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Native Chemical Ligation of Hydrolysis-Resistant 3'-Peptidyl–tRNA Mimics

Anna-Skrollan Geiermann,[†] Norbert Polacek,[‡] and Ronald Micura^{*,†}[†]Institute of Organic Chemistry and Center for Molecular Biosciences, University of Innsbruck, Austria[‡]Innsbruck Biocenter, Division of Genomics and RNomics, Medical University of Innsbruck, Austria

S Supporting Information

ABSTRACT: Hydrolysis-resistant 3'-peptidyl–RNA conjugates that mimic tRNA termini represent a remarkable synthetic challenge, particularly if they contain amino acids with complex side-chain functionalities, such as arginines. Here we demonstrate a novel approach that combines solid-phase synthesis and bioconjugation to obtain these derivatives with high efficiency and purity. The key step is native chemical ligation of 3'-cysteinyl–RNA fragments to highly soluble peptide thioesters. The so-prepared 3'-peptidyl–RNA conjugates relate to resistance peptides that can render the ribosome resistant to macrolide antibiotics by a yet unknown ribosomal translation mechanism.

Hydrolysis-resistant RNA–peptide conjugates that mimic acylated tRNA termini are highly requested compounds for structural and functional studies of the ribosomal elongation cycle.¹ Such conjugates in their simplest form are represented by puromycin derivatives.^{2,3} They were positioned as stable substrates in the peptidyl transferase center (PTC) and gave rise to the first detailed mechanistic insights for ribosomal peptide bond formation based on high-resolution X-ray structures.^{3,4} In a very recent example, synthetic 3'-aminoacyl–RNA conjugates significantly contributed to our understanding of how the growing peptide chain communicates within the PTC in ribosomal stalling phenomena.⁵ Facile experimental access to this type of conjugate is expected to stimulate further investigations and functional characterization of different states of ribosomal translation, in particular of pre- and postpeptidyl transfer states⁶ and tRNA hybrid states⁷ as well as translation initiation,⁸ elongation,⁹ and termination.¹⁰ In addition, such conjugates will enable the exploration of peptide-mediated macrolide antibiotic resistance phenomena.¹¹

The synthesis of 3'-aminoacyl– and 3'-peptidyl–RNA conjugates represents a serious bottleneck for such studies, and therefore, our research program is devoted to the elaboration of straightforward routes to achieve these derivatives.¹² A solid foundation for their chemical synthesis has been presented that relies on the assembly of both the peptide and the RNA on the same functionalized solid support followed by cleavage and deprotection of the whole conjugate.^{13,14} This approach is convenient for most RNA and peptide sequences, but it suffers from certain limitations concerning the side-chain flexibility of the peptide moiety. For instance, partial oxidation of the N-terminal methionines of the resistance peptide–RNA targets has been a drawback of the original pathway. Moreover, attempts to obtain arginine-containing 3'-peptidyl–RNA conjugates by this approach have failed to date.¹⁵ One important class of 3'-peptidyl–RNA conjugates that frequently contain arginines function as so-called

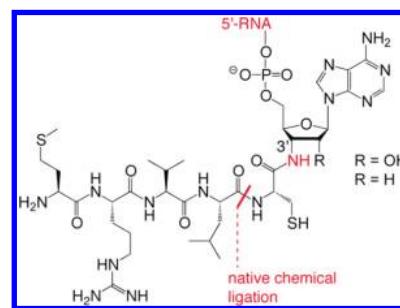


Figure 1. Novel strategy involving NCL for the synthesis of hydrolysis-resistant 3'-peptidyl–tRNA mimics. The peptide shown was chosen exemplarily and relates to a typical antibiotic resistance peptide.¹¹

resistance peptides.¹¹ When these peptides are translated, they can render the ribosome resistant to macrolide antibiotics by a mechanism yet to be explored. The prospect of an efficient synthetic access to enable investigations that can shed light onto this process, particularly in context with ribosomal X-ray structure determinations, prompted us to target arginine-containing 3'-peptidyl–tRNA mimics.

