

Supporting Information

Spontaneous Formation of RNA Strands, Peptidyl RNA, and Cofactors

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1 Materials and Methods

1.1 Reagents and Instrumentation

Reagents

Nicotinamide mononucleotide (NMP⁺) and flavin mononucleotide (FMN) were purchased from Sigma Aldrich (Deisenhofen, Germany). Acetylglycine and phenylalanine ethyl ester were from Bachem (Bubendorf Switzerland); glycine and phenylalanine were from Aldrich. All other reagents, oligonucleotides, buffers and solvents were as described in the Supporting Information of the accompanying manuscript.^[S1]

MALDI-TOF mass spectrometry

MALDI-TOF mass spectra were acquired as described for the accompanying manuscript,^[S1] except for spectra of free peptide in positive mode, for which a matrix of α -cyano-4-hydroxycinnamic acid in water/acetonitrile (0.025 M, 2:1 v/v) was used.

HPLC

The HPLC monitoring of the formation of the dipeptide, isolated from the ethyl acetate extract of the reaction mixture, was performed on a silica column (Merck, LiChrospher 250 x 4 mm, 5 μ m particle size) with hexane/*isopropanol* (75:25 v/v). Ion-exchange HPLC was performed as described in the accompanying paper.^[S1] Other details are given in the General Protocols, below.

NMR

NMR spectra were acquired on a Bruker ARX 300 spectrometer (300 MHz) or a Bruker AVANCE 500 spectrometer (500 MHz).

1.2 General Protocols

General Protocol 1. *Oligomerization reactions*

Oligomerization reactions were performed as described in the Supporting Information of the accompanying publication,^[S1] except that more than one nucleotide were used, and/or amino acid building blocks were included. Briefly, an aqueous solution of the ribonucleotide(s) (0.15 M, 9.75 μ mol) in buffer (0.5 M HEPES, 0.08 M MgCl₂ and 0.15 M 1-ethylimidazole) at pH 7.5 was used to dissolve a sample of EDC hydrochloride (10 mg, 52 μ mol), and the resulting solution was allowed to react at 0 °C. Samples (2 μ L) were drawn at stated intervals, diluted with buffer (200 μ L; 5 mM Tris in acetonitrile/water, 20:80, v/v; pH 7.5), and analyzed by anion-exchange HPLC, followed by MALDI-TOF MS of fractions.

General Protocol 2. *Cofactor-forming reactions*

To AMP (17.5 mg; 48 μ mol) and the appropriate phosphate species (sodium pyrophosphate, 12.8 mg, 48 μ mol, nicotinamide mononucleotide, 16 mg, 48 μ mol, or flavin mononucleotide, 23 mg, 48 μ mol) was added an aliquot of buffer (320 μ L) containing HEPES (0.5 M, 160 μ mol), MgCl₂ (0.08 M, 25.6 μ mol), and 1-ethylimidazole (0.15 M, 48 μ mol). The solution was brought to pH 7.5 with NaOH and cooled to 0 °C. To start the reaction, EDC (48 mg, 256 μ mol) was added. The reaction mixture was vortexed for 10 s and kept at 0 °C. Flavin mononucleotide was only partially dissolved in the reaction mixture and in the case of sodium pyrophosphate, a precipitate formed. In either case, the solid was removed by centrifugation prior to NMR analysis. Samples were drawn (1 μ L) and analyzed by TLC (1-propanol/H₂O/NH₄OH 25%, 55:10:35 v/v/v) or diluted with water (500 μ L) and analyzed by MALDI-TOF mass spectrometry and/or NMR. For NMR measurements, 120 μ L of D₂O were added to the solution. For representative NMR and mass spectra for the cofactor-forming reaction of NAD⁺ see chapter 2.2, below.

General Protocol 3. *Simultaneous primer extension, oligomerization of ribonucleotides and peptide formation.*

To a suspension of beads bearing capture oligonucleotide (5 μ L, 5 mg/mL) in HEPES buffer (0.5 M with 0.08 M MgCl₂, pH 7.5) were added solutions of template (0.6 μ L, 100 μ M, 60 pmol) and primer (0.5 μ L, 100 μ M, 50 pmol), and the strands were allowed to hybridize at 0 °C for 15 min. The supernatant was aspirated and 5 μ L of a freshly prepared solution of EDC (0.77 mg, 4 μ mol) in buffer (HEPES, 0.5 M; MgCl₂, 0.08 M; 1-ethylimidazole, 0.15 M) containing AMP (0.15 M), GMP (0.15 M), CMP (0.15 M), UMP (0.15 M), Ac-Gly-OH (0.125 M), and H-Phe-OEt (0.125 M) at pH 7.5 was added. The mixture was transferred to a vessel containing 5 nmol of GUCG, vortexed (5 s) and incubated at 0 °C. At 5 d intervals, the supernatant was drawn, analyzed for peptide, peptidyl RNA, and RNA strands, and replaced with a freshly prepared solution. Extraction of supernatants with CH₂Cl₂ (40 μ L) and HPLC (hexane/isopropanol, 75:25 v/v) allowed for monitoring peptide formation. Oligomerization products were analyzed by SAX-HPLC of the aqueous supernatant.

2 Supplementary Data

2.1 Oligomerization of Ribonucleotides to Give RNA Strands

Representative Mass Spectra from Assays on Oligomerization of AMP in the Presence of Other Nucleotides

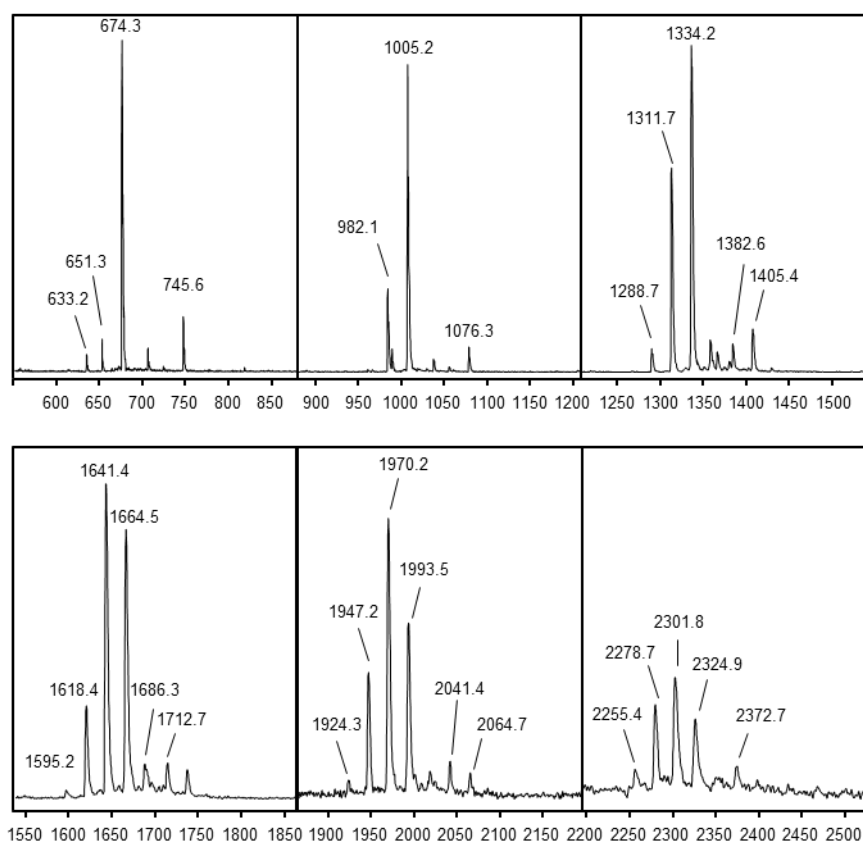


Figure S1. Expanded view of spectra, as shown in Figure 2 of the main manuscript: MALDI-TOF mass spectra of mixed oligomers of AMP and UMP as identified in HPLC fractions after a reaction time of 30 d. The calculated average masses, and the found maxima of the unresolved isotope pattern are listed below.

