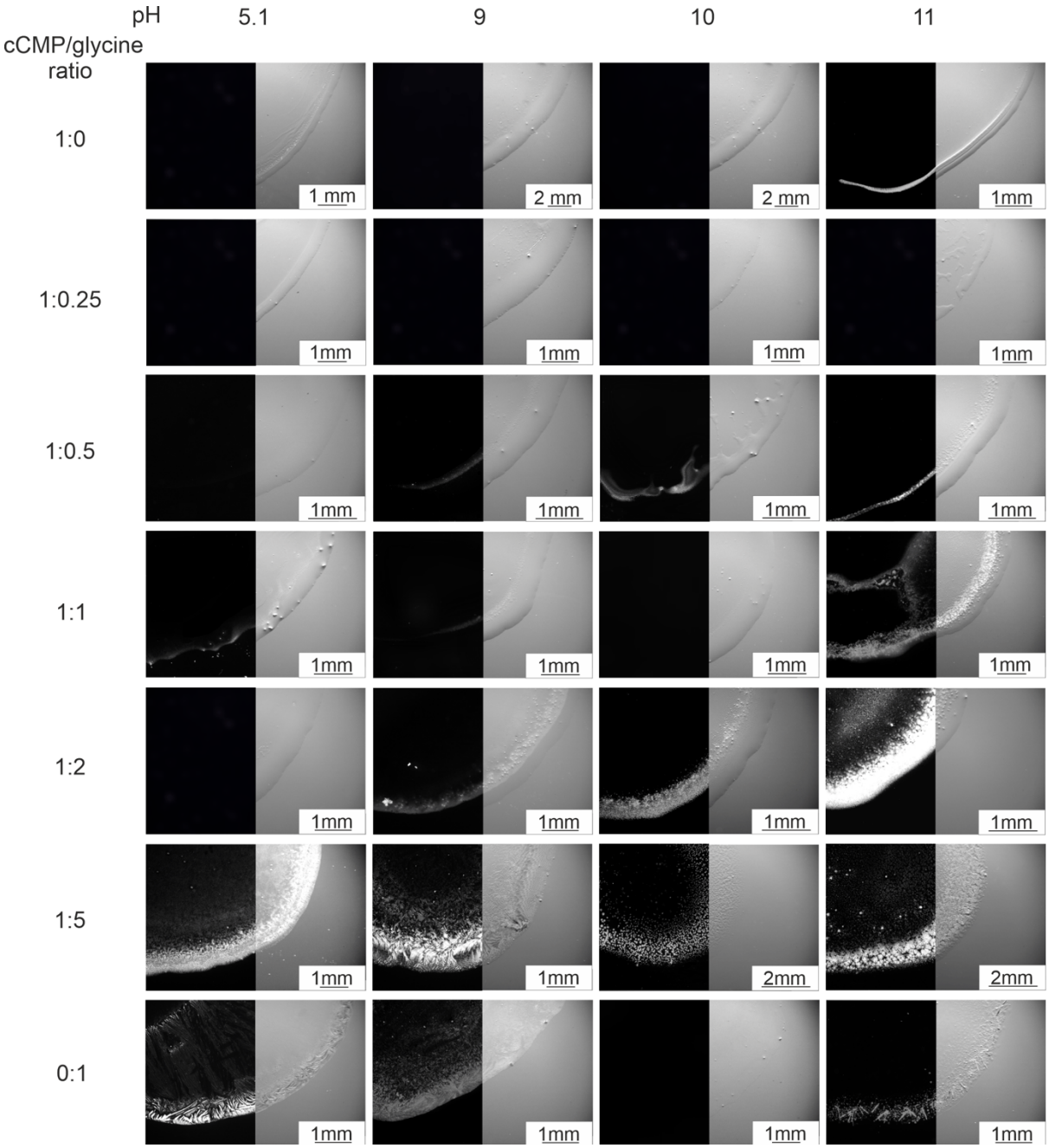


Figure S14. Morphological characterization of cCMP and glycine mixtures with varying ratios across different pH ranges by optical microscopy. The solutions containing cCMP and glycine mixed in various ratios were prepared within pH 5.1-11 and subsequently incubated for 20 hours at room temperature. The left half of the images displays the internal structure of the samples viewed in cross-polarized light. In the experiment, 100 nmol of nucleotides and 25-500 nmol of valine were used.

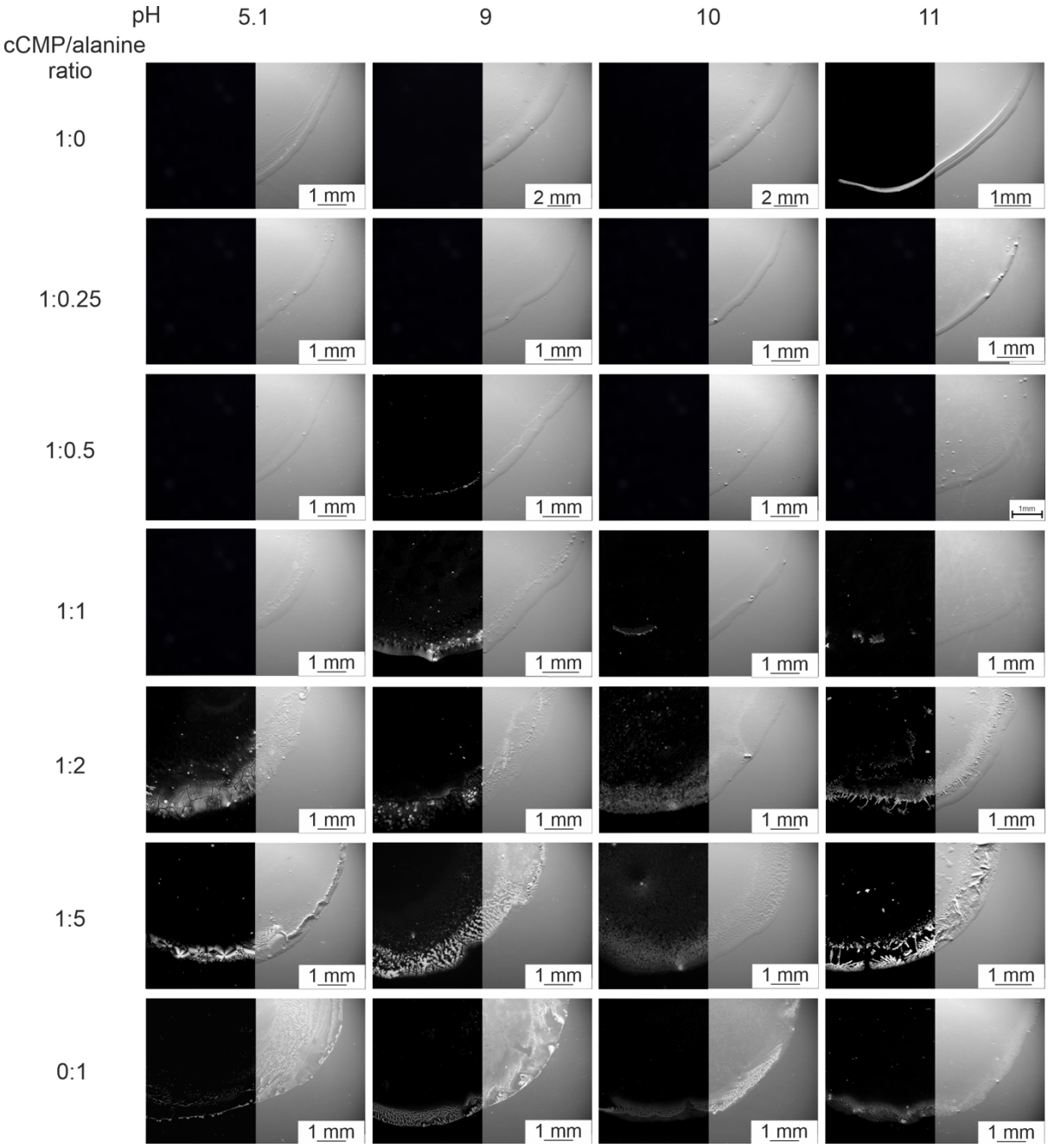


Figure S15. Morphological characterization of cCMP and alanine mixtures with varying ratios across different pH ranges by optical microscopy. The solutions containing cCMP and alanine mixed in various ratios were prepared within pH 5.1-11 and subsequently incubated for 20 hours at room temperature. The left half of the images displays the internal structure of the samples viewed in cross-polarized light. In the experiment, 100 nmol of nucleotides and 25-500 nmol of valine were used.

