Amino-functionalized DNA: the properties of C5-amino-alkyl substituted 2′-deoxyuridines and their application in DNA triplex formation

John A. Brazier, Takayuki Shibata, John Townsley, Brian F. Taylor, Elaine Frary, Nicholas H. Williams and David M. Williams*

Centre for Chemical Biology, Department of Chemistry, Krebs Institute, University of Sheffield, Sheffield S3 7HF, UK

ABSTRACT

The incorporation of C5-amino-modified 2′-deoxyuridine analogues into DNA have found application in nucleic acid labelling, the stabilization of nucleic acid structures, functionalization of nucleic acid aptamers and catalysts, and the investigation of sequence-specific DNA bending. In this study, we describe the physicochemical properties of four different C5-amino-modified 2′-deoxyuridines in which the amino group is tethered to the base via a 3-carbon alkyl, *Z*- or *E*-alkenyl or alkynyl linker. Conformational parameters of the nucleosides and their pK_a values were deduced using ^{1}H NMR. All of them display the expected *anti*-conformation of the nucleoside with 2′-*endo* sugar puckers for the deoxyribose ring. A preference for the *cisoid* conformation for the *Z*-alkenyl analogue is found, while the *E*-alkenyl analogue exists exclusively as its *transoid* conformation. The pK_a values range from 10.0 for the analogue with an aliphatic propyl linker to 8.5 for the propargylamino analogue. The analogues have been used for the synthesis of triple-helix forming oligonucleotides (TFOs) in which they replace thymidine in the natural sequence. Oligonucleotides containing the propargylamino analogue display the highest stability especially at low pH, while those containing analogues with propyl and especially *Z*-alkenyl linkers are destabilized to a great extent. TFOs containing the analogue with the *E*-alkenyl linker have stability similar to the unmodified structures. The chemical synthesis of TFOs containing the analogue, 5-(3-hydroxyprop-1-ynyl)-2′-deoxyuridine that possesses a neutral but polar side chain show a remarkable stability, which is higher than that of all TFOs containing the alkylamino or alkenylamino analogues and only slightly lower than that of TFOs containing the propargylamino analogue. Both the hydroxyl and propargylamino substitutions impart enhanced triple-helix stability relative to the analogous sequences containing C5-propynyl-2′-deoxyuridine. Furthermore, a similar dependence of stability on pH is found between TFOs containing the hydroxypropynyl modifications and those containing the propargylamino side chains. This suggests that the major factor responsible for stabilizing such triple helices is due to the presence of the alkyne with an attached electronegative group.

INTRODUCTION

Pyrimidine nucleosides functionalized at C5 with aminoalkyl side chains have been used extensively for attaching labels and reporter groups to both nucleotides and oligonucleotides (1). There has also been great interest in exploiting the properties of such amino-modified DNA, which at the appropriate pH displays cationic side chains. The chemical syntheses and properties of such 'zwitterionic DNA' containing C5-aminohexyl-modified cytosines (1,2) and C5-aminohexyl (2) and C5-aminopropyl-modified uracil (**3a**) (2,3) bases were first reported by Switzer *et al*. They observed reduced mobilities for zwitterionic oligonucleotides during PAGE, suggesting that the tethered amino groups are largely protonated below pH 8. At very low-salt concentrations, the relative stabilities of duplexes containing C5-amino modifications of U and C were generally higher than those of the natural duplexes (2,3), but at higher ionic strength this situation is reversed. In contrast,

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C5-propyl and hexyl-modified 2′-deoxyuridines (i.e. simple alkyl chains in the place of cationic side chains) result in a marked destabilization of a DNA duplex (2,3). Most recently, the stabilizing properties of C5-propargylamino-2′-deoxyuridine (**4a**) when placed within the third strand of DNA triple helices (4,5) have been described. Such stabilization is largely independent of salt concentration but does show a pH-dependence. Further stabilization of triplexes can be achieved by attachment of a 2-aminoethoxy side chain at the 2′-position of this analogue (5). DNA duplexes containing C5-propargylamino-2′-deoxyuridine also show enhanced stabilities relative to the unmodified duplexes (6–8), a trend also found for the corresponding analogue C5-propargylamino-2′-deoxycytidine (7).

C5-Amino-modified pyrimidine nucleosides (Figure 1) have also been used to investigate the processes of DNA bending that may be induced following multiple incorporation of several such analogues (8–12). Interestingly, DNA containing C5-propargylamino-2′-deoxyuridine shows no propensity to undergo sequence-specific bending in contrast to that containing the corresponding C5-propylamino analogue (8).

