Modified RNA Derivatives

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

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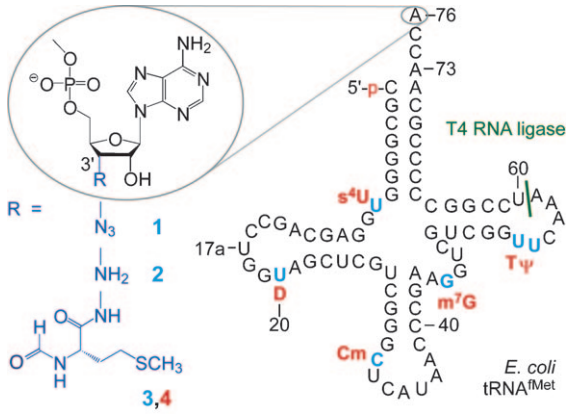
Dedicated to Professor Karl Grubmayr on the occasion of his 60th birthday

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′-NH_2 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′-NH_2-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′-N_3- and 3′-NH_2-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^{fMet} target structures **1**– **4** in this study. The aim was a flexible synthetic strategy to allow efficient access to 3′-N_3-, 3′-NH_2-, 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 2 B).

The 3′-N_3-RNA was quantitatively reduced by overnight incubation with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Figure 1 B) 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 1 C, 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′-NH_2-RNA, no unspecific aminal 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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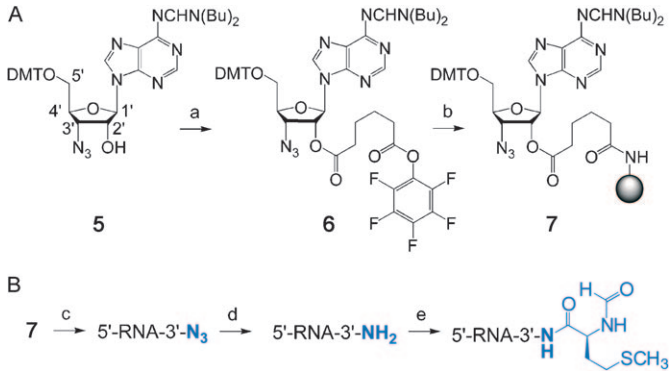

Scheme 2. Synthesis of the 3′-azido-3′-deoxyadenosine-derivatized solid support **7** and its use in RNA solid-phase synthesis. A) Reaction conditions: a) 4.7 equiv $PfpOOC(CH_2)_4COOPfp$, 1 equiv DMAP, in DMF/pyridine (1:1), room temperature, 1 h, 74%; b) 3 equiv (w/w) amino-functionalized support (GE 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_{RNA} = 20 μM, 0.5 mM TCEP, 100 mM Tris·HCl, pH 8, 1 d, −20 °C, 95–98%; e) c_{RNA} = 100 μM, 25 mM fMet-OPfp, 100 mM Tris·HCl, pH 8, DMSO/H_2O (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.

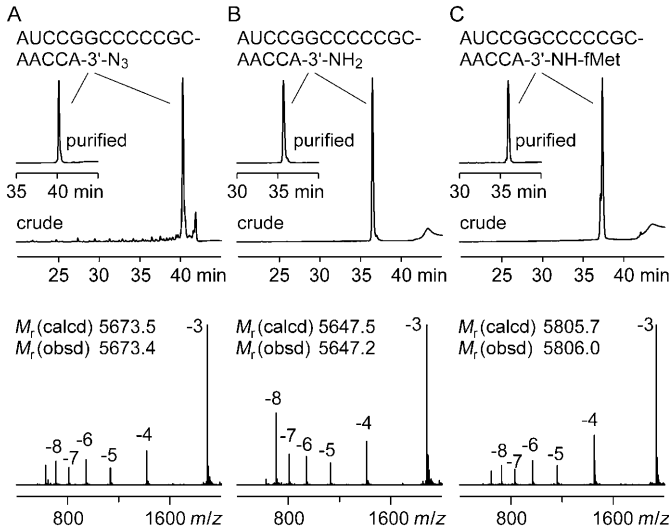

Figure 1. Characterization of 3′-modified 18 nt oligoribonucleotides; anion-exchange HPLC traces and LC-ESI mass spectra. A) RNA-3′-N_3. B) RNA-3′-NH_2. C) RNA-3′-NH-fMet. For conditions see the Supporting Information.

yields with 3′-NH_2-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′-N_3-, 3′-NH_2-, 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′-N_3- and 3′-NH_2-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′-N_3 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 (0.1 M; 120 μL per coupling) were activated by benzylthiotetrazole/acetonitrile (0.3 M; 360 μL per coupling); capping (3× 0.4 min): A: Ac_2O/*sym*-collidine/acetonitrile (2:3:5), B: 4-(dimethylamino)pyridine/acetonitrile (0.5 M), A/B = 1:1; oxidation (1.0 min): I_2 (10 mM) in acetonitrile/*sym*-collidine/H_2O (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.

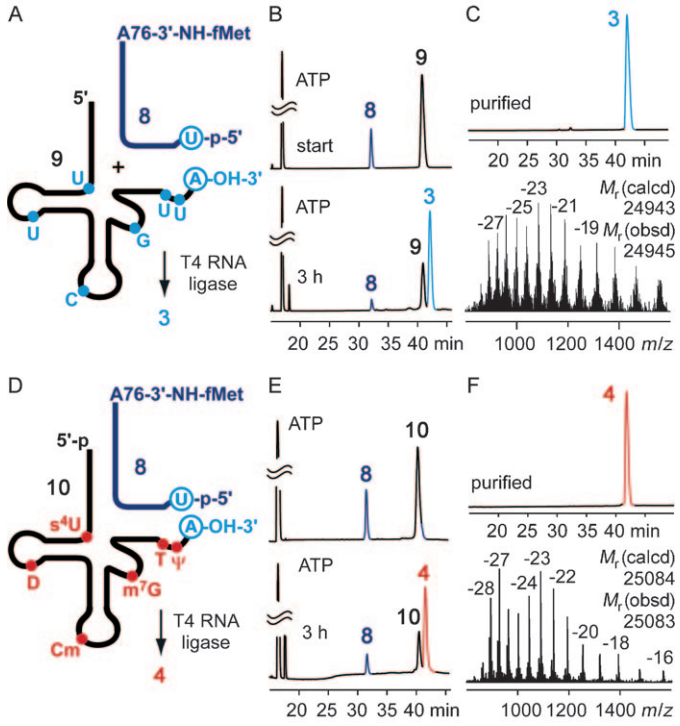

Figure 2. Enzymatic ligation of donor conjugate **8** using T4 RNA ligase to prepare $tRNA^{fMet}$ derivatives **3** and **4**. Acceptor: Synthetic 5′-tRNA fragment **9** (A–C) or natural counterpart **10** containing genuine nucleoside modifications (D–F). HPLC profiles and LC-ESI mass spectra. For 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 CH_3NH_2 in EtOH (8 M, 0.65 mL) and CH_3NH_2 in H_2O (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 1.0 M solution of tetrabutylammonium fluoride (TBAF)·$3H_2O$ in THF (1.0 mL) for 16 h at 37 °C. The reaction was quenched by the addition of triethylammonium acetate buffer (1.0 M, pH 7.3, 1.0 mL). The volume of the solution was reduced to 0.5 mL and directly applied on a Hi*Prep* 26/10 desalting column (GE Healthcare). The crude oligonucleotide was eluted with H_2O 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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