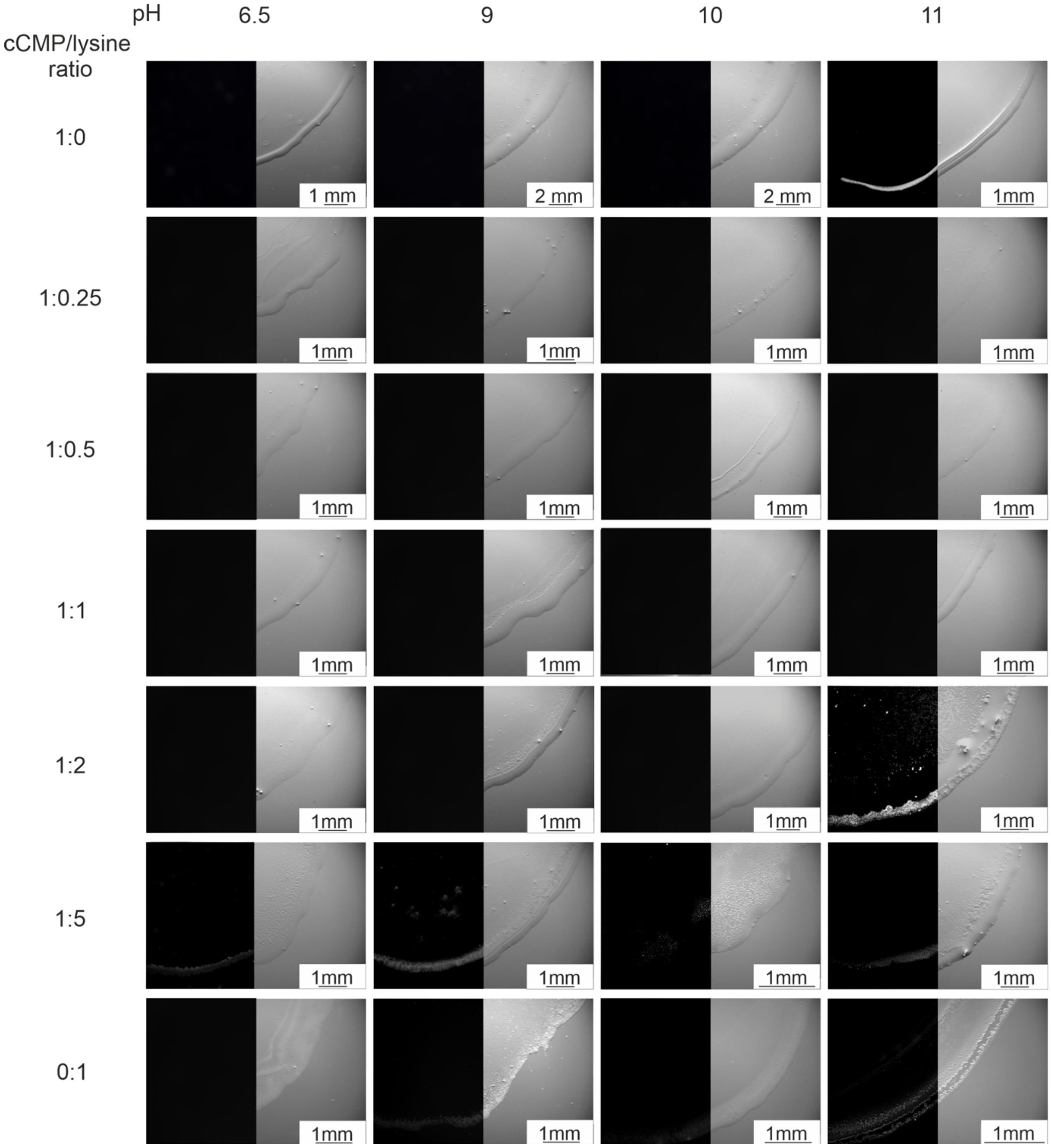


Figure S16. Morphological characterization of cCMP and lysine mixtures with varying ratios across different pH ranges by optical microscopy. The solutions containing cCMP and lysine mixed in various ratios were prepared within pH 6.5-11 and subsequently incubated for 20 hours at room temperature. The left half of the images displays the internal structure of the samples viewed in cross-polarized light. In the experiment, 100 nmol of nucleotides and 25-500 nmol of valine were used.

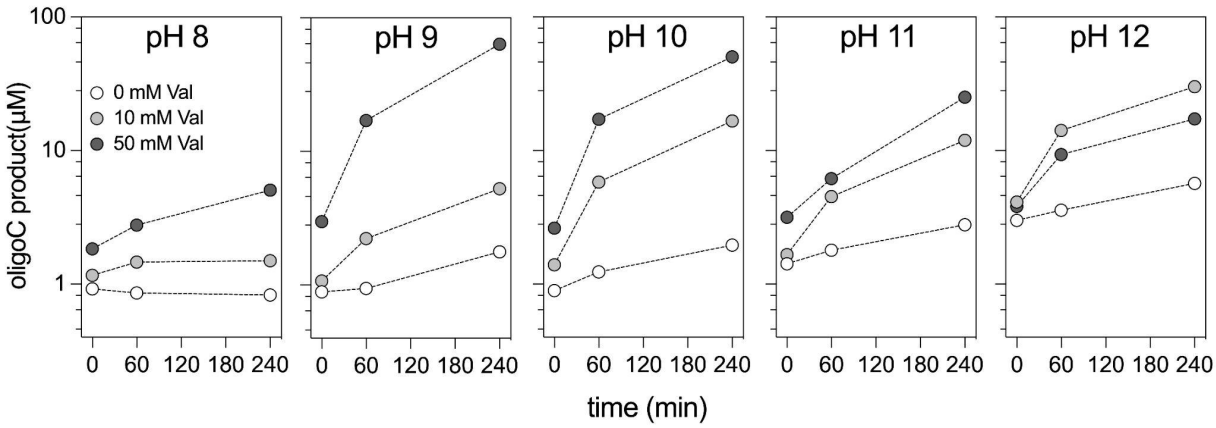


Figure S17. Kinetics of valine-assisted cyclic nucleotide oligomerization at various pH ranges. Product evolution from cCMP oligomerization is plotted (log-scale) in the absence and presence of valine across pH 8-12. All reactions were performed with 10 mM cNMP and 10-50 mM valine, with drying at room temperature (up to 4h). Quantitation was done on a reverse phase HPLC column coupled to ESI-TOF and by a custom-written LabView program. The products at different time points were used to calculate the average rate of the dry-state oligomerization.

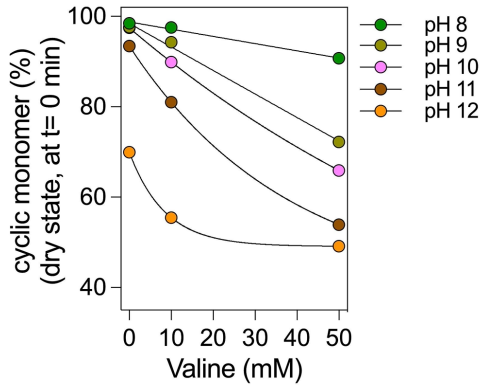


Figure S18. Effective cyclic nucleotide concentrations at the beginning of the dry state reactions. Cyclic monomers are plotted as a function of pH (8-10) and valine concentrations. The solutions of cCMP (10 mM) and valine (10-50 mM) at various pH were dried at room temperature and promptly resuspended in nuclease-free water for MS analysis to determine the cyclic monomer hydrolysis. Higher pH and valine concentrations result in a reduction in the amount of cyclic nucleotides present at t=0 minutes of the dry state.

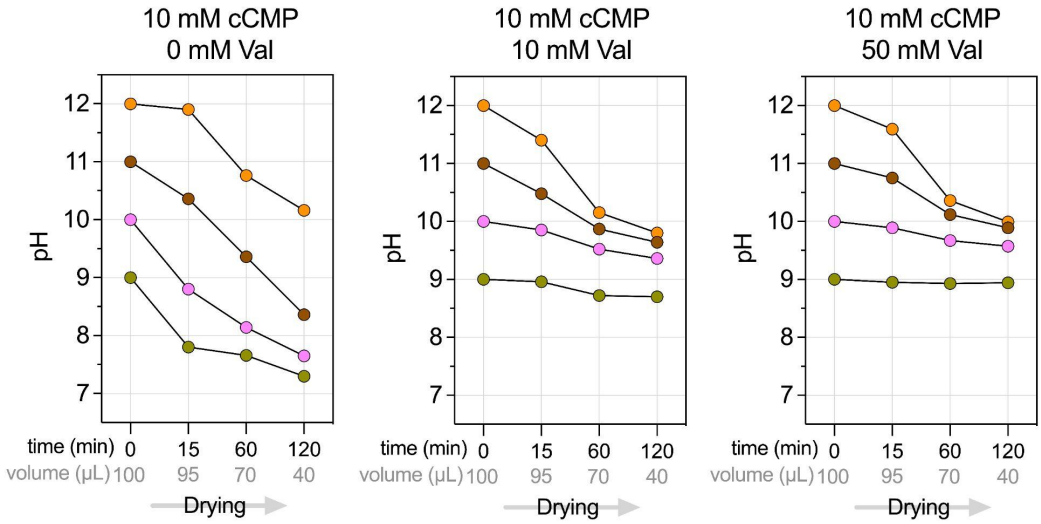


Figure S19. The pH of nucleotide and valine mixtures during the drying process. A 100 µL mixture of cCMP (10 mM) and valine (0-50 mM) at varying initial pH (9-12) was subjected to drying under airflow conditions at room temperature. The pH of the solution was measured at different drying times or volumes. The decrease in pH arises from air dissolution and the hydrolysis of cyclic monomers. While cCMP alone has no buffering effect due to the lack of an alkaline pK_a, valine resists pH changes in the nucleotide solution and tends to stabilize the pH closer to its amine pK_{aH}.