The 5′-triphosphates of several C5-amino-modified pyrimidine nucleosides have also been investigated for use in SELEX (13–25) for which the cationic side chains add additional functionality to nucleic acids designed to function as aptamers or catalysts. In a comparative study of the substrate properties of C5-amino-modified dUTPs, in which the amino group is tethered to the nucleobase via 3-carbon linkers of differing flexibility (alkyne, alkene and alkane), we found that both the rigidity and conformation (*Z*- versus *E*-alkenyl) of the linker is very important (13). Thus, analogues containing alkynyl and *E*-alkenyl linkers were considered as the best substrates, while

C5-propylamino dUTP was not incorporated by a variety of polymerases.

Clearly, the properties of nucleotides and DNA containing C5-amino-modified-2′-deoxyuridines depend on the type of linker between the amino group of the side chain and the nucleobase. In this regard, the nucleoside conformation and ionization states of the amino group are relevant to studies concerned with nucleotide and nucleic acid labelling, SELEX and the structural properties of such modified nucleic acids. However, there has so far been no detailed comparative study investigating the effect of a variety of linkers differing in both flexibility and conformation on the properties of C5-amino-modified 2′-deoxyuridines and oligonucleotides containing them. In this study, we investigate the physicochemical properties of four different C-5 amino-modified 2′-deoxyuridines in which the amino group is tethered to the nucleobase by one of four different 3-carbon linkers, such as propyl, *E*-propenyl, *Z*-propenyl and propargyl, and compare the stabilities of DNA structures that contain these analogues in the place of thymidine in the third strand of triple helices. Furthermore, we investigate the basis of the stabilizing role of the propargylamino side chain in triple helices by comparison with analogous DNA structures containing the novel analogue C5-(3-hydroxypropyn-1-yl)-2′-deoxyuridine.

MATERIALS AND METHODS

Chemicals and reagents

All reagents were obtained from commercial suppliers and used without purification. Dichloromethane, pyridine and acetonitrile were dried under reflux from calcium hydride,
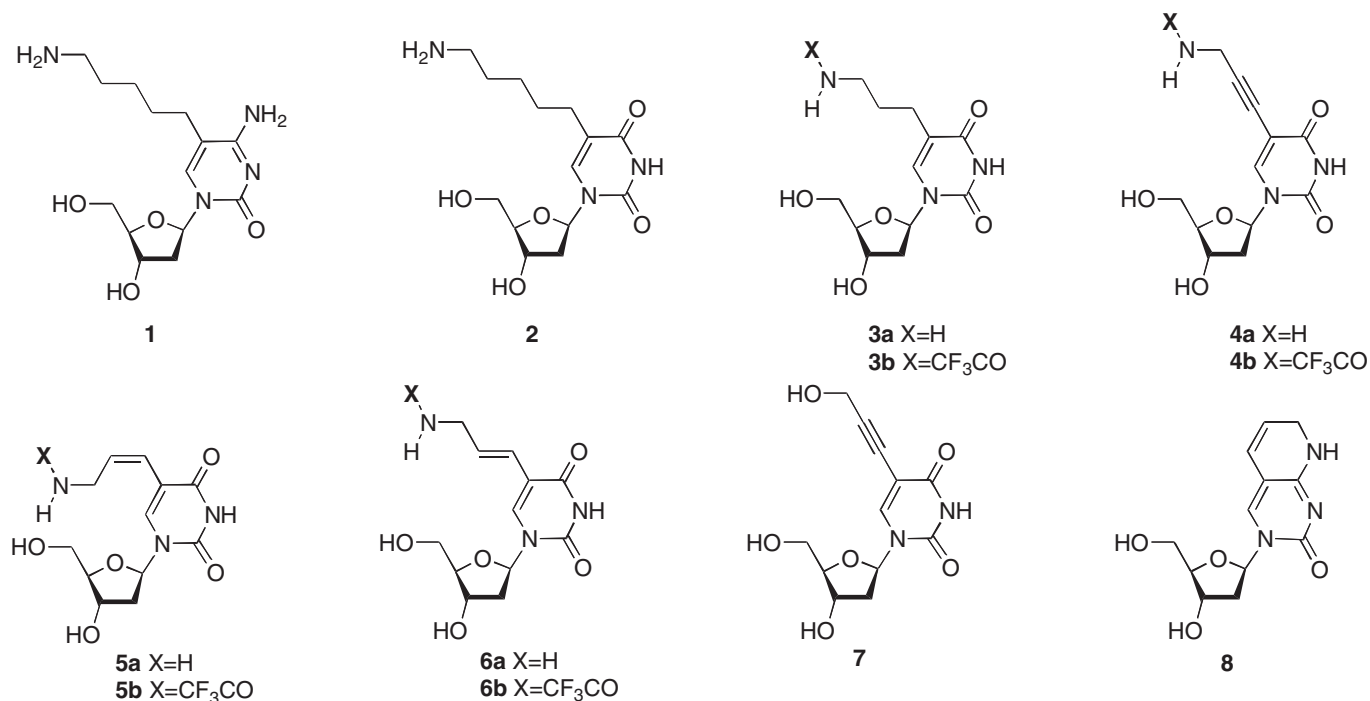