A_2 , $[M-H]^-$ calculated for $C_{20}H_{26}N_{10}O_{13}P_2$ 675.1, found 674.3

AU, $[M-H]^-$ calculated for $C_{19}H_{25}N_7O_{15}P_2$ 652.1, found 651.3

A_3 , $[M-H]^-$ calculated for $C_{29}H_{37}N_{12}O_{21}P_3$ 1004.2, found 1005.2

A_2U , $[M-H]^-$ calculated for $C_{28}H_{36}N_{15}O_{19}P_3$ 981.2, found 982.1

A_4 , $[M-H]^-$ calculated for $C_{40}H_{50}N_{20}O_{25}P_4$ 1333.2, found 1334.2

A_3U , $[M-H]^-$ calculated for $C_{39}H_{49}N_{17}O_{27}P_4$ 1310.2, found 1311.7

A_2U_2 , $[M-H]^-$ calculated for $C_{38}H_{48}N_{14}O_{29}P_4$ 1287.2, found 1288.7

A₅, [M-H]⁻ calculated for C₅₀H₆₂N₂₅O₃₁P₅ 1662.3, found 1664.5
A₄U, [M-H]⁻ calculated for C₄₉H₆₁N₂₂O₃₃P₅ 1639.3, found 1641.4
A₃U₂, [M-H]⁻ calculated for C₄₈H₆₀N₁₉O₃₅P₅ 1616.3, found 1618.4
A₂U₃, [M-H]⁻ calculated for C₄₇H₅₉N₁₆O₃₇P₅ 1593.3, found 1595.2
A₆, [M-H]⁻ calculated for C₆₀H₇₄N₃₀O₃₇P₆ 1991.3, found 1993.5
A₅U, [M-H]⁻ calculated for C₅₉H₇₃N₂₇O₃₉P₆ 1968.3, found 1970.2
A₄U₂, [M-H]⁻ calculated for C₅₈H₇₂N₂₄O₄₁P₆ 1945.3, found 1947.2
A₃U₃, [M-H]⁻ calculated for C₅₇H₇₁N₂₁O₄₃P₆ 1922.3, found 1924.3
A₇, [M-H]⁻ calculated for C₇₀H₈₆N₃₅O₄₃P₇ 2320.4, found 2324.9
A₆U, [M-H]⁻ calculated for C₆₉H₈₅N₃₂O₄₅P₇ 2297.4, found 2301.8
A₅U₂, [M-H]⁻ calculated for C₆₈H₈₄N₂₉O₄₇P₇ 2274.4, found 2278.7
A₄U₃, [M-H]⁻ calculated for C₆₇H₈₃N₂₆O₄₉P₇ 2251.4, found 2255.4.

2.2 Peptide Formation

Short-Hand Notation for Amino Acidyl and Peptidyl RNAs. For the peptidyl RNA phosphoramidates, the *C*-terminal substituent is given first, for lack of a better nomenclature. So, EtO-Phe-A stands for EtO-CO-CH(Ph)-NH- as the substituent at the 5'- phosphate of AMP.

Peptide Formation

The following protocol is for an assay performed to establish the methodology and to test for the compatibility with a phospholipid. The assay results showed that both peptide and phosphoramidate formed under condensation conditions and that even high concentration of phospholipid do not prevent the formation of either species.

Ac-Gly-Phe-OEt. An aqueous solution of *N*-acetyl glycine (40 μ L, 0.25 M, 10 μ mol), phenylalanine ethyl ester (40 μ L, 0.25 M, 10 μ mol) and AMP (48 μ L, 0.25 M, 12 μ mol) was dried by lyophilization. The residue was dissolved in buffer (80 μ L) containing HEPES (0.5 M, 40 μ mol), MgCl₂ (0.08 M, 6.4 μ mol), 1-ethylimidazole (0.15 M, 12 μ mol) at pH 7 to give the following concentrations: 0.125 M of the amino acid derivatives and 0.15 M AMP. The mixture was adjusted to pH 7.5 with NaOH, cooled to 0 °C and then added to EDC (12.4 mg, 64 μ mol) to give an EDC concentration of 0.8 M. The mixture was vortexed for 10 s and stored on ice. The reaction was monitored by TLC and HPLC. After full conversion, the mixture was extracted with ethyl acetate (5 x 200 μ L). The combined organic layers were concentrated *in vacuo* to give a colorless solid. Yield 1.7 mg (6 μ mol, 60% as determined gravimetrically); TLC (CH₂Cl₂:*isopropanol*, 9:1 *v/v*, ninhydrin): R_f = 0.34 (product), R_f = 0.48 (H-Phe-OEt); HPLC (*hexane:isopropanol*, 75:25 *v/v*): t_R = 7.0 min (product), 4.6 min (H-Phe-OEt); ¹H NMR (300.1 MHz, D₂O): δ = 7.42–7.20 (m, 5 H, *Ph*), 4.69 (dd, J = 5.9 Hz, J = 8.5 Hz, 1H, *CH*), 4.18 (dq, J = 1.0 Hz, 7.1 Hz, 2H, O-CH₂-CH₃), 3.82 (s, 2H, CH₂), 3.20 (dd, J = 5.9 Hz, J = 14.0 Hz, 1H, -CH_aH_bPh) and 3.05 (dd, J = 8.5 Hz, J = 14.0 Hz, 1H, -CH_aH_bPh), 2.01 (s, 3H, CH₃), 1.22 (t, J = 7.1 Hz, 3H, O-CH₂-CH₃). MALDI-TOF MS in positive mode (*m/z*): calcd for C₁₅H₂₀N₂O₄ [M+H]⁺ 293.3, found 293.1; no signals for nucleotide-containing products were detected in the organic extract. MALDI-TOF MS of the aqueous layer after extraction with ethyl acetate measured in negative mode (*m/z*): 346 (AMP, 15%), 521 (EtO-Phe-A, 10%), 675 (diadenosyl 5'-pyrophosphate and/or A₂, 100%), 850 (EtO-Phe-A₂, 5%), 1005 (A₃, 8%); MALDI-TOF MS of the aqueous phase measured in positive mode (*m/z*): 523 (EtO-Phe-A, 100%), 677 (diadenosyl 5'-pyrophosphate and/or A₂, 87%), 852 (EtO-Phe-A₂, 10%), 1006 (A₃, 15%).

