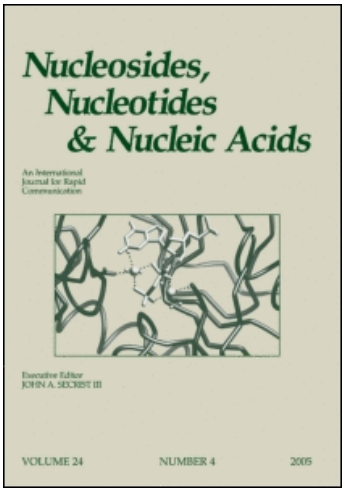

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Syntheses and Interactions of Oligodeoxyribonucleotides Containing 2'-Amino-2'-Deoxyuridine

Paul S. Miller[a]; Purshotam Bhan[b]; Lou-Sing Kan[c]
[a] Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, MD, USA [b] Dyad Pharmaceuticals, Columbia, MD, USA [c] Institute of Chemistry, Academica Sinca, Taipei, Taiwan, ROC

To cite this Article Miller, Paul S. , Bhan, Purshotam and Kan, Lou-Sing(1993) 'Syntheses and Interactions of Oligodeoxyribonucleotides Containing 2'-Amino-2'-Deoxyuridine', Nucleosides, Nucleotides and Nucleic Acids, 12: 8, 785 — 792
To link to this Article: DOI: 10.1080/07328319308018549
URL: http://dx.doi.org/10.1080/07328319308018549

NUCLEOSIDES & NUCLEOTIDES, 12(8), 785-792 (1993)

SYNTHESES AND INTERACTIONS OF OLIGODEOXYRIBONUCLEOTIDES CONTAINING 2'-AMINO-2'-DEOXYURIDINE

Paul S. Miller*†, Purshotam Bhan‡, and Lou-Sing Kan§

†Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, 615 North Wolfe Street, Baltimore, MD, USA;
‡Dyad Pharmaceuticals, 7101 Riverwood Drive, Columbia, MD, USA;
§Institute of Chemistry, Academica Sinca, Nankang,Taipei, Taiwan, ROC

Abstract: Oligodeoxyribonucleotides containing 2'-amino-2'-deoxyuridine (dU) were synthesized and their ability to form duplexes with complementary DNA or RNA oligonucleotides was studied. Substitution of dU with dU in these oligomers results in lowered Tms of the duplexes.

The recent interest in the use of antisense and anticode oligonucleotides for controlling gene expression and their potential as therapeutic agents has lead to efforts to design oligonucleotide analogs which show increased resistance to hydrolysis by nucleases and which are capable of binding to complementary nucleic acids[1]. In addition to modifications of the phosphodiester backbone such as the introduction of nuclease resistant phosphorothioate[2] or methylphosphonate[3] linkages, modifications to the sugar such as introduction of 2'-O-alkylribonucleosides can also impart nuclease resistance to the oligomer, while not affecting its ability to hybridize to complementary nucleic acids[4,5,6].

Oligomers which contain 2'-amino-2'-deoxyribonucleosides would also appear to be potential candidates for antisense applications. Previous studies have reported that polynucleotides which contain 2'-amino-2'-deoxycytidine (dC) or 2'-amino-2'-deoxyuridine (dU) show increased resistance to alkaline hydrolysis and enzymatic hydrolysis by pancreatic ribonuclease, micrococcal nuclease or snake venom phosphodiesterase[7]. Recent studies by Eckstein and coworkers have shown that hammerhead ribozymes which contain 2'-amino-2'-deoxyribonucleoside substitutions have increased resistance to enzymatic degradation, and in some cases still retain catalytic properties similar to those of the parent ribozyme[8,9,10]. Although these results are encouraging, the effects of 2'-aminonucleoside substitution on oligomer hybridization properties have not been explored. In this paper we describe the syntheses of a series of oligodeoxyribonucleotides which contain one or more 2'-amino-2'-deoxyuridine residues and studies on the effects of

this substitution on the ability of these oligomers to form duplexes with complementary DNA and RNA targets.