Figure 1. Structures of C5-amino-modified pyrimidine nucleosides (**1–8**).

distilled and stored under argon, over 3 Å molecular sieves. MeOH was dried under reflux from magnesium methanolate, distilled and stored under argon, over molecular sieves. Triethylamine was dried under reflux from potassium hydroxide, distilled and stored over potassium hydroxide pellets under argon. Anhydrous *N,N*-dimethylformamide was purchased from Aldrich. NMR spectra were recorded on a Bruker AC250 spectrometer and chemical shifts are reported in δ values relative to tetramethylsilane (^{1}H and ^{13}C) or 85% phosphoric acid (^{31}P) as an external standard. ^{1}H NMR, ^{13}C NMR and ^{31}P NMR were recorded at 250, 60 and 101 MHz, respectively. Described syntheses were used for phosphoramidites **10b** (4) and **12b** (26,27).

Full characterization for all the compounds described below can be found in the Supplementary Material.

General method for the synthesis of nucleosides 3a, 4a and 6a from 3b (13), 4b (13,28) and 6b (17,29). The protected nucleoside (100 mg) was stirred in concentrated aqueous ammonia (5 ml) overnight at room temperature and evaporated as oil (quantitative yield).

5-(Z-3-Aminoprop-1-enyl)-2′-deoxyuridine (5a). Compound **5b** (30) (0.1 g, 0.265 mmol) was stirred in concentrated aqueous ammonia (5 ml) for 45 min at room temperature and the solution was evaporated. The residue was again dissolved in water (1 ml) and acidified to pH 4 by the dropwise addition of trifluoroacetic acid. Evaporation gave yellow crystals (quantitative yield).

3-β-D-(2′-Deoxyribofuranosyl)-7,8-dihydropyrido[2,3-d] pyrimidine (8). Prepared in the manner similar to **5a** but stirred overnight in ammonia solution followed by evaporation.

5′-O-(4,4′-Dimethoxytrityl)-5-(3-trifluoroacetamidopropyl)-2′-deoxyuridine (9a). To a solution of 5-(3-trifluoroacetamidopropyl)-2′-deoxyuridine (13) (0.260 g, 0.682 mmol) in anhydrous pyridine (5 ml), anhydrous Et₃N (0.12 ml, 0.887 mmol) and dimethylaminopyridine (0.004 g, 0.034 mmol) were added. Dimethoxytrityl chloride (0.277 g, 0.819 mmol) was then added and the reaction was stirred for 4 h at room temperature. MeOH (1 ml) was added and the solution was evaporated, diluted in CH₂Cl₂ (100 ml) and the organic layer was washed with saturated aqueous NaHCO₃ solution (150 ml). The organic layer was dried (MgSO₄) and the solution was evaporated. Purification by silica column chromatography [CH₂Cl₂ (1% Et₃N) followed by 5% MeOH/CH₂Cl₂ (1% Et₃N)] resulted in a pale yellow foam (0.370 g, 0.542 mmol, 79%).

5′-O-(4,4′-Dimethoxytrityl)-5-(3-trifluoroacetamidopropyl)-2′-deoxyuridine-3′-(2-cyanoethyl-N,N-diisopropyl) phosphoramidite (9b). To a solution of 5′-O-(4,4′-dimethoxytrityl)-5-(3-trifluoroacetamidopropyl)-2′-deoxyuridine (**9a**) (0.350 g, 0.512 mmol) in anhydrous CH₂Cl₂ (2.3 ml), anhydrous diisopropylethylamine (0.36 ml, 2.05 mmol) was added followed by the dropwise addition of 2-cyanoethyl-*N,N*-diisopropylchlorophosphoamidite (0.23 ml, 1.02 mmol). After 1 h at room temperature, MeOH (0.1 ml) was added and the mixture was stirred for 1 min. EtOAc (100 ml) was added and the organic layer was washed with 5% aqueous NaHCO₃ solution (50 ml) followed by saturated aqueous sodium chloride solution (50 ml). The organic layer was then dried (MgSO₄) and

evaporated as foam. Purification by silica column chromatography using high-performance liquid chromatography (HPLC) grade solvents and a positive pressure of argon (50% EtOAc, 40% CH₂Cl₂ and 10% Et₃N) resulted in the cream-coloured foam (0.350 g, 0.396 mmol, 87%).