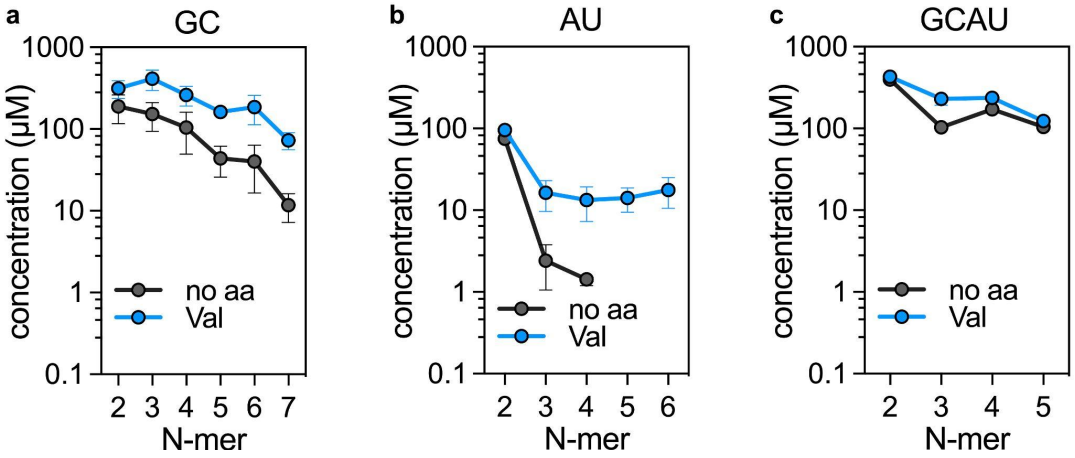


Figure S20. G/C, A/U, and G/C/A/U oligomers from 2',3'-cyclic nucleotides catalysed by Valine. Concentrations of the oligomers of different lengths are plotted in a log scale for G/C/A/U oligomerization in the absence and presence of Valine. All reactions were performed with 40 mM cNMP and 100 mM valine at pH 10, incubated at room temperature for 20 hours,. Quantitations were done on a reverse phase HPLC column coupled to ESI-TOF and by a custom-written LabView program. Errors are given as S.D. of three independent experiments.

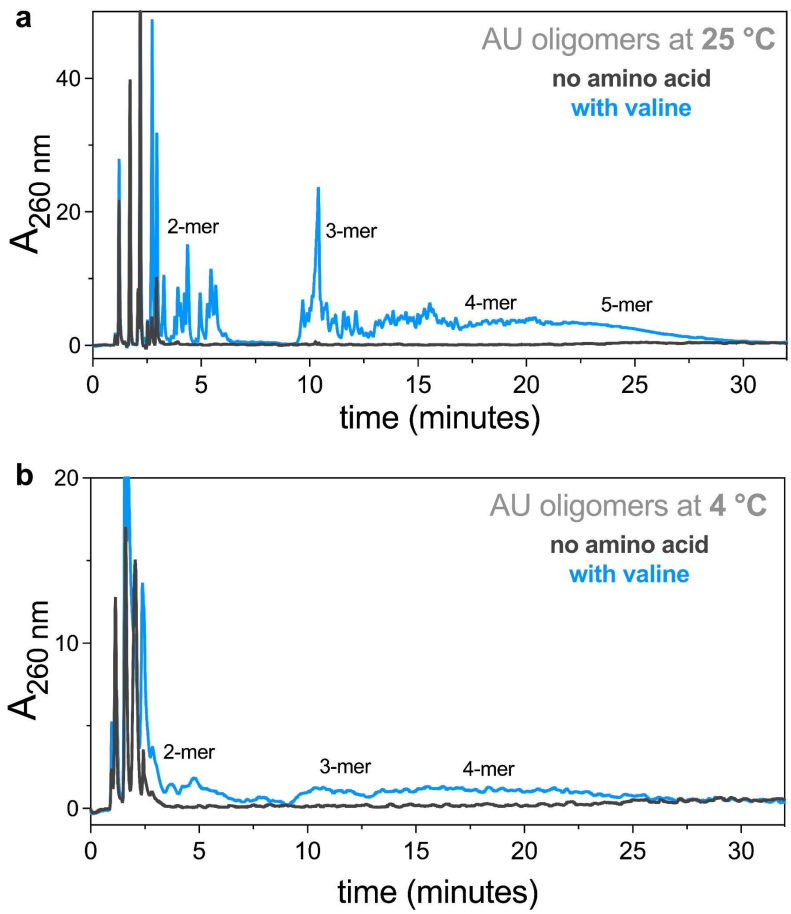


Figure S21. Analysis of the valine-catalysed A/U oligomerization using HPLC. a, HPLC chromatograms at 260 nm illustrating AU oligomerization at room temperature. **b**, HPLC chromatograms of AU oligomerization at 4 °C. In the experiments, 40 mM cyclic nucleotides (20 mM each of A and U) and 100 mM valine at pH 10 were rapidly dried and incubated for 20 h. Approximate elution times of oligomeric RNA with varying lengths and compositions are indicated on the plot. Quantitative estimations were conducted via ESI-TOF after ethanol precipitation of the oligomer products.

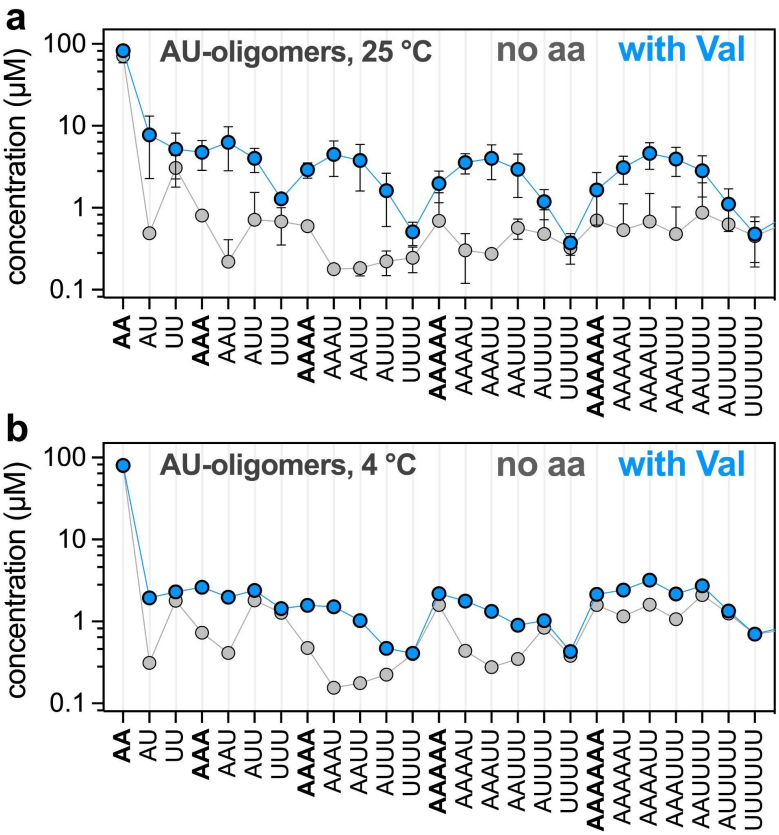


Figure S22. Valine-catalysed A/U oligomerization. a, Concentrations of the AU-oligomers of different lengths and compositions from reactions conducted with and without valine at 25°C are plotted in a log scale. Errors are calculated as s.d. of results obtained from three independent experiments. **b,** Concentrations of the AU-oligomers of different lengths and compositions from reactions at 4°C. In the experiments, 40 mM cyclic nucleotides and 100 mM valine at pH 10 were rapidly dried and incubated for 20h at room temperature. The resulting products underwent an ethanol precipitation step before the quantitation by HPLC-ESI-TOF.