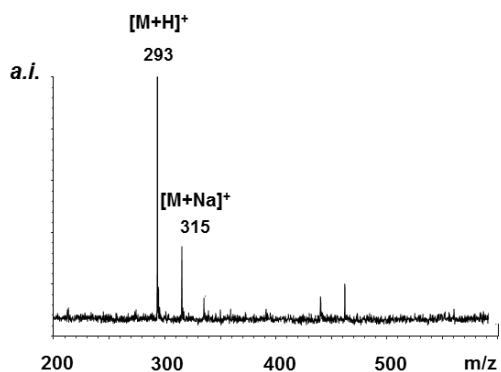


Figure S2 MALDI-TOF mass spectrum of Ac-Gly-Phe-OEt in positive mode.

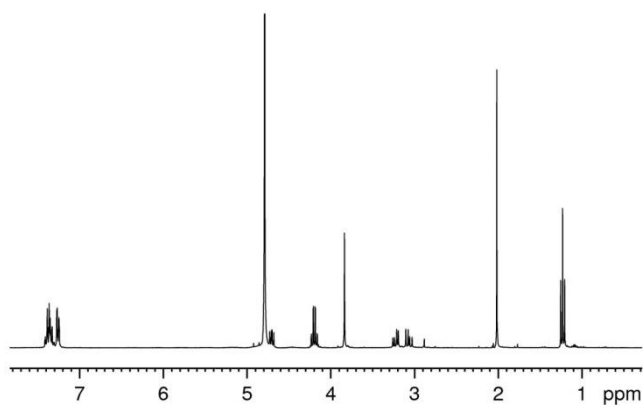


Figure S3. ¹H NMR of Ac-Gly-Phe-OEt (D₂O, 300 MHz), as obtained by extraction with ethyl acetate.

Formation of Covalently Linked Species with Nucleotides

The following spectrum demonstrated that besides a 'free' peptide, a covalently linked species was formed between the free amine of the ethyl ester of phenylalanine and AMP in condensation buffer.

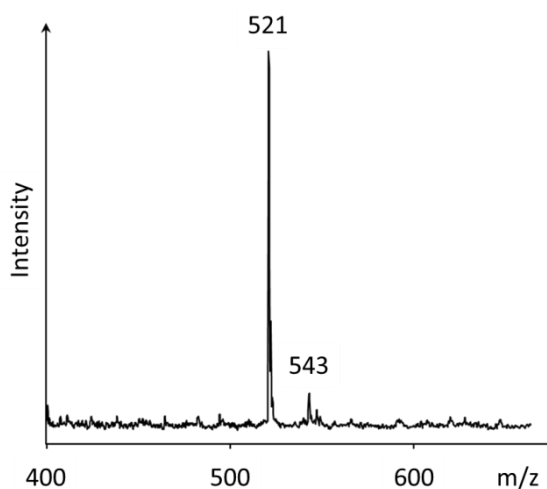


Figure S4. MALDI-TOF mass spectrum of EtO-Phe-A phosphoramidate in positive mode, as detected in the aqueous solution.

Formation of Ac-Gly-Phe-OEt in the presence of the phospholipid DPPC. An assay was performed as described for Ac-Gly-Phe-OEt, above. To the solution was also added DPPC (7.4 mg, 10 μ mol, final concentration 0.125 M). The analytical data for the dipeptide were in agreement with those given for Ac-Gly-Phe-OEt, above.

Formation of Mixed Oligomers in the Presence of Peptides

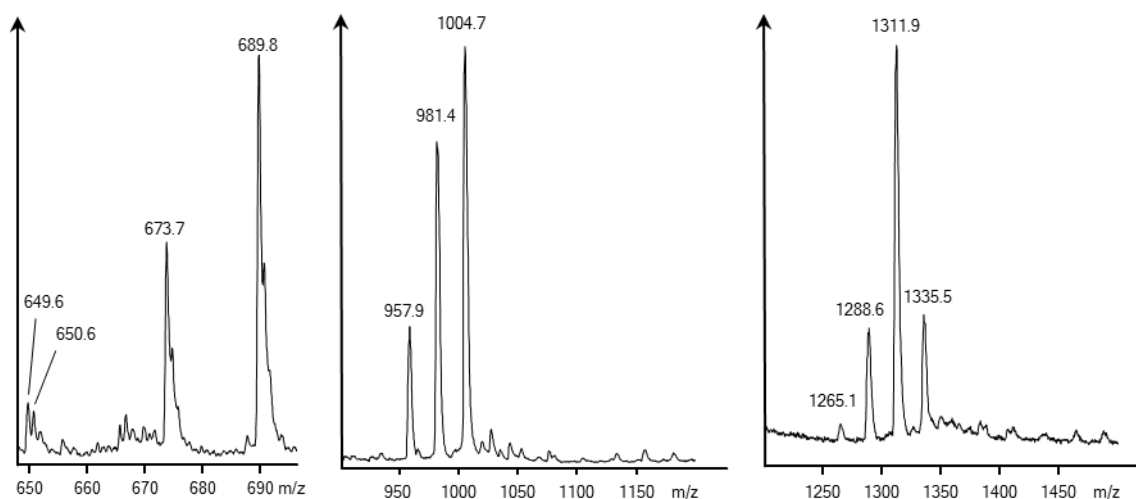


Figure S5. Expanded view of spectra shown in Figure 3 of the manuscript: MALDI-TOF mass spectra of HPLC fractions showing mixed oligomers of AMP, GMP, CMP and UMP, formed in an assay performed according to General Protocol 1. Since the mass difference of CMP and UMP is 1 Da, individual peaks for either were resolved for the dimer fraction only. Calculated masses are average masses, masses found are maxima of the unresolved isotope patterns.

A₂, [M-H]⁻ calculated for C₂₀H₂₆N₁₀O₁₃P₂ 675.1, found 673.7

AU, [M-H]⁻ calculated for C₁₉H₂₅N₇O₁₅P₂ 653.1, found 650.6

AC, [M-H]⁻ calculated for C₁₉H₂₆N₈O₁₄P₂ 652.1, found 649.6

AG, [M-H]⁻ calculated for C₂₀H₂₆N₁₀O₁₄P₂ 691.1, found 689.8

A₃, [M-H]⁻ calculated for C₃₀H₃₈N₁₅O₁₉P₃ 1004.2, found 1004.7

A₂U, [M-H]⁻ calculated for C₂₉H₃₇N₁₂O₂₁P₃ 981.1, found 981.4

A₄, [M-H]⁻ calculated for C₄₀H₅₀N₂₀O₂₅P₄ 1333.2, found 1335.5

A₃U, [M-H]⁻ calculated for C₃₉H₄₉N₁₇O₂₇P₄ 1310.2, found 1311.9

A₂U₂, [M-H]⁻ calculated for C₃₈H₄₈N₁₄O₂₉P₄ 1287.2, found 1288.6

AU₃, [M-H]⁻ calculated for C₃₇H₄₇N₁₁O₃₁P₄ 1264.2, found 1265.1.