Since many sequences of resistance peptides contain cysteines beside arginines,¹¹ retrosynthetic analysis suggested a convergent pathway that involves native chemical ligation (NCL) as a potential route (Figure 1). NCL was originally designed to link unprotected peptide fragments under mild conditions.¹⁶ The process involves a reaction between a weakly activated C-terminal thioester and an unprotected N-terminal cysteine residue.¹⁷ The thermodynamic strength of an amide bond over a thioester bond is the driving force behind this reaction, which is made possible through a proximity-driven S-to-N acyl migration.

To achieve our specific targets, one fragment must be an RNA–peptide conjugate providing the 3'-terminal cysteine moiety while the other fragment, the arginine-containing peptide, must be functionalized as a thioester. On the basis of our previously elaborated synthetic approach for generating 3'-aminoacylamino-3'-deoxyadenosine derivatives,¹⁴ we synthesized the novel 3'-cysteinylamino-3'-deoxyadenosine-modified solid supports **1** and **2** by analogy (Figure 2A).

Starting from commercially available 3'-amino-2',3'-dideoxyadenosine, we obtained support **1** in four steps in good yields [Figure 2A; also see the Supporting Information (SI)]. Thereby, the cysteine thiol was masked as a *tert*-butyl disulfide group. This functional moiety was stable during RNA assembly and, as the sole protecting group, remained attached under the optimized conditions for cleavage of the conjugate from the solid support

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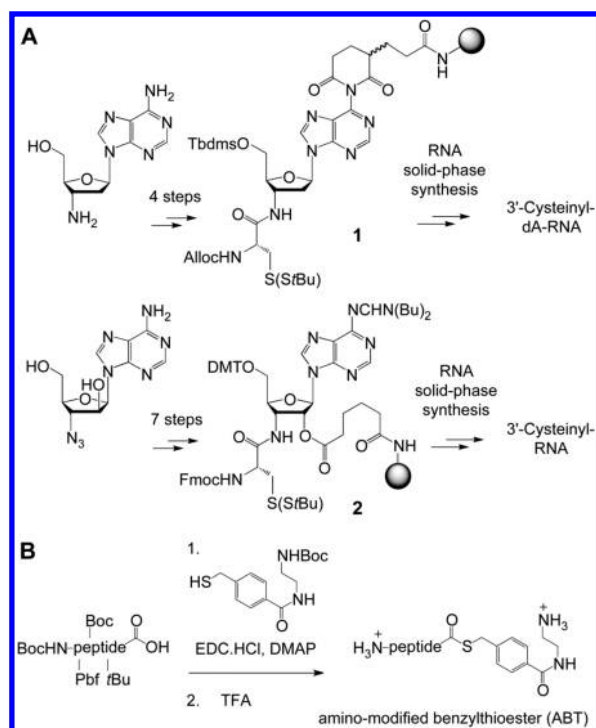


Figure 2. Fragment synthesis for NCL of 3'-peptidyl-RNA conjugates. (A) Novel solid supports **1** and **2** for the solid-phase synthesis of 3'-cysteinylamino-2',3'-dideoxy- and 3'-cysteinylamino-3'-deoxy-RNA (for synthetic details, see the SI). (B) Preparation of peptide thioesters with increased solubility in aqueous solution.

and deprotection (Figure 3A). Likewise, the corresponding 2'-deoxyadenosine solid support **2** was synthesized using *tert*-butyl disulfide side-chain protection [seven steps starting from readily available 9-(3'-azido-3'-deoxy- β -D-arabinofuranosyl)adenine;¹⁸ Figure 2A and the SI] and yielded the corresponding *S*-*t*Bu-masked 3'-cysteinyl conjugates (Figure 3A and Table 1).

