RNA-directed Peptide Synthesis Across a Nicked Loop

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Abstract

Ribosomal translation at the origin of life requires controlled aminoacylation to produce mono-aminoacyl esters of tRNAs. Herein, we show that transient annealing of short RNA oligo:amino acid mixed anhydrides to an acceptor strand enables the sequential transfer of aminoacyl residues to the diol of an overhang, first forming aminoacyl esters then peptidyl esters. Using *N*-protected of aminoacyl esters prevents unwanted peptidyl ester formation in this manner. However, *N*-acylaminoacyl transfer is not stereospecific.

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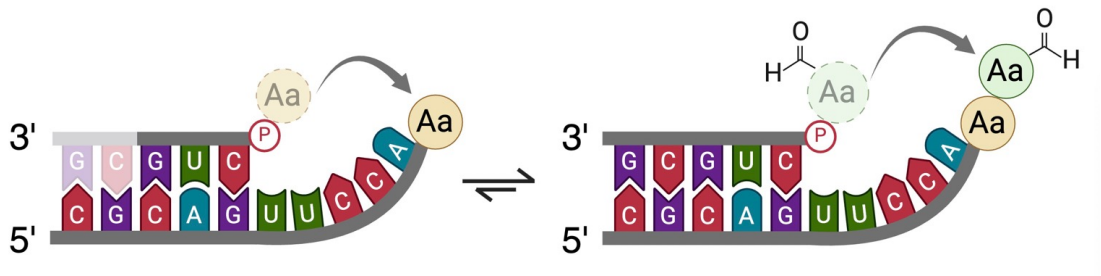

6 Examples, 23-91% Yields

Introduction

Modern RNA-coded peptide synthesis is inordinately complicated, including the ribosome and various translation factors to enable peptidyl transfer at the (*N*-acyl-)aminoacylated 3'-CCA termini of two juxtaposed tRNAs.[1] Although the details of this mechanism have been elucidated in recent years,[2,3] it is not known how translation could have first emerged.

According to the RNA world hypothesis,[4] RNA was the first biological macromolecule to appear in the prebiotic environment. It is capable of both storing genetic information and catalysing essential biochemical reactions. Given RNA's key role in the synthesis of proteins there has been a long-standing interest in understanding how translation developed on early Earth. Several cases of RNA-catalysed/mediated peptide synthesis have been reported. A 196-nt ribozyme was evolved to perform a peptidyl transfer reaction to yield Met-Phe dipeptide.[5,6] A triphenylalanyl-RNA ester was formed in a nicked RNA/DNA duplex without additional ribozymes.[7] Oligophenylalanine up to a pentapeptide was detected using a five-nucleotide ribozyme and phenylalanyl adenosine monophosphate.[8] Interestingly, in all these cases the amino acids studied are not considered prebiotically plausible.[9,10,11] Crucially, no relationship between RNA sequence and peptide sequence has been demonstrated with these systems. Two examples of RNA-templated peptide synthesis, in a nicked duplex or across a duplex terminus, were published recently.[12,13] A condensation buffer containing carbodiimide (EDC) or 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM) was required in either case, thus, the coupling reactions are not spontaneous. Furthermore, an adequate hypothesis for how either of these mechanisms could have transitioned to the extant mechanism is yet to be proposed, calling into question their relevance to the origin and early evolution of life.

We have previously reported an aminoacyl-transfer reaction within a stem-overhang RNA structure (Fig. 1a).[14] Starting from a chemically synthesised aminoacyl-phosphate mixed anhydride at the 5'-terminus of an RNA strand, in combination with a longer overhanging complementary RNA, the aminoacyl-residue is spontaneously transferred to the 3'-terminus forming a 2'/3'-aminoacyl-ester. This mechanism has clear parallels to that of extant biology which utilises an aminoacyl-adenylate mixed anhydride and

aminoacyl tRNA synthetases to build 2'/3'-aminoacyl-ester tRNAs. We have also reported recently how the transfer varies with RNA sequence and different amino acids and the possible consequences for the origin of the genetic code.[15] We were interested to see if further equivalents of mixed anhydride would allow subsequent aminoacyl transfer to the aminoacyl-ester amino group to give a 2'/3'-dipeptidyl-ester.

