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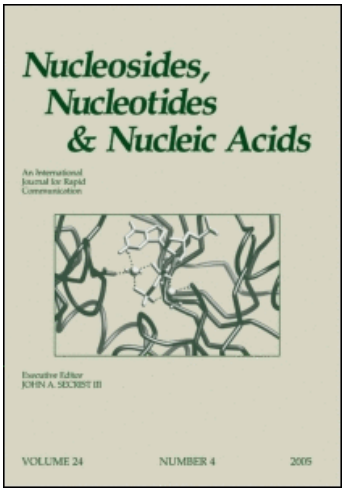

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Synthesis and Activity of Modified Cytidine 5'-Monophosphate Probes for T4 RNA Ligase 1

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SYNTHESIS AND ACTIVITY OF MODIFIED CYTIDINE 5′-MONOPHOSPHATE PROBES FOR T4 RNA LIGASE 1

Anilkumar R. Kore, Irudaya Charles, Litao Yang, and Scott Kuersten

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□ *We describe the synthesis of a series of unique base modified ligation probes such as p(5′)C-4-ethylenediamino **3**, p(5′)C-4-biotin **4**, and pre-adenylated form A(5′)pp(5′)C-4-biotin **6** and tested their biological activity with T4 RNA ligase 1 using a standard pCp probe **1** as a control. The intermolecular ligation assay was developed using a 5′-FAM labeled 24 mer single-stranded (ss) RNA and the average ligation efficiencies for pCp **1**, p(5′)C-4-ethylenediamino **3**, p(5′)C-4-biotin **4**, and pre-adenylated form A(5′)pp(5′)C-4-biotin **6** were found to be 44%, 81%, 39% and 16% respectively, as determined using a denaturing gel analysis. Furthermore, confirmation of the ligation activity of the biotinylated probes to the RNA substrate was confirmed by streptavidin conjugation and analysis by nondenaturing gel electrophoresis. These results strongly suggest that the new probes are valid substrates for T4 RNA ligase 1 and therefore could be useful for developing a miRNA detection system that includes rapid isolation, efficient labeling and detection of miRNAs on sensitivity-enhanced microarrays.*

Keywords T4 RNA ligase; pCp; p(5′)C-4-biotin; A(5′)pp(5′)C-4-biotin; base modified cytidine

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INTRODUCTION

The T4 RNA ligase 1 enzyme catalyzes the 3′→5′ phosphodiester bond formation of RNA molecules with concomitant hydrolysis of ATP to AMP and PP_i. While T4 RNA ligase 1 is useful in catalyzing the formation of a phosphodiester bond between an acceptor oligonucleotide and a corresponding donor and can also be used with some success with similar 2′-deoxyoligonucleotides, its effectiveness is limited in that it does not work equally well with all oligonucleotide sequences.[1–7] Generally, acceptors with a high purine content and pyrimidine-containing donors result in the

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highest yields of joined product. However, acceptor molecules of the same nucleoside composition but differing sequence can nevertheless react with a common donor and result in vastly different products.[7] The mechanism of phosphodiester bond formation as catalyzed by the enzyme is similar to that which has been elucidated for DNA ligase, although the substrate specificities are different.[8,9] DNA ligase requires double-stranded DNA substrates and T4 RNA ligase 1 is more active with single-stranded nucleic acids (both DNA and RNA). In both cases, three distinct and reversible steps are involved in phosphodiester bond formation.[10,11] In the first step, the enzyme reacts with ATP to form an adenylated enzyme intermediate with the release of pyrophosphate. In the second step, the adenyl group from the enzyme is transferred to the 5′-terminal phosphate of the donor to form an adenylated donor molecule A(5′)pp(5′)Np. The final step involves the nucleophilic attack of the 3′-hydroxyl group of the acceptor to the adenylated 5′-phosphoryl group of the donor to form a phosphodiester bond with the elimination of AMP. While several compounds of the class, Ado-5′PP-X (where X is any pyrimidine and purine base) have been tested for ligation,[6] to the best of our knowledge, no examples of using a biotin-containing or other modified pyrimidine base as X has been reported. In this class, the enzyme would catalyze the transfer of the nonadenylated portion of Ado-5′PP-X to the acceptor and would be considered an active ATP-independent donor. T4 RNA ligase 1 can tolerate certain modifications in the ribose, base, and 5′-P parts of donor molecules. For example, 2′-*O*-methylcytidine, 5′-thiophosphoryl-pNp derivatives, and 5′-phosphorothioate donor p(s)Ap are substrates.[2,12,13] In order to further explore the fidelity and substrate specificity of T4 RNA ligase 1 enzyme, we have synthesized the base modified cytidine and pre-adenylated forms of A(5′)pp(5′)C-4-biotin for substrate validation. The modified ligation probes may be useful for labeling and detection of RNA molecules with fluorescein or biotin moieties for use in microarray applications or perhaps as probes for in situ hybridization and detection.[14] In this communication, we report an efficient synthesis of modified ligation probes and their validation as legitimate substrates for T4 RNA ligase 1 using a 5′-FAM labeled 24 mer ssRNA.