MATERIAL AND METHODS

Reactions were monitored by thin layer chromatography (TLC) on precoated thin layer (0.25 mm) silica gel 60 F-254 plates purchased from EM Reagents. High performance liquid chromatography (HPLC) was carried out on Whatman C-18 reversed phase columns (ODS-3) using linear gradients of acetonitrile in 0.1 M sodium phosphate buffer (pH 5.8) at a flow rate of 1.0 ml/min for the analytical columns and 2.5 ml/min for the preparative columns. All solvents were dried by refluxing over calcium hydride followed by distillation under anhydrous conditions. The RNA oligonucleotide, r-GGUCAACUAG, was prepared by GENTA, Inc. Proton and phosphorous nmr spectra were recorded on a Bruker WM 300 MHz spectrometer.

Synthesis of 2'-amino-2'-deoxyuridine 2'-Amino-2'-deoxyuridine was prepared from uridine (15 g; 61 mmol) according to literature procedures[11,12]. The nucleoside was purified by ion exchange chromatography on a Dowex 50Wx8 (H^+ form) column and was obtained in 40% overall yield: mp 197-199 °C. ^1H NMR: (DMSO-d6), 7.71 (H-6, d, 8.0 Hz, 1H) 5.63 (H-1', d, 7.9 Hz, 1H), 5.54 (H-5, d, 8.0 Hz, 1H), 3.88 (H-3', q, 5.3 Hz/2.2 Hz, 1H), 3.82 (H-4', m, 3.7 Hz/2.2 Hz, 1H), 3.53 (H-5', m, 11.7 Hz/3.7 Hz, 1H), 3.51 (H-5", m, 11.7Hz/3.7Hz, 1H) 3.26 (H-2', q, 7.9 Hz/5.3 Hz, 1H).

Synthesis of 5'- O-(4,4'-Dimethoxytrityl)-2'-trifluoroacetamido-2'-deoxyuridine 2'-Amino-2'-deoxyuridine (3.1g; 12.76 mmol) was suspended in dry methanol (260 ml) and S-ethyl trifluorothioacetate (2.5 ml; 19.67 mmol) added to it. The mixture was stirred at room temperature for 2 hr until homogenous and the clear solution was allowed to stand at room temperature for 24 h. This solution was next bubbled with nitrogen for 1 hr followed by the removal of solvent under reduced pressure to give 4 g of 2'-trifluoroacetamido-2'-deoxyuridine intermediate which was used without purification. This intermediate (1.5 g; 4.62 mmol) was dried by co-evaporation with dry pyridine (2x50 ml) and dissolved in dry pyridine (50 ml). Dimethoxytrityl chloride (1.725 g; 5.1 mmol) was added with stirring in five one hour installments followed by stirring for five more hours. The reaction mixture was diluted with methanol (10 ml) and the solvents removed under reduced pressure. The residue was dissolved in chloroform (200 ml) and washed with 5% sodium bicarbonate solution (1x100 ml), brine (1x100 ml) and dried over anhydrous sodium sulfate. Removal of solvent gave the crude product which was purified on silica gel (60 g). Elution with 5%

methanol-chloroform gave 2.2 g of pure product (75.8 % overall yield). ^{1}H NMR: (DMSO-d6), 7.69 (H-6, d, 8.1 Hz, 1H), 7.32 (DMTr, m, 5H), 7.25 (DMTr, d, 8.7 Hz, 4H), 6.89 (DMTr, d, 8.7 Hz, 4H), 6.01, (H-1', d, 6.1 Hz, 1H), 5.46 (H-5, d, 8.1 Hz, 1H), 4.59 (H-2', m, 1H), 4.25 (H-3', m, 1H), 4.04 (H-4', m, 1H), 3.72 (-OCH_3, s, 6H), 3.25 (H-5', m, 2H).

