New Uridine Derivatives for Systematic Evolution of RNA Ligands by Exponential Enrichment

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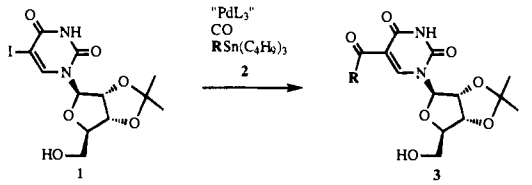
Systematic evolution of ligands by exponential enrichment (SELEX) is a procedure that generates nucleic acid ligands capable of high-affinity binding to both protein and small molecule targets.[1] Recent examples of evolved RNA binding to proteins include R17 coat protein,[2] HIV reverse transcriptase,[3] HIV rev protein,[4] basic fibroblast growth factor,[5] vascular endothelial growth factor,[6] thrombin,[7] and *Escherichia coli* ρ factor.[8] Several examples of RNA ligands binding small molecules have also been reported including ATP,[9] theophylline,[10] nicotinamide,[11] and stereoselective binding to tryptophan,[12] arginine,[13] and valine.[14] Although SELEX is remarkably effective at generating highly specific nucleic acid ligands, the scope of potential SELEX targets could be expanded if the physical properties of the nucleic acids were altered systematically. Introduction of modified uridine triphosphates, which carry appended carbonyl groups capable of a variety of interactions,[15] would increase significantly the domain of "shape space" from which high-affinity ligands are isolated.

Nucleosides with appended carbonyl groups are able to form *reversible* covalent cross-links.[16] Ligands containing hydrophobic uridine analogs (**2b,d**) are expected to exhibit, in addition to covalent interactions, altered intramolecular contacts and, thereby, generate new secondary and higher order structural motifs. Performing SELEX with a UTP analog appended with a cross-linking or hydrophobic group might afford high-affinity RNA ligands to targets that otherwise would not be possible.

The chemistry described below is the first example of palladium-mediated carbonylative carbon−carbon bond forming reactions at the 5-position of uridine and further extends our efforts from the synthesis of DNA nucleoside[17] analogs to RNA nucleotides ready for introduction into SELEX. To make introduction of new functionality into the SELEX process useful it is important that the number of steps involved in the synthesis of a modified UTP be as streamlined as possible. Previous synthetic schemes to prepare 5-acyluridines required several steps.[18] In addition, current synthetic methodology used to make UTP involves first blocking the 5′-hydroxyl group with DMT followed by 3′,2′ hydroxyl protection using acetate groups. After removal of the DMT group, the 5′-hydroxyl is converted to the triphosphate. Simplifying this protection/deprotection scheme by using a protecting group that is selective for the 3′,2′ hydroxyls eliminates two steps in *any* UTP synthetic protocol. It was necessary to use a protecting group compatible with palladium-mediated CO insertion that could be removed easily after triphosphate synthesis without product degradation. One obvious solution to this regioselective protection problem was formation of the 2′,3′-isopropylidene.[19]

It was desirable to prepare 5-carbonyluridine analogs by Stille[20] one-step carbonylative coupling methods. We were concerned about the success of these reactions with **1** since the 5′-hydroxyl was unprotected, poised for intramolecular cyclization as suggested by earlier literature precedent for carbonylative coupling of aryl halides in the presence of alcohols.[21] While commercially available $Pd(PPh_3)_4$ gave low yields of **3a−d**, combining palladium(II) acetate with 3 equiv of triphenylphosphine and copper(I) iodide in THF provided an *in situ* route for the formation of an active catalytic species. This palladium catalyst favored carbonylative carbon−carbon bond formation to **1** of vinyl- and aryl-stannanes giving **3a−d** in high yields with no detectable ester formation due to reaction of the 5′-hydroxyl.