Concerning the required peptide thioester fragments, we first prepared standard benzylthioesters using commercially available preloaded sulfamylbutyryl resins, Fmoc chemistry for peptide assembly, and *N*-*tert*-butoxycarbonyl (Boc) protection of the N-terminal methionine. Subsequent alkylation of the sulfamyl group followed by thiolysis and acidic deprotection provided the corresponding peptide benzylthioesters. However, the limited solubility of the synthesized thioesters restricted their efficient application for NCL. Therefore, to obtain thioesters with increased solubility, we synthesized amino-modified benzylthioesters (ABTs), which were introduced by Suga and co-workers¹⁹ in the context of flexizyme-catalyzed aminoacylation reactions of tRNAs. We synthesized the required *N*-(2-aminoethyl)-4-mercaptomethylbenzamide along the reported lines and prepared side-chain-protected peptide acids with an N-terminal Boc protecting group using Fmoc chemistry on a 2-chlorotriptyl chloride resin (Figure 2B).²⁰ Carbodiimide-promoted esterification resulted in the desired peptide 4-(*N*-(2-aminoethyl)carbamoyl)benzylthioesters, and indeed, these novel peptide thioester derivatives were endowed with significantly increased solubility in aqueous buffer solutions (Figure 3B and Table 1).

Having the 3'-cysteinyl-RNA and peptide-ABT fragments in hand, we attempted their NCL, which finally resulted in the workflow illustrated in Figure 4. Starting with a 22 nucleotide (nt) *S*-*t*Bu-protected 3'-cysteinyl-RNA minihelix and a 10-fold excess of leucine-ABT (Figure 5), we immediately observed conversion to

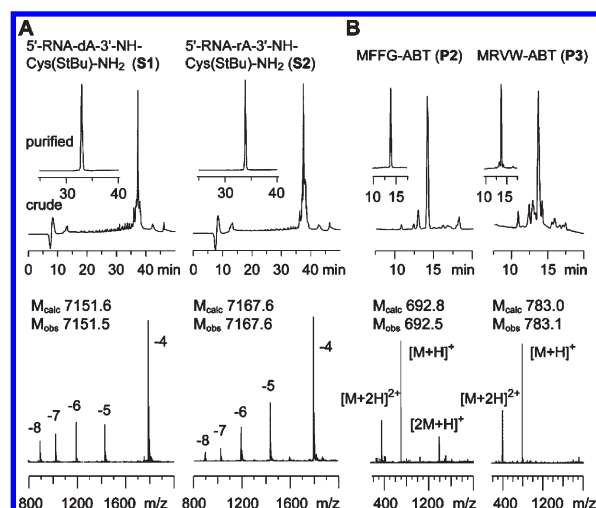


Figure 3. Characterization of 3'-cysteinyl-RNA and peptide thioesters. (A) Anion-exchange HPLC traces of (top) crude and (inset) purified Cys-RNAs **S1** and **S2** and (bottom) negative-ion-mode LC-ESI-mass spectra. (B) RP HPLC traces of (top) crude and (inset) purified thioesters **P2** and **P3** and (bottom) positive-ion-mode ESI mass spectra. For detailed conditions, see the SI.

