RNA Modifications

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Electrophilic RNA for Peptidyl-RNA Synthesis and Site-Specific Cross-Linking with tRNA-Binding Enzymes

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Abstract: *RNA functionalization is challenging due to the instability of RNA and the limited range of available enzymatic reactions. We developed a strategy based on solid phase synthesis and post-functionalization to introduce an electrophilic site at the 3′ end of tRNA analogues. The squarate diester used as an electrophile enabled sequential amidation and provided asymmetric squaramides with high selectivity. The squaramate-RNAs specifically reacted with the lysine of UDP-MurNAc-pentapeptide, a peptidoglycan precursor used by the aminoacyl-transferase $FemX_{Wv}$ for synthesis of the bacterial cell wall. The peptidyl-RNA obtained with squaramate-RNA and unprotected UDP-MurNAc-pentapeptide efficiently inhibited $FemX_{Wv}$. The squaramate unit also promoted specific cross-linking of RNA to the catalytic Lys of $FemX_{Wv}$ but not to related transferases recognizing different aminoacyl-tRNAs. Thus, squaramate-RNAs provide specificity for cross-linking with defined groups in complex biomolecules due to its unique reactivity.*

Chemical modification of RNA for conjugation with peptides, proteins, and other biomolecules is notoriously underdeveloped in spite of increasing interest in the field of chemical biology.[1] The most common methods are based on solid-phase synthesis (SPS) of RNA enabling the site-specific incorporation of reactive nucleotide analogues for post-functionalization.[2] Alternatively, modified nucleotides can be enzymatically incorporated into RNA but this strategy is limited by the specificity of RNA polymerases. Several approaches have successfully overcome this problem, including priming of in vitro transcription with modified nucleotides and dinucleotides,[3] 3′ extension with poly(A) polymerase,[4] post-transcriptional modifications by S-adenosylmethionine-dependent methyltransferases,[5] and expansion of the genetic code using unnatural bases both in the DNA template and RNA transcript.[6] Reaction of benzyl-guanine-RNA with the deaminase domain of the enzyme ADAR fused to the protein of interest provides RNA–protein adducts.[7] T4 RNA ligase offers flexibility, but this approach is restricted to modification of the 3′ extremity.

An initial challenge for RNA conjugation is the design of chemical groups that are compatible with SPS and RNA stability. A second challenge is the choice of a chemoselective reaction for the post-functionalization step. In the literature, nucleotide precursors were designed to incorporate azido or alkyne groups for Huisgen–Sharpless cycloaddition in RNA[3d,8] and DNA.[9] Aldehyde groups have also been incorporated for hydrazone formation.[10] Other reactions, such as Staudinger ligation,[11] or Michael addition,[12] have only been applied to DNA. Incorporation of amino or thiol nucleophiles is widely used because they are unreactive with the RNA core and their synthesis is based on commercially available phosphoramidites. However, this approach requires introduction of an electrophilic site or a thiol group into the biomolecule partner. For proteins, this can be achieved by site-directed mutagenesis to introduce a cysteine residue at the desired position. The incorporation of electrophilic groups has been based on modifications of natural amino acids, such as lysine residues, for introduction of a maleimide group,[13] or cysteine residues for conversion to dehydroalanine.[14] In contrast, the reverse approach based on electrophilic RNAs potentially provides access to cross-linking with unmodified peptides and proteins, which naturally contain

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nucleophilic sites. In that case, the main limitation is the lack of specificity for a defined residue in peptides and proteins. Herein, we report a new electrophilic RNA derivative based on a squarate group, which specifically reacts with amino groups (Figure 1). We report the synthesis of a peptidyl–RNA