Synthesis of 5'- O-(4,4'-Dimethoxytrityl)-2'-trifluoroacetamido-2'-deoxyuridine 3'-O-(2-Cyanoethyl-N,N'-diisopropylphosphoramidite) A solution of 5'-dimethoxytrityl-2'-trifluoroacetamido-2'-deoxyuridine (1.34 g; 2.14 mmol) in dry CH_2Cl_2 (40 ml) was treated with 2-cyanoethyl chloro-N-N'-diisopropylphosphoramidite (0.75 ml; 2.95 mmol) in the presence of diisopropylethylamine (0.76 ml; 4.3 mmol) for 1 hr at room temperature. The mixture was diluted with methylene chloride (75 ml) and washed with a solution of 5% sodium bicarbonate (1x50 ml) and brine (1x50 ml). The aqueous solutions were back-extracted with ethyl acetate (1x50 ml) and the combined organics dried over anhydrous sodium sulfate. Removal of solvents furnished the product (1.6 g, 96% yield) which was found to be homogenous on TLC. Proton-decoupled ^{31}P NMR: ($CDCl_3$), 149.00 (s), 150.45 (s).

Synthesis of Oligodeoxyribonucleotides Oligodeoxyribonucleotides were synthesized on a Biosearch 8700 DNA synthesizer on 1 μmol scales on controlled pore glass supports using protected deoxynucleoside-3'-O-(2-cyanoethyl-N,N'-diisopropylphosphoramidite) synthons following standard procedures[13]. For oligomers containing 2'-amino-2'-deoxyuridine, the coupling step was extended to 30 min. The synthesizer was programmed to remove the dimethoxytrityl group at the end of the synthesis. The oligomers were deprotected and cleaved from the support by treatment of the support with 1 ml of 50% concentrated ammonium hydroxide for 5 h at 55 °C. The support was filtered, washed with water (5xlml) and the combined washings were evaporated. The residue was dissolved in water and purified by reversed phase HPLC. The oligomers were desalted on Sep-Pak cartridges (Waters Associates) and were eluted from the cartridge with 3 ml of acetonitrile/water (1:1 v/v).

Characterization of the Oligomers The base ratios of the oligonucleotides containing 2'-amino-2'-deoxyuridine were determined by HPLC analysis of nuclease digests of the oligomers. Each oligomer (0.15 A_{254} unit) was treated with 0.006 units of snake venom phosphodiesterase and 2.4 units of calf intestinal phosphatase in a buffer containing 50 mM Tris (pH 8.2), 2 mM magnesium chloride at 37 °C for 16 hrs. The calf intestinal phosphatase contained adenine deaminase activity which converted deoxyadenosine to deoxyinosine. Each digest was analyzed by reversed phase HPLC using a gradient 2%-3% acetonitrile (12 min) followed by 3%-20% acetonitrile in 50 mM sodium phosphate (pH 5.8) at a flow rate of 1.0 ml/min. The results are shown in TABLE 1. The extinction coefficients of the oligomers were also determined. Each

TABLE 1. Base Ratios and Molar Extinction Coefficients of Oligodeoxyribonucleotides

Oligomer		dA	dC	dG	dU	dU	ϵ_{254}
d-CUAGUUGACC	1	2.0	2.8	2.0	2.8	--	76400
d-CUAGUUGACC	2	1.9	2.9	2.0	2.1	0.8	75100
d-CUAGUUGACC	3	2.0	2.9	2.1	1.0	2.1	78700
d-CUAGUUGACC	4	2.0	3.2	2.0	--	3.2	79800

oligomer (0.15 A_{254} unit) was dissolved in 48 μl of 50 mM Tris (pH 8.2), 2 mM magnesium chloride solution. The solution was treated with 2 μl of water or 2 μl snake venom phosphodiesterase (0.006 units) for 16 hrs at 37 °C. The spectra of the enzyme treated oligomer and the water treated oligomer were recorded and the extinction coefficient was determined. The results are shown in TABLE 1.