Results and Discussion

When taking a close look at the HPLC trace of the products of one such transfer reaction of alanyl (Ala) residues, (3 eq. 5'-L-Ala-pCUG mixed anhydride as donor strand mixed with 1 eq. 5'-CGCAGUUCCA as acceptor stand, Fig. 1a) we discovered a new peak with a longer retention time than the 2'/3'-Ala-ester acceptors (Fig. 1b). The peak reached its maximum yield of 8% after 140 min, compared to 70 min for the known 2'/3'-Ala-esters (Fig. 1c, Table S1, Fig. S1). This new peak decreased in intensity slowly with a half-life of 14 h at 16°C, more than double that of the 2'/3'-Ala-esters. After isolation of species responsible for the new peak, MALDI-TOF characterization was consistent with a 10-mer strand plus two Ala residues (Fig. 1d). Although it is possible in theory to produce a bis-2',3'-Ala,Ala-diester (with Ala residues both at terminal or internal 2' and terminal 3' positions), we would expect such species to have comparable lability to the 2'/3'-Ala-esters. However, we expected the 2'/3'-dialanyl-esters (Ala-Ala) to be more stable than the monoalanyl esters and therefore tentatively assigned the new peak to result from the formation of the 2'/3'-Ala-Ala-ester acceptor strands.

We had previously used RNAse A digestion to analyse (*N*-acetyl)-aminoacylation of RNA.[14] RNAse A cleaves the terminal adenosine nucleoside of the acceptor stem whilst maintaining any aminoacyl ester bonds. By comparison against synthetic standards, the 2'/3'-(*N*-acetyl)-Ala-ester adenosines could be identified in the digestion products of (*N*-acetyl)-aminoacylated RNA when analysed by HPLC, and thus the presence of terminal 2'/3'-(*N*-acetyl)-aminoacyl esters in the undigested RNA could be deduced. To provide further verification that the new peak we had observed indicated the formation of 2'/3'-Ala-Ala-esters, we HPLC purified the species responsible for the peak, then lyophilised and digested it with RNAse. The chromatogram of the digested material contained peaks consistent with both 2'- and 3'-Ala-Ala-ester adenosines (Fig.

S2, S3) when compared to synthetic standards. The region of the chromatogram corresponding to the bis-2',3'-Ala,Ala-diester adenosine standard was quite congested in the digested sample, but very low in intensity. We conclude the new peak to result from the formation of 2'/3'-Ala-Ala-esters. Furthermore, we were able to identify a mass peak corresponding to the 10-mer strand plus three alanine residues by MALDI (Fig. 1d), which is logically even more likely to include at least one peptide bond.

In prokaryotes, peptide synthesis starts with *N*-formylmethionine (fMet) encoded by the start codon AUG. The formyl group allows the corresponding aminoacyl-tRNA to be accommodated at the P site of the ribosome. *N*-formylaminoacids have been demonstrated to be prebiotically plausible species on early Earth.[16] Therefore, we studied *N*-formyl-alanyl (fAla) and *N*-formyl-glycyl transfer (fGly) (Fig. 2a, b, Table S2, Fig. S4, S5). First, we demonstrated that both fAla and fGly transferred to the 2',3'-diol of a 10-mer acceptor in our system (maximum observed yields 16% and 21% at pH 8.0, Fig. 2a, b Ctrl lines). Next, we tested for peptide formation. With the same 10-mer acceptor strand, an Ala residue was first transferred to the 10-mer 3'-terminus using a free Ala mixed anhydride-tetramer donor (5'-L-Ala-pCUGC). When the yield of 2'/3'-Ala-esters reached their peak, 2 eq. fAla- or fGly-mixed anhydride-pentamer donor (5'-fAla-/fGly-pCUGCG) was added to the system.

After the addition of formylaminoacyl mixed anhydride, the peak corresponding to the 2'/3'-Ala-esters was observed to decrease much faster (half-life = 1.9 h for fGly and 3.3 h for fAla) than without the formylaminoacyl mixed anhydride (half-life = 6.6 h). New peaks with similar kinetic profiles to each other were detected (Fig. S4, S5) as well as peaks identifiable as 2'/3'-formylaminoacyl-esters by comparison with a control reaction (Fig. 2a, b Ctrl lines). After isolation, the species responsible for the new peaks produced MALDI peaks consistent with RNA plus both Ala- and fGly/fAla- residues (Fig. 2c). Despite our previous assumption that no bis-2',3'-products were formed, we entertained the possibility these new peaks may correspond to the 2',3'-Ala-,fAla-/Ala-,fGly-diesters. We would expect that both 2',3'-Ala-,fAla- and 2',3'-Ala-,fGly-diesters would first hydrolyse to 2'/3'-fAla- or 2'/3'-fGly-esters. However, the corresponding peaks for these species did not increase as the new peaks degraded, supporting formation of 2'/3'-dipeptidyl esters instead.

As there was always a substantial proportion of unreacted 10-mer acceptor strand after the first step, we also observed the direct formation of 2'/3'-fAla-/fGly-esters from said acceptor. Due to the increased nucleophilicity of a 2',3'-diol compared to a single hydroxyl, we would expect more 2'/3'-fAla-/fGly-ester to form than any bis-species. However, in the case of fAla transfer (Fig. 2a, Table S2), we found the rate constants of formation of 2'/3'-fAla-ester and the new species to be nearly identical (0.0066 min^{-1} and 0.0067 min^{-1} respectively). In the case of fGly transfer (Fig. 2b. Table S2), the integration of the new peaks was 2-fold higher than the integration of the 2'/3'-fGly-ester peak. All these observations suggest that the new peaks did not correspond to bis-species, but instead indicated the formation of 2'/3'-formyldipeptidyl-esters.