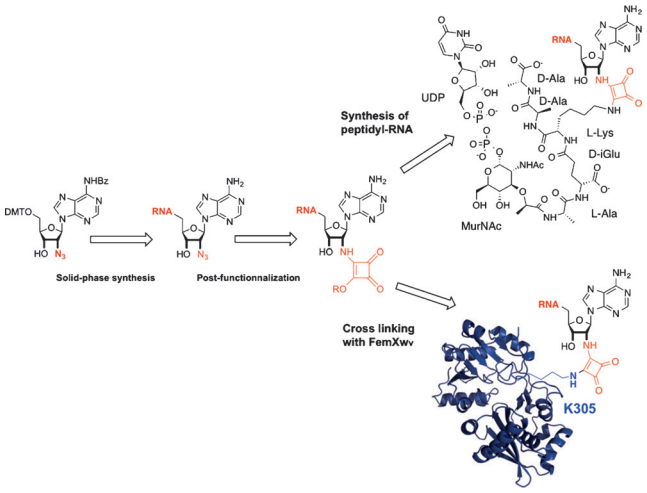
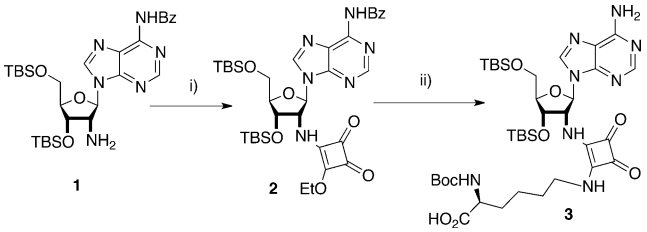


Figure 1. Synthesis of squaramate-RNA for peptidyl-RNA synthesis and cross-linking with active site K^{305} of the aminoacyl-transferase $FemX_{Wv}$. MurNAc, *N*-acetyl muramic acid; UDP, uridine-diphosphate.

conjugate and its biological evaluation as an inhibitor of the $FemX_{Wv}$ aminoacyl-transferase involved in bacterial cell wall synthesis.[15] We also show that the reactivity of the squaramate-RNA with surface-exposed non-catalytic lysine residues is limited. This enables specific formation of a covalent adduct between the squaramate-RNA and a catalytic lysine of the $FemX_{Wv}$ transferase. The unprecedented advantage of our methodology is that it does not require any modification of the protein or peptide partner used for the post-functionalization step.

Our strategy to access squaramate-RNA is based on incorporation of 2′-azido-2′-deoxyadenosine[16] at the 3′ extremity of RNA by SPS, reduction of the azido group, and addition of a squarate diester for subsequent ligation with amine containing partners (Figure 1). The relevant feature of the squarate diester group is the reduced reactivity of the mono-squaramide (also called squaramate) resulting from the first amidation step of the diester. This enables sequential amidation leading to asymmetric squaramides with high selectivity and in high yields. Diester-squarate-mediated coupling has been applied to synthesis of carbohydrate conjugates,[17] peptide-protein conjugates,[18] and oligodeoxyribonucleotide[19] or poly U-carbohydrate[20] conjugates. To investigate the relevance of this strategy for the synthesis of RNA–peptide conjugates, we first tested the two-step reaction using nucleoside and lysine as surrogates of RNA and peptide, respectively (Scheme 1).

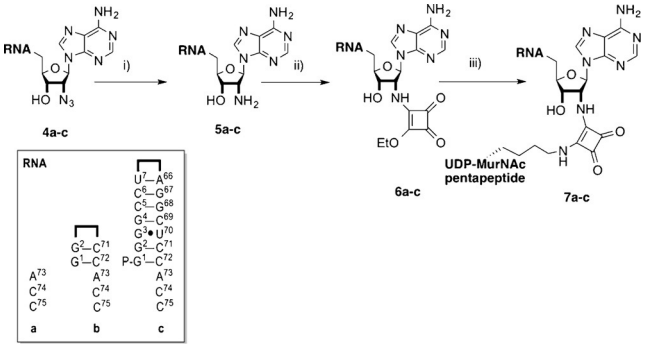
The synthesis started by addition of commercially available diethylsquarate to the 2′-deoxy-2′-amino-adenosine **1**[21] in basic conditions. The reaction provided 2′-squaramate adenosine **2** in 80% yield. As expected,[22] no symmetric bis-



Scheme 1. Synthesis of 2′-squaramate-adenosine **2** and bis-squaramide **3**. Conditions: i) Diethylsquarate, DIPEA, EtOH, RT, 1 h, 80%. ii) Boc-Lys-OH, DIPEA, EtOH, 40°C, 24 h, 73%.

squaramide formation was observed. Reaction of **2** with Boc-Lys in harsher conditions yielded the asymmetric bis-squaramide **3** in 73% yield. In those conditions, loss of the benzoyl protecting group of the nucleobase occurred but did not interfere with the reaction.