2.3 Co-Oligomerization of Nucleotides and Amino Acids

Co-Oligomerization of one Amino Acid and AMP

Oligomerization of H-Gly-OH in the presence of AMP. Solutions of glycine (80 μ L, 0.25 M, 20 μ mol) and AMP (48 μ L, 0.25 M, 12 μ mol) were lyophilized, and the mixture was then treated with freshly prepared condensation buffer [EDC (12.4 mg, 64 μ mol) in buffer (80 μ L) containing HEPES (0.5 M, 40 μ mol), $MgCl_2$ (0.08 M, 6.4 μ mol), 1-ethylimidazole (0.15 M, 12 μ mol), pH 7.5] at 0 °C. The conversion of the amino acid was monitored by TLC (silica, *n*-butanol/ H_2O /HOAc, 10:5:5 *v/v/v*, ninhydrin staining). After 5 d, complete conversion of glycine was detected. MALDI-TOF MS (linear negative mode) of the reaction mixture after 5 d (*m/z*): 403 (HO-Gly-A, 50%), 460 [HO-(Gly)₂-A, 25%], 517 [HO-(Gly)₃-A, 13%], 573 [HO-(Gly)₄-A, 10%], 630 [HO-(Gly)₅-A, 5%], 675 (adenosine 5'-pyrophosphate and/or A₂, 100%), 687 [HO-(Gly)₆-A, 2%], 1005 (A₃, 4%). After 10 d, a sample (2 μ L) was drawn, diluted with buffer (200 μ L, Tris, 5 mM; in acetonitrile:water, 20:80, *v/v*; pH 7.5) and analyzed via SAX-HPLC. Samples (0.5 μ L) of individual fractions were analyzed by MALDI-TOF mass spectrometry.

Oligomerization of phenylalanine and AMP. Solutions of phenylalanine (80 μ L, 0.25 M, 20 μ mol) and AMP (48 μ L, 0.25 M, 12 μ mol) were lyophilized and the residue was then treated with freshly prepared condensation buffer [EDC (12.4 mg, 64 μ mol) in buffer (80 μ L) containing HEPES (0.5 M, 40 μ mol), $MgCl_2$ (0.08 M, 6.4 μ mol), 1-ethylimidazole (0.15 M, 12 μ mol), pH 7.5 at 0 °C], resulting in a slurry. TLC monitoring of the conversion of the amino acid (*n*-butanol/ H_2O /HOAc, 10:5:5 *v/v/v*, ninhydrin staining): R_f (phenylalanine) 0.81. After 14 d at 0 °C, the starting material had reacted almost quantitatively while more solid precipitated. The solid was then separated using centrifugation, and washed twice with H_2O (2 x 100 μ L). For MALDI-TOF MS, a small sample of the precipitate was dissolved in H_2O (500 μ L), aided by sonication, and then analyzed in linear negative mode (*m/z*): 640 (HO-(Phe)₂-A, 10%), 675 (adenosine 5'-pyrophosphate and/or A₂, 65%), 787 [HO-(Phe)₃-A, 8%], 822 (HO-Phe-A₂, 8%), 934 [HO-(Phe)₄-A, 10%]; 968 [HO-(Phe)₂-A₂, 2%], 1081 [HO-(Phe)₅-A, 40%], 1228 [HO-(Phe)₆-A, 60%], 1375 [HO-(Phe)₇-A, 30%], 1522 [HO-(Phe)₈-A, 3%], 1557 [HO-(Phe)₆-A₂, 4%], 1704 [HO-(Phe)₇-A₂, 4%], 1739 [HO-(Phe)₅-A₃, 3%], 1851 [HO-(Phe)₈-A₂, 3%], 1886 [HO-(Phe)₆-A₃, 3%], 1998 [HO-(Phe)₉-A₂, 2%], 2033 [HO-(Phe)₇-A₃, 1%].

Selection of lipophilic peptidyl RNAs with *n*-octanol. A sample of the products that precipitated during oligomerization of phenylalanine in presence of AMP (1 mg) was suspended in H_2O (100 μ L), treated with *n*-octanol (100 μ L), and vortexed for 30 s. Phase separation was aided by centrifugation. MALDI-TOF MS of the octanol solution (linear negative mode) gave (*m/z*): 1081, 1228, 1375, 1522, 1669 [HO-(Phe)₅-A up to HO-(Phe)₉-A]; 1704, 1851, 1998, 2145, 2292, 2439, 2586, 2733 [HO-(Phe)₇-A₂ up to HO-(Phe)₁₄-A₂]; 2474, 2621, 2768, 2915, 3062 [HO-(Phe)₁₀-A₃ up to HO-(Phe)₁₄-A₃].

Acidic release of peptides from peptidyl RNA. A sample of the products precipitated during the oligomerization of phenylalanine and AMP (1 mg) was treated with aqueous acetic acid (50 μ L, 80%) at r.t. for 5 d. The reaction mixture was then diluted with H₂O (50 μ L), treated with octanol (100 μ L), and vortexed. The phases were separated using centrifugation. The organic phase was analyzed by MALDI-TOF MS (linear positive mode, with α -cyano-4-hydroxycinnamic acid as matrix) (m/z) = 754 (Phe)₅, 50%; 901 [(Phe)₆, 100%], 1049 [(Phe)₇, 100%], 1196 [(Phe)₈, 20%].

