

Efficient Access to Nonhydrolyzable Initiator tRNA Based on the Synthesis of 3'-Azido-3'-Deoxyadenosine RNA**

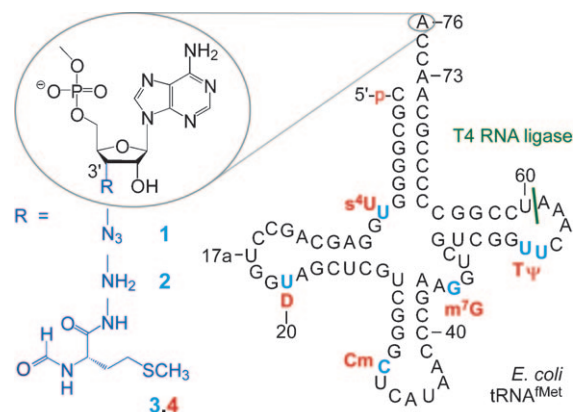
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Dedicated to Professor Karl Grubmayr on the occasion of his 60th birthday

3'-Aminoacyl-tRNA conjugates with a hydrolysis-resistant amide linkage instead of the natural ester represent valuable substrates for biochemical studies of ribosomal processes. Access to such conjugates is currently a serious bottleneck for the investigation and functional characterization of pre- and post-peptidyl-transfer states,^[1] of the tRNA hybrid states,^[2] of translation initiation^[2] and termination,^[3] as well as of phenomena like ribosome stalling.^[4] For the preparation of 3'-aminoacyl-tRNA with a stable amide linkage, an approach was originally developed in the 1970s that involved enzymatic degradation of the tRNA ..CCA76 3'-terminus to yield tRNA ..CC75 intermediates for the enzymatic attachment of 3'-amino-3'-deoxyadenosine using tRNA nucleotidyl transferase.^[5] The resulting tRNA provided a reactive 3'-NH₂ group, which was charged enzymatically with an amino acid by using the cognate tRNA synthetase. This method has been applied occasionally;^[1b,5c,d] however, it is rather inefficient. An early report on the acylation of 3'-NH₂-modified tRNA by N-protected amino acid hydroxysuccinimide esters or anhydrides had only faint resonance, most likely because of the poor selectivity and incomplete coupling.^[6]

Here, we introduce a novel combined chemical and enzymatic concept for the preparation of the *E. coli* initiator tRNA derivatives 3'-(*N*-formylmethionyl)amino-tRNA^{fMet} **3** and **4**. En route, our flexible approach allows access to the respective 3'-N₃- and 3'-NH₂-functionalized tRNAs **1** and **2** (Scheme 1).

The starting point for our undertaking was the 3'-azido-3'-deoxyadenosine derivative **5**, which is readily available according to a previously introduced synthesis.^[7] This compound was transformed into the pentafluorophenyl (Pfp) adipic acid ester **6** and finally into the functionalized solid support **7** (Scheme 2 A). We suspected the sterically hindered azide group of **7** to have limited reactivity in reactions with



Scheme 1. 3'-Modified *E. coli* tRNA^{Met} target structures 1–4 in this study. The aim was a flexible synthetic strategy to allow efficient access to 3'-N₃-, 3'-NH₂-, and 3'-(*N*-formylmethionyl)amino-derivatized tRNA with and without natural nucleoside modifications.

phosphoramidites and indeed found that this matrix was applicable to standard RNA solid-phase synthesis using nucleoside phosphoramidites as building blocks (Scheme 2B).

The 3'-N₃-RNA was quantitatively reduced by overnight incubation with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Figure 1B) and subsequently charged with fMet using the activated amino acid Pfp ester. Amide bond formation proceeded within 15 to 45 min and in 77–94 % yield depending on the length of the RNA (Figure 1C, Table 1; see the Supporting Information). To show that there is no unspecific reaction of the Pfp ester with the nucleobases, control experiments were conducted using RNA oligonucleotides with the genuine 3'-OH; no adducts were obtained under the conditions used. Also, when free fMet was incubated with 3'-NH₂-RNA, no unspecific aminor or imino adducts with the formyl group were observed. We further mention that *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) amino acid Pfp esters reacted significantly slower and only in poor