Melting Experiments Melting experiments were carried out in a buffer containing 0.1 M sodium chloride, 50 mM sodium citrate, pH 5.0 or 0.1 M sodium chloride, 50 mM Tris, pH 8.0. The buffers used in experiments with the RNA target were prepared using deionized water which had been treated with diethylpyrocarbonate and then autoclaved to ensure removal of ribonuclease activity. All glassware, including the UV cuvettes were baked overnight at 90 °C to inactivate ribonucleases. The duplexes were prepared by mixing equal volumes of 2 μM stock solutions of the component oligomers at room temperature. The solutions were stored overnight at 4 °C. Absorbance versus temperature profiles were measured on either a Cary 219 or Cary 3 spectrophotometer fitted with a programmable, thermostatted cell compartment and a temperature probe. The solutions were heated from 0 to 60 °C at a rate of 0.5 °C per minute. The melting temperature (Tm) of duplex was calculated as the midpoint in the sigmoidal shaped thermal transition profile. The melting profiles were reversible and did not show hysteresis effects.

RESULTS AND DISCUSSION

Oligodeoxyribonucleotides containing 2'-amino-2'-deoxyuridine were prepared (Figure 1). The sequences of the oligodeoxyribonucleotides are shown in Table 1. 2'-Amino-2'-deoxyuridine was prepared in a three step procedure similar to that described by Verheyden *et. al.*[11] and by Hobbs and Eckstein[12]. This involved conversion of uridine to its 2,2'-anhydro derivative by treatment with diphenylcarbonate. The anhydro derivative

was converted to 2'-azido-2'-deoxyuridine by reaction with sodium azide and the azide intermediate was reduced by hydrogenation on Pd/carbon to give d-U. The amino nucleoside was then converted to its protected 2-cyanoethyl-N,N'-diisopropylphosphoramidite synthon, which was used to prepare the oligonucleotides by standard phosphoramidite chemistry on a controlled pore glass support. The oligomers were deprotected by treatment with 50% ammonium hydroxide and purified by reversed phase HPLC. The oligomers could be phosphorylated by polynucleotide kinase and ATP and the phosphorylated oligomers migrated as single bands when subjected to polyacrylamide gel electrophoresis. The oligomers were completely digested to their component nucleosides upon overnight treatment with a combination of snake venom phosphodiesterase and bacterial alkaline phosphatase and gave the expected ratio of nucleosides as shown in TABLE 1. The extinction coefficients of the dU containing oligomers were determined by enzymatic hydrolysis[14] and were similar to that of the dU containing parent oligomer.