We further studied the transfer by RNAse digestion of the transfer of fGly onto preformed 2'/3'-Ala-esters. By comparison of the HPLC chromatograms of the digestion products with synthetic standards, we identified peaks corresponding to the 2'/3'-fGly-Ala-esters of adenosine, and none of the bis-2',3'-Ala-,fGly-diesters. (Fig. S3, S6)

The question remained, in the case of the fAla transfer onto preformed 2'/3'-Ala-esters why were there two new peaks with significant differences in retention time? When a fAla-residue was transferred from a pentamer donor to the 2',3'-diol of the 10-mer acceptor as a control experiment, two overlapping peaks arose (Fig. 2a). This could be consistent with racemization of fAla during the chemical synthesis of the mixed anhydride followed by transfer of both D- and L-fAla-residues. Previous experiments had demonstrated free D-Ala mixed anhydrides would only transfer inefficiently whilst D-*N*-acetylalanyl (D-AcAla) mixed anhydrides transferred equally well as L- across a nicked loop.[14,15] Alternatively, the two new peaks could be due to a mixture of 2'/3'-aminoacyl-esters resolving differently on the column. In the case of fGly transfer to a 2',3'-diol, only one peak was identified, which could suggest either the transfer was regioselective for one alcohol or that both 2'/3'-aminoacyl-esters have equal retention times.

The yield of peptide bond formation differed between these formylaminoacyl residues (2'/3'-fAla-Ala-esters 23%, 2'/3'-fGly-Ala-esters 57%). Unsurprisingly, due to the difference in charge state, 2'/3'-fAla-Ala-esters were less labile to cleavage ($t_{½}$ = 4.1 d

at 10°C, pH 6.8) than 2'/3'-Ala-Ala-esters (t½ = 1 d at 10°C, pH 6.8) which can additionally cleave to form diketopiperazines. The half-life of the 2'/3'-fGly-Ala-esters under the same conditions was t½ = 3.4 d, a bit shorter than the 2'/3'-fAla-Ala-esters.

Next, we broadened our aminoacyl-mixed anhydride scope in the first step to include leucyl- and prolyl-residues (Leu and Pro respectively). For the second transfer, fAla- or fGly-mixed anhydride pentamer donor strand were used as with previous experiments (Fig. 3, Table S2, Fig. S7-S10). Transfers of fAla onto Leu- or Pro-acceptors produced four new HPLC peaks with identical kinetics, whilst transfers of fGly onto Leu or Pro produced two new ones. This is consistent with both the racemisation of fAla and a resolution of the mixture of 2'/3'-esters in the products. We did not further differentiate these peaks. HPLC and MALDI analysis confirmed that all four 2'/3'-formyl-dipeptidyl-esters were correctly synthesized, with distinct yields and synthesis/hydrolysis kinetics. In the first step, Ala transferred better than Leu and Pro as reported previously.[15] In the second step, fGly generally transferred better or as well as fAla, depending on the identity of the first amino acid transferred.

Both the constituent amino acids in the formyl-dipeptidyl ester affected the ester lability, despite their inability to form diketopiperazines. It has previously been shown that a Leu-tRNA ester was more stable than an Ala-tRNA ester or a Pro-tRNA ester.[17] We observed the same trend. Furthermore, the 2'/3'-formyldipeptidyl-esters containing fAla are always found to be more stable than those containing fGly.

The 2'/3'-formyldipeptidyl-esters are equivalent to the acceptor stem of a tRNA at the A-site after peptide bond formation but before translocation. In extant translation, releasing the mature peptides is assisted by the Gly-Gly-Gln motif in the Class I release factors.[18] But hydrolysis of 2'/3'-peptidyl-esters in a prebiotic context has not been documented to the best of our knowledge. In this work, with the help of aminoacyl transfer chemistry, we have built a small but diverse series of 2'/3'-(*N*-formyl)-dipeptidyl-esters and characterized their hydrolytic stability (Table S2). The longest half-life among the six 2'/3'-formyldipeptidyl-esters is 2'/3'-fAla-Leu-ester (t½ = 6.6 days at 10°C, pH 6.8). This suggests that peptidyl RNA conjugates can survive and become enriched in the prebiotic environment without auxiliary macromolecules.