We then investigated squarate-mediated ligation for the synthesis of peptidyl–RNA conjugates. The RNA partners were oligonucleotides mimicking various portions of the tRNA acceptor arm (Scheme 2, inset). The peptidyl partner



Scheme 2. Synthesis of squaramate-RNAs and peptidyl-RNAs. Conditions: i) TCEP, Tris-HCl pH 8.0, H_2O, −20°C, 12 h, **5a** 51%, **5b** 44%, **5c** 47%; ii) Diethylsquarate (100 equiv.), HEPES pH 7.5, 20% DMF in H_2O, RT, 3 h, **6a** 79%, **6b** 75%, **6c** 75%; iii) UDP-MurNAc-pentapeptide, borate buffer pH 9.2, 10% DMF in H_2O, 37°C, 72 h, **7a** 23%, **7b** 40%, **7c** 45%. The inset shows the sequence of the oligonucleotides linked to the 5′ position of **4**, **5**, **6**, and **7**. Base-pairing in **b** and **c** is stabilized by a phosphoethylene glycol linker introduced by SPS.[25]

was UDP-MurNAc-pentapeptide, the native cytoplasmic precursor for synthesis of bacterial cell wall peptidoglycan[23] (Scheme 2). For optimization, we first tested the 2′-azido-ACCA tetranucleotide **4a**, which was obtained by SPS (Supporting Information). The ACCA sequence of **4a** corresponds to the single-strand 3′ extremity of most tRNAs. TCEP-mediated chemical reduction of the azido group[24] of **4a** afforded the 2′-amino-tetranucleotide **5a** in 51% yield. The addition of diethylsquarate onto **5a** led to squaramate **6a** in 79% yield. We then investigated the reaction of the squaramate **6a** with UDP-MurNAc-pentapeptide, which contains a single amino group (Figure 1). The RNA-UDP-MurNAc-pentapeptide **7a** was obtained in 23% yield in borate buffer (500 mM; pH 9.2) with an excess of UDP-MurNAc-pentapeptide (10 equivalents). The same

approach was used to obtain RNA-UDP-MurNAc-pentapeptide conjugates **7b** and **7c** containing larger portions of the tRNA acceptor arm. The conjugates **7b** and **7c** contained 2 and 7 base pairs of the acceptor arm, respectively, in addition to the ACCA extremity present in **7a**. Increasing the size of the RNA moiety did not reduce the yield of the reactions (Scheme 2). These results show that the squarate-mediated ligation provides a versatile route to peptidyl–RNA adducts. It is fully compatible with unprotected UDP-MuNAc-pentapeptide, which contains phosphate groups, carbohydrate hydroxyls, and carboxyl groups (Figure 1).

The RNA-UDP-MurNAc-pentapeptide conjugate **7c** was biologically evaluated as an inhibitor of the aminoacyl transferase $FemX_{Wv}$ from *Weissella viridescens*, which catalyzes the transfer of L-Ala from Ala-$tRNA^{Ala}$ to the peptidoglycan precursor UDP-MurNAc-pentapeptide[15] (Figure 2A). The conjugate **7c** was expected to inhibit $FemX_{Wv}$

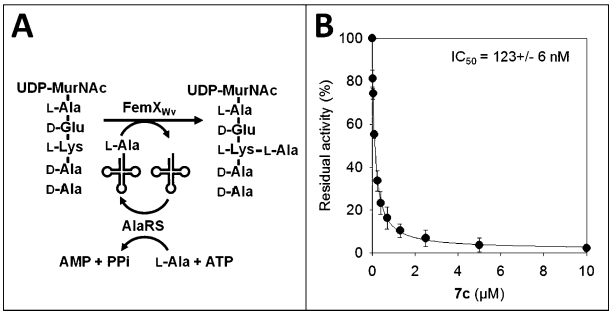
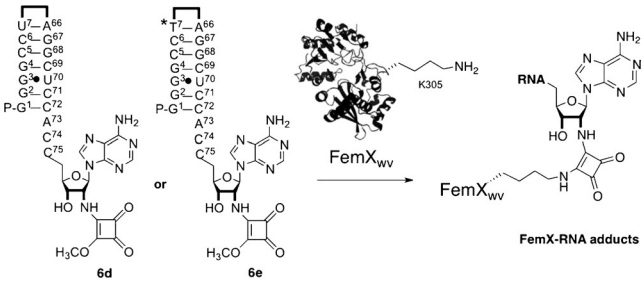