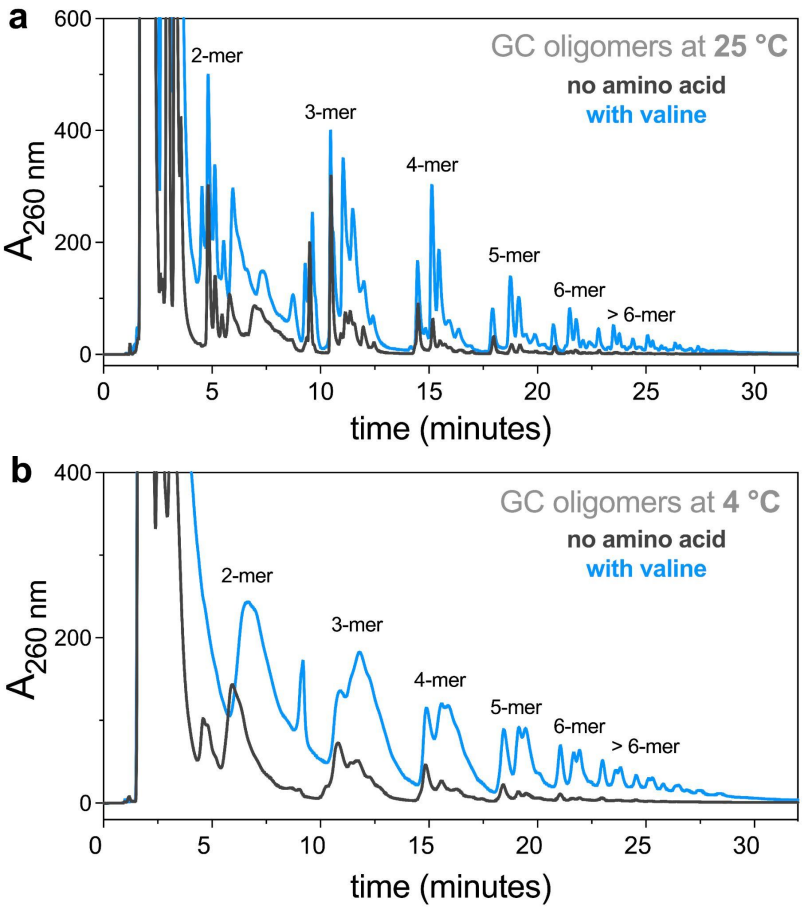


Figure S23. Analysis of the valine-catalysed G/C oligomerization using HPLC. a, HPLC chromatograms at 260 nm illustrating GC oligomerization at room temperature. **b**, HPLC chromatograms of GC oligomerization at 4 °C. In the experiments, 40 mM cyclic nucleotides (20 mM each of G and C) and 100 mM valine at pH 10 were rapidly dried and incubated for 20h. Approximate elution times of oligomeric RNA with varying lengths and compositions are indicated on the plot. Quantitative estimations were conducted via ESI-TOF after ethanol precipitation of the oligomer products.

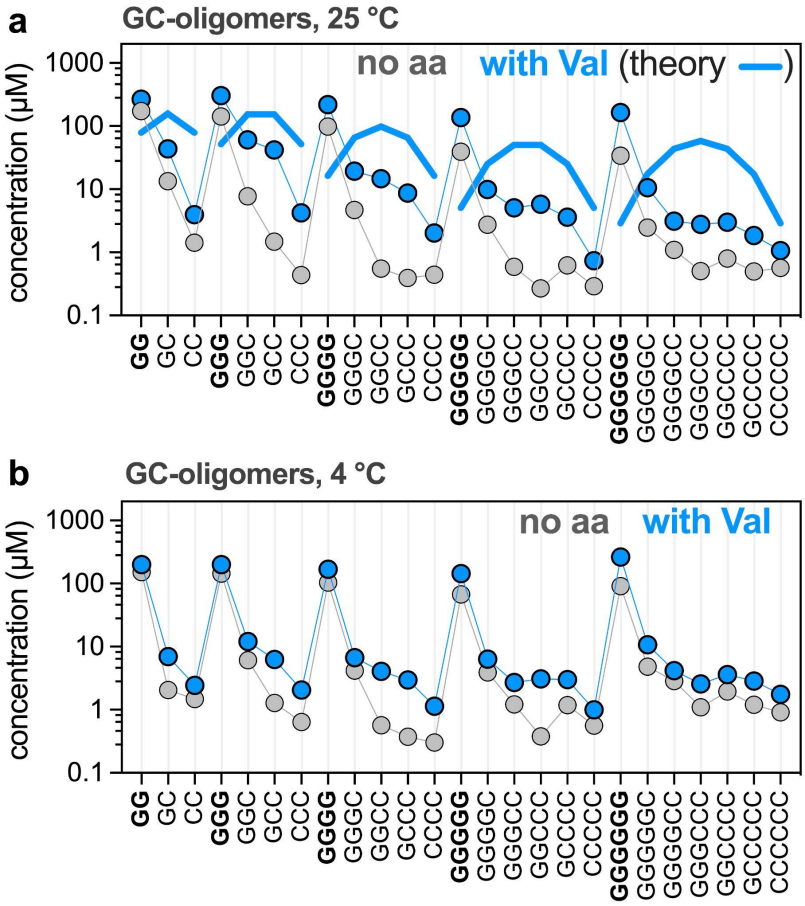


Figure S24. Valine-catalysed G/C oligomerization. a, Concentrations of the GC-oligomers of different lengths and compositions from reactions conducted with and without valine at 25 °C are plotted in a log scale. Results represent the average from three independent experiments. The blue trace represents random oligomerization in which G and C are equally efficient. **b,** Concentrations of the GC-oligomers of different lengths and compositions from reactions at 4°C. In the experiments, 40 mM cyclic nucleotides and 100 mM valine at pH 10 were rapidly dried and incubated for 20h at room temperature. The resulting products underwent an ethanol precipitation step before the quantification by HPLC-ESI-TOF.

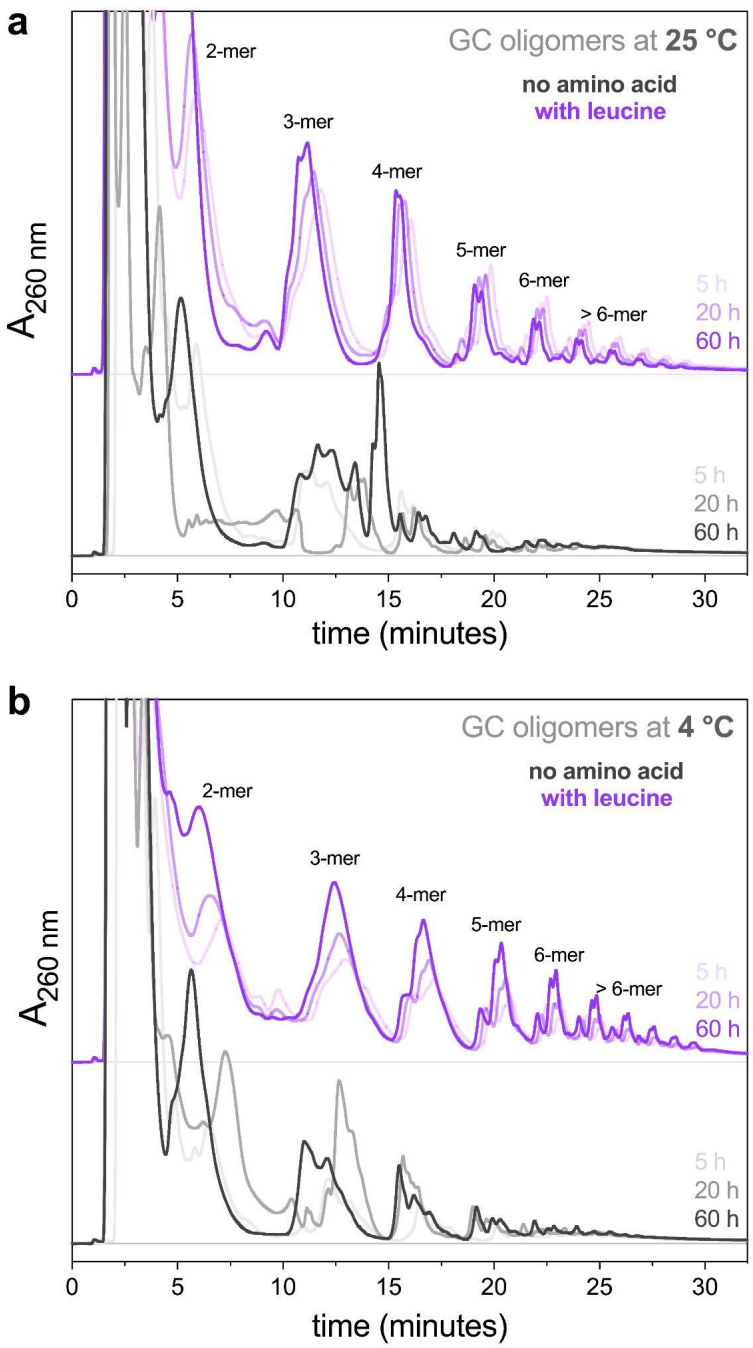


Figure S25. Analysis of the leucine-catalysed G/C oligomerzation using HPLC. a, HPLC chromatograms at 260 nm illustrating GC oligomerization conducted with and without leucine at room temperature. **b**, HPLC chromatograms of GC oligomerization at 4 °C. In the experiments, 40 mM cyclic nucleotides (20 mM each of G and C) and 100 mM leucine at pH 10 were rapidly dried and incubated for 5h, 20h and 60h. Approximate elution times of oligomeric RNA with varying lengths and compositions are indicated on the plot. Quantitative estimations were conducted via ESI-TOF after ethanol precipitation of the oligomer products.

