Interaction of tRNA (uracil-5-)-methyltransferase with NO₂Ura-tRNA

Xiangrong Gu 1, Akira Matsuda 2, Kathryn M. Ivanetich 1,3 and Daniel V. Santi 1,*

1Departments of Biochemistry and Biophysics and of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0448, USA, 2Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan and 3Biomolecular Resource Center, University of California, San Francisco, CA 94143-0541, USA

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ABSTRACT

tRNA in which uracil is completely replaced by 5-nitro-uracil was prepared by substituting 5-nitro-UTP for UTP in an in vitro transcription reaction. The rationale was that the 5-nitro substituent activates the 6-carbon of the Ura heterocycle towards nucleophiles, and hence could provide mechanism-based inhibitors of enzymes which utilize this feature in their catalytic mechanism. When assayed shortly after mixing, the tRNA analog, NO₂Ura-tRNA, is a potent competitive inhibitor of tRNA-Ura methyl transferase (RUMT). Upon incubation, the analog causes a time-dependent inactivation of RUMT which could be reversed by dilution into a large excess of tRNA substrate. Covalent RUMT–NO₂Ura-tRNA complexes could be isolated on nitrocellulose filters or by SDS–PAGE. The interaction of RUMT and NO₂Ura-tRNA was deduced to involve formation of a reversible complex, followed by formation of a reversible covalent complex in which Cys 324 of RUMT is linked to the 6-position of NO₂Ura 54 in NO₂Ura-tRNA.

INTRODUCTION

Escherichia coli tRNA (uracil-5-)-methyltransferase (RUMT) catalyzes the S-adenosylmethionine (AdoMet)-dependent methylation of a specific Urd residue to form the m⁵U residue found in the T-loop of most prokaryotic and eukaryotic tRNA. The catalytic mechanism of RUMT is analogous to the mechanisms of thymidylate synthase (TS) and DNA-(m⁵C)-methyltransferase (1–3). The mechanism involves initial formation of a covalent Michael adduct between the thiol of Cys 324 of the enzyme and the 6-carbon of U54 of tRNA (2,4) which serves to activate the 5-position of U54 for the subsequent one-carbon transfer. We have previously reported that, in the absence of AdoMet, RUMT forms binary covalent complexes with unmodified tRNA²⁵⁵e or synthetic tRNA²⁵⁵e containing 5-fluoroUra substituted for Ura (FUra-tRNA), which may be isolated on nitrocellulose filters or by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (5).

The inhibitory and substrate properties of a number of 5-substituted dUMPs with TS suggested that electron withdrawing groups at the 5-position increase the affinity of such analogs for the enzyme, probably through increasing the reactivity of the 6-carbon toward nucleophiles (6–8). Indeed, C-6 of the analog 5-nitro-2-deoxyuridine monophosphate (NO₂dUMP) forms a sufficiently strong covalent attachment with thiols, that a covalent TS-NO₂dUMP complex can be physically isolated (9). Although covalent, the TS-NO₂dUMP complex is reversible, and free enzyme and inhibitor can be restored by β-elimination of the enzyme from C-6 of the pyrimidine. It was reasonable to believe that other enzymes whose mechanisms involve covalent attachment of a nucleophilic catalyst to C-6 of pyrimidine substrates would likewise be inhibited by substrates containing the NO₂Ura substituent. In this paper, we describe the synthesis and characterization of tRNA containing NO₂Ura, and its potent mechanism-based inhibition of RUMT.

MATERIALS AND METHODS

General

5-Nitro-uridine triphosphate (NO₂UTP) was prepared by a slight modification of the method of Huang and Torrence (10). Briefly, the trisodium salt of UTP (Yamasa, Choshi, Japan; 550 mg, 1 mmol) was mixed with a solution of nitronium tetrafluoroborate (Aldrich, 0.5 M, 10 ml) in sulfolane. The solution was stirred for 48 h at room temperature. Chloroform (50 ml) was added to the mixture, and the resulting precipitate was collected by centrifugation, washed with chloroform (2 × 20 ml), and dissolved in water (20 ml). The pH of the solution was adjusted to ~7 by the addition of concentrated ammonium hydroxide. The solution was applied to a DEAE–cellulose A-200m column (Chisso, Japan; 2 × 30 cm), which was washed with 0.05 M triethylammonium bicarbonate (pH 7.9, total volume 3 l). Appropriate triphosphate fractions were pooled, evaporated, and co-evaporated several times with water in vacuo to give NO₂UTP (~20% yield as a yellow glass).