In all three examples where fGly-mixed anhydride was used in the second transfer, we identified two peaks with longer retention time and greater stability toward hydrolysis (peaks marked with * in Fig. S5, S8, S10). These peaks may correspond to 2'/3'-tripeptidyl-esters terminating in fGly. This would require fGly-mixed anhydride to react with the 2'/3'-dipeptidyl-esters we identified at the beginning of this communication (Fig. 1b, S1). Due to the lack of material, we were unable to characterise these peaks further.

We investigated the mechanism of the multi-step (*N*-formyl)-aminoacyl transfer. We prepared the alanyl mixed anhydride donor in three different lengths, trimer (5'-L-Ala-pCUG), tetramer (5'-L-Ala-pCUGC) and pentamer (5'-L-Ala-pCUGCG). As the first transfer reached its maximum, two equivalents of a fGly-mixed anhydride pentamer (5'-fGly-pCUGCG) were added. With the increasing length of the original free alanyl-mixed anhydride donor, it becomes harder for the fGly-mixed anhydride pentamer donor to replace it, thus reducing the yield and rate of dipeptidyl ester formation. The 5'-L-Ala-pCUG followed by 5'-fGly-pCUGCG transfer gave the highest dipeptide yield (65%) in the shortest time (325 min) (Fig. 4, Table S3). The 5'-L-Ala-pCUGC followed by 5'-fGly-pCUGCG transfer yield was 57% (at 410 min), whilst the 5'-L-Ala-pCUGCG followed by 5'-fGly-pCUGCG transfer was the slowest, reaching to maximum yield of 40% after 1000 min. These data indicate the necessity of strand exchange beefore (*N*-formyl)-dipeptidyl ester formation.

When *N*-acyl-amino acids are activated at their C termini they form oxazolones which are reactive electrophiles.[19] It is possible the formylaminoacyl-mixed anhydrides could be reversibly cleaved to from 5(*4H*)-oxazolones, which then subsequently react intermolecularly with the nucleophilic amine of the 2'/3'-aminoacyl-ester. To test this hypothesis we incubated the 10-mer acceptor strand and a pentamer (pCUGCG) containing a 5'-phosphate then added the water-soluble carbodiimide EDC and fGly/fAla to provide *in situ* formation of oxazolone (Fig. S11, S12). This failed to produce any acyl transfer products, mixed anhydride or other RNA esters. Repeating the reaction instead using preformed Ala 5(*4H*)-oxazolone up to 2000 eq. also failed to produce any peaks where we would expect transfer products nor mixed anhydride, in contrast to previous studies with nucleosides (Fig. S13, S14).[19] Similar addition of Ala

5-(*4H*)-oxazolone to preformed 2'/3'-Ala-esters did not produce new peaks with the correct retention time or stability (Fig. S15). As no transfer to the 10-mer strand is observed at such high concentrations of oxazolone and given the relative stability of the formylaminoacyl-mixed anhydrides under these conditions, we think it unlikely that 5-(*4H*)-oxazolone is implicated in the formation of *N*-formyldipeptidyl-2'/3'-esters or *N*-formylaminoacyl-2'/3'-esters presented above. These experimental results confirmed our hypothesis that the strand replacement must occur, even though the experiment is performed below the melting temperature of the RNA duplex.

Oxazolones are known to racemise.[20] The synthetic chemistry used to make formylaminoacyl mixed anhydrides in this work would likely produce 5-(*4H*)-oxazolones as reactive intermediates, so we would expect both diastereoisomers of the formylaminoacyl mixed anhydrides to be formed. Stereoselectivity of transfer of (*N*-acyl)-aminoacyl residues would have been important to nascent translation to ensure diasteromerically pure proteins. We and others have previously demonstrated that the transfer of aminoacyl residues from a mixed anhydride in a nicked loop[14] or nicked duplex[21] configuration is stereoselective (with some exceptions for specific sequences[15]). However, nicked loop transfer of AcAla residues is not stereospecific.[14] This last observation is interesting in comparison to a previous report that AcAla residues do infact transfer stereoselectively in a nicked duplex.[22]

As diastereoselectivity of transfer had only been studied for the transfer of AcAla residues, we studied the transfer of the more prebiotically plausible fAla residues used in this work. A 5-mer mixed anhydride synthesised from L-fAla as a donor strand was mixed was mixed with a 10-mer acceptor strand (1 eq. 5'-fAla-pAGCGA mixed anhydride with 1 eq. 5'-UCGCUUUCCA forming a nicked loop) or with a 10-mer template strand and 8-mer acceptor strand (1 eq. 5'-fAla-pAGCGA mixed anhydride with 1 eq. 5'-UCGCUUUCCA template and 1 eq. 5'-UAAUGGAA acceptor making a nicked duplex). fAla transfer to their acceptors was identified in (Fig S16, 16%) across a nicked loop or (Fig S17, 61%) nicked duplex respectively by the presence of new peaks with increased retention time consistent with our previous experiments. The crude mixtures were digested by RNAse A as before and analysed by HPLC (Fig. S18, S19). Comparison of these chromatograms with those of synthetic standards confirmed the

presence of nearly equal amounts of both L- and D-2'/3'-fAla-ester adenosines. These results were consistent with racemisation of the formylalanyl residue during synthesis of the fAla mixed anhydride 5-mer, followed by the transfer of both L- and D-fAla residues. Repeating these experiments, but starting with D-fAla, afforded similar transfer yields (13% nicked loop and 50% nicked duplex, Fig. S16, S17) and resulted in nearly identical chromatograms (Fig. S18 and S19).