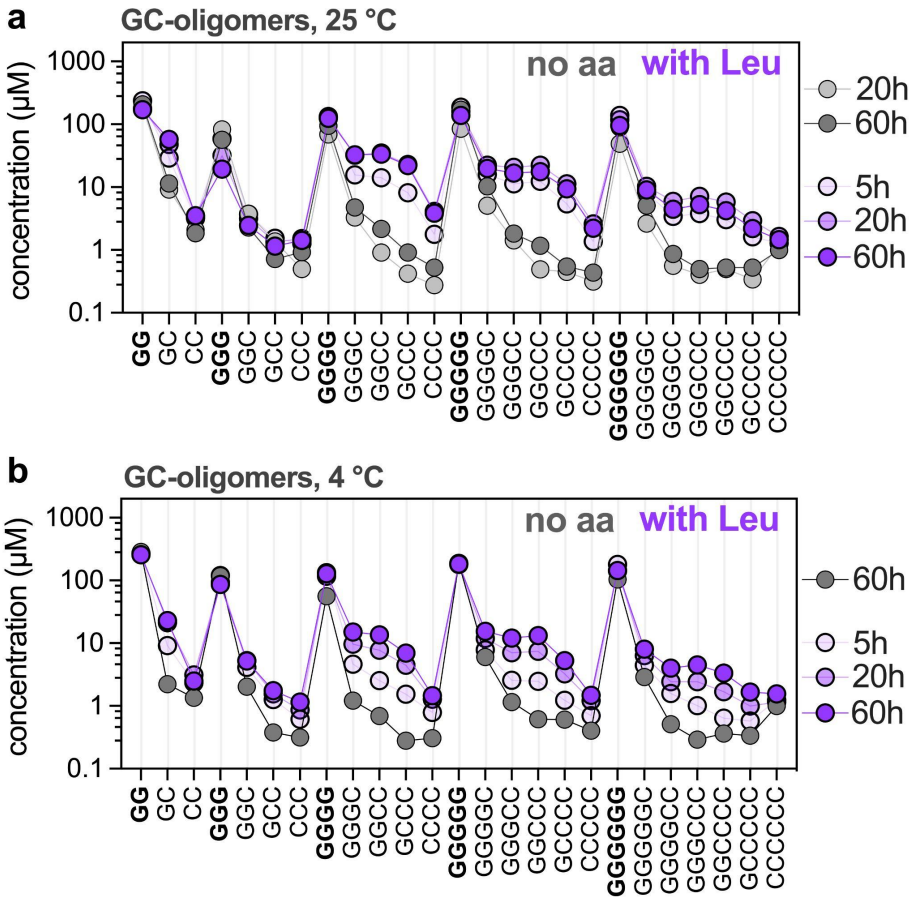


Figure S26. Leucine-catalysed G/C oligomerization. a, Concentrations of the GC-oligomers of different lengths and compositions from reactions conducted with and without leucine at 25 °C are plotted in a log scale. **b,** Concentrations of the GC-oligomers of different lengths and compositions from reactions at 4°C. In the experiments, 40 mM cyclic nucleotides and 100 mM valine at pH 10 were rapidly dried and incubated for 5h, 20h and 60h at room temperature. The resulting products underwent an ethanol precipitation step before the quantification by HPLC-ESI-TOF.

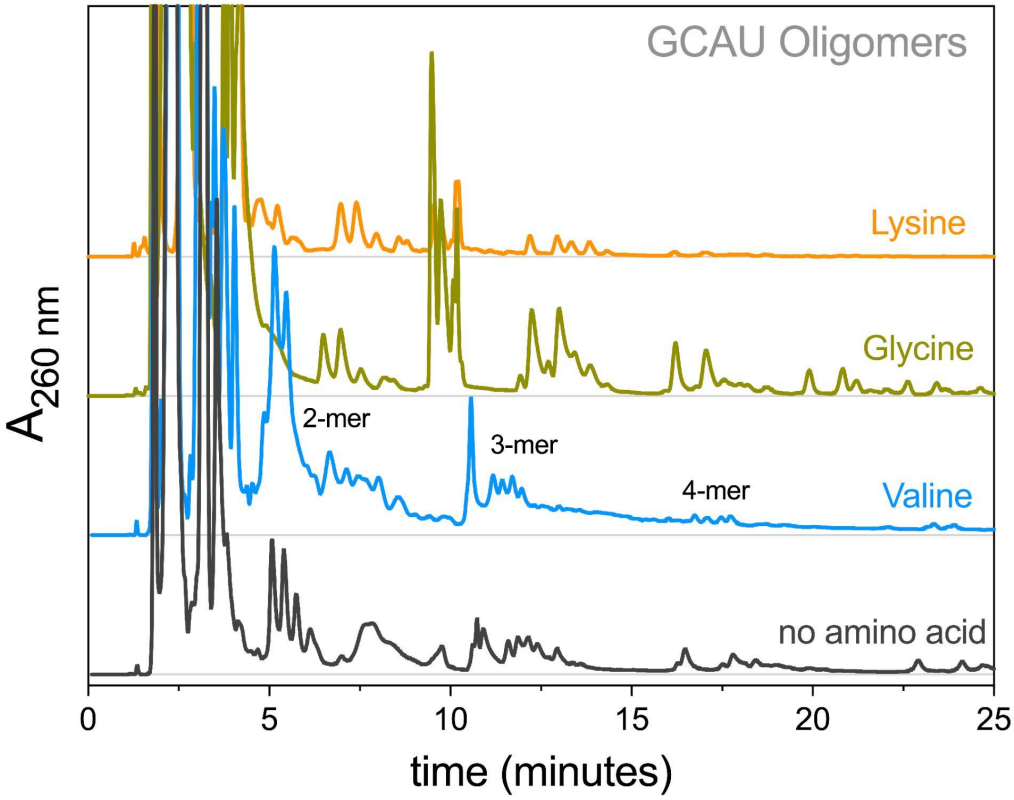


Figure S27. Analysis of the amino acid-catalysed G/C/A/U oligomerization using HPLC. HPLC chromatograms at 260 nm illustrating GCAU oligomerization conducted in the absence or presence of amino acids (valine, glycine and lysine) at room temperature. In the experiments, 40 mM cyclic nucleotides (10 mM each of G, C, A and U) and 100 mM valine at pH 10 were rapidly dried and incubated for 20h. Approximate elution times of oligomeric RNA with varying lengths and compositions are indicated on the plot. Quantitative estimations were conducted via ESI-TOF after ethanol precipitation of the oligomer products.