Formation of Peptidyl Chains is more Efficient in the Presence of AMP

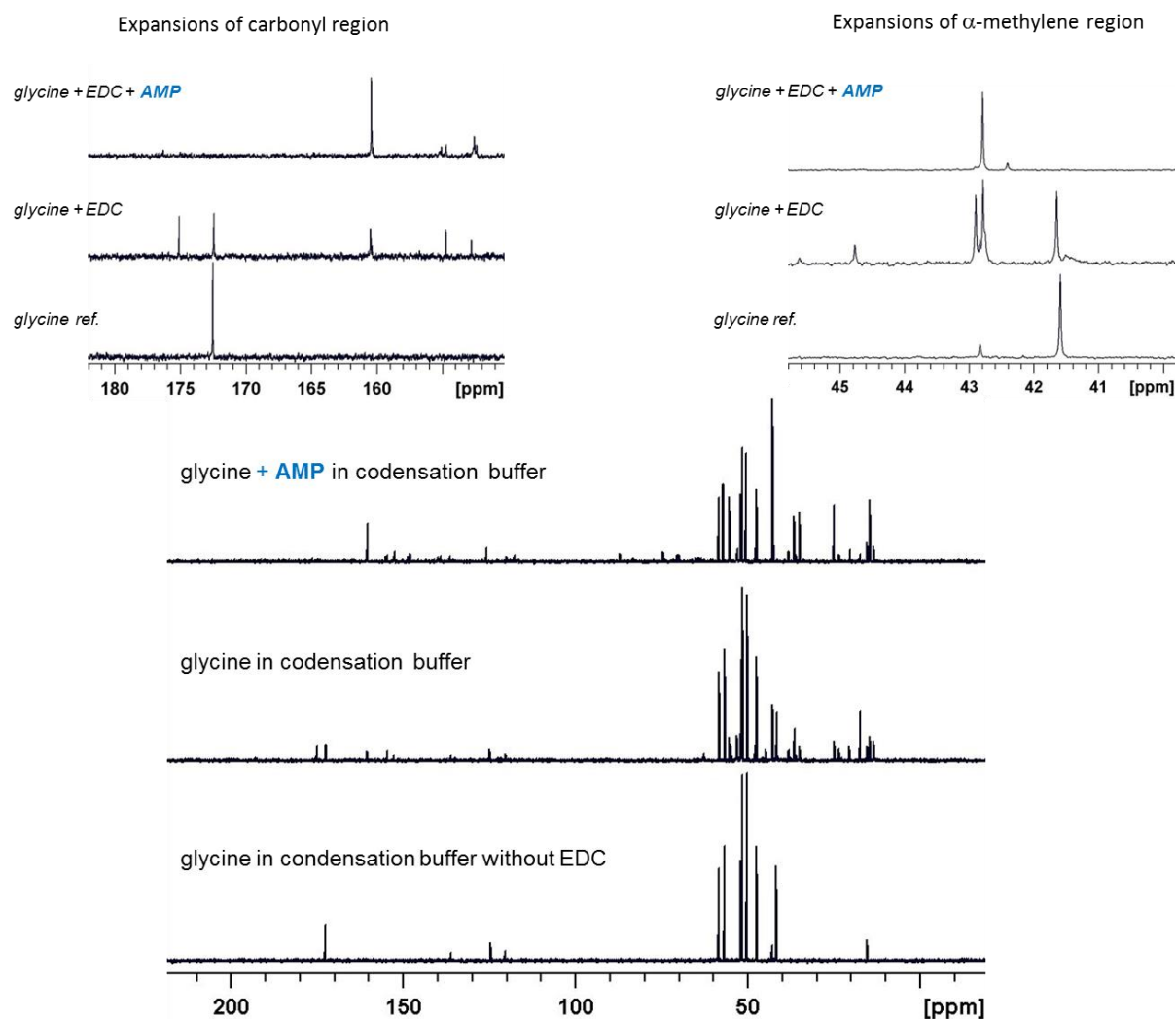


Figure S6. The reactivity pattern of glycine in condensation buffer changes in the presence of AMP, as detected by ^{13}C NMR spectroscopy (75 MHz). Overview spectra with full spectral width are shown in the bottom part. Spectra of glycine alone in buffer lacking EDC are shown at the very bottom. The spectra above them are from glycine in condensation buffer after 2 d at 0 °C. The spectra at the top of each overlay are those of a solution of glycine in condensation buffer with AMP after 2 d at 0 °C. Conditions: $\text{H}_2\text{O}/\text{D}_2\text{O}$ (4:1) as solvent for the condensation buffer containing 0.3 M glycine with or without 0.8 M EDC and with or without 0.3 M AMP. The expansions in the upper part of the figure show diagnostic regions with carbonyl resonances of glycine (180 to 150 ppm) and methylene resonances of glycine (45 to 40 ppm). Further, in the presence of AMP, one predominant peptidyl product is formed, whereas in the absence of AMP the reaction gives a distribution of peptides and side products.

Co-Oligomerization of Two Amino Acids and AMP

Oligomerization of glycine, phenylalanine, and AMP. To a mixture of EDC hydrochloride (10 mg, 52 μmol) was added an aqueous solution (65 μL) containing HEPES (0.5 M) MgCl_2 (0.08 M), 1-ethylimidazole (0.15 M), AMP (0.15 M), phenylalanine (0.1 M) and glycine (0.1 M) at pH 7.5. Samples (2 μL) were drawn at stated intervals, diluted with buffer (200 μL ; Tris, 5 mM; in acetonitrile:water, 20:80, v/v; pH 7.5), and analyzed via SAX-HPLC. Samples (0.5 μL) of individual or combined fractions were analyzed by MALDI-TOF mass spectrometry. (m/z): 1026.4 (HO-Gly-(Phe) $_2$ -A $_2$), 1061.1 (HO-Gly-A $_3$), 1083.4 (HO-(Gly) $_2$ -(Phe) $_2$ -A $_2$), 1118.1 (HO-(Gly) $_2$ -A $_3$), 1140.1 (HO-(Gly) $_2$ -(Phe) $_2$ -A $_2$), 1151 (HO-Phe-A $_3$), 1173.7 (HO-(Gly) $_3$ -A $_3$), 1208.5 (HO-Gly-Phe-A $_3$), 1231 (HO-(Gly) $_4$ -A $_3$), 1265 (HO-(Gly) $_2$ -Phe-A $_3$), 1298.7 (HO-(Phe) $_2$ -A $_3$), 1322 (HO-(Gly) $_3$ -Phe-A $_3$), 1356 (HO-Gly-(Phe) $_2$ -A $_3$), 1377.9 (HO-(Gly) $_4$ -Phe-A $_3$), 1412.9 (HO-(Gly) $_2$ -(Phe) $_2$ -A $_3$), 1435.6 (HO-(Gly) $_5$ -Phe-A $_3$), 1721.8 (HO-Gly-Phe-A $_5$), 1868.5 (HO-Gly-Phe-A $_5$), 1924.2 (HO-(Gly) $_2$ -Phe-A $_5$), 2071.8 (HO-(Gly) $_2$ -(Phe) $_2$ -A $_5$), 2128.3 (HO-(Gly) $_3$ -(Phe) $_2$ -A $_5$), 2276.3 (HO-(Gly) $_3$ -(Phe) $_3$ -A $_5$), 2333.7 (HO-(Gly) $_4$ -(Phe) $_3$ -A $_5$).