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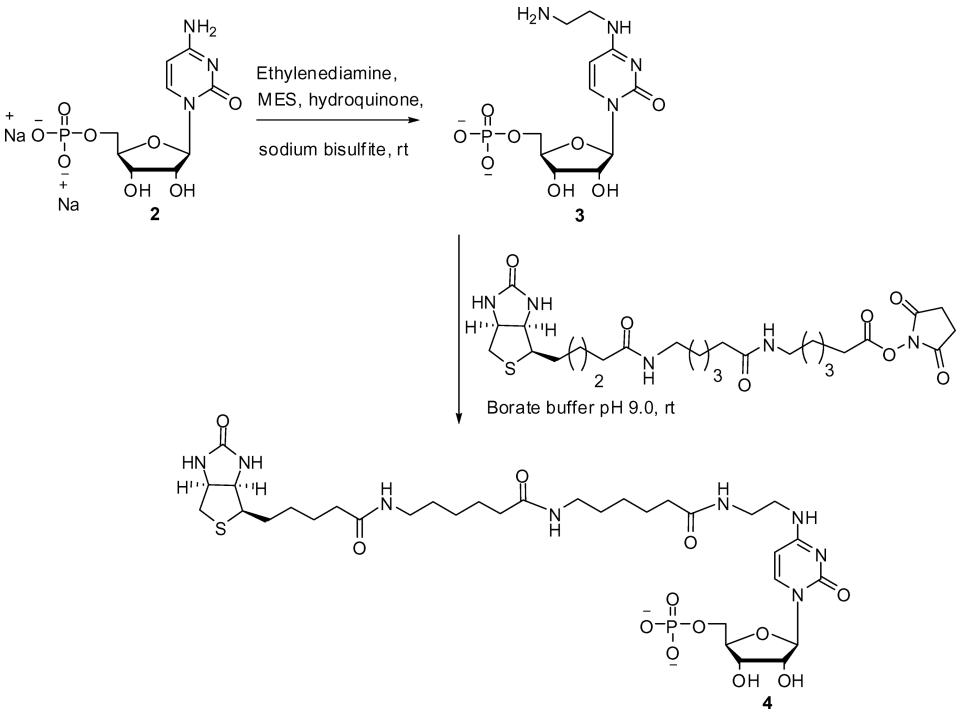
RESULTS AND DISCUSSION

The reaction pathway leading to the formation of desired probes **3** and **4** for biological evaluation is depicted in Scheme 1. The first step involves the introduction of ethylenediamino group at N^4-position of cytidine 5′-monophosphate. Thus, the reaction of cytidine monophosphate **2** with ethylenediamine in the presence of sodium bisulfite as the catalyst, MES as the buffer, and hydroquinone as the radical scavenger afforded the



SCHEME 1 Synthesis of p(5′)C-4-ethylenediamino **3** and p(5′)C-4-Biotin **4**.