Figure 2. Inhibition of $FemX_{Wv}$ by the peptidyl-RNA **7c.** A) Amino-acyl transfer reaction catalyzed by $FemX_{Wv}$. AlaRS, alanyl-tRNA synthetase. B) Residual activity of $FemX_{Wv}$ in the presence of increasing concentrations of **7c**.

because it combines the two substrates of the aminoacyl-transfer reaction linked by a stable squaramide, as previously shown for similar bi-substrate inhibitors generated by Huisgen-Sharpless cycloaddition.[26] $FemX_{Wv}$ was inhibited by the conjugate **7c** with an IC_{50} of 123 ± 6 nM (Figure 2B). This value is three orders of magnitude higher than the IC_{50} of 0.15 ± 0.01 nM previously reported for the bi-substrate containing the same RNA and UDP-MurNAc-pentapeptide moieties linked by a triazole ring.[26a] Together, these results indicate that the squarate-mediated ligation generated a potent inhibitor, although the triazole linker is clearly favored within the $FemX_{Wv}$ active site. Nonetheless, the squaramide expands the repertoire of linkers available to generate bi-substrate inhibitors.

Our next objective was to test the cross-linking of squaramate-RNAs with $FemX_{Wv}$. Ala-$tRNA^{Ala}$ recognition by $FemX_{Wv}$ was previously shown to mainly involve interactions with the $tRNA^{Ala}$ acceptor arm.[26b,27] The $FemX_{Wv}$ protein partner of the ligation reaction contains 18 lysine residues in addition to the catalytic residue K^{305}, which is thought to stabilize the tetrahedral intermediate generated by nucleophilic attack of the carbonyl of Ala-$tRNA^{Ala}$ by the amine of UDP-MurNAc-pentapeptide.[26b] Incubation of $FemX_{Wv}$ with squaramate-RNA **6c** (Scheme 2) led to marginal cross-linking (data not shown). In contrast, squaramate-RNA **6d**, containing a methyl-ester-squarate instead of an ethyl-ester-squarate (Scheme 3), was efficiently ligated to $FemX_{Wv}$ (Figure 3A). The reaction was carried out in the conditions previously used for the formation of the RNA-UDP-MurNAc-pentapeptide adduct (500 mM borate buffer, pH 9.2, 5 h, 20 °C) using $FemX_{Wv}$ (50 μM) and **6d** (25 μM) in a 2:1 molar ratio. PAGE under denaturing conditions was used to monitor covalent cross-linking (Figure 3A). RNA–protein adducts were detected both by Coomassie blue staining and immuno-detection of $FemX_{Wv}$. Cross-linking of $FemX_{Wv}$ $K^{305}A$ mutant was not observed, indicating that none of the 18 other lysine residues of the protein reacted with squaramate-RNA **6d** (Figure 3A). Thus, the observed specificity for K^{305} likely results from binding of squaramate-RNA **6d** into the $FemX_{Wv}$ active site.

A fluorescein was introduced onto uracil of the U^7–A^{66} base pair in order to generate fluorescent probe **6e** (Scheme 3) for direct detection of the RNA moiety in RNA–protein adducts. The same RNA–protein adduct was observed by Coomassie blue staining, fluorescence, and



Scheme 3. Covalent cross-linking of squaramate-RNAs **6d** or **6e** with aminoacyl-transferase $FemX_{Wv}$. Conditions: 500 mM borate buffer, pH 9.2, 5 h, 20 °C, $FemX_{Wv}$ (50 μM), **6d** or **6e** (25 μM). (See the *T structural formula in the Supporting Information).