5′-O-(4,4′-Dimethoxytrityl)-5-(Z-3-trifluoroacetamidoprop-1-enyl)-2′-deoxyuridine (11a). Prepared in an analogous way similar to **9a**. Yield 77%.

5′-O-(4,4′-Dimethoxytrityl)-5-(Z-3-trifluoroacetamidopropenyl)-2′-deoxyuridine-3′-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (11b). Prepared in an analogous way similar to **9b**. Yield 77%.

5′-O-(4,4′-Dimethoxytrityl)-5-(3-tert-butyldimethylsilyloxyprop-1-ynyl)-2′-deoxyuridine (13a). Copper(I)iodide (0.035 g, 0.183 mmol) was added to a stirred solution of 5′-O-(4,4′-dimethoxytrityl)-5-iodo-2′-deoxyuridine (7) (0.300 g, 0.458 mmol) in anhydrous DMF (10 ml), in the absence of light, and the mixture was stirred for 10 min. Propargyl alcohol (0.160 ml, 2.75 mmol), anhydrous Et₃N (0.13 ml, 0.916 mmol) and tetrakis(triphenylphosphine)palladium(0) (0.106 g, 0.092 mmol) were then added sequentially and the reaction was left for overnight stirring. The solvent was removed by evaporation and the reaction was diluted with excess MeOH. The precipitate formed was removed by filtration through hyflo filter aid and the filtrate was evaporated. Treatment with excess MeOH and the subsequent filtration was repeated until no further precipitate was formed. Purification by silica column chromatography (gradient of 0–5% MeOH in CH₂Cl₂/ 0.5% pyridine) gave a white foam of 5′-O-(4,4′-dimethoxytrityl)-5-(3-hydroxyprop-1-ynyl)-2′-deoxyuridine (0.440 g, 0.755 mmol, 50%). To the stirred solution of 5′-O-(4,4′-dimethoxytrityl)-5-(3-hydroxyprop-1-ynyl)-2′-deoxyuridine (0.350 g, 0.600 mmol) in anhydrous DMF (10 ml), *tert*-butyldimethylsilylchloride (0.095 g, 0.630 mmol) and imidazole (0.205 g, 3.00 mmol) were added. After 3.5 h, the reaction was diluted with CH₂Cl₂ (100 ml) and washed with 5% aqueous sodium carbonate solution (100 ml) and saturated aqueous sodium chloride solution (100 ml). The organic layer was dried (MgSO₄), evaporated and purified by silica column chromatography [2% MeOH/CH₂Cl₂ (0.5% pyridine)] to give **13a** as a white foam (0.274 g, 0.393 mmol, 65%).

5′-O-(4,4′-Dimethoxytrityl)-5-(3-tert-butyldimethylsilyloxyprop-1-ynyl)-2′-deoxyuridine-3′-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (13b). Prepared in an analogous way similar to **9b**. Polymer-bound benzyl alcohol [0.31g (2–3 mmol/g), 0.775 mmol] was then added, the reaction was stirred for 10 min then diluted with EtOAc (10 ml) and the mixture was filtered washing the solid filtrate with further EtOAc (25 ml). The filtrate was washed with 2 M aqueous sodium carbonate solution (2 × 50 ml), water (4 × 30 ml) and saturated aqueous sodium chloride solution (50 ml), and the organic layer was dried (MgSO₄) and evaporated to give a white foam (0.340 g, 0.378 mmol, 97%).

DNA synthesis

Oligonucleotides were synthesized using standard protocols on an Applied Biosystems 394 automated synthesizer using