Plasmid p67YF0 used for preparation of unmodified yeast tRNA²⁵⁵e was a gift from O. C. Uhlenbeck (Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO).
T7 RNA polymerase was isolated from E.coli BL21 harboring the plasmid pAR1219 (J. J. Dunn, Brookhaven National Laboratory, Upton, New York). [5-32P]pCp (3000 Ci/mmol) was from Amersham. [32P]RNA was purified on urea–PAGE (11). RUMT was purified as previously described (12,13).

RNA synthesis

T7 RNA polymerase-catalyzed in vitro synthesis of tRNA\textsuperscript{Phe} containing 5-nitroUra substituted for Ura (NO\textsubscript{2}Ura-tRNA) was performed using Bst\textsubscript{NI}-digested p67YFO and 100 \muM NO\textsubscript{2}UTP in place of 1 mM UTP as described (14). Products were purified using 7 M urea–20% PAGE. Unmodified tRNA, FUra-tRNA and m\textsuperscript{3}U54-tRNA were prepared as described (5). The concentrations of tRNA and NO\textsubscript{2}Ura-tRNA were calculated using 1.6 nmol per 1 A\textsubscript{260} (15).

3′-Labeling of RNA

In vitro synthesized NO\textsubscript{2}Ura-tRNA was labeled at the 3′-end with T4 RNA ligase (New England Biolabs) and varying concentrations of RUMT (0.05–4 \muM) in binding buffer [50 mM N-tris (hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES), pH 6.6, 5 mM dithiothreitol, 2 mM MgCl\textsubscript{2}, 1 mM EDTA and 50 mM NaCl] (2) were incubated at 15°C for 60 min, and assayed by nitrocellulose filtration (5,17). With saturating RUMT, ~50% of the NO\textsubscript{2}Ura-tRNA was trapped, which we assume represents the filtration efficiency for the combination of noncovalent and covalent complexes. The apparent dissociation constant (K\textsubscript{app}) for NO\textsubscript{2}Ura-tRNA was obtained by nonlinear least-squares fit of the binding data to the equation:

\[
\frac{\text{RNA}_{\text{Bound}}}{\text{RNA}_{\text{Total}}} = \frac{1}{1 + \frac{\text{K}_{\text{app}}}{\text{E}_{\text{Total}}}}
\]

The nitrocellulose binding assay was used to determine the bimolecular rate constant (k\textsubscript{1}) for association of RUMT and NO\textsubscript{2}Ura-tRNA to form isoform binary complexes. The [32P]NO\textsubscript{2}Ura-tRNA (1 nM) was incubated with specified concentrations of RUMT (50–150 nM) at 15°C in binding buffer. The initial rate was obtained from plots of bound RNA versus time over the first 10% of the reaction (5). The dissociation of total noncovalent plus covalent bound complexes obtained by adding a large excess of unlabelled, unmodified tRNA to equilibrated RUMT–[32P]NO\textsubscript{2}Ura-tRNA complexes, and measuring the bound radioactivity by the nitrocellulose filtration assay with time. The rate constants were calculated from the half lives for dissociation of total noncovalent plus covalent complexes obtained from a plot of bound RNA versus time (5).

K\textsubscript{app} was obtained by non-linear least squares fit of the binding data in Figure 4 to an equation that calculates free ligand concentration after correction for depletion of NO\textsubscript{2}Ura-tRNA by complex formation (18).

Gel shift assay

Gel shift assays were performed as previously described (2,5). Reaction mixtures (400 \muM) containing 50 nM [32P]NO\textsubscript{2}Ura-tRNA (2 × 10\textsuperscript{5} c.p.m.) and 8 \muM RUMT in binding buffer, were incubated at 15°C. Aliquots (20 \muM) were removed at the indicated times and the components were separated on SDS–12% PAGE. The [32P]-containing bands were excised, extracted and counted in 5 ml Aquasol II (5). Covalent complexes were quantitated as the fraction of total tRNA that was covalently bound.