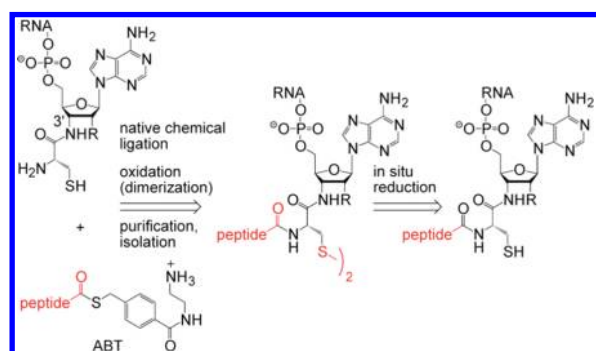
a major product under conditions typically applied for NCL reactions [involving a high concentration of urea, tris(carboxyethyl)phosphine for *S*-*t*Bu removal *in situ*,²¹ and thiophenol to form more reactive thioesters²² in sodium phosphate buffer at pH 7.5]. Yields were ~65% for ligations with 3'-cysteinylamino-2',3'-dideoxyadenosine-based thiol donors and ~55% for 3'-cysteinylamino-3'-deoxyadenosine donors, which may suggest that the 2'-OH is slightly disfavorable for the ligation reaction for either steric, conformational, or mechanistic reasons or a combination of these. Interestingly, we found by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) that the major peak in the analytical anion-exchange HPLC chromatogram corresponds to the disulfide-bridged homodimer of the desired 3'-leucylcysteinyl-RNA ligation product (Figure 5). The dimer was formed under the conditions of the HPLC buffer system, which contained up to 0.5 M LiClO₄. Replacement of LiClO₄ by NaCl led to a significant reduction in dimer formation. However, we considered the dimers advantageous because of their significantly slower migration and hence facile HPLC purification from unreacted 3'-cysteinyl-RNA, *S*-*t*Bu-protected starting material, and potential byproducts. Moreover, subsequent reduction of the dimers to the corresponding thiol monomers with dithiothreitol (DTT) proceeded cleanly and in quantitative yields. Excess DTT could be efficiently removed by flash chromatography on a short Sep-Pak cartridge filled with reversed-phase (RP) material. Because of the ease of handling, this synthesis/purification strategy was applied to all 3'-peptidyl-RNAs prepared here (Table 1). These conjugates were purified and isolated as dimers by anion-exchange chromatography, thus resulting in very high purities as evaluated by LC-ESI-MS (see the SI). We point out that we currently test mildly oxidative reagents as additives to the HPLC buffers for further optimization of the yields for dimer formation.

For NCL of our main targets carrying pentapeptide resistance sequences, we further optimized the ligation conditions and found that higher buffer concentrations of 1 M Tris·HCl and a pH value of 8.0 were advantageous for yields and reproducibility (Figure 6). We also tested conditions recently published by the Seitz group

Table 1. NCL of 3'-Cysteinylligonucleotides and Amino Acid/Peptide Thioesters to 3'-Peptidyl-tRNA Mimics

3'-cysteinylligonucleotide ^a	peptide thioester ^b	conditions ^c	product conjugate ^d	yield (%) ^e	<i>M</i> _{obs} (<i>M</i> _{calc}) (amu) ^f
GGGUGAUUUCGAUCACCC- ACCDa-3'-NH-Cys(StBu)-NH ₂ (S1)	Leu-ABT (P1)	A	RNA-ACCDa-3'-NH-CysLeu-NH ₂ (C1)	65	14352.0 (14351.1)
GGGUGAUUUCGAUCACCC- ACCA-3'-NH-Cys(StBu)-NH ₂ (S2)	Leu-ABT (P1)	A	RNA-ACCA-3'-NH-CysLeu-NH ₂ (C2)	55	14364.8 (14365.1)
GGGUGAUUUCGAUCACCC- ACCA-3'-NH-Cys(StBu)-NH ₂ (S2)	MFFG-ABT (P2)	B	RNA-ACCA-3'-NH-CGFFM-NH ₂ (C3) (clarythromycin)	54	15122.0 (15122.5)
GGGUGAUUUCGAUCACCC- ACCA-3'-NH-Cys(StBu)-NH ₂ (S2)	MRVW-ABT (P3)	B	RNA-ACCA-3'-NH-CWVRM-NH ₂ (C4) (telithromycin)	72	15302.2 (15303.3)
GGGUGAUUUCGAUCACCC- ACCA-3'-NH-Cys(StBu)-NH ₂ (S2)	MLLT-ABT (P4)	B	RNA-ACCA-3'-NH-CTLLM-NH ₂ (C5) (roxythromycin)	45	12840.6 (12839.1)
GGGUGAUUUCGAUCACCC- ACCA-3'-NH-Cys(StBu)-NH ₂ (S2)	MVLW-ABT (P5)	B	RNA-ACCA-3'-NH-CWLVM-NH ₂ (C6) (roxythromycin)	66	15074.0 (15073.8)
GGGUGAUUUCGAUCACCC- ACCA-3'-NH-Cys(StBu)-NH ₂ (S2)	MRVL-ABT (P6)	B	RNA-ACCA-3'-NH-CLVRM-NH ₂ (C7) (RU64399)	24	15216.2 (15216.2)
p-CUCCGAACGCGCC- UCCA-3'-NH-Cys(StBu)-NH ₂ (S3)	MRVW-ABT (P3)	B	RNA-UCCA-3'-NH-CWVRM-NH ₂ (C8) (telithromycin)	51	15156.1 (15159.2)