reagents, columns and phosphoramidites from Applied Biosystems, except for the mild/fast deprotection phosphor-amidites and C5-propynyl-2′-deoxyuridine amidite that were purchased from Glen Research. Oligonucleotide syntheses were performed with DMT-ON using 0.1 M solutions of normal phosphoramidites and 0.15 M solutions of modified phosphoramidites. Natural oligonucleotides were deprotected at 65°C overnight while those containing modified dU ana-logues and *N*4-acetyl-dC were deprotected at room temperat-ure overnight. All oligonucleotides were purified using reverse-phase HPLC (ODS C18, 300 × 4.6 mm²; Alltech) using a flow rate of 1 ml/min with a gradient of 5–65% CH₃CN in 0.1 M triethylammonium acetate, pH 6.5 for more than 30 min. Purified oligonucleotides were then detritylated (20% AcOH at room temperature for 1 h, followed by evaporation) and re-purified by reverse-phase HPLC as above using either gradient A [5–30% CH₃CN in 30 min (ODN D1–D3, ODN T41, ODN T61–T63, ODN T71 and ODN T72; see Table 2 for sequences)] or gradient B [*t* = 0 min, 5% CH₃CN; *t* = 20, 14% CH₃CN; *t* = 30, 14% CH₃CN; *t* = 40, 17% CH₃CN; and *t* = 45, 17% CH₃CN (all other ODNs)]. Retention times were 20–25 min for gradient A and 30–40 min for gradient B. Solutions of purified oligonuc-leotides were then evaporated, resuspended in 1 ml of water and dialysed. All the oligonucleotides were characterized using matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) mass spectra and displayed molecular weights within 4 Da of the expected masses.

UV-melting experiments

UV-melting experiments were carried out on a Cary 500 UV–visible spectrometer with a Cary temperature controller attached. UV-melting profiles were obtained over a pH range of 5.0–9.0 using an appropriate buffer for each pH. For pH 5.0–7.0, pH 7.5–8.0 and pH 8.5–9.0, sodium cacodylate, sodium phosphate and sodium borate buffers were used, respectively. The buffers used consist of 10 mM of the appro-priate buffer, 300 mM sodium chloride and 0.1 mM Na₂EDTA. Each sample had a total volume of 1 ml and con-tained 1 nmol of each oligonucleotide strand. Samples were annealed by heating to 90°C and cooling to 15°C at a rate of 2°C/min. The UV-melting curves were measured at 260 nm (in duplicate) while heating the sample from 15 to 90°C at a rate of 1°C/min. Measured melting temperature (T_m) values of duplicate runs were all within 0.5°C of one another. At heating rates of both 0.5 and 1°C/min there were no notable differences in T_m values, suggesting that the system is in equilibrium.

NMR studies of nucleosides

Nuclear Overhauser Enhancement (NOE) experiments. All NOE spectra were obtained at 400 MHz and were performed at 298 K with D₂O as the solvent, except the experiment to determine double bond orientation within 5-(*E*-3-aminoprop-1-enyl)-2′-deoxyuridine, which was performed in (CD₃)₂SO owing to spectral overlap of the alkenyl peaks in D₂O. 5-(*Z*-3-Trifluoroactamidoprop-1-enyl)-2′-deoxyuridine was measured in CD₃OD because of solubility problems. d₆-Acetone (10%) was introduced into each sample measured in D₂O as an internal lock. The nucleoside solutions at ~0.05 M were

degassed with argon for 20 min before use. 5-(3-Amino-prop-1-ynyl)-2′-deoxyuridine, 5-(*E*-3-aminoprop-1-enyl)-2′-deoxyuridine in D₂O, 5-(*Z*-3-trifluoroactamidoprop-1-enyl)-2′-deoxyuridine and 5-(3-aminopropanyl)-2′-deoxyuridine were all performed under identical processing spectral para-meters applying the NOEMULT pulse sequence of the Bruker software package. An irradiation time of 5 s with an irradiation power of 76 dB <50 W gave saturation of ⩽95%. 5-(*Z*-3-Aminoprop-1-enyl)-2′-deoxyuridine was subjected to an irradiation time of 0.2 s and 5-(*E*-3-aminoprop-1-enyl)-2′-deoxyuridine in (CD₃)₂SO was subjected to an irradiation time of 10 s. Analysis of the spectral data was performed by sequential exponential multiplication (line broadening 3) and Fourier transform of two FIDs (one for a desired irradi-ation point and one for the resonance control point) followed by subtraction of the two spectra. All NOE percentages were obtained by peak height measurements from the difference spectra divided by peak height measurements from the control spectra.

pK$_a$ determinations. All spectra were obtained at 400 MHz with D₂O as the solvent, using its deuterium as an internal lock and a nucleoside concentration of ~0.05 M. The pH of each solution was adjusted by the addition of 0.1 M sodium deuteroxide or deuterium chloride solution and measured using a pH meter calibrated to pH 4.01 and 7.04 using known standards.