At this point, it seemed odd that only one out of the four possible combinations of *N*-acetyl-/*N*-formyl-aminoacyl transfers across either a nicked-loop or nicked-duplex was stereoselective. We synthesised 5-mer mixed anhydrides using either L- or D-AcAla as a donor strand, then added them to a nicked duplex scenario containing the same 10-mer template strand and 8-mer acceptor strand as our earlier experiments (1 eq. 5'-AcAla-pAGCGA mixed anhydride with 1 eq. 5'-UCGCUUUCCA template and 1 eq. 5'-UAAUGGAA acceptor). We identified the transfer of AcAla residues across a nicked duplex by formation of a new peak as before (starting from Ac-L-Ala 25%, starting from Ac-D-Ala 22%; Fig. 20). The species responsible for this peak were collected, lyophilised and then digested with RNAse. Subsequent HPLC analysis and comparison to synthesised standards demonstrated the presence of equal amounts of L- and D- 2'/3'-AcAla-ester adenosines (Fig. 21).

In contrast to the previous report,[22] we conclude that *N*-acylaminoacyl residues do not transfer stereoselectively in either nicked-loop or nicked-duplex scenarios. Due to the lack of experimental detail reported by Tamura *et al.*[22], it is difficult to see where our conflicting results originate. It is possible that the different sequences used demonstrate different levels of selectivity, something we have seen in some exceptional cases with the transfer of aminoacyl residues in a nicked loop.[15] Alternatively, simple experimental errors in the previous work[22] such as differing loading onto gels (no loading control is shown) or significantly hydrolysed mixed anhydride starting material synthesised from D-AcAla, are potential causes.

Conclusion

Our results here demonstrate the possibility of assembling prebiotic peptidyl-RNA via strand replacement and transfer from (*N*-formyl)-aminoacyl mixed anhydrides. We

note dipeptides have been shown to have sequence specific activities relevant to the origins of life, such as vesicle growth.[23] Stable duplex and strand replacement are the prerequisites for peptidyl assembly. Here, the peptide directly forms at the CCA-ending overhang, as in extant tRNA. The hydrolytic stability of formyl-dipeptidyl-RNA varies with amino acid sequence. Our investigations into the mechanism of *N*-formylamino acid transfer suggest that 5-(*4H*)-oxazolones are not implicated in the mechanism of transfer. The transfer of *N*-acylamino acids onto 2',3'-diol termini in a nicked-duplex or a nicked-loop, and onto aminoacyl esters in nicked loops show no stereoselectivity. Due to the similarity in chemistry, it is conceivable that a transition from trimer-mixed anhydride donors to adenylate-mixed anhydrides which we see in modern biology is simpler than those template chemistries presented previously.[12,13] Our results also highlight the difficulty of avoiding peptidyl-transfer in systems capable of transferring aminoacyl residues from free amino acid:phosphate mixed anhydrides to 2',3'-diol termini of RNA.

ASSOCIATED CONTENT

Supporting Information

The data that support the findings of this study are available within its Supporting Information.

The Supporting Information is available free of charge at

Materials and methods, supplementary data and figures (PDF)

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Notes

The authors declare no competing financial interest.