(1)

2a, 3a: R = $CHCH_2$ 89 %
2b, 3b: R = *E*-$CHCHSi(CH_3)_3$ 73 %
2c, 3c: R = $C(OC_2H_5)CH_2$ 81 %
2d, 3d: R = C_6H_5 90 %

The synthesis of one example of a 5-position-modified dUTP and its incorporation into a DNA evolution protocol has been reported recently.[22] However, the inclusion of 5-position-modified UTP in RNA evolution protocols was unknown, and it was uncertain whether T7 RNA polymerase would accept these modified uridine 5′-triphosphates as substrates. It was decided to prepare the 5′-triphosphate from **3d** because it contains the most sterically demanding substituent and would be most likely to fail as a substrate for T7 RNA polymerase. In addition, the binding domains of antibodies are rich in aromatic residues,[23] which might be a useful property to include in RNA ligands. For these reasons **3d** was chosen as a primary candidate for triphosphate synthesis. Using a modified Eckstein protocol,[24] **3d** was converted to the corresponding 5′-triphosphate **4** (32% not optimized).[19]

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(1) Gold, L.; Tuerk, C.; Allen, P.; Binkley, J.; Brown, D.; Green, L.; MacDougal, S.; Schneider, D.; Tasset, D.; Eddy, S. In *The RNA World*; Gespeland, A., Atkins, J., Eds.; Cold Springs Harbor Laboratory Press: Plainview, NY, 1993; Chapter 19.
(2) Schneider, D.; Tuerk, C.; Gold, L. *J. Mol. Biol.* **1992**, *228*, 862.
(3) Tuerk, C.; MacDougal, S.; Gold, L. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6988.
(4) (a) Tuerk, C.; MacDougal-Waugh, S. *Gene* **1993**, *137*, 33. (b) Jensen, K. B.; Green, L.; MacDougal-Waugh, S.; Tuerk, C. *J. Mol. Biol.* **1994**, *235*, 237.
(5) Jellinek, D.; Lynott, C. K.; Rifkin, D. B.; Janjic, N. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11227.
(6) Jellinek, D.; Green, L. S.; Bell, C.; Janjic, N. *Biochemistry* **1994**, *33*, 10450.
(7) Bock, L.; Griffin, L.; Latham, J.; Vermaasm, E.; Toole, J. *Nature* **1992**, *355*, 564.
(8) Schneider, D.; Gold, L.; Platt, T. *FASEB J.* **1993**, *7*, 201.
(9) Sassanfar, M.; Szostak, J. W. *Nature* **1993**, *364*, 550.
(10) Jenison, R. D.; Gill, S. C.; Pardi, A.; Polisky, B. *Science* **1994**, *263*, 1425.
(11) Lauhon, C. T.; Szostak, J. W. *J. Am. Chem. Soc.* **1995**, *117*, 1246.
(12) Famulok, M.; Szostak, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 3990.
(13) Connell, G. J.; Illangesekare, M.; Yarus, M. *Biochemistry* **1993**, *32*, 5497.
(14) Majerfield, I.; Yarus, M. *Nature Struct. Biol.* **1994**, *1*, 287.
(15) (a) An example of a Schiff's base formation is given by Kryak and Bobst: Kryak, D. D.; Bobst, A. R. *Nucleosides Nucleotides* **1990**, *9*, 1015. (b) 1,4-Conjugate addition has been observed for carbonyl-appended 2-deoxyuridine analogs: Kirschenheuter, G.; Rollman, B.; Zyzniewski, C.; Dewey, T.; Crouch, G. J.; Eaton, B. E. Unpublished results.
(16) Crouch, G. J.; Mundt, A. A.; Rollman, B. R.; Eaton, B. E. Unpublished results.
(17) Crouch, G. J.; Eaton, B., E. *Nucleosides Nucleotides* **1994**, *13*, 939.
(18) Hayakawa, H.; Tanaka, H.; Miyasaka, T. *Tetrahedron* **1985**, *41*, 1675.
(19) Synthetic procedures and spectroscopic data are given in supporting information.
(20) Stille, J. K. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 508.
(21) Stille. J. K.; Wong, P. K. *J. Org. Chem.* **1975**, *40*, 532.
(22) Latham, J. A.; Johnson, R.; Toole, J. J. *Nucleic Acids Res.* **1994**, *22*, 2817.
(23) Sigurskjold, B. W.; Bundle, D. R. *J. Biol. Chem.* **1992**, *267*, 8371.