The pK$_a$ values were determined following non-linear regression fitting to the following equation using Kaleidagraph software (Synergy Software, Reading, PA).

$$y = m2 + (m3 - m2) \times \left[10^{-m1} / \left(10^{-m0} + 10^{-m1} \right) \right], \qquad \textbf{1}$$

where m0 is the ppm at a given pH, m1 is pK$_a$, m2 is the ppm of H6 at low pH and m3 is the ppm of H6 at high pH. For fitting of two pK$_a$ values to the data, the equation used is as follows:

$$\begin{aligned} y = & \left(m3 \times 10^{-m1} \times 10^{-m2} \right) + \left(m4 \times 10^{-m1} \times 10^{-m0} \right) \\ & + \left(m5 \times 10^{-m0} \times 10^{-m0} \right) / \left(10^{-m1} \times 10^{-m2} \right) \\ & + \left(10^{-m1} \times 10^{-m0} \right) + \left(10^{-m0} \times 10^{-m0} \right), \qquad \textbf{2} \end{aligned}$$

where m0 is the ppm at given pH, m1 is pK$_a$1, m2 is pK$_a$2, m3 is the ppm of H6 at high pH (double deprotonated species), m4 is the ppm of H6 of monodeprotonated species (double deprotonation) and m5 is the ppm of H6 of monodeprotonated species (double deprotonation).

RESULTS AND DISCUSSION

Synthesis of C5-amino-modified 2′-deoxyuridines

The trifluoroacetylated derivative of 5-(propargylamino)-2′-deoxyuridine (**4b**) was prepared as described by Hobbs (28) and was used as the precursor for the corresponding *Z*-alkenyl (**5b**) and 5-(3-aminopropyl) (**3b**) analogues as described previously (13,30). The 5-aminoallyl derivative (**6a**) was prepared according to the modification described by Dey and Sheppard (29) from the original palladium-catalysed coupling procedure described by Sakthivel and Barbas (17).

Treatment of the trifluoroacetyl protected nucleosides **3b**, **4b** and **6b** with aqueous ammonia solution at room temperature overnight afforded the corresponding amino nucleosides **3a**, **4a** and **6a**. In contrast, when the *cis*-alkenyl derivative **5b** was treated with ammonia, a novel bicyclic nucleoside **8** was formed which was characterized using both ^{1}H NMR and mass spectrometry. Analysis of the reaction mixture by reverse-phase HPLC indicated that the amount of the less polar, later-eluting peak corresponding to nucleoside **8** was ~90% after ammonolysis for 4 h at room temperature. However, the deprotected compound **5a** could be obtained as its ammonium salt by reducing the time of the ammonolysis to 45 min, followed by subsequent acidification to pH 4 with trifluoroacetic acid. Compound **5a** was found to be stable as its ammonium salt, but underwent complete conversion into the bicyclic nucleoside **8** if the sample was left overnight in aqueous solution above pH 8.

Conformational analysis of C5-amino-modified 2′-deoxyuridines

The glycosidic bond angle (χ) defined as the torsion angle between the O4′–C1′ and N1–C2 bonds of pyrimidine nucleosides defines two major conformations in dynamic equilibrium, either *anti* or *syn* in which the base H6 or O2 atoms, respectively, lie above the sugar. The normal conformation in pyrimidine nucleosides is *anti*. Seela and co-workers (31) have described a simple semi-quantitative method for estimating the population of molecules of a given nucleoside occupying these conformations, which may be derived from proton NOE values between the H-6 proton (for pyrimidines) and the sugar H1′, H2′ and H3′ protons. By using this method, the determined NOE values for the nucleosides **3a–6a** (see Table 1 in Supplementary Material) were used to estimate the percentage of the *anti* conformer for each respective nucleoside (Table 1). The amount of *anti* conformer estimated for thymidine using this method (31) is 78 ± 10%. As shown in Table 1, all compounds **3a–6a** exist predominantly in the *anti* conformation within the normal expected range. Thus, the side chains do not

have any appreciable effect on the glycosylic torsion angles that these analogues display compared with thymidine. All the analogues also display the same conformational preference of the deoxyribose ring for the 2′-*endo* (*S*-conformation) that is found for thymidine (32) (see Table 1).