Author Contributions

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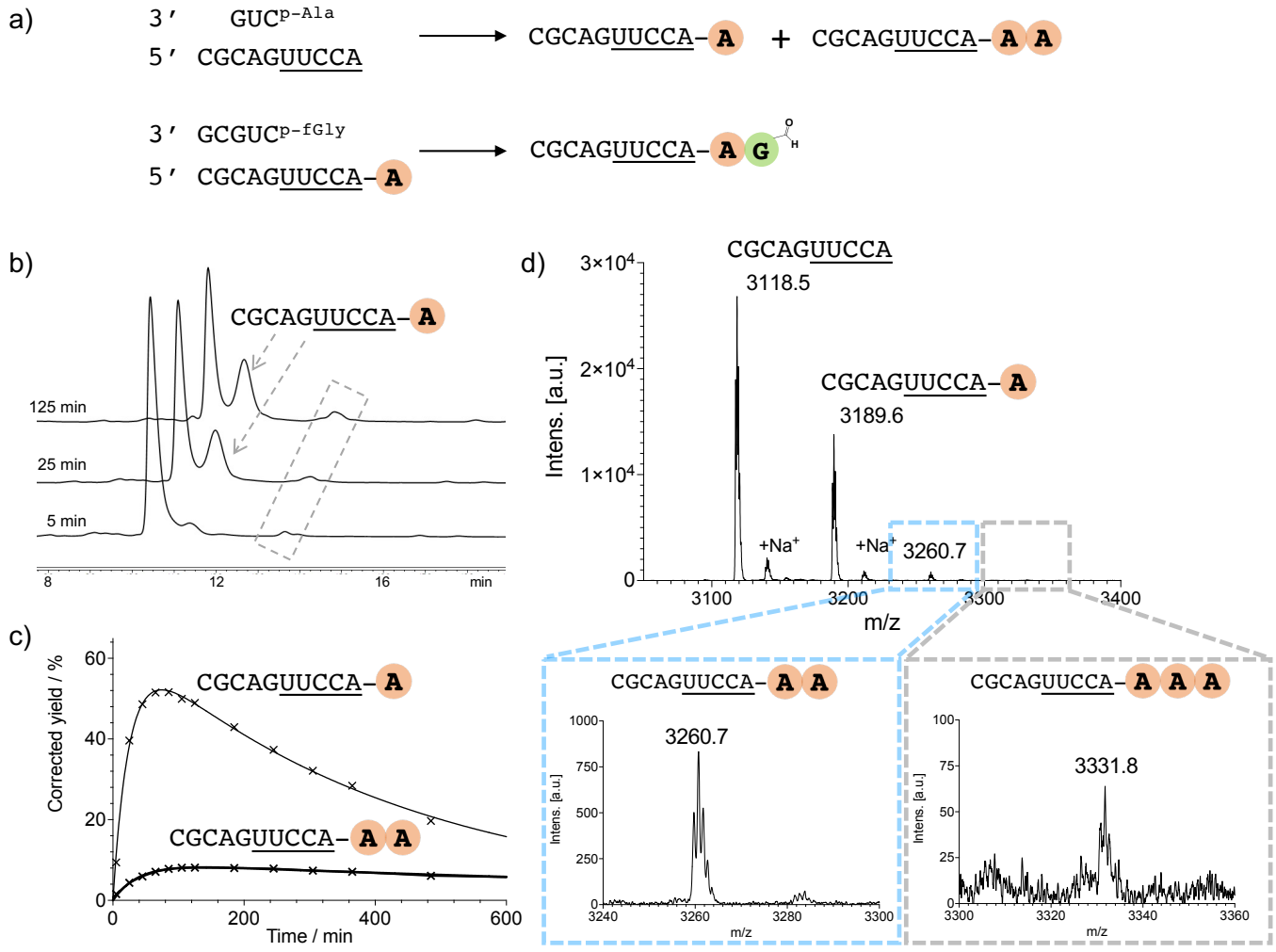

Fig. 1. Peptide synthesis from free alanine transfer. a) Schematic representation of aminoacyl-transfer; b) HPLC trace of alanyl mixed anhydride transfer over time. The broken box shows a new peak other than the 10-mer acceptor strand and its 2'/3'-Ala-esters , (indicated by the dashed arrow); c) Time courses of the 2'/3'-Ala-esters (thin line) and the 2'/3'-dipeptidyl-esters (putatively) corresponding to the new peaks over time (corrected for the mixed anhydride yield); d) MALDI-TOF results for the aminoacyl-transfer reaction, 2'/3'-Ala-Ala-esters, calculated 3260.6, found 3260.7; 2'/3'-trialanyl-esters, calculated 3331.7, found 3331.8. Conditions: Ala-mixed anhydride donor strand 3 eq., acceptor strand 100 μM, HEPES 50 mM, NaCl 100 mM, $MgCl_2$ 5 mM, pH 6.8, 16°C.