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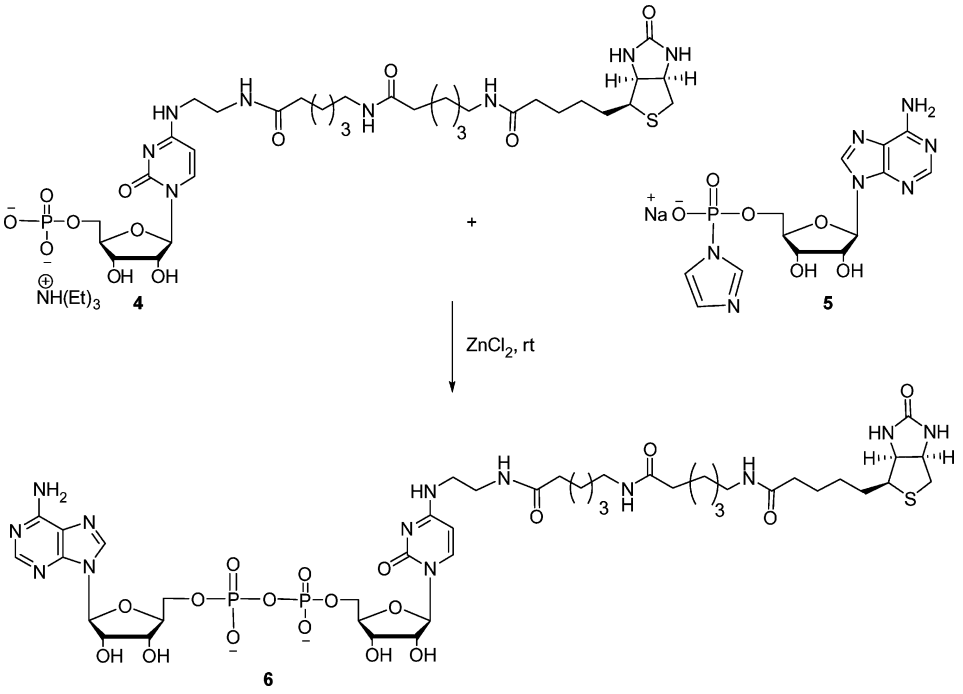
corresponding p(5′)C-4-ethylenediamino **3** in 76% yield. It is likely that the bisulfite adds across the 5,6 double bond of the pyrimidine, followed by the transamination at N^4 of the resulting bisulfite adduct of cytidine 5′-monophosphate with ethylenediamine and subsequent removal of bisulfite group to regenerate the double bond and afford the desired product **3**. It is noteworthy that this probe **3** contains a dangling linker arm with free reactive primary amine group at the end that will be available for postligation labeling with either biotin or fluorescein moiety. Finally, the reaction of **3** with EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce Biotechnology Inc., Rockford, IL, USA) in the presence of borate buffer, pH 9.0 affords the corresponding p(5′)C-4-biotin **4** in 56% yield. The structure of **3** and **4** was thoroughly characterized by ^{1}H and ^{31}P NMR and mass data. The compounds **3, 4**, and **6** were desalted as per reported procedure and NMR were recorded.[18]

The preparation of A(5′)pp(5′)C-4-biotin **6** is depicted in Scheme 2. The required starting material ImAMP **5** was prepared in 63% yield by the reaction of TEA salt of AMP with imidazole, triphenyl phosphine, and aldrithiol. Treatment of ImAMP **5** with TEA salt of p(5′)C-4-biotin **4** in the presence of $ZnCl_2$ as the catalyst furnished the final A(5′)pp(5′)C-4-biotin **6** in 56% yield.



SCHEME 2 Synthesis of A(5′)pp(5′)C-4-Biotin **6**.

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Ligation of New Probes to RNA Substrates Using T4 RNA Ligase 1

Labeling RNA 3′-termini is important for studies of gene regulation at the RNA level and RNA biological function in vitro or in vivo for clinical tests and research. In order to test whether the modified probes are legitimate substrates for T4 RNA ligase 1, we have optimized a convenient method for labeling a synthetic 5′-FAM labeled oligodeoxyribonucleotide at the 3′-hydroxyl termini with the enzyme T4 RNA ligase 1 using the modified cytidine probes. The conditions of this assay were chosen to mimic standard reactions used to label miRNA substrates for use in microarray-type experiments and involves a prolonged incubation time and a fixed enzyme concentration to insure maximal labeling of the substrates. Therefore, this type of 'end-point' assay is only designed to test the relative ability of each probe to ligate to a substrate under identical conditions and not to measure distinct kinetic parameters of the enzyme or substrates.

Three independent ligation experiments were carried out and the average ligation efficiencies for pCp **1**, p(5′)C-4-ethylenediamino **3**, p(5′)C-4-biotin **4**, and pre-adenylated form A(5′)pp(5′)C-4-biotin **6** were 44%, 81%, 39% and 16%, respectively (Figure 2B). From these results it was clear that the probe p(5′)C-4-ethylenediamino **3** was a very good substrate for T4 RNA ligase 1. This probe also has a free amine group available for

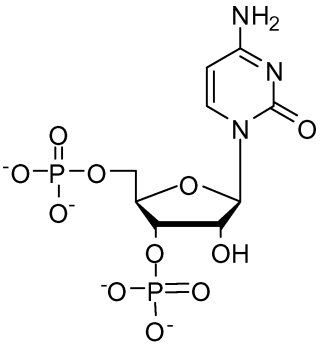

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FIGURE 1 Structure of standard pCp.