Enzyme assay

Typically, reaction mixtures (20 \muM) containing 1 \muM unmodified tRNA, 25 \muM [\textsuperscript{3}H-Me]AdoMet (2 Ci/mmol) and 0.2 \muM RUMT in binding buffer were incubated at 15°C. An aliquot (18 \muM) was removed at a specified time for DEAE–paper disk assay (5). The assay efficiency was ~60% for \textsuperscript{3}H-methyl incorporation into tRNA (data not shown).

Data

Data points in the figures represent duplicate determinations.

RESULTS

Preparation and characterization of NO\textsubscript{2}Ura-tRNA

NO\textsubscript{2}Ura-tRNA was prepared by T7 RNA polymerase-catalyzed in vitro transcription of the yeast tRNA\textsuperscript{Phe} gene contained within p67YFO using NO\textsubscript{2}UTP instead of UTP. With NO\textsubscript{2}UTP, the reaction mixture yielded 0.5 A\textsubscript{260} of transcript/ml, which corresponds to 20 mol RNA/mol template. After the transcript was labeled with 5′-32PpCp, analysis on 7 M urea–20% PAGE showed a major radioactive RNA product that migrated as did unmodified tRNA. Complete RNase T2 digestion of this labeled product followed by separation of the products on two-dimensional
Figure 2. Competitive inhibition of RUMT by NO₂Ura-tRNA. Reaction mixtures (40 µl) contained varying concentrations of unmodified tRNA and NO₂Ura-tRNA, 25 µM [³H-Me]AdoMet (4 Ci/mmol) and 0.1 µM RUMT in binding buffer. The reactions were initiated by adding enzyme. After incubation at 15°C for 1–2 min, aliquots (18 µl) were removed for DEAE–paper disk assay. The NO₂Ura-tRNA concentrations were zero (○), 1 µM (●) and 3 µM (□). The lines were derived from a weighted fit of the data to the Michaelis–Menten equation. Kinetic constants were derived from weighted fits of the lines to the Michaelis–Menten equation or the linear competitive inhibitor equation (22).

TLC showed that ~90% of the transcripts had the expected 3' terminal adenosine (19). NO₂Ura-tRNA showed a sharp Tₘ of 84°C, which was 14°C higher than unmodified tRNA (Fig. 1).

**Inactivation of RUMT by NO₂Ura-tRNA**

NO₂Ura-tRNA was not methylated by RUMT (data not shown), but did inhibit the methylation of unmodified tRNA by RUMT. NO₂Ura-tRNA was a competitive inhibitor with respect to tRNA when the reaction was initiated with enzyme (Fig. 2). The following constants were calculated from Figure 2: Kₘ = 0.37 µM, kₗ = 1.9 min⁻¹ and Kᵢ = 1.0 µM.

For time-dependent inhibition studies, RUMT was preincubated with NO₂Ura-tRNA for the times indicated; at specified times, the solution was added to a standard assay mixture containing [³H-Me]AdoMet and unmodified tRNA, and initial velocities were determined. There was a time-dependent pseudo first order loss of enzyme activity (kₗ = 1.9 × 10⁻³ s⁻¹; Fig. 3). To demonstrate reversibility of the complex, 3.3 µM NO₂Ura-tRNA (15 × Kᵢ) was preincubated with 0.33 µM RUMT in binding buffer at 15°C for 30 min, then 30 µM unmodified tRNA was added. Aliquots were removed with time, and mixed with [³H-Me]AdoMet to determine the initial velocities. The activity of enzyme was restored following incubation of the NO₂Ura-tRNA–RUMT complex with unmodified tRNA for 60 min (data not shown).

**Interaction of NO₂Ura-tRNA with RUMT**

A minimal mechanism for the interaction of RUMT with NO₂Ura-tRNA is proposed in Scheme 1. First, there is reversible formation of the noncovalent RUMT–NO₂Ura-tRNA binary complex I characterized by rate constants k₁ and k₋₁ and dissociation constant K₁. Secondly, there is unimolecular conversion of I to one or more covalent complexes 2 characterized by apparent rate constants k₂ and k₋₂ and apparent dissociation constant K₂ (K₂ = [nonspecific complex]/[covalent complex] = k₂/k₁). We assessed these constants as described for the analogous RUMT–tRNA binary complexes (20).