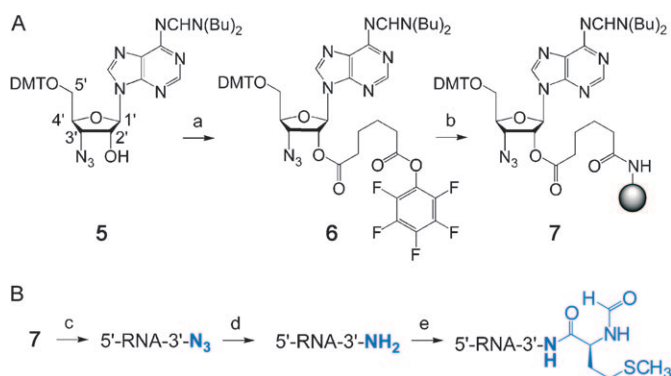
Table 1: Selection of synthesized 3'-azido-3'-deoxyadenosine RNA.

Sequence	Amount [nmol]	M_r (calcd) [amu]	M_r (obsd) [amu]
5'-ACCA-3'-N ₃	128	1231.8	1231.6
5'-AUC ₂ G ₂ C ₅ GCA ₂ C ₂ A-3'-N ₃	264	5673.5	5673.4
5'-p-AUC ₂ G ₂ C ₅ GCA ₂ C ₂ A-3'-N ₃	225	5753.5	5752.9
5'-p-UC ₂ G ₂ C ₅ GCA ₂ C ₂ A-3'-N ₃	370	5424.3	5424.0

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Scheme 2. Synthesis of the 3'-azido-3'-deoxyadenosine-derivatized solid support **7** and its use in RNA solid-phase synthesis. A) Reaction conditions: a) 4.7 equiv PfpOOC(CH₂)₄COOPfp, 1 equiv DMAP, in DMF/pyridine (1:1), room temperature, 1 h, 74%; b) 3 equiv (w/w) amino-functionalized support (GE Healthcare, Custom Primer Support 200 Amino), 2 equiv pyridine, in DMF, room temperature, 22 h, loading: 76 μmol g⁻¹. B) Reaction conditions: c) standard RNA solid-phase synthesis and deprotection; d) $c_{\text{RNA}} = 20 \mu\text{M}$, 0.5 mM TCEP, 100 mM Tris-HCl, pH 8, 1 d, -20°C, 95–98%; e) $c_{\text{RNA}} = 100 \mu\text{M}$, 25 mM fMet-OPfp, 100 mM Tris-HCl, pH 8, DMSO/H₂O (1:1), 37°C, 15–45 min, 77–94%. DMAP = 4-(dimethylamino)pyridine, DMF = *N,N*-dimethylformamide, DMSO = dimethyl sulfoxide. For details see the Supporting Information.

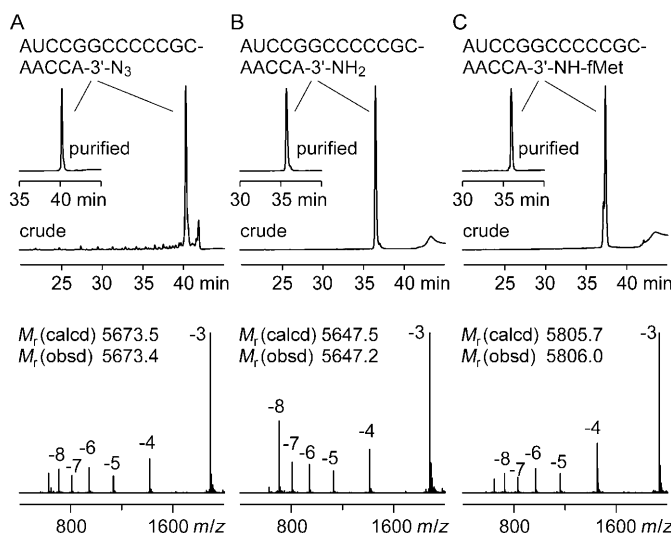


Figure 1. Characterization of 3'-modified 18 nt oligoribonucleotides; anion-exchange HPLC traces and LC-ESI mass spectra. A) RNA-3'-N₃. B) RNA-3'-NH₂. C) RNA-3'-NH-fMet. For conditions see the Supporting Information.

yields with 3'-NH₂-RNA. We also tested amino acids that were activated as thioesters and observed slow and incomplete coupling at best (see the Supporting Information). We considered that the electron-withdrawing properties of the *N*-formyl group ($-I$ effect) can be the reason for the advanced performance of the fMet-OPfp ester.