Oligonucleotides containing an amino group tethered by an alkenyl linker are likely to have very different properties depending on the stereochemistry of the double bond and its orientation with respect to the heterocyclic base. Thus, for compounds **5a** and **6a** both *cisoid* and *transoid* orientations are possible (Figure 2) owing to restricted rotation around the C5–C7 single bond resulting from the conjugated π system in these molecules. [1]H NOE was used to estimate the populations of the respective conformations. For the *E*-alkene (**6a**), 1% NOE was seen between H_b and H6, while no NOE was observed between H_a and H6, demonstrating a strong preference for the *cisoid* conformation. In contrast, for the *Z*-alkene (**5a**), upon irradiation of H_a, NOEs to both H6 and the methylene protons (1.3%) indicate a slight preference for the *cisoid* conformation. The corresponding NOEs between H6 and H_a (2.6%) and the methylene protons (3.5%) for the trifluoroacetyl protected compound **5b** indicate the same preference of the protected *Z*-alkene for the *cisoid* conformation. The cyclization of **5a** to give nucleoside **8** must occur through the *transoid* conformation, suggesting a low-energy barrier to the interconversion between this and the *cisoid* conformation.

Determination of the pK_a values of C5-amino-modified 2′-deoxyuridines

To our knowledge, the C5-amino-modified 2′-deoxyuridines, **3a–6a**, are expected to be cationic at physiological pH but their pK_as have not been determined to date. The respective NMR signals associated with the H6 protons of C5-aminoalkyl-2′-deoxyuridines are highly sensitive to the nature of the C5 aminoalkyl side chain (see Materials and Methods). For this reason, we chose to determine the pK_a values of these compounds using ^{1}H NMR by measuring the chemical shift of H6 over a range of pH values. The respective plots are shown in Supplementary Figure 1 and the pK_a values derived from them using Equation 1 (see Materials and Methods) are shown in Table 1. At high pH (>11), a second transition appears to be present in the titration curve for the *Z*-alkenyl analogue **5a**. This presumably arises owing to the cyclization of **5a** to form the bicyclic compound **8**.

The reported pK_a values for propargylamine, allylamine and propylamine are 8.15 (33), 9.49 (34) and 10.53 (34), respectively, and may be interpreted based on the alkynyl group being the best electron withdrawing group, thereby producing the least basic amino group. Although the same trend is observed for the nucleosides, those bearing the alkyne- and alkene-tethered amino groups are more basic than expected, while the pK_a of the aminopropyl-modified analogue **3a** is less basic than expected. The determined pK_a of thymidine under our experimental conditions was found to be 10.42 (data not shown). The pK_as measured in these experiments represent macroscopic pK_a values for these nucleosides and cover the double-deprotonation event, i.e. from cationic nucleoside to anionic nucleoside in which the side chain is neutral and the N1 imino proton has dissociated. Our attempts to fit the

Table 1. Physical properties of C5-modified 2′-deoxyuridines determined using ^{1}H NMR

Compound	Percentage of anti-conformation	Percentage of *S*-conformer[a] (2′-endo)	Orientation of alkenyl group	pK_a value[b]
3a (alkane)	78	59	—	10.00 ± 0.04 (8.96)
4a (alkyne)	71	58	—	8.53 ± 0.09 (8.05)
5a (*Z*-alkene)	89	56	Slight *cisoid* preference	9.79 ± 0.15 (9.31)
6a (*E*-alkene)	76	58	*Cisoid*	9.69 ± 0.04 (9.34)
Thymidine	78[c]	62[d]	—	10.42 ± 0.04 (9.96)

[a]Calculated from H1′, H2′ and H2″ coupling constants as described previously (32).
[b]The pK_a value shown in parentheses is the lower value obtained after fitting the data to two pK_as.
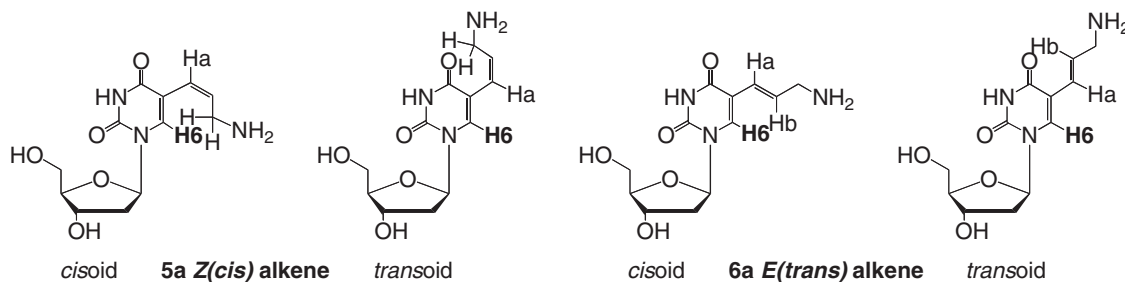[c]Data taken from (31).
[d]Data taken from (42).