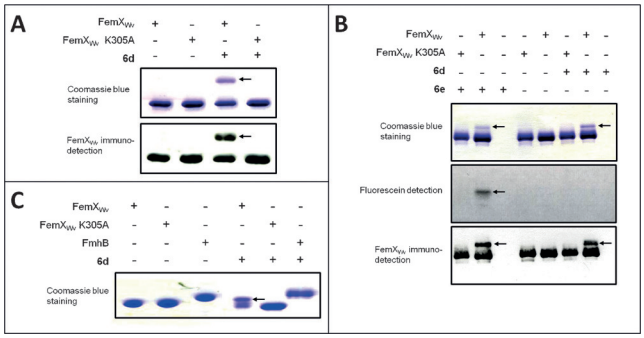


Figure 3. Analyses of the cross-linking of Fem transferases and squaramate-RNAs. A) Proteins and cross-linking adducts were separated by SDS-PAGE and detected by Coomassie blue staining and immuno-detection of $FemX_{Wv}$. B) Detection of cross-linking products obtained with squaramate **6d** and fluorescent squaramate **6e** by Coomassie blue staining, fluorescence, and Western blot. Immunodetection was performed with rabbit anti-$FemX_{Wv}$ primary antibodies and goat anti-rabbit secondary antibodies conjugated to horse radish peroxidase. C) Cross-linking of $FemX_{Wv}$, $FemX_{Wv}$ $K^{305}A$, and FmhB with squaramate **6d**. The arrows indicate the position of the $FemX_{Wv}$-squaramide adducts.

immuno-detection of $FemX_{Wv}$ (Figure 3B). This observation confirmed the presence of the $FemX_{Wv}$ and RNA moieties in the protein band detected by Coomassie blue staining. These results indicate that functionalization of RNA with fluorescein was fully compatible with the synthetic route of the squaramate-containing electrophilic RNA.

The role of RNA-binding in the cross-linking reaction involving K^{305} of $FemX_{WV}$ and the squaramate-RNA was further investigated by testing another member of the Fem aminoacyl-transferase family that does not recognize Ala-$tRNA^{Ala}$. We chose FmhB from *Staphylococcus aureus*, which specifically transfers a glycyl residue from Gly-$tRNA^{Gly}$ to peptidoglycan precursors and efficiently discriminate between Gly-$tRNA^{Gly}$ and Ala-$tRNA^{Ala}$.[28] In spite of the presence of the conserved K^{305} residue in the FmhB active site and of 49 additional lysine residues, no cross-linking of squaramate-RNA **6d** with FmhB was observed (Figure 3C). These observations confirm that binding of **6d** to the $FemX_{Wv}$ active site is required for cross-linking.

In conclusion, we have developed a versatile route for the synthesis of electrophilic RNAs based on post-functionalization of an azido group incorporated by SPS. The post-functionalization step involved the addition of a squarate diester following reduction of the azido group. This strategy was fully compatible with the introduction of other functionalities in the RNA moiety, including a fluorescent probe for the detection of RNA–enzyme adducts and a hairpin loop linker for stabilization of RNA base-pairing. The squaramate-RNAs specifically reacted with the primary amine of unprotected UDP-MurNAc-pentapeptide in spite of the presence of phosphate, hydroxyl, and carboxylic acid groups in this biomolecule. The first application of this methodology for peptidyl-RNA synthesis provided a potent inhibitor of $FemX_{Wv}$. A squaramate-RNA containing the acceptor arm of $tRNA^{Ala}$ was also found to specifically react with the catalytic Lys residue of $FemX_{Wv}$ (K^{305}), despite the presence of several additional lysines, including surface exposed residues. RNA binding into the $FemX_{Wv}$ catalytic cavity was essential for cross-linking because adducts were not observed with FmhB, which recognizes Gly-$tRNA^{Gly}$ instead of Ala-$tRNA^{Ala}$.

The unique cross-linking features of the squarate moiety provide a powerful and versatile tool for synthesis of peptidyl-RNAs. The only prerequisite is that the RNA carries an aliphatic primary amino group, which was generated from an azido group in the current study, but could also be incorporated using commercially available protected 2′-amino or 2′-aminoalkyl nucleoside building blocks, thereby providing access to bioconjugation at internal RNA positions. In contrast to others methods, natural peptides and proteins can be used for cross-linking without any modification. The assay conditions can be designed to provide specificity for lysine residues within enzyme active sites, as documented for $FemX_{Wv}$. The squaramate RNAs could therefore potentially be used to probe the reactivity of lysine residues in tRNA-dependent enzymes, generate stable RNA–protein adducts for X-ray diffraction analyses, and identify unknown tRNA-interacting proteins.

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