0002-7863/95/1517-8474$09.00/0

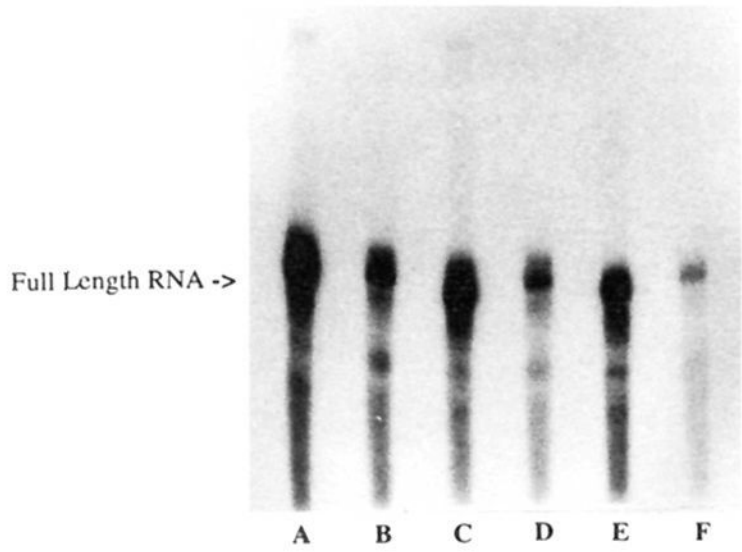

Figure 1. PAGE analysis of T7 transcripts at a constant concentration of ATP (100 μM), CTP (1 mM), and GTP (1 mM) with varying concentrations of UTP (A, 1 mM; C, 200 μM; E, 40 μM) or **4** (B, 1 mM; D, 200 μM; F, 40 μM).

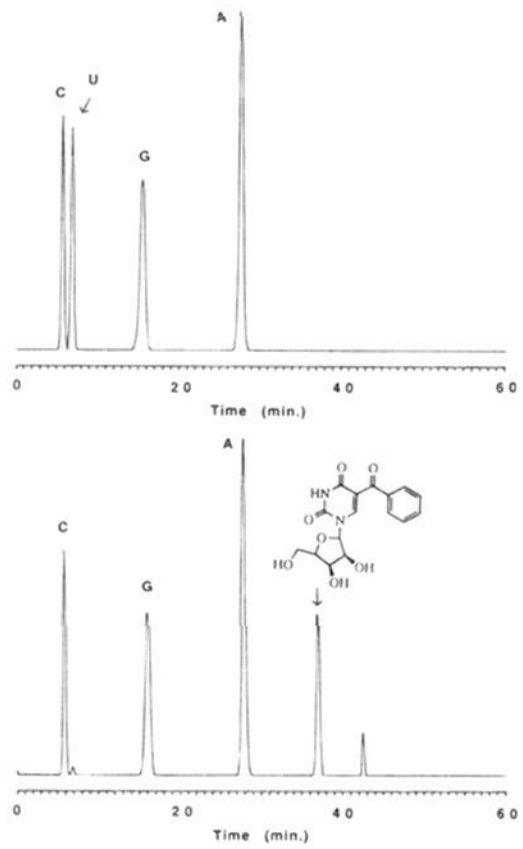

Figure 2. HPLC chromatograms (Vydac C18) of RNA digests: (top) native RNA; (bottom) 5-benzoyl-U-RNA.

Modified UTP **4** was tested at three concentrations for its transcription efficiency with T7 RNA polymerase using an 87 nucleotide dsDNA template with a 40 nucleotide random region. Control experiments were also performed using UTP under the same conditions. After purification by denaturing polyacrylamide gel electrophoresis (DPAGE) the amount of full-length product was determined by UV. The amount of full-length transcript varied, as compared to UTP, depending on the concentration of **4** used.[19] T7 transcription of an identical concentration (200 pmol) of dsDNA template using either 2.0 mM UTP or **4** gave 6.23 nmol of RNA and 2.17 nmol of 5-benzoyl-U-RNA (35% relative yield) as determined by UV absorbance at 260 nm. Decreasing the concentration of UTP and **4** to 0.5 mM gave 3.70 nmol of RNA and 1.68 nmol of 5-benzoyl-U-RNA (45% relative yield). The best relative yield of full-length transcript was obtained at 1.0 mM UTP or **4** yielding 4.31 nmol of RNA and 3.36 nmol 5-benzoyl-U-RNA (78% relative yield). High concentrations (>2 mM) of **4** were found to be inhibitory, giving significantly lower relative yields of 5-benzoyl-U-RNA (<10%). Transcription reactions incubated for 5−20 h showed no apparent difference in yield of 5-benzoyl-U-RNA. In addition, regardless of the concentration