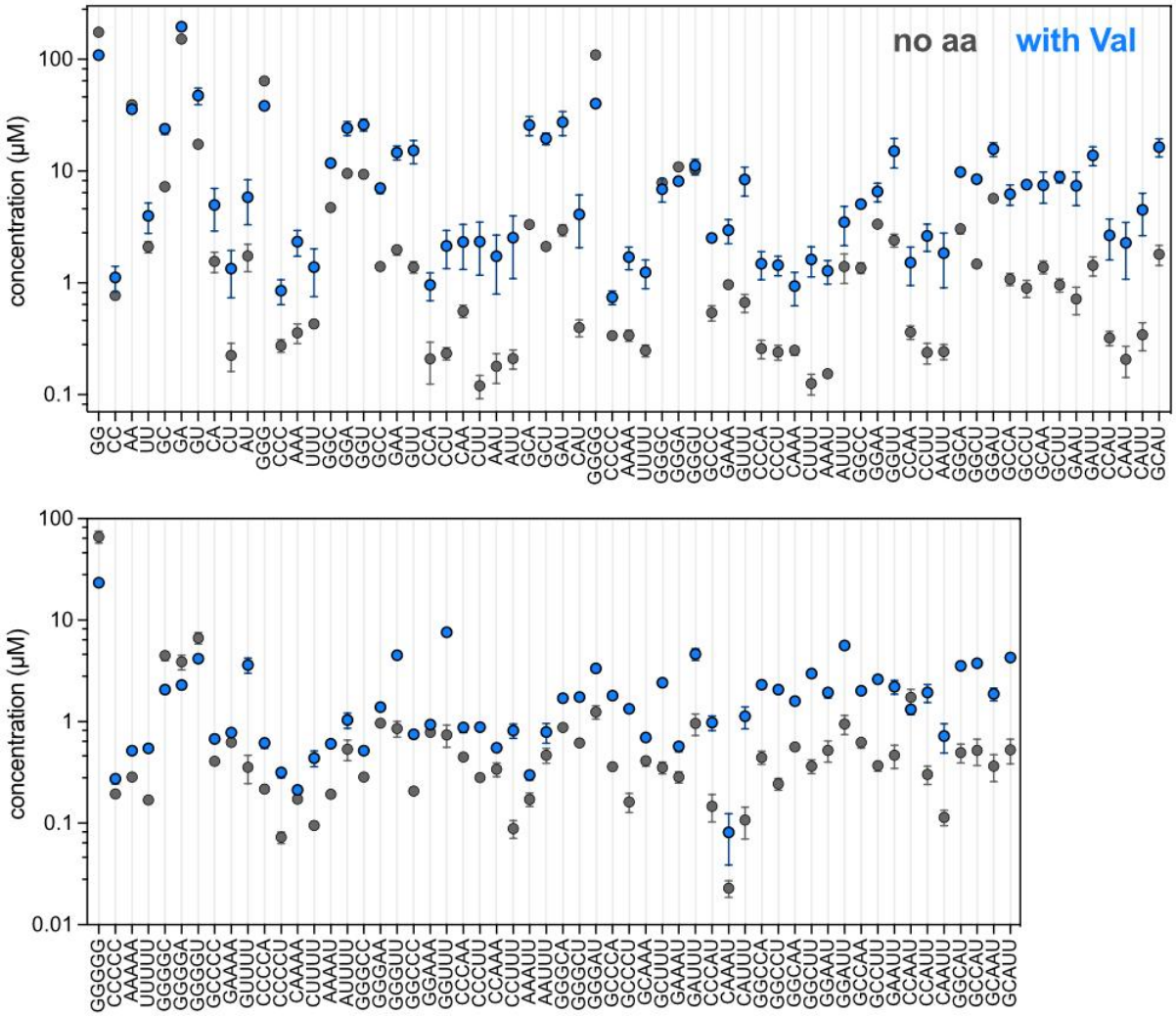


Figure S28. **Enhanced compositional diversity in the valine-catalyzed co-oligomerization of G/C/A/U nucleotides.** The concentrations of RNA oligomers (2-5 mer) are presented for various compositions for the GCAU reactions conducted with and without valine. In the experiments, 40 mM cyclic nucleotides (10 mM each of G, C, A and U) and 100 mM valine at pH 10 were rapidly dried and incubated for 20 h at room temperature. The quantifications were done by LC-MS and a self-written LabView program. The results are the mean with s.d of three independent experiments.

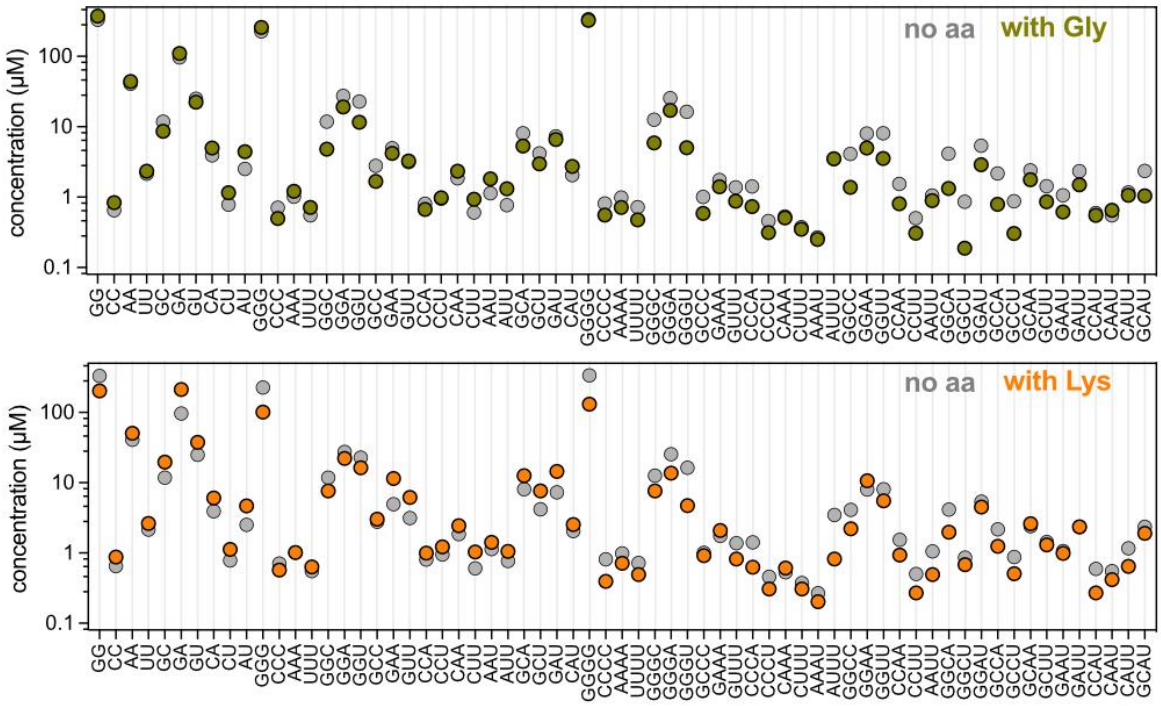


Figure S29. **Enhanced compositional diversity in the glycine- and lysine-catalyzed co-oligomerization of G/C/A/U nucleotides.** The concentrations of RNA oligomers (2-5 mer) are presented for various compositions for the GCAU reactions conducted in the presence of glycine and lysine. In the experiments, 40 mM cyclic nucleotides (10 mM each of G, C, A and U) and 100 mM glycine or lysine at pH 10 were rapidly dried and incubated for 20 h at room temperature. The quantifications were done by LC-MS and a self-written LabView program. The results are the mean of two independent experiments.

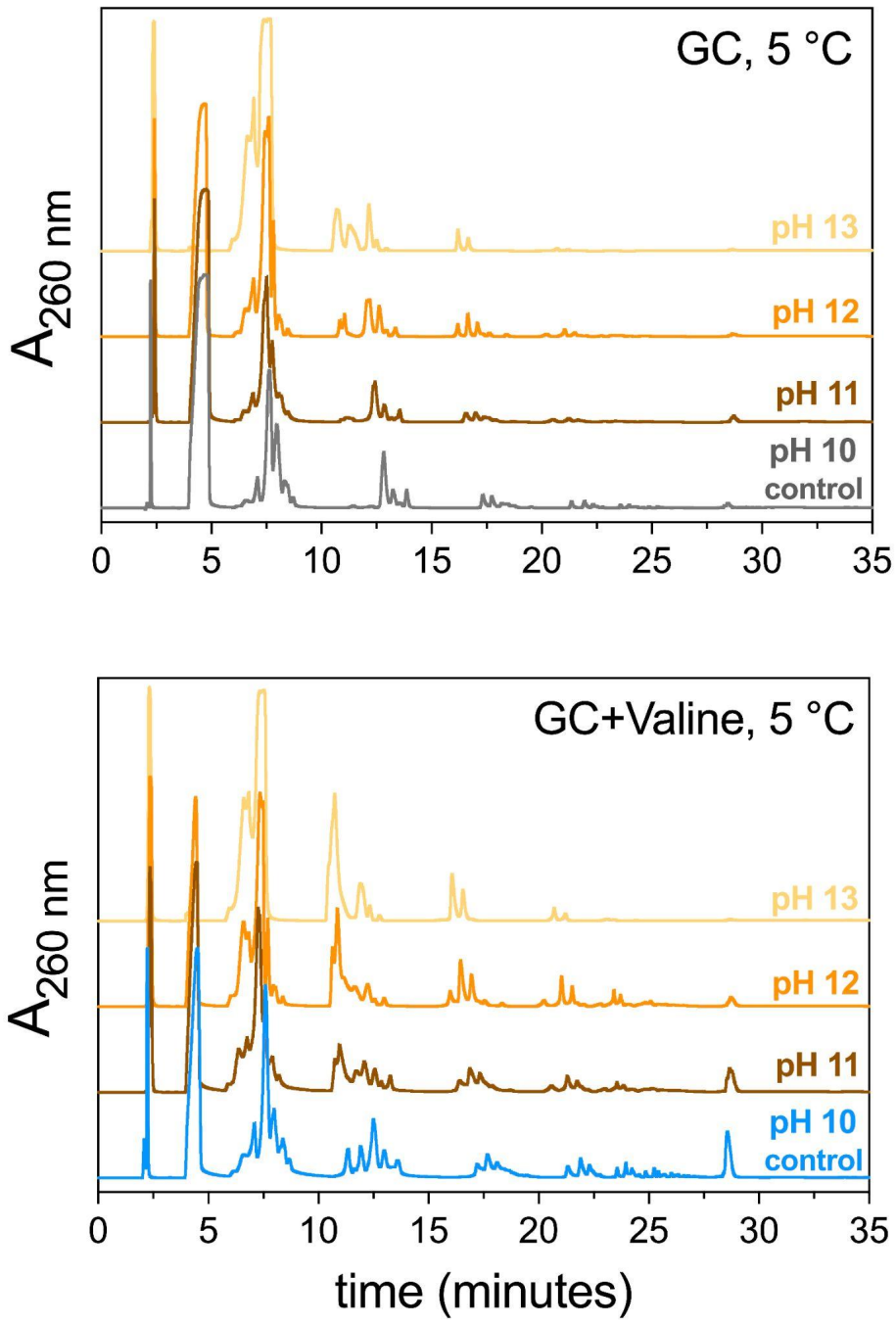


Figure S30. Hydrolysis of GC-oligomers at 5 °C investigated by HPLC. HPLC chromatograms at 260 nm illustrating hydrolysis of GC oligomers incubated with 0.5M KCl at pH 11-13 and 5°C for 24 h. The oligomers were synthesized at pH 10 using 40 mM cyclic nucleotides (20 mM each of G and C), with or without 100 mM valine at pH 10, after drying for 20 hours. The hydrolysis at higher pH is evident from the reduction of peak areas at higher elution times, corresponding to longer oligomers and an increase of peak areas at lower elution times, indicative of shorter oligomers and monomers. Further quantitative estimations were conducted by ESI-TOF.