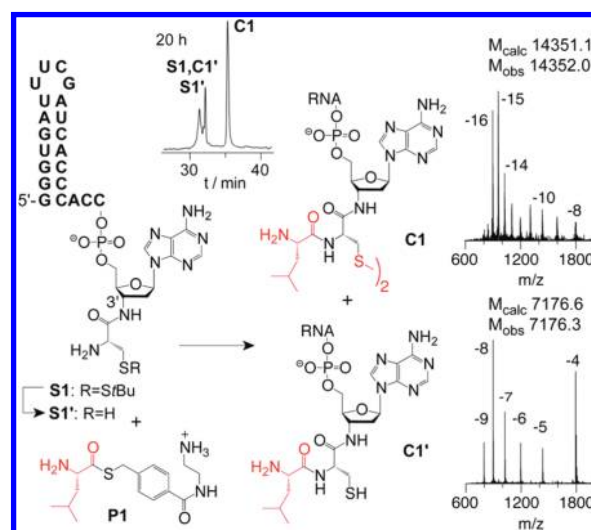
^a Oligonucleotide sequence in the 5'-to-3' direction. ^b Peptide sequence from the N-terminus to the C-terminus. ^c Conditions A: 0.25 mM **S_n**, 2.5 mM **P_n**, 0.1 M sodium phosphate (pH 7.5), 0.1 M TCEP, 2% (v/v) thiophenol. Conditions B: 0.25 mM **S_n**, 8 mM **P_n**, 7 M urea, 1 M Tris·HCl (pH 8.0), 0.1 M TCEP, 2% (v/v) thiophenol. ^d Isolated as the disulfide-bridged homodimer; the macrolide antibiotic to which resistance is conferred is noted in parentheses. Peptide sequence from the C-terminus to the N-terminus. ^e Determined from areas in HPLC profiles. ^f Molecular weights *M* of disulfide-bridged homodimers as obtained using LC-ESI ion-trap MS.

**Figure 4.** Workflow elaborated for NCL of 3'-cysteinyll-RNA and peptide thioesters to obtain high-quality 3'-peptidyl-RNA conjugates.

that rely on the sole additive of sodium 2-mercaptoethanesulfonate (MESNa)²³ but could not further increase the yields.

According to the strategy presented here, we synthesized a series of RNA-peptide conjugates that mimic 3'-acylated tRNA termini carrying pentapeptides associated with macrolide antibiotic resistance (C3–C8; Table 1). One of them, arginine-containing **C8**, was further successfully ligated to the chemically synthesized 56 nt 5' fragment of *Escherichia coli* tRNA^{Cys} using T4 RNA ligase¹² to obtain the corresponding full-length tRNA-peptide conjugate (Figure 7 left).

Furthermore, by employing ribosome chemical probing experiments, we demonstrated that this peptidyl-tRNA and the corresponding tRNA 3' fragment, the 18 nt RNA-3'-NH-CWVRM-NH₂ (**C8'**), bind to their expected binding sites in the peptidyl transferase center (PTC) of the ribosome. The location of interacting conjugates can be identified through analysis of diagnostic nucleobase protections in the 23S rRNA. P-site-bound tRNA protects characteristic nucleobases from chemical modifications: in particular, modification of U2585 with 1-cyclohexyl-3-(2-(*N*-methylmorpholinoethyl)carbodiimide *p*-toluenesulfonate (CMCT) is sensitive for tRNA binding to the

**Figure 5.** NCL of 3'-cysteinyll-RNA **S1** and leucine thioester **P1** to obtain the corresponding 3'-di-peptidyl-RNA conjugate. Substantial dimerization of the ligation products was observed. Conditions: see Table 1.