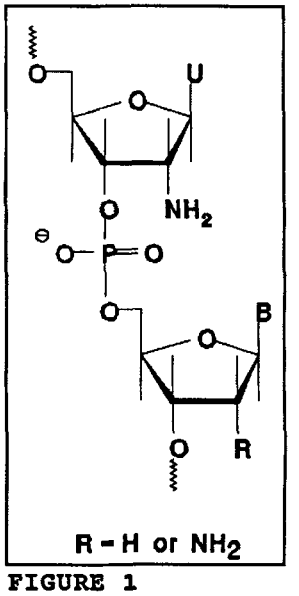

FIGURE 1

Previous investigations by Guschlbauer and Jankowski have shown that the pk of the 2'-amino group of dU is 6.2 and that the sugar exists primarily in the 2'-endo conformation[15]. We used proton nmr to determine the pk of the 2'-amino group and to examine the conformation of the sugars in d-UpU. Sugar proton assignments were carried out using 1-D and 2-D COSY analysis. Examination of the effect of pH on the chemical shifts of the 1',2',3' and 4' protons of the dU residue indicated that the pk of the 2'-amino group is 6.0 in the dimer. This value is similar to that of dU and is in good agreement with that observed for poly(dU)[7]. As shown in Table 2, both sugars in the dimer preferentially exist in the 2'-endo conformation over the pH range 1.7 to 7.5[16]. This conformation would place the protonated amino group in close proximity to the negatively charged phosphodiester linkage and potentially allow formation of an ion pair. Previous studies on poly (dA) suggested that interactions between the 2'-amino group and the phosphodiester backbone could contribute to the unusual stacking interactions observed by circular dichroism spectroscopy at pH 5.7 for this polynucleotide[17].

The stabilities of duplexes formed between oligomers 1 - 4 and a complementary DNA target, d-GGTCAACTAG, or RNA target, r-GGUCAACUAG, were studied. These experiments were carried out at Ph 5.0, conditions under which the 2'-amino groups should be protonated, and at Ph 8.0,

TABLE 2. Effect of pH on Sugar Conformation in d-UpU

Ph	$J_{1'-2'}$ (Up)	%^{2}E (Up)	$J_{1'-2'}$ (pU)	%^{2}E (pU)
1.7	7.7 Hz	71.3	6.6 Hz	61.1
6.0	7.9 Hz	73.1	6.6 Hz	61.1
7.5	7.5 Hz	69.4	6.6 Hz	61.1

TABLE 3 Melting Temperatures of Duplexes Containing 2'-Amino-2'-deoxyuridine

		Tm (°C)			
		DNA Target		RNA Target	
Oligomer		Ph 5.0	Ph 8.0	Ph 5.0	Ph 8.0
d-GUAGUUGACC	1	33	35	32	32
d-GUAGUUGACC	2	30	34	26	26
d-GUAGUUGACC	3	16	21	19	23
d-GUAGUUGACC	4	<5	14	10	18

conditions under which the 2'-amino groups should exist in the free amine form. As shown in Table 3, all four oligomers formed duplexes with both the DNA and RNA targets. The stabilities of these duplexes varied with both the number of dU residues and the pH.

Duplexes formed between the parent oligomer, 1, and the DNA or RNA targets, had similar melting temperatures (Tms). A similar result was observed by Inoue *et. al.* in a study of hybrids formed between DNA and RNA oligonucleotides, although the relative Tms appear to depend on the sequences of the oligonucleotides[6].

Introduction of a single dU near the 5'-end of the oligomer, as illustrated by oligomer 2, results in a small, 1-3 °C, reduction in the Tm of the duplex formed with the DNA target and a greater, 6 °C, reduction of the Tm of the duplex formed with the RNA target. This effect may be related to the sugar conformation of dU. It is generally found that DNA/DNA duplexes tend to adopt a B-form geometry in which the sugars have a 2'-endo conformation, whereas DNA/RNA duplexes appear to adopt an A-form geometry in which the nucleoside sugars are in a 3'-endo conformation[18]. Thus introduction of a single nucleoside unit which has a strong preference for the 2'-endo sugar conformation may perturb the ability of the oligomer to bind to a complementary RNA target.

An even greater reduction in Tm is seen when the two central dU nucleosides are replaced by dU, as in 3 and 4. In this case the stabilities of the duplexes formed with the DNA target are reduced to a greater extent than are those formed with the RNA target, a result which is opposite that observed for duplexes formed by oligomer 1 which contains a single dU substitution. Consistent with these results is the observation that $(dU)_{10}$ did not form a duplex with d-GG$(A)_{10}$CC whereas $(dU)_{10}$ formed a duplex with a Tm of 12 °C at pH 8.0. Thus it appears that other factors in addition to conformational preference contribute to the overall stability of dU-containing duplexes.