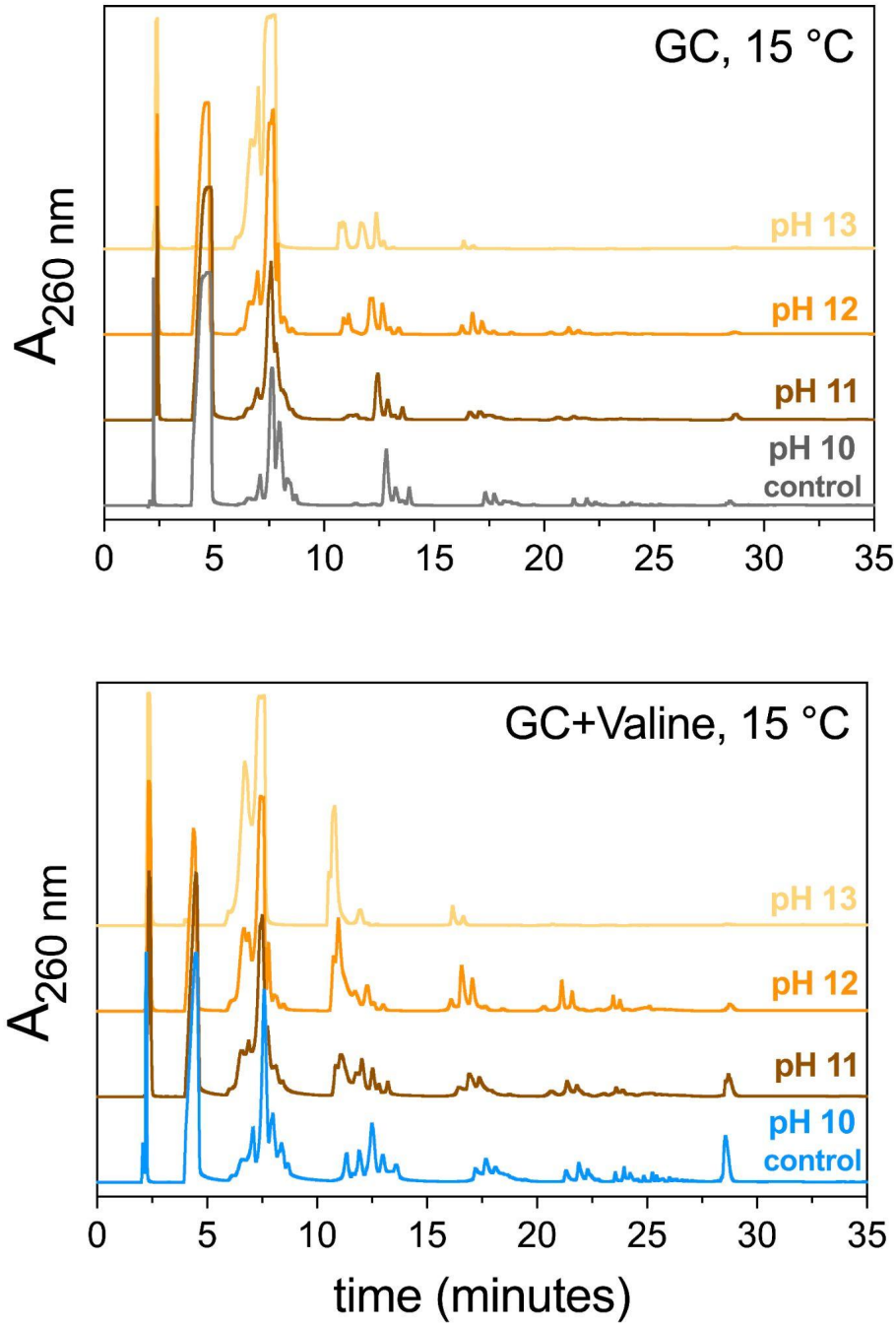


Figure S31. Hydrolysis of GC-oligomers at 15 °C investigated by HPLC. HPLC chromatograms at 260 nm illustrating hydrolysis of GC oligomers incubated with 0.5M KCl at pH 11-13 and 15°C for 24 h. The oligomers were synthesized at pH 10 using 40 mM cyclic nucleotides (20 mM each of G and C), with or without 100 mM valine at pH 10, after drying for 20 hours. The hydrolysis at higher pH is evident from the reduction of peak areas at higher elution times (for longer oligos) and an increase of peak areas at lower elution times (shorter oligos and monomers). Further quantitative estimations were conducted by ESI-TOF.

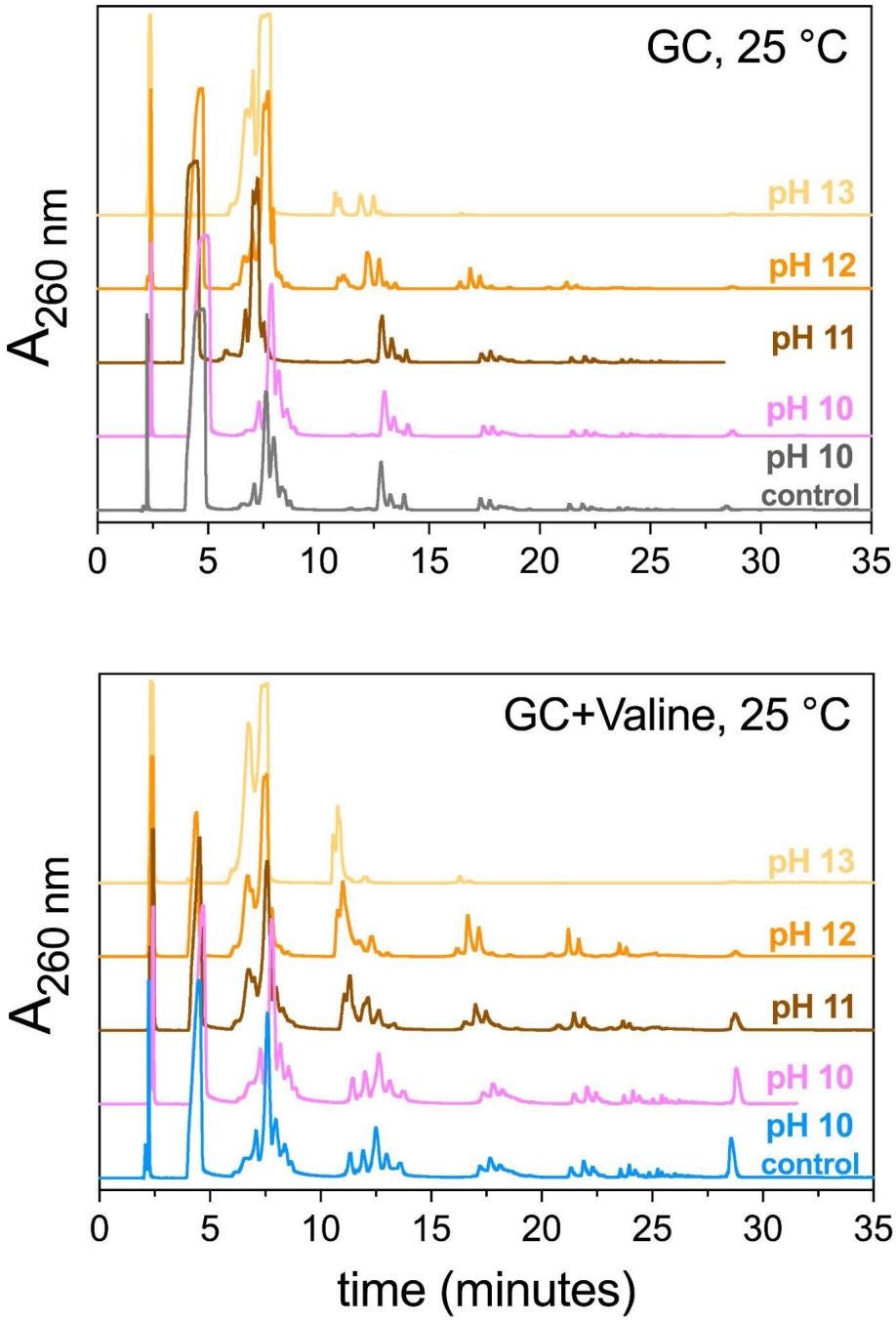


Figure S32. Hydrolysis of GC-oligomers at 25 °C investigated by HPLC. HPLC chromatograms at 260 nm illustrating hydrolysis of GC oligomers incubated with 0.5M KCl at pH 10-13 and 25°C for 24 h. The oligomers were synthesized at pH 10 using 40 mM cyclic nucleotides (20 mM each of G and C), with or without 100 mM valine at pH 10, after drying for 20 hours. The hydrolysis at higher pH is evident from the reduction of peak areas at higher elution times (for longer oligos) and an increase of peak areas at lower elution times (shorter oligos and monomers). Further quantitative estimations were conducted by ESI-TOF.