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postligation modification with either biotin or fluorescein moieties; a useful feature if this substrate is used for a hybridization based detection system like microarray technologies. The pre-adenylated probe A(5′)pp(5′)C-4-biotin **6** was not incorporated efficiently compared to probes **1**, **3**, and **4** possibly due to the modification having a bulky biotin group with a long linker arm. Interestingly, the pre-adenylated probe A(5′)pp(5′)C-4-biotin **6** was less efficient than p(5′)C-4-biotin **4** despite requiring only the final step of the three reaction events needed for complete ligation (the nucleophilic attack of the 3′-hydroxyl group of the acceptor to the adenylated 5′-phosphoryl group of the donor). This might be explained one of two ways. First, the presence of ATP in the reaction may have slightly prevented the pre-adenylated probe from reacting with the RNA substrate due to competitive inhibition with the enzyme. A second possibility is that the presence of the biotin moiety may reduce the ability of the enzyme to bind to the pre-adenylated probe; while in the context of the p(5′)C-4-biotin **4** reaction the complex is already assembled and is therefore not a limiting step.

Confirmation of Biotinylated Ligation Product Using Streptavidin Conjugation

To determine whether the ligated probes actually contain a biotin group as predicted, we incubated the ligated products with streptavidin protein and tested whether this would alter the molecular weight of the components as determined by a relative shift in mobility on a native polyacrylamide gel. A total of 2 μL of the above ligation mixtures were incubated with or without 15 μL 0.2 mM streptavidin and separated the products using a nondenaturing polyacylamide gel (Figure 3). This analysis clearly demonstrated that the FAM-labeled RNA substrates contained a biotin moiety following ligation and resulted in a mobility shift of the product to a higher molecular weight in the presence of streptavidin. Quantification

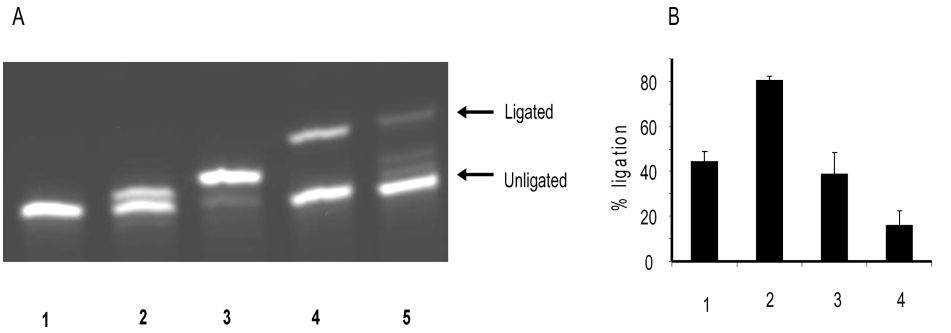

FIGURE 2 5′-FAM labeled 24 mer RNA oligonucleotide ligation with modified probes and T4 RNA ligase. A) 15% denaturing PAGE gel; lane 1 is a control ligation reaction where no probe was added, lane 2 represents ligation with standard pCp probe **1**, lane 3 was ligated with p(5′)C-4-ethylenediamino **3**, lane 4 was ligated with p(5′)C-4-biotin **4**, and lane 5 was ligated with A(5′)pp(5′)C-4-biotin **6**. B) Quantitation of the ligation efficiency. Lane 1 shows ligation efficiency of standard pCp 1 (44%), lane 2 shows ligation efficiency of p(5′)C-4-ethylenediamino **3** (81%), lane 3 shows ligation efficiency of p(5′)C-4-Biotin **4** (39%), and lane 4 shows ligation efficiency of A(5′)pp(5′)C-4-biotin **6** (16%).

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of the streptavidin shifted products indicated the ligation efficiencies of p(5′)C-4-biotin **4**, and pre-adenylated form A(5′)pp(5′)C-4-biotin **6** were 30% and 21%, respectively. The quantitated ligation efficiencies were lower than those obtained from the denaturing gel (Figure 3) probably due to the distinct mobility difference of the products in the two gel systems resulting in a strong compression of the bands representing biotinylated RNA conjugated with streptavidin, thus causing a weaker fluorescence intensity than in the denaturing gels.