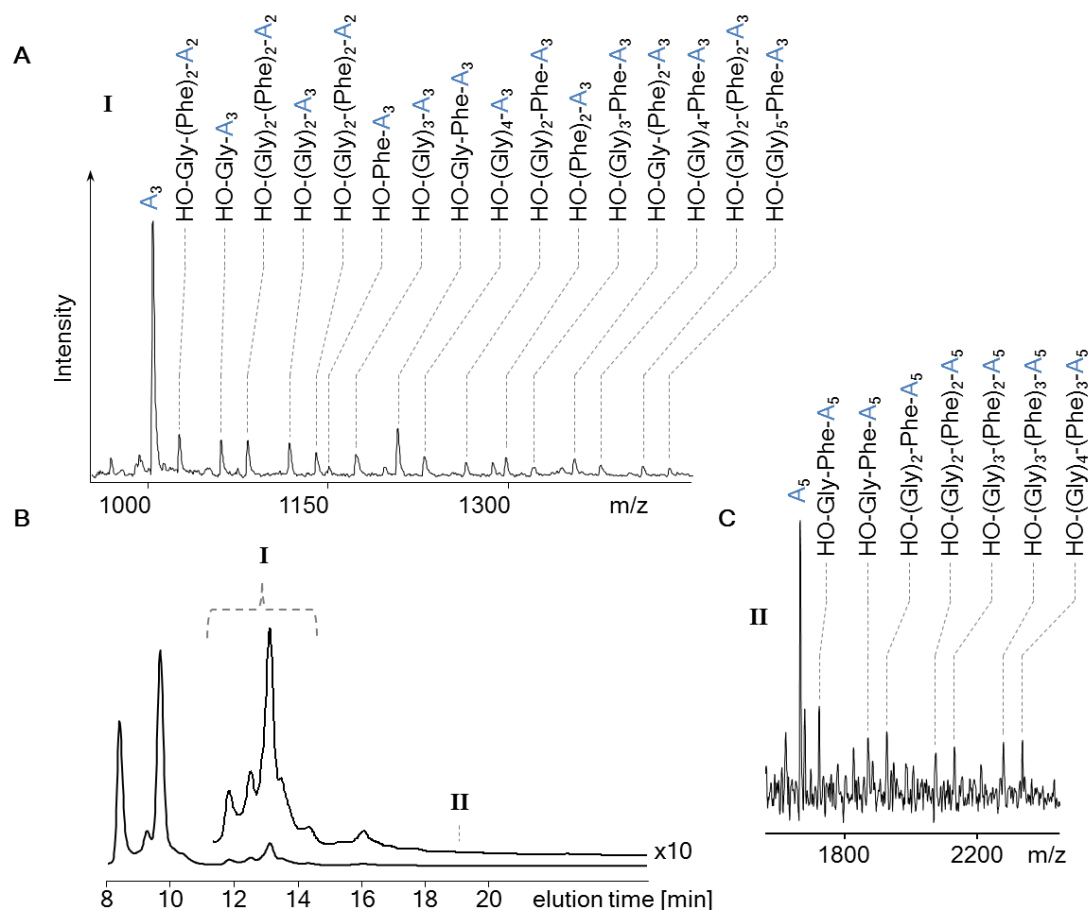


Figure S7. a) MALDI-TOF mass spectrum of a region containing A_3 , and its peptidyl derivatives. b) Underlying ion-exchange HPLC chromatogram of the reaction mixture after 7 d at 0 $^{\circ}\text{C}$. c) MALDI-TOF mass spectrum of a sample containing A_5 with peptidyl residues of up to 7 amino acids.

Simultaneous Oligomerization of two Amino Acids, and two Ribonucleotides Gives a Divers Set of Mixed-sequence Peptidyl RNAs

Formation of peptidyl RNAs from glycine, phenylalanine, UMP and AMP. To a mixture of EDC hydrochloride (10 mg, 52 μmol) was added a solution (65 μL) containing HEPES (0.5 M) MgCl_2 (0.08 M), 1-ethylimidazole (0.15 M), AMP (0.15 M), UMP (0.15 M), phenylalanine (0.1 M) and glycine (0.1 M) at pH 7.5. Samples (2 μL) were drawn at stated intervals, diluted with buffer (200 μL , Tris 5 mM, pH 7.5, containing 20% MeCN), and analyzed via SAX-HPLC. Samples (0.5 μL) of individual or combined fractions were analyzed by MALDI-TOF mass spectrometry; (m/z): 1026.1 (HO-Gly-(Phe)₂-A₂), 1037.9 (HO-Gly-UA₂), 1061 (HO-Gly-A₃), 1083 (HO-(Gly)₂-(Phe)₂-A₂), 1095 (HO-(Gly)₂-UA₂), 1118 (HO-(Gly)₂-A₃), 1151 (HO-Phe-A₃), 1174 (HO-(Gly)₃-A₃), 1185 (HO-Gly-Phe-UA₂), 1208 (HO-Gly-Phe-A₃), 1242 (HO-(Gly)₂-Phe-UA₂), 1265.6 (HO-(Gly)₂-Phe-A₃), 1276 (HO-(Phe)₂-UA₂), 1298.7 (HO-(Phe)₂-A₃), 1332.8 (HO-Gly-(Phe)₂-UA₂), 1355.4 (HO-Gly-(Phe)₂-A₃), 1390 (HO-(Gly)₂-(Phe)₂-UA₂), 1412.4 (HO-(Gly)₂-(Phe)₂-A₃), 1676 (HO-Gly-U₂A₃), 1699 (HO-Gly-UA₄), 1823.3 (HO-Gly-Phe-U₂A₃), 1846.2 (HO-Gly-Phe-UA₄).

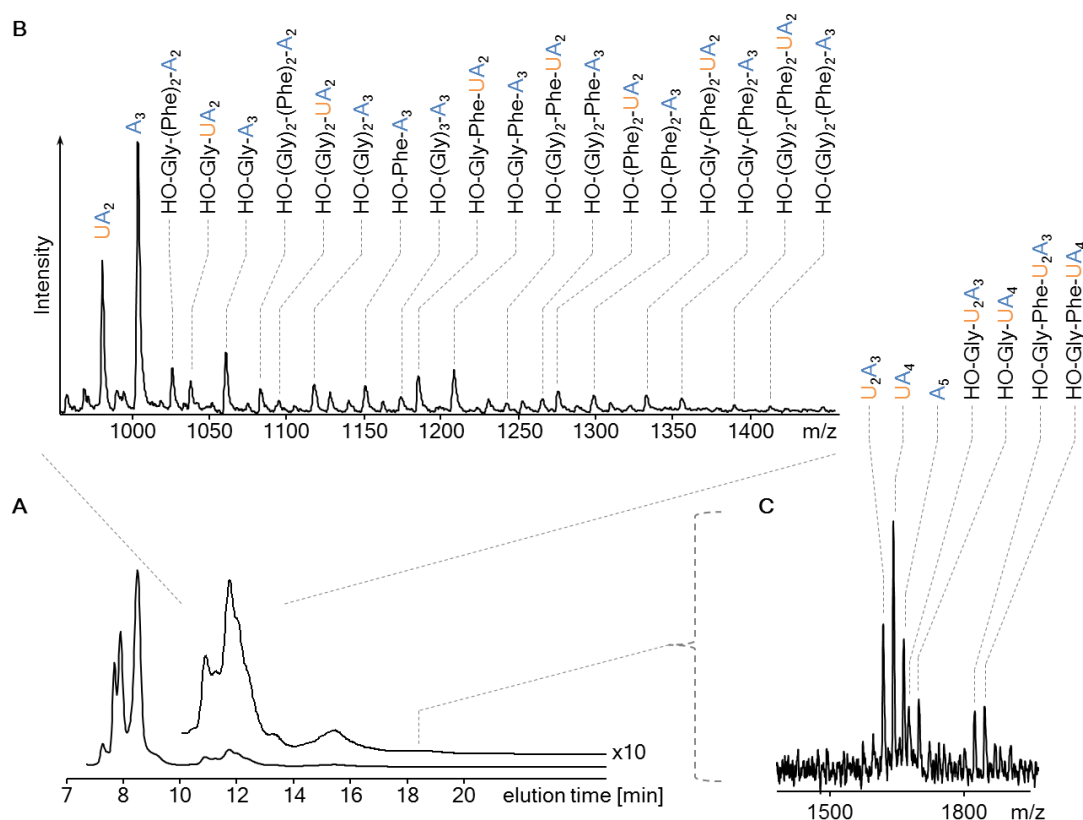


Figure S8. a) Ion-exchange HPLC chromatogram of the reaction mixture after 7 d at 0 °C; b) MALDI-TOF mass spectrum of combined chromatographic fractions of a region showing trinucleotides with different peptidyl chains; c) MALDI-TOF mass spectrum of a late-eluting fraction containing mixed pentanucleotides with amino acid or peptidyl chains.