(24) Ludwig, J.; Eckstein, F. *J. Org. Chem.* **1989**, *54*, 631.

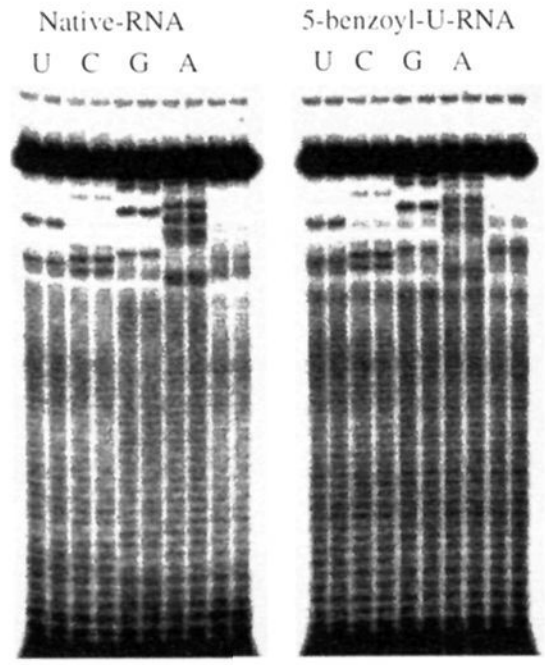

Figure 3. AMV RT dideoxy sequencing gel analysis of native RNA and 5-benzoyl-U-RNA. Duplicates of each sequencing lane were run.

of UTP or **4**, the major product was the full-length RNA with no increase in aborted transcripts (Figure 1). Apparently, T7 RNA polymerase can accommodate relatively large groups at the 5-position of uridine.

Of course it was also possible that full-length transcripts could be obtained in the absence of actual incorporation of **4.** It was desirable to determine if only a small fraction (≪25%) of **4** had been incorporated into the random region. Base hydrolysis and alkaline phosphatase digestion followed by HPLC analysis (normalized by the extinction coefficient of the nucleosides, Figure 2) revealed no difference between the extent of incorporation of **4** as compared to UTP. Replicate HPLC analysis of several transcripts prepared at 0.5, 1.0, and 2 mM UTP or **4** showed the same level of incorporation of **4** relative to UTP within experimental error.[19]

The final requirement for inclusion of **4** into the SELEX protocol was that the modified RNA must serve as a template for AMV reverse transcriptase (AMV RT) to make cDNA. Both the modified and native RNA transcripts gave good yields of the full-length cDNA products (60−90%). AMV RT dideoxy sequencing (Figure 3) also showed good fidelity in the 5′-fixed region and no difference in intensity of the bands in the random region as compared to UTP, demonstrating that the SELEX cycle could be completed using **4** in place of UTP without significant bias in sequence space.

In summary, simple modification of Stille-type carbonylative C−C coupling chemistry provides an expeditious route to several 5-position carbonyl-appended uridine nucleosides. The uridine analogs described here are converted easily to nucleoside triphosphates using procedures that avoid unwanted protection/deprotection steps. The modified UTP with the most sterically demanding group at the 5-position was shown to transcribe with T7 RNA polymerase giving comparable incorporation for both **4** and UTP. The modified RNA served as a template for cDNA transcription by AMV RT, and dideoxy sequencing showed good fidelity in the fixed regions of the template. SELEX experiments are being performed on several protein targets using ligands containing these modified uridine analogs.

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Supporting Information Available: Experimental procedures and analytical data (10 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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