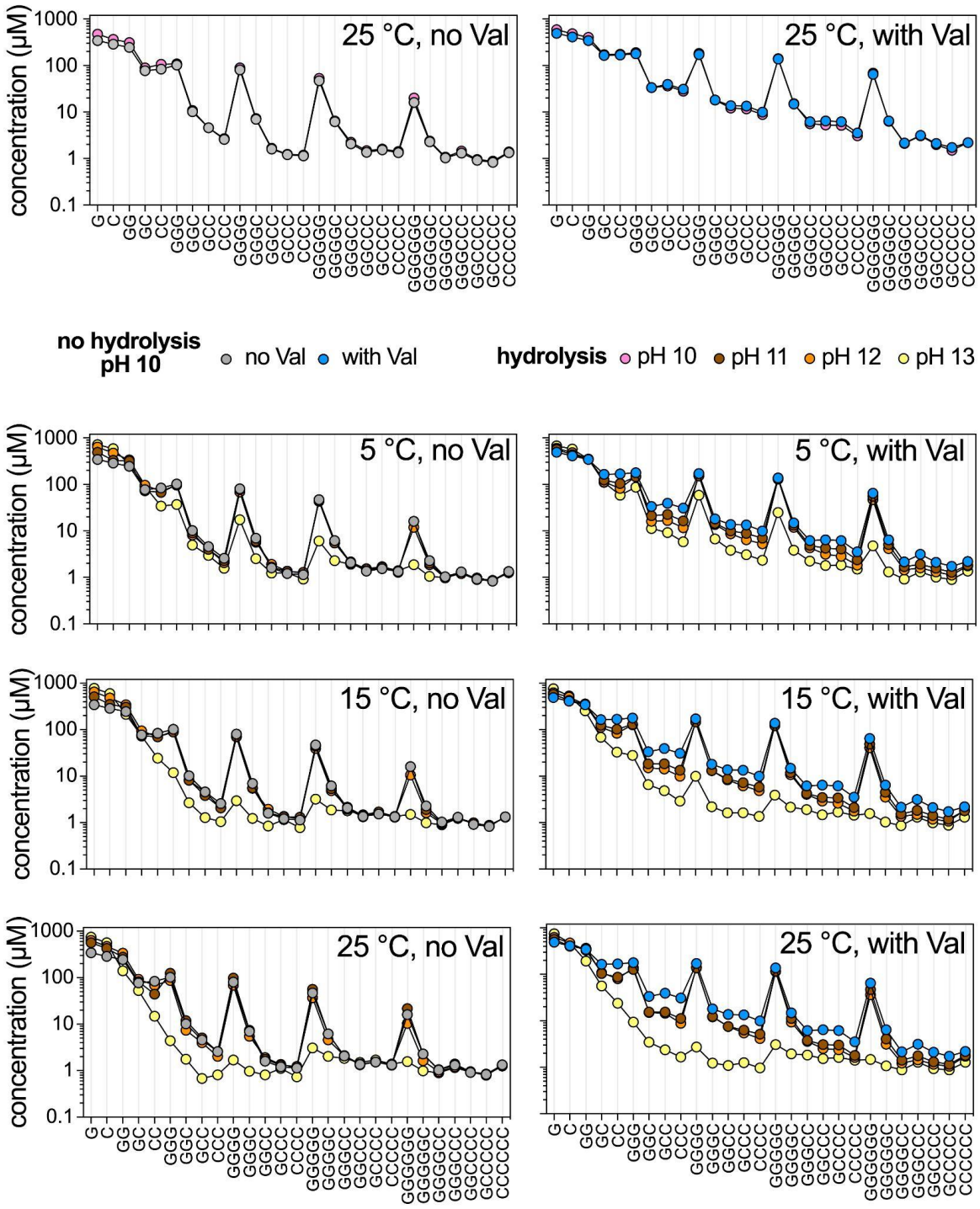


Figure S33. Temperature and pH-dependent hydrolysis of GC-oligomers. The concentrations of GC-oligomers in the absence (left panel) and presence (right panel) of valine are presented for various hydrolytic conditions (initial pH:10-13; temperature: 5 °C, 15 °C and 25 °C). The oligomers used for hydrolysis were synthesized at pH 10 using 40 mM cyclic nucleotides (20 mM each of G and C), with or without 100 mM valine at pH 10, after drying for 20 hours. These products were then incubated at different pH and temperature with 0.5M KCl for 24 h. The hydrolysis is evident from the reduction of longer oligo concentration and an increase of shorter oligo and monomer concentration at higher pH and temperature. All quantitative estimations were conducted by ESI-TOF.

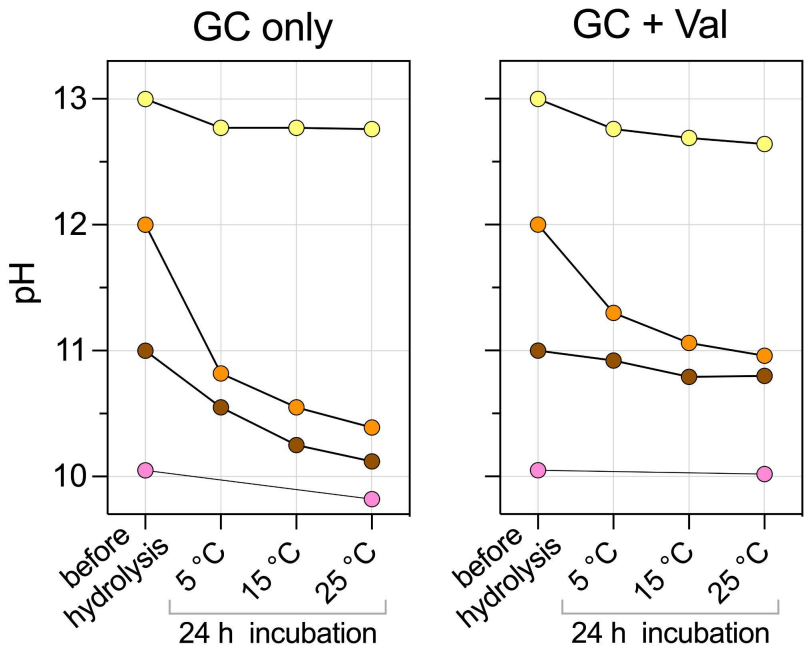


Figure S34. pH-drop as a result of hydrolysis. The pH of every hydrolytic condition explored in Figure S32 was measured for GC-oligomers, both in the absence (left) and presence (right) of valine, after a 24-hour incubation. Compared to GC-oligos without valine, valine appears to have a buffering effect and resists the pH shift more.

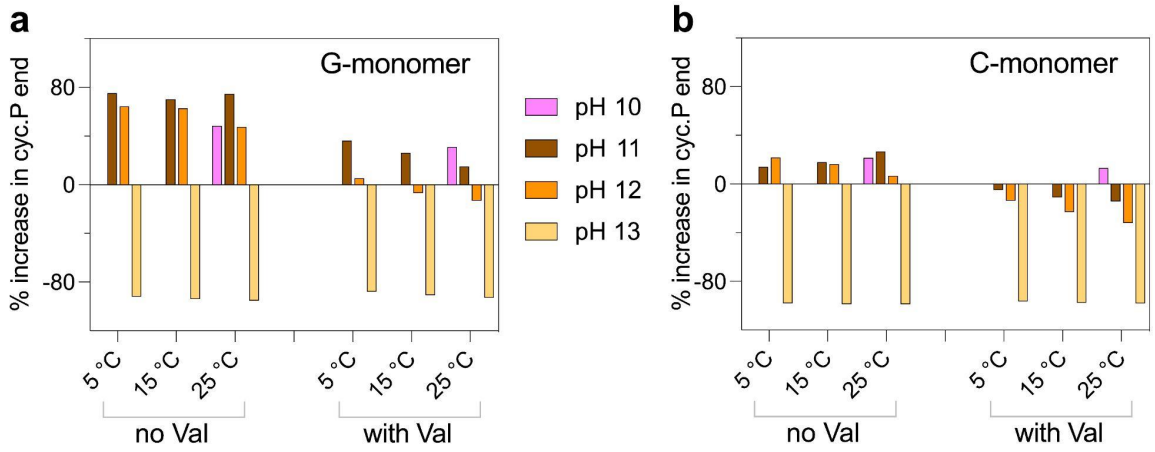


Figure S35. Increased cyclic monomers (cNMP) as a result of hydrolysis. a, The percentage increase in concentrations of cyclic G in GC-oligomer mixtures with and without valine is plotted for all hydrolytic conditions investigated in Figure S32. **b,** The percentage increase in concentrations of cyclic C is plotted, similar to **a**. All quantitative estimations were conducted by ESI-TOF.

References

1. Kessner, D. et al. ProteoWizard: open source software for rapid proteomics tools development. *Bioinformatics*, **24**, 2534-2536 (2008).
2. Łącki, M. K., Valkenborg, D. & Startek, M. P. Isospec2: Ultrafast fine structure calculator. *Anal. Chem.* **92**, 9472–9475 (2020).