The Peptidyl RNAs Formed are Phosphoramidates

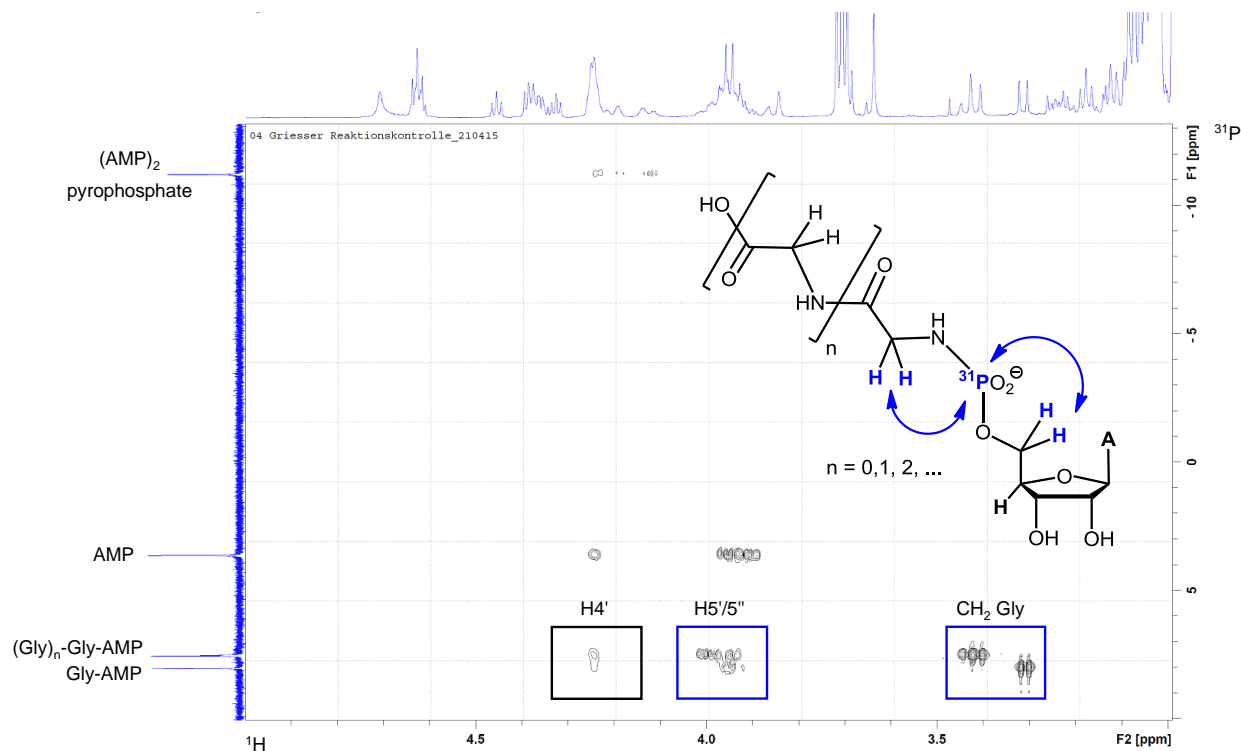


Figure S9. ^{31}P - ^1H HMBC NMR spectrum (500 MHz, D_2O) showing long-range correlation between phosphate and ribose nuclei of the AMP portion, and the same phosphate and the neighboring α -methylene protons of the glycyl portion of the peptidyl chain. The spectrum was acquired at the end of an assay (21 d) involving glycine (0.25 M) and AMP (0.25 M) in condensation buffer. The sample was prepared by lyophilizing the reaction mixture and taking it up again in D_2O for NMR spectroscopy.

Reactivity of AMP in the Presence of Glycine is Similar for Different Activation Modes

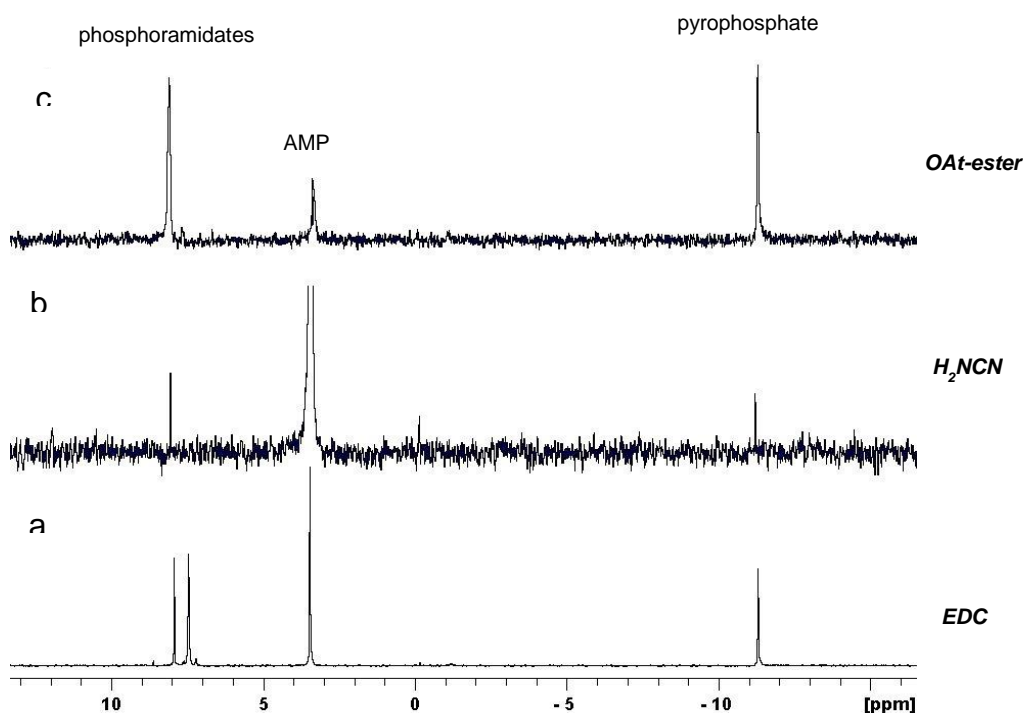


Figure S10. ³¹P NMR spectra (125 MHz) of samples from reactions of AMP and glycine, induced by different modes of activation. Samples were measured after addition of D₂O (100 μL) for locking of the NMR spectrometer. See Figure 5 for a series of related spectra for EDC and cyanamide, with an expansion of the region with phosphodiester peaks. We note that phosphoramidate formation competes with chain growth. a) Condensation of AMP (0.15 M) and glycine (0.2 M) induced by EDC (0.8 M), after a reaction time of 13 days at 0 °C. b) Condensation of AMP (0.15 M) and glycine (0.2 M), activated by cyanamide (H₂NCN, 0.8 M), after a reaction time of 2 days at 50 °C. c) Reaction of the oxyazabenzotriazolide of AMP (0.08 M) with glycine (0.2 M), without carbodiimide, after a reaction time of 2 days at 0 °C.