The apparent dissociation constant (Kᵢ) for total RUMT–NO₂Ura-tRNA complexes was determined by measurement of the fraction of bound NO₂Ura-tRNA using a fixed concentration of [³²P]NO₂Ura-tRNA and a 1- to 80-fold molar excess of RUMT (Fig. 4). Kᵢ was 4-fold greater for NO₂Ura-tRNA than for unmodified tRNA (Table 1). Kᵢ is described in terms of the

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**Scheme 1**

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**Figure 3.** Inactivation of RUMT by NO₂Ura-tRNA. The preincubation mixtures (150 µl) containing 1.25 µM NO₂Ura-tRNA and 0.25 µM RUMT in binding buffer, were incubated at 15°C. Aliquots (32 µl) were removed when specified and mixed with 8 µl unmodified tRNA plus [³H-Me]AdoMet (2 Ci/mmol) for measurement of the initial velocity of methylation. The final concentrations in initial velocity experiments were 1 µM NO₂Ura-tRNA, 0.2 µM RUMT, 1 µM unmodified tRNA and 25 µM AdoMet. Aliquots (18 µl) were removed at 1 and 2 min for the DEAE–paper disk assay (○). Controls omitted NO₂Ura-tRNA from the preincubation mixture (□).

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**Figure 4.** Titration of NO₂Ura-tRNA with RUMT. Fraction of NO₂Ura-tRNA bound is plotted versus RUMT concentration. Reaction mixtures (20 µl) containing 50 nM [³²P]NO₂Ura-tRNA (1.4 × 10⁴ c.p.m.) and varying concentrations of RUMT (0.05–4 µM) were incubated at 15°C for 60 min, and assayed by nitrocellulose filtration. Data are corrected for filtration efficiencies (see Results).

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The apparent dissociation constant (Kᵢ) for total RUMT–NO₂Ura-tRNA complexes was determined by measurement of the fraction of bound NO₂Ura-tRNA using a fixed concentration of [³²P]NO₂Ura-tRNA and a 1- to 80-fold molar excess of RUMT (Fig. 4). Kᵢ was 4-fold greater for NO₂Ura-tRNA than for unmodified tRNA (Table 1). Kᵢ is described in terms of the...
equilibrium and kinetic constants of Scheme 1 by equations 1 and 2.

\[
K_{\text{app}} = \frac{K_1K_2}{1 + K_2} \quad 1
\]

\[
K_{\text{app}} = \frac{k_1 - k_{-2}}{k_1(k_{-2} + K_2)} \quad 2
\]

Nitrocellulose binding assay

A nitrocellulose filter binding assay commonly used to trap protein–RNA complexes was used to measure native noncovalent plus covalent RUMT–NO\textsubscript{2}Ura-tRNA complexes (5). In this assay, free \textsuperscript{[\textit{32}P]}\textit{NO}_2\textit{Ura}-tRNA is almost completely removed (counts <150 c.p.m.) (data not shown). At an 80-fold excess of RUMT, 50% of the added NO\textsubscript{2}Ura-tRNA was trapped, which represents the filtration efficiency of the assay (21). This is slightly lower than the filtration efficiency (65%) for the RUMT–tRNA complex (5).

Rates of association of RUMT and NO\textsubscript{2}Ura-tRNA were measured by mixing excess RUMT with \textsuperscript{[\textit{32}P]}\textit{NO}_2\textit{Ura}-tRNA, and measuring the nitrocellulose-bound radioactivity. Initial rates were obtained over the first 10% of the reaction from plots of bound RNA versus time (Fig. 5), and \(k_1\) values were calculated from the equation for a bimolecular reaction. The \(k_1\) was 4-fold lower for NO\textsubscript{2}Ura-tRNA than for unmodified tRNA (Table 1).