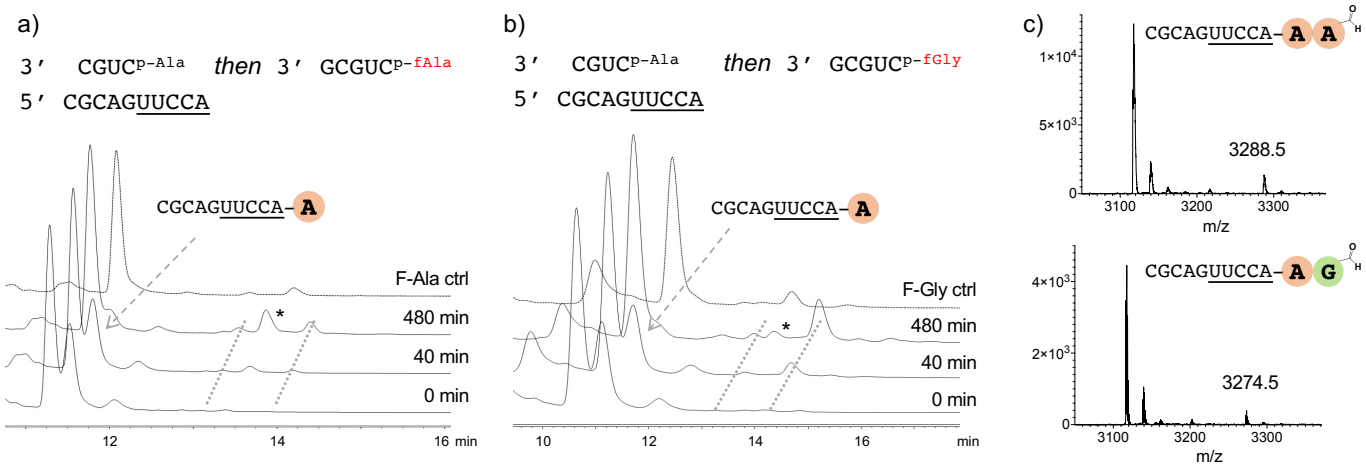

Fig. 2. Peptide synthesis with free alanine followed by formyl amino acids transfer. HPLC traces of a) fAla, and b) fGly transfer to 2′/3′-Ala-ester acceptors. The dotted lines show two new peaks corresponding to 2′/3′-*N*-formyldipeptidyl-ester acceptors. fAla and fGly ctrl indicate the reaction of fAla-/fGly-mixed anhydride donor transferred directly to the 2′/3′-diol of a 10-mer acceptor to give 2′/3′-fAla/fGly-esters. * indicates 2′/3′-fAla/fGly-esters. c) the respective MALDI spectra and data. Conditions: Ala-mixed anhydride donor strand 1 eq., *N*-formylaminoacyl-mixed anhydride donor strand 2 eq., acceptor strand 100 μM, HEPES 50 mM, NaCl 100 mM, $MgCl_2$ 5 mM, pH 6.8, 10°C. fAla/fGly Ctrl was performed at pH 8.0.