2.4 Cofactor Formation

Representative NMR and Mass Spectra for Cofactor-forming Reaction

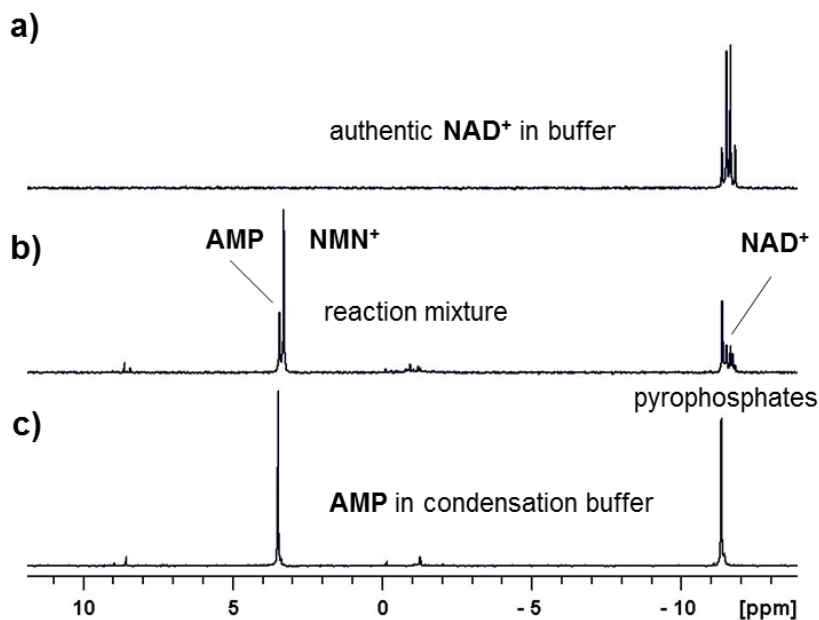
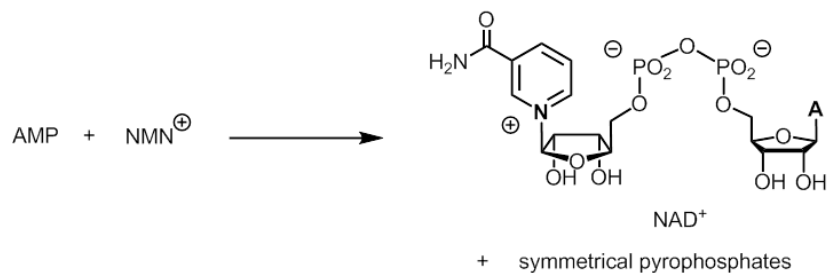


Figure S11. Formation of a coenzyme (NAD⁺) from AMP and nicotinamide mononucleotide (NMN⁺) under condensation conditions. a-c) ³¹P NMR spectra (D₂O, 125 MHz). **a)** Solution of authentic NAD⁺ in reaction buffer. **b)** Sample of reaction mixture with AMP (150 mM) and NMN⁺ (150 mM) after a reaction time of 7 days at 0 °C. **c)** Sample of reaction mixture with AMP alone (150 mM) after a reaction time of 7 days at 0 °C.

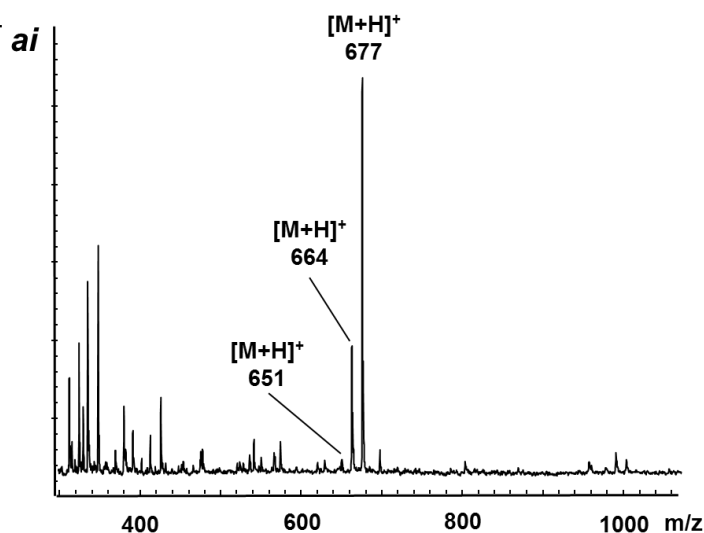


Figure S12. Full-width MALDI-TOF mass spectrum of a sample from a solution containing AMP (0.15 M) and NMN⁺ (0.15 M) in condensation buffer, after a reaction time of 12 h at 0 °C. Masses found include (*m/z*): 335 (NMN⁺, 50%), 348 (AMP, 55%), 651 [(NMN⁺)₂, approx. 5%], 664 (NAD⁺, 35%), 677 [(AMP)₂, 100%], 981 [(NMN⁺)₂A, <5%], 993 [(NMN⁺)A₂, <5%], and 1007 (A₃, <5%). We note that chain growth has set in after 12 h. Cationic species, containing NMN⁺, probably give weak signals in negative mode due to unfavorable ionization properties. In the low-mass region, matrix peaks dominate. For a spectrum acquired after 4d, please see Figure 4a of the main manuscript.

Analytical data

ATP: TLC (1-propanol/H₂O/NH₄OH 25%, 55:10:35 *v/v/v*) R_f = 0.25; MALDI-TOF MS, negative mode (*m/z*): calcd for C₁₀H₁₆N₅O₁₃P₃ [M-H]⁻ 506.2, found 505.8.

NAD⁺: TLC (1-propanol/H₂O/NH₄OH 25%, 55:10:35 *v/v/v*) R_f = 0.57; MALDI-TOF MS in positive mode (*m/z*): calcd for C₂₁H₂₈N₇O₁₄P₂⁺ [M]⁺ 664.4, found 664.1.

FAD: TLC (1-propanol/H₂O/NH₄OH 25%, 55:10:35 *v/v/v*) R_f = 0.56; MALDI-TOF MS in negative mode (*m/z*): calcd for C₂₇H₃₃N₉O₁₅P₂ [M-H]⁻ 784.5, found 784.1. An expected byproduct, as described by Tanaka^[S2] or Forrest et al.,^[S3] is the 4',5'-cyclic phosphate of FMP at *m/z* = 436. TLC (1-propanol/H₂O/NH₄OH 25%, 55:10:35 *v/v/v*; R_f = 0.50). This was the only product observed in the absence of AMP. A control reaction performed without MgCl₂ showed improved solubility for flavin mononucleotide and a higher yield of FAD, as detected by MALDI-TOF MS.

References for Supporting Information

[S1] M. Jauker, H. Griesser, C. Richert, Copying of RNA sequences without pre-activation, *submitted* (accompanying manuscript).

[S2] Tanaka, *Yakugaku Zasshi* **1958**, 78, 627-630.

[S3] H.S. Forrest, , H.S. Mason, A.R. Todd, *J. Chem. Soc.* **1952**, 75, 2530-2534.