P-site.²⁴ The CMCT modifications cause a stop when the RNA is reverse-transcribed, and this results in a specific band on a polyacrylamide gel when the reverse-transcription products are separated by electrophoresis. The right panel of Figure 7 depicts an example of a typical polyacrylamide gel of primer extension analysis of *E. coli* ribosomes exposed to tRNA^{Cys}-3'-NH-CWVRM-NH₂, **C8'**, and the 18 nt RNA-3'-NH-Cys-NH₂ (**S3'**). From the protection pattern of U2585, it is evident that tRNA^{Cys}-3'-NH-CWVRM-NH₂ protects the P-site uridine to a comparable extent as the reference tRNA^{Tyr} while the footprint is less for the short single-stranded RNA-pentapeptide **C8'**. In contrast, the same RNA with a single cysteine, **S3'**, provided no footprint, indicating no binding to the ribosomal P-site under the used conditions.

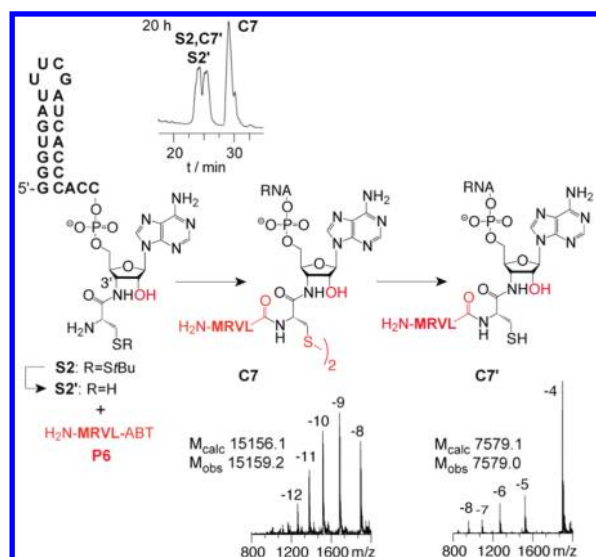


Figure 6. NCL of a 3'-peptidyl-tRNA mimic with an arginine-containing resistance peptide (MRVLC). Conditions: see Table 1.

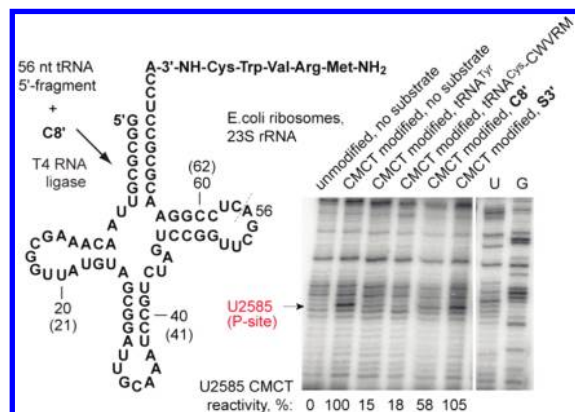


Figure 7. (left) Enzymatic ligation of the full-length tRNA^{Cys}-3'-NH-CWVRM-NH₂ and (right) ribosomal binding assay using chemical probing (by CMCT) and primer extension analysis of *E. coli* 23S rRNA. The footprint at U2585 indicates efficient tRNA-peptide conjugate binding to the ribosomal P-site of the peptidyl transferase center (PTC). For details, see the main text.

Taken together, these results indicate that our approach creates efficient access to hydrolysis-resistant, biologically active 3'-peptidyl-tRNA mimics. With the use of native chemical ligation, greater side-chain flexibility is achieved in comparison with the previously introduced route that relied exclusively on solid-phase synthesis.¹⁴ Using this novel approach, we have obtained five resistance peptide-RNA conjugates that are awaited for structural and functional ribosomal studies to shed light on that specific antibiotic resistance phenomenon.

■ ASSOCIATED CONTENT

Supporting Information. Procedures and characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author
ronald.micura@uibk.ac.at

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