With this facile access to 3'-N₃-, 3'-NH₂-, and 3'-NH-fMet-modified RNA in our hands, we moved on to the synthesis of *E. coli* tRNA^{fMet} targets which we intended to achieve by

enzymatic ligation using T4 RNA ligase. We synthesized the 5'-phosphorylated 17 nucleotide (nt) fMet-RNA conjugate **8** and the respective 60 nt 5'-tRNA fragment **9** (Figure 2A). These RNAs aligned properly into a sufficiently stable pre-ligation complex so that the 5'-phosphate of the donor **8** came into close vicinity of the 3'-OH of the acceptor **9** to allow efficient ligation (77% yield, Figure 2B). The full-length tRNA-fMet conjugate was isolated by anion-exchange chromatography (1.9 nmol of purified **3**), and the correct mass was confirmed by LC-ESI mass spectrometry (Figure 2C). Likewise, when we applied the 5'-terminal tRNA^{fMet} fragment **10** carrying all genuine nucleoside modifications, a satisfying ligation yield of almost 70% was achieved (0.6 nmol of purified **4**, Figure 2D–F). Fragment **10** was readily obtained by cleavage of tRNA^{fMet} in the TΨC loop using a 10–23 DNA enzyme and subsequent dephosphorylation (see the Supporting Information). We mention that this particular generation of natural 5'-tRNA fragments with all nucleoside modifications is applicable also to other tRNA species, as demonstrated very recently in the context of nonhydrolyzable 3'-peptidyl-tRNAs.^[8]

In this study, we have demonstrated a novel approach for the efficient access to hydrolysis-resistant fMet-tRNA^{fMet} with and without the natural modification pattern. Moreover, we stress that the 3'-N₃- and 3'-NH₂-modified *E. coli* tRNA^{fMet} variants, **1** and **2**, respectively, were prepared in equally efficient manner (see the Supporting Information); thus, this approach is highly flexible, also for other types of tRNA, and from different organisms. Many potential applications are conceivable, since the 3'-amino group can be charged with other amino acids including nonnatural ones, by using either an appropriate chemical activation or potentially, also the flexizyme methodology.^[9] Another promising aspect is the use of 3'-azido-modified tRNA for cellular studies that focus on the action of tRNA modification enzymes.^[10] Since the 3'-azido group is bioorthogonal and generally does not affect cellular functions, direct isolation and/or labeling of these metabolized tRNA derivatives from cell lysates by means of one of the modern bioconjugation strategies, such as the Staudinger ligation or click chemistry,^[11] are within reach. Lastly, we mention that these studies have encouraged us to envisage and realize the synthesis of RNA with site-specific 2'-N₃ groups as potential siRNA reagents, on which we will report in near future.

Experimental Section

RNA solid-phase synthesis on the azido-modified support **7:** All oligonucleotides were synthesized on a Pharmacia Gene Assembler Special or Pharmacia Gene Assembler Plus following standard synthesis protocols. Detritylation (2.0 min): dichloroacetic acid/1,2-dichloroethane (4:96); coupling (3.0 min): phosphoramidites/acetonitrile (0.1M; 120 μL per coupling) were activated by benzylthiotetrazole/acetonitrile (0.3M; 360 μL per coupling); capping (3 × 0.4 min): A: Ac₂O/*sym*-collidine/acetonitrile (2:3:5), B: 4-(dimethylamino)pyridine/acetonitrile (0.5M), A/B = 1:1; oxidation (1.0 min): I₂ (10 mM) in acetonitrile/*sym*-collidine/H₂O (10:1:5). Solutions of amidites, tetrazole solutions, and acetonitrile were dried over activated molecular sieves (4 Å) overnight. All sequences were synthesized trityl-off.