The dissociation of total noncovalent plus covalent bound complexes was monitored by adding a 20-fold excess of unlabelled competitor tRNA to pre-formed, equilibrated RUMT–\textsuperscript{[\textit{32}P]}\textit{NO}_2\textit{Ura}-tRNA complexes, and measuring the loss of bound radioactivity with time. As previously observed with RUMT–tRNA complexes (20), dissociation of the RUMT–\textsuperscript{[\textit{32}P]}\textit{NO}_2\textit{Ura}-tRNA complex was biphasic (Fig. 6). This occurs because the reaction is initiated from equilibrated covalent plus noncovalent complexes, and there is an initial relatively rapid depletion of noncovalent complex 1 (t_\text{1/2} \sim 0.55 min), followed by slower depletion of covalent complexes 2 (t_\text{1/2} \sim 21 min). The dissociation rate constants for the first and second phases, viz., \(k_1\) and \(k_{-2}\) respectively, were calculated from the half lives obtained from Figure 6. The \(k_1\) was 4-fold greater for NO\textsubscript{2}Ura-tRNA than for tRNA, while the \(k_{-2}\) values were comparable. Values for \(k_1\) and \(k_{-2}\) were also calculated from corresponding rate constants for association and equilibrium constants. For both dissociation rate constants, the calculated and experimental values were in good agreement (Table 1).

Table 1. Formation of the RUMT binary complex\textsuperscript{a}

<table>
<thead>
<tr>
<th>(\text{NO}_2\text{Ura-tRNA}\text{Phe})</th>
<th>(\text{tRNA}\text{Phe}\text{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_1)</td>
<td>(1.7 \times 10^{-4} \text{ M}^{-1} \text{s}^{-1})</td>
</tr>
<tr>
<td>(k_{-1}\text{c})</td>
<td>(21 \times 10^{-3} \text{s}^{-1})</td>
</tr>
<tr>
<td>(k_{-1}\text{d})</td>
<td>((12 \times 10^{-3} \text{s}^{-1}))</td>
</tr>
<tr>
<td>(K_1\text{e})</td>
<td>((68 \times 10^{-8} \text{ M}))</td>
</tr>
<tr>
<td>(k_2\text{f})</td>
<td>((14 \times 10^{-4} \text{s}^{-1}))</td>
</tr>
<tr>
<td>(k_{-2}\text{c})</td>
<td>((5.3 \times 10^{-4} \text{s}^{-1}))</td>
</tr>
<tr>
<td>(k_{-2}\text{f})</td>
<td>((5.5 \times 10^{-4} \text{s}^{-1}))</td>
</tr>
<tr>
<td>(K_2\text{h})</td>
<td>0.39</td>
</tr>
<tr>
<td>(K_{\text{app}}\text{i})</td>
<td>((19 \times 10^{-8} \text{ M}))</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Reactions were performed at pH 6.6 and 15°C. Values not in parentheses are from a single experiment. Values in parentheses were calculated from other constants.

\textsuperscript{b}Data for tRNA\text{Phe} is from reference (5).

\textsuperscript{c}Calculated from t\textsubscript{1/2} from \textsuperscript{[\textit{32}P]}\textit{NO}_2\textit{Ura-tRNA}\text{Phe} from Fig. 6.

\textsuperscript{d}Calculated from t\textsubscript{1/2} = K_1 \times k_1.

\textsuperscript{e}From equation 1.

\textsuperscript{f}From SDS–PAGE measurements of initial rate of covalent complex formation (data for \textsuperscript{[\textit{32}P]}\textit{NO}_2\textit{Ura-tRNA}\text{Phe} from Fig. 7).

\textsuperscript{g}From \(k_{-1} = K_1 \times k_1\).

\textsuperscript{h}From SDS–PAGE measurements of [noncovalent]/[covalent] complexes at equilibrium (data for \textsuperscript{[\textit{32}P]}\textit{NO}_2\textit{Ura-tRNA} from Fig. 7).