EXPERIMENTAL

General Methods

All of the commercial reagents and solvents are used as such without further purification. ^{1}H NMR spectra were recorded in D_2O on a Bruker 400 MHZ and ^{31}P NMR were recorded on a Bruker 162 MHz. Chemical shifts are reported in ppm, and signals are described as s (singlet), d (doublet), t(triplet), q (quartet), and m (multiplet). ESI mass was recorded on an Applied Biosystems/Sciex MDX API 150 model and MALDI-TOF was recorded on an Applied Biosystems Voyager DE-PRO model.

Synthesis of Standard pCp 1

The synthesis of standard pCp **1** (Figure 1) was done on an automatic synthesizer by following conventional phosphoramidite protocols.[15] In a typical 5 μmol scale synthesis, the 3′-phosphate CPG was added to cytidine phosphoramidite followed by 5′-chemical phosphorylation Reagent II

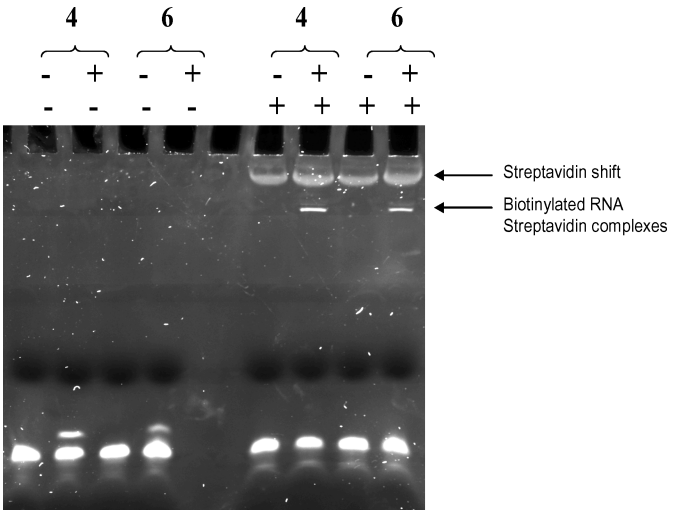

FIGURE 3 Streptavidin conjugation of biotinylated RNA oligonucleotides. The ligation products were incubated with or without streptavidin at 16°C for 16 hours. Biotinylated and unlabeled RNA were separated using a nondenaturing 12.5% PAGE gel. "−" and "+" on the top row indicate without (−) and with (+) p(5′)C-4-biotin **4** or A(5′)pp(5′)C-4-biotin **6** and those on the second row are reactions without (−) or with (+) streptavidin addition.

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(Glen Research, Sterling, VA, USA), that is, [3-(4,4′-Dimethoxytrityloxy)-2,2-dicarboxyethyl]propyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite] using standard phosphoramidite chemistry on a MerMade-12 and was purified by anion exchange FPLC. MS (m/z): 403 $[M\text{-}H]^-$.

Synthesis of p(5′)C-4-Ethylenediamino 3

Synthesis of p(5′)C-4-ethylenediamino, **3** was achieved as per reported procedure with little modifications.[16] To a stirred solution of ethylenediamine (5.19 mL, 78.00 mmol) in 3.00 mL water, MES (0.439 g, 2.247 mmol) and hydroquinone (0.012 g, 0.109 mmol) were added and the pH of the solution was adjusted to 7.0 using concentrated NaOH solution. To this solution, sodium bisulfite (1.460 g, 14.03 mmol) was added and the pH of the resulting solution was readjusted to 7.0. Then, a solution of cytidine monophosphate (1.00 g, 2.72 mmol) in 4.00 mL was added and the pH of the solution was adjusted to 6.2. The resulting reaction mixture was allowed to stir at room temperature for 24 hours. The reaction was quenched by adjusting the pH of the solution to 9.0 using NaOH solution and allowed to stir for 1 hour. Then, the pH of the resulting reaction mixture was adjusted to 5.5 and loaded on a DEAE Sepharose column. The desired product was eluted with 25–100% 1M triethylammonium bicarbonate buffer, pH 7.5 and the fractions containing the product were pooled, evaporated and dried in a vacuum desiccator over phosphorous pentoxide to obtain pure TEA salt

of p(5′)C-4-ethylenediamino, **3** (0.85 g, 76% yield) as a white solid. ^{1}H NMR (400 MHz, D_2O) δ 8.11 (d, $J = 7.6$ Hz, 1H), 6.15 (d, $J = 8.0$ Hz, 1H), 6.03 (d, $J = 4.0$ Hz, 1H), 4.36 (m, 2H), 4.26 (m, 1H), 4.07–3.94 (m, 2H), 3.72 (t, $J = 5.2$ Hz, 2H), 3.25 (t, $J = 5.6$ Hz, 2H); ^{31}P NMR (162 MHz, D_2O) δ 4.86; MS (m/z): 365 $[M\text{-}H]^-$.