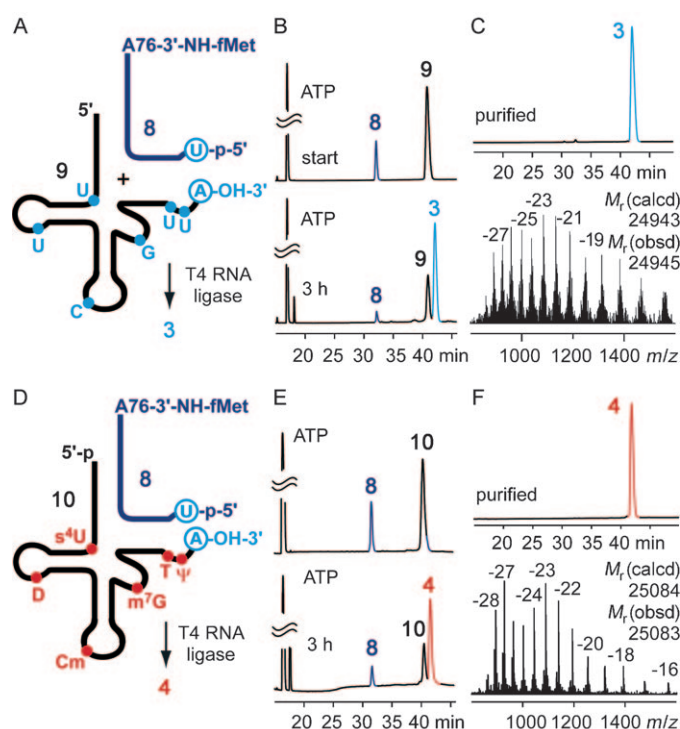


Figure 2. Enzymatic ligation of donor conjugate **8** using T4 RNA ligase to prepare tRNA^{fMet} derivatives **3** and **4**. Acceptor: Synthetic 5'-tRNA fragment **9** (A–C) or natural counterpart **10** containing genuine nucleoside modifications (D–F). HPLC profiles and LC-ESI mass spectra. For conditions see the Supporting Information.

Deprotection of RNA strands synthesized on the azido-modified support **7**: The beads were transferred into an Eppendorf tube, and equal volumes of CH₃NH₂ in EtOH (8M, 0.65 mL) and CH₃NH₂ in H₂O (40%, 0.65 mL) were added. The mixture was kept at room temperature for 8 h. After the supernatant was filtered and evaporated to dryness, the 2'-O-silyl ethers were removed by treatment with a 1.0M solution of tetrabutylammonium fluoride (TBAF)·3H₂O in THF (1.0 mL) for 16 h at 37°C. The reaction was quenched by the addition of triethylammonium acetate buffer (1.0M, pH 7.3, 1.0 mL). The volume of the solution was reduced to 0.5 mL and directly applied on a HiPrep 26/10 desalting column (GE Healthcare). The crude oligonucleotide was eluted with H₂O and subsequently evaporated to dryness. For analysis and purification of the 3'-azido-modified RNA see the Supporting Information.

Reduction of 3'-azido-3'-deoxyoligonucleotides: One equivalent of 3'-azido-3'-deoxyoligonucleotide (final concentration = 20 μM) and 25 equivalents of TCEP (tris(2-carboxyethyl)phosphine hydrochloride, final concentration = 0.5 mM) were dissolved in 100 mM Tris·HCl (pH 8.0). For crude 3'-azido-3'-deoxyoligonucleotides (not purified before reduction) the yield of the oligonucleotide (1 μmol scale) was estimated to be 400 nmol. After 24 h at –20°C, the reaction solution was desalted on a C18 SepPak Plus cartridge (Waters). The reduction, purification, and analysis of 3'-amino-3'-deoxyoligonucleotides were monitored by anion-exchange chromatography and LC-ESI mass spectrometry (for details see the Supporting Information).

For experimental procedures for fMet loading onto 3'-amino-3'-deoxyoligonucleotides and enzymatic ligation to obtain the corresponding tRNA derivatives **1–4** see the Supporting Information.

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