\textsuperscript{i}From nitrocellulose filter measurements of total bound complex (data for \textsuperscript{[\textit{32}P]}\textit{NO}_2\textit{Ura-tRNA} from Fig. 4).
The NO\textsubscript{2} Ura-tRNA was characterized by SDS–PAGE at varying times (Fig. 7). With the concentrations of rapid, and \( k_2 \) could be measured as the appearance of covalent components used, the formation of noncovalent complexes was assessed by mixing NO\textsubscript{2} Ura-tRNA with an excess of RUMT, and aliquots removed at varying times for assay. The total enzyme-bound NO\textsubscript{2} Ura-tRNA was measured by the nitrocellulose filter assay, the covalent component was estimated from the free NO\textsubscript{2} Ura-tRNA on SDS–PAGE. As indicated, the NO\textsubscript{2} Ura-tRNA was completely bound to RUMT (Table 1).

The experimental evidence for the mechanism of interaction of NO\textsubscript{2} Ura-tRNA with RUMT is as follows. First, NO\textsubscript{2} Ura-tRNA initially inhibits the methylation activity of RUMT competitively with respect to the substrate tRNA (Fig. 2). This implicates formation of a reversible RUMT–NO\textsubscript{2} Ura-tRNA complex (Scheme 1) analogous to the normal RUMT–tRNA binary complex. Secondly, upon preincubation of RUMT with NO\textsubscript{2} Ura-tRNA, there is a time-dependent loss of enzyme activity (Fig. 3). The enzyme inactivation is consistent with conversion of the noncovalent complex to a much tighter complex, such as covalent complex 2 (Scheme 1). Scheme 2 shows the conversion of the reversible complex to the covalent complex. Thirdly, the kinetics of dissociation of the RUMT–NO\textsubscript{2} Ura-tRNA complexes are biphasic (Fig. 6), suggesting the presence of at least two distinct chemical species which we have assigned to the non-covalent and covalent forms of the complex (1 and 2). Finally, SDS–PAGE allows isolation of a RUMT–NO\textsubscript{2} Ura-tRNA complex which is most consistent with covalent complex 2. Since Cys 324 of RUMT has been identified as the covalent catalyst of the normal reaction with unmodified tRNA, it is most reasonable to suggest that the Cys 324 thiol is the enzyme nucleophile attached to NO\textsubscript{2} Ura-tRNA in the covalent complex.

It is of interest to compare the kinetic and thermodynamic parameters of RUMT binding to NO\textsubscript{2} Ura-tRNA versus tRNA. The initial noncovalent complex of RUMT with the NO\textsubscript{2} Ura-tRNA analog is tight, but is ~10-fold weaker than the RUMT–tRNA complex. This is caused by a 4-fold lower \( k_{on} \) (\( k_1 \)) and a 4-fold higher \( k_{off} \) (\( k_{-1} \)) (Table 1). Since the interaction of tRNA with RUMT probably requires a conformational change to expose the T-arm (X. R. Gu, unpublished results), these parameters may be explained by the higher stability of the tertiary structure of NO\textsubscript{2} Ura-tRNA, as reflected by its higher melting temperature (Fig. 1). The NO\textsubscript{2} Ura-tRNA noncovalent complex undergoes covalent bond formation (\( k_2 \)) with the enzyme 6-fold faster than the tRNA noncovalent complex, and the resultant covalent complex is ~3-fold more stable (\( k_2 \)) (Table 1). Both effects may be explained by the electron withdrawing effects of the 5-NO\textsubscript{2} group on the electrophilicity of C-6, and the stability of resonance forms of 5-NO\textsubscript{2} 5,6-dihydro uracils (3). The latter may also explain why RUMT-catalyzed methylation of NO\textsubscript{2} Ura-tRNA by AdoMet does not occur.

**Scheme 2.**
In summary, NO₂Ura-tRNA represents a mechanism-based inhibitor of RUMT which was designed on the basis of knowledge of the binding and mechanistic properties of the enzyme, the chemical properties of the inhibitor, and precedent from analogous studies of the interaction of a small molecule counterpart with TS. NO₂Ura-tRNA is quite different from the other known inhibitor of RUMT, FUra-tRNA, in that it is not methylated at C-5. The relative simplicity of the RUMT–NO₂Ura-tRNA interaction and the stability of the covalent complex indicates that NO₂Ura-tRNA will be a useful tool for future investigation of this and other enzymes which bind nucleic acids.

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