Synthesis of p(5′)C-4-Biotin 4

The compound ethylenediamino CMP **3**, (0.05 g, 0.122 mmol) was dissolved in 2.00 mL of 0.1 M sodium borate (pH 9.0) and the solution was stirred for 15 minutes at room temperature. Then, the pH of the solution was adjusted to 9.0 using concentrated NaOH solution. To this solution, a freshly prepared solution of EZ-Link Sulfo-NHS-LC-LC-Biotin (0.098 g, 0.146 mmol) in DMSO (2 mL) was added slowly over a period of 10 minutes. The pH of the reaction mixture was re-adjusted to 9.0 and allowed to stir at room temperature overnight. After stirring overnight, the pH of the reaction mixture was adjusted to 5.5 and loaded on an AMBERCHROM XT20 RP Column (Buffer A: 50 mM TEAA, Buffer B: 50 mM TEAA + 25% Acetonitrile). The desired product was eluted with 2–50% buffer B and the fractions containing the product were pooled, evaporated and dried in a vacuum desiccator over phosphorous pentoxide to obtain pure p(5′)C-4-biotin **4** (0.052 g, 0.069 mmol, 56.3% yield) as a white solid. ^{1}H NMR (400 MHz, D_2O) δ 8.00 (d, $J = 7.2$ Hz, 1H), 6.07 (d, $J = 7.2$ Hz, 1H), 6.01 (d, $J = 4.0$ Hz, 1H), 4.61 (dd, $J = 4.8$, 8.0 Hz, 1H), 4.42 (dd, $J = 4.6$, 7.6 Hz, 1H), 4.33 (m, 2H), 4.24 (m, 1H), 4.07–3.95 (m, 2H), 3.61–3.47 (m, 4H), 3.33 (m, 1H), 3.16 (m, 4H), 2.99 (dd, $J = 4.8$, 13.2 Hz, 1H), 2.78 (d, $J = 12.8$ Hz, 1H), 2.23 (m, 6H), 1.77–1.23 (m, 18H); ^{31}P NMR (162 MHz, D_2O) δ 4.43; MS (m/z): 818 $[M]^+$.

Preparation of ImAMP 5

Synthesis of the imadazolide salt of AMP **5** was prepared as per a reported procedure.[17] Commercially available sodium salt of AMP (Sigma-Aldrich, St. Louis, MO, USA) was passed through DEAE sepahadex column and eluted with 1M TEAB buffer (pH 7.5) to obtain the corresponding triethylamine salt of AMP. To a stirred solution of triethylamine salt of AMP (0.25 g, 0.455 mmol) in 5.00 mL DMF, triphenylphosphine (0.239 g; 0.910 mmol), imidazole (0.155 g; 2.274 mmol), and aldrithiol (0.200 g; 0.910 mmol) were added. To this solution, triethylamine (0.070 mL, 0.500 mmol) was added and the solution was stirred overnight. Then, the reaction mixture was centrifuged and the supernatant solution was collected and kept at 4°C. To a solution of 1.0 g of $NaClO_4$ in 100 mL of acetone maintained at −20°C for 2 hours was added the above supernatant solution in order to precipitate ImAMP. The heterogeneous solution was centrifuged

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and the supernatant solution was discarded. The precipitate was washed with 3 × 50 mL of acetone to remove yellow colored impurities/byproducts and residual sodium perchlorate. The resulting solid was dried under vacuum over P_2O_5 for 2 hours to afford pure ImAMP **5** (0.12 g, 62.9% yield) as a white solid. This compound was stored at −20°C.