The Tms of duplexes formed between oligomers 3 or 4 and either the DNA or the RNA target are reduced to a greater extent at pH 5.0 than they are at pH 8.0. The magnitude of this reduction is greater for oligomer 4 which contains three dU residues. The 2'-amino groups of dU should be protonated at pH 5.0. Such protonation would reduce the net charge of the oligomer and thus reduce the charge repulsion between the oligomer backbones, an effect which would be expected to stabilize the duplexes. The observed destabilizing effect of lower pH may be a result of electrostatic interactions between the 2'-amino group of the sugar and the negatively charged phosphodiester internucleotide bond which would tend to increase the conformational rigidity of the sugar-phosphate backbone.

It appears that dU substitution, particularly multiple substitutions, can influence the ability of oligodeoxyribonucleotides to interact with complementary DNA and RNA targets. Depending upon the conditions of the experiment, these effects could arise from conformational changes and/or electrostatic interactions within the oligomer. These factors could play a role when 2'-amino-2'-deoxyuridine nucleosides are considered for use in antisense oligonucleotides or are inserted into biochemically active nucleic acids such as ribozymes.

ACKNOWLEDGEMENT

This work was supported by a grant from the National Institutes of Health (GM 45012). We wish to thank Ms. Cynthia Cushman for running the DNA synthesizer and Mr. David Noll for obtaining the ^{31}P NMR spectrum.

REFERENCES

1. Uhlmann, E. & Peymann, A. (1990) *Chem. Rev.* ***90***, 544-579.
2. Stein, C.A., Tonkinson, J.L. & Yakubov, L. (1991) *Pharmac. Ther.* ***52***, 365-384.
3. Miller, P.S. (1992) in Antisense RNA and DNA, Murray, J.A.H., ed. J. Wiley, & Sons, New York, pp. 241-253.
4. Iribarren, A.M., Sproat, B.S., Neuner, P., Sulston, I., Ryder, U., & Lamond, A.I. (1990) *Proc. Natl. Acad. Sci. (USA)* ***87***, 7747-7751.

5. Paolella, G., Sproat, B.S. & Lamond, A.I. (1992) *EMBO J. 11*, 1913-1919.
6. Inoue, H., Hayase, Y., Imura, A., Iwai, S., Miura, K. & Ohtsuka, E. (1987) *Nucleic Acids Res. 15*, 6131-6149.
7. Hobbs, J., Sternbach, H., Sprinzle, M. & Eckstein, F. (1973) *Biochemistry 12*, 5138-5145.
8. Pieken, W.A., Olsen, D.B., Benseler, F., Aurup, H. & Eckstein, F. (1991) *Science 253*, 314-317.
9. Olsen, D.B., Benseler, F., Aurup, H., Pieken, W.A. & Eckstein, F. (1991) *Biochemistry 30*, 9735-9741.
10. Williams, D.M., Pieken, W.A. & Eckstein, F. (1992) *Proc. Natl. Acad. Sci. (USA) 89*, 918-921.
11. Verheyden, J., Wagner, D. & Moffatt, J. (1971) *J. Org. Chem. 36* 250-254.
12. Hobbs, J. & Eckstein, F. (1977) *J. Org. Chem. 42*, 714-719.
13. Brown, T. & Brown, D.J.S. (1991) Oligonucleotides and Analogues. A Practical Approach, Eckstein, F., ed. IRL Press, New York, pp. 1-24.
14. Miller, P.S. & Cushman, C.D. (1992) *Bioconjugate Chemistry 3*, 74-79.
15. Guschlbauer, W. & Jankowski, K. (1980) *Nucleic Acids Res. 8*, 1421-1433.
16. Altoona, C. & Sundaralingam, M. (1972) *J. Am. Chem. Soc. 94*, 8205-8212.
17. Ikehara, M., Toshikazu, F. & Kakiuchi, N. (1977) *Nucleic Acids Res. 4*, 989-1000.
18. Saenger, W. Principles of Nucleic Acid Structure (1984) Springer-Verlag, New York.

Received 2/26/93
Accepted 6/10/93