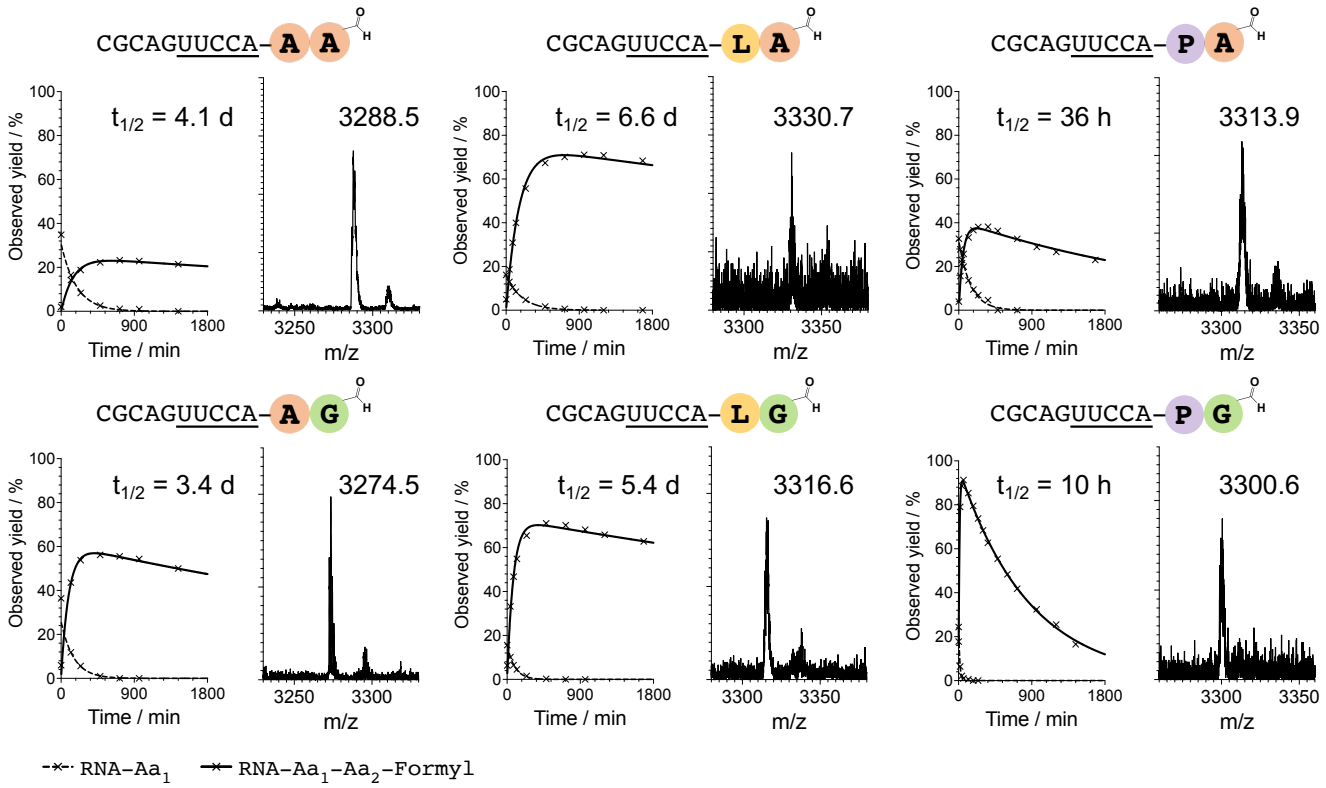

Fig. 3. Time courses and MALDI characterization of 2'/3'-*N*-formyldipeptidyl RNA. Timepoint 0 was set at the peak yield of 2'/3'-aminoacyl-esters at which point the formylaminoacyl-mixed anhydride donor was added. The solid line in the time course represents the percentage yield of 2'/3'-fGly-/fAla-dipeptidyl-esters relative to the 2'/3'-aminoacyl-esters. The broken line represents the consumption doe to hydrolysis and formylaminoacylation of the 2'/3'-aminoacyl-esters. Conditions: aminoacyl-mixed anhydride tetramer donor strand 1 eq., *N*-formyl-aminoacyl-mixed anhydride pentamer donor strand 2 eq., acceptor strand 100 μM, HEPES 50 mM, NaCl 100 mM, $MgCl_2$ 5 mM, pH 6.8, 10°C.

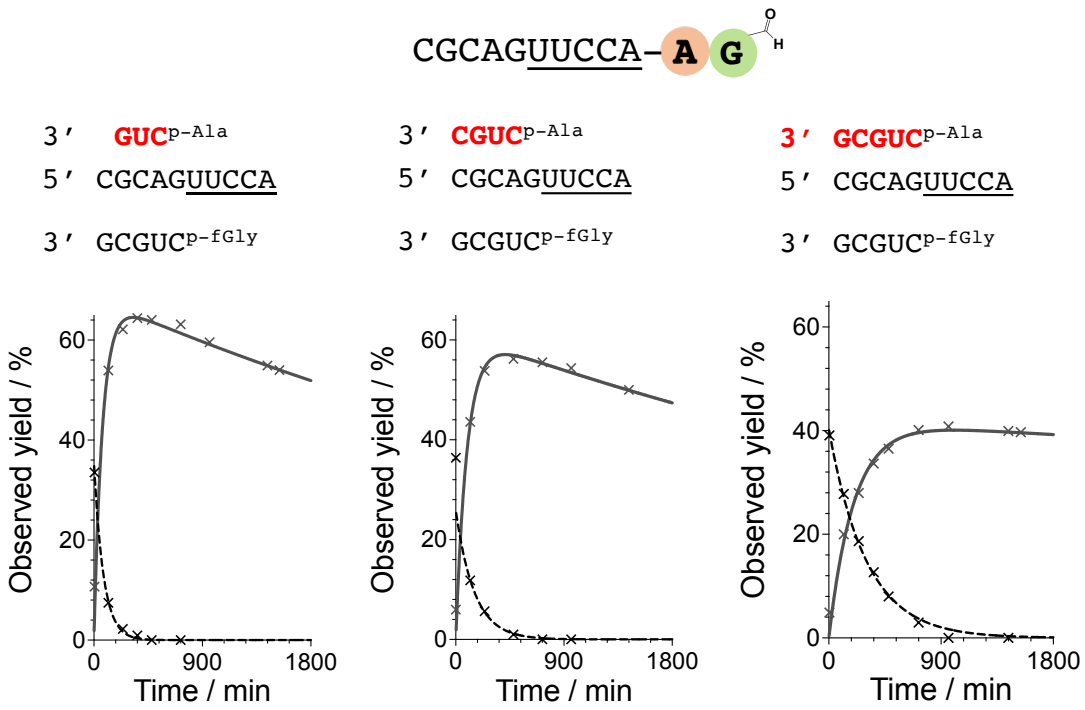

Fig. 4. Peptide synthesis is achieved by donor strand replacement. Time courses for the formation of 2′/3′-fGly-Ala-esters using different length Ala-mixed anhydride donor strands. Conditions: Ala-mixed anhydride donor strand 1 eq., *N*-formylglycyl-mixed anhydride donor strand 2 eq. added at the peak formation of 2′/3′-Ala-esters, acceptor strand 100 μM, HEPES 50 mM, NaCl 100 mM, $MgCl_2$ 5 mM, pH 6.8, 10°C.

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