Preparation of A(5′)pp(5′)C-4-Biotin 6

The final conjugation was carried out as per previously reported procedure.[18] To a stirred solution of TEA salt of p(5′)C-4-biotin **4** (0.05 g; 0.060 mmol) and ImAMP **5** (0.028 g; 0.066 mmol) in 2.0 mL of DMF, zinc chloride (0.00822 g; 0.060 mmol) was added and the reaction mixture was stirred overnight. Then, the reaction mixture was added to a solution of EDTA (0.020 g; 0.060 mmol) in 10.0 mL of water and stirred for 10 minutes. The resulting aqueous solution was adjusted to pH 5.5 and loaded on a DEAE Sepharose column. The desired product was eluted with 25–100% 1M triethylammonium bicarbonate buffer, pH 7.5 and the fractions containing the product were pooled, evaporated and dried in vacuum desiccator over phosphorous pentoxide to obtain A(5′)pp(5′)C-4-biotin **6** (0.035 g, 56% yield) as a white solid. ^{1}H NMR (400 MHz, D_2O) δ 8.42 (s, 1H), 8.20 (s, 1H), 7.59 (d, J = 7.6 Hz, 1H), 6.08 (d, J = 5.2 Hz, 1H), 5.87 (d, J = 4.0 Hz, 1H), 5.73 (d, J = 7.2 Hz, 1H), 4.75 (m, 1H), 4.60 (m, 1H), 4.51 (m, 1H), 4.40 (m, 2H), 4.32–4.15 (m, 7H), 3.41–3.26 (m, 5H), 3.14 (m, 4H), 2.97 (dd, J = 4.2, 12.8 Hz, 1H), 2.77 (d, J = 12.4 Hz, 1H), 2.22 (m, 6H), 1.73–1.21 (m, 18H); ^{31}P NMR (162 MHz, D_2O) δ −10.14; MS (m/z): 1148 $[M+H]^+$.

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Intermolecular Ligation Assay for Modified Probes with T4 RNA Ligase 1

The RNA oligonucleotide for ligation with modified probes **3**, **4**, and **6** was purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The sequence of 24 mer 5′-FAM labeled RNA oligonucleotide: 5′-FAM-AUGCAAGCUUCACCAUGAGAUAUC-3′. T4 RNA ligase 1 and RNase inhibitors are from Applied Biosystems (Foster City, CA, USA). Streptavidin (Thermo Scientific, Rockford, IL, USA) powder was reconstituted in 20 mM potassium phosphate (pH 6.5) based on the manufacturer's instruction. A typical 20 μL final volume reaction contains the following reagents at the final concentrations indicated. A solution of 5′-FAM labeled oligodeoxyribonucleotide (2.28 μM in water); 1X T4 RNA ligase buffer [0.5 M Tris-HCl, pH 7.8, 0.1 M $MgCl_2$, 0.1 M DTT, 10 mM ATP] (2 μL); acetonitrile, 5 μL; RNase inhibitor, (1 μL, 0.1 units/μL); and water was added to adjust the final volume of 17.4 μL. The above resulting mixture was first denatured at 65°C for 5 minutes and then cooled to room temperature. The modified

ligation probes **1**, **3**, **4**, and **6** were added (0.6 μL, 0.5 mM of each probe) and finally 5 units of T4 RNA ligase 1 (One unit (U) of T4 RNA ligase 1 is the amount of enzyme required to catalyze the formation of 1 nmol of 5′-[32P]-rA12–18 into a phosphatase-resistant form in 30 minutes at 37°C) of enzyme was added to each reaction and ligation reactions were carried out at 16°C for 16 hrs and terminated by heating at 95°C for 5 minutes in 1X Gel Loading Buffer II (Applied Biosystems). Ligated and unligated species were separated on denaturing (7 M urea) 15% polyacrylamide gels or 12.5% Native polyacrylamide gels. Gel images were taken using a FluorChem SP imaging system (Alpha Innotech, San Leanodro, CA, USA) and band intensities were analyzed using Quantity One software (Biorad, Hercules, CA, USA).

CONCLUSION

We have successfully synthesized and characterized base modified cytidines bearing 5′ monophosphate **1**, **3**, **4** and a pre-adenylated form of A(5′)pp(5′)C-4-biotin **6** analogs as new probes for T4 RNA ligation. The assay for RNA ligation clearly indicated that the new probes are valid substrates for T4 RNA ligase. The new base modified cytidine 5′-monophosphate ligation probes with and without biotin could be useful for developing a miRNA detection system involving the rapid isolation, efficient labeling and detection of miRNAs using sensitivity-enhanced microarrays.

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