Figure 2. Structures of *cisoid* and *transoid* conformations of C5-amino-alkenyl modified 2′-deoxyuridines **5a** and **6a**.
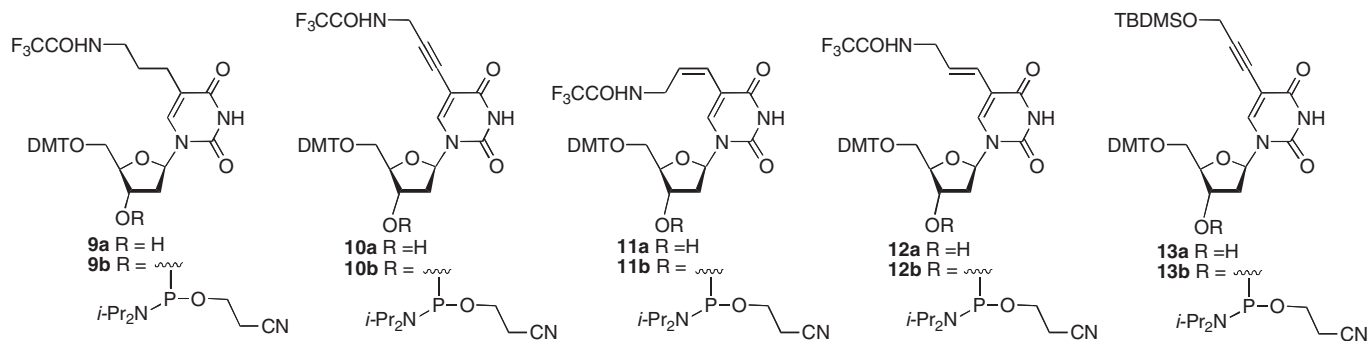

Figure 3. Structures of phosphoramidites of C5-modified 2′-deoxyuridines (**9b–13b**) used in DNA synthesis.

titration data to two pK_a values provides an estimate of the lower value of these two pK_as, below which the side chain is protonated and the base is neutral. This value is also displayed in Table 1. The range of pK_a values calculated indicates that during UV-melting studies of DNA triple helices containing modified oligonucleotides (see below) the base analogues are largely protonated over the range of pH values investigated (pH 5–7).

Chemical synthesis of oligonucleotides containing C5-modified 2′-deoxyuridines

The trifluoroacetyl protected nucleosides **3b–6b** were converted into their corresponding 5′-dimethoxytrityl derivatives (**9a–12a**) and the phosphoramidites **9b–12b** (Figure 3) were then prepared by phosphitylation with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite. The alkyne **10b** and *E*-alkene derivative **12b** have been prepared previously by others (4,26,27) and together with compounds **9b** and **11b** showed the presence of two diastereoisomers using ^{31}P NMR. All of the phosphoramidites display the expected accurate masses following analysis using electrospray mass spectrometry (see Materials and Methods). The phosphoramidite **13b** was prepared from 5′-*O*-dimethoxytrityl-5-iodo-2′-deoxyuridine, by palladium-catalysed coupling with propargyl alcohol. This was followed by protection of the side chain alcohol function as its *t*-butyldimethylsilyl ether to give **13a**, which was subsequently phosphitylated as described above. ^{31}P NMR and accurate mass measurement were used to characterize compound **13b**.

In order to investigate the effects of C5 modifications on the stability of DNA triple helices, we synthesized the oligonucleotides shown in Table 2. The triple-helix structure chosen for our study has been used previously by others (35) and provides clear melting transitions for the triplex and duplex components. The polypyrimidine third strand of the triplex-forming structures shown in Table 2 contained the bases cytosine, thymine and one or more C5-modified uracil. The phosphoramidites **9b–13b** were employed in standard oligonucleotide synthesis, using 0.15 M rather than the standard 0.1 M solutions in acetonitrile and typically resulted in coupling yields ∼95%. To avoid normal deprotection conditions, which were particularly problematic for oligonucleotides containing analogue **5** (see below), *N*4-acetyl-protected 2′-deoxycytidine phosphoramidite was used and deprotection of DMT-ON oligonucleotides following synthesis was achieved using concentrated aqueous ammonia solution at room temperature for 12 h (4 h for oligonucleotides containing **5a**). The crude oligonucleotides containing analogues **3**, **4**, **6** and **7** were all purified by reverse-phase HPLC and detritylated using 20% aqueous acetic acid, re-purified by HPLC and then dialysed. However, when oligonucleotides containing 5-(Z-3-aminoprop-1-enyl)-2′-deoxyuridine **5** were deprotected, multiple peaks were observed using HPLC owing to the formation of compound **8** within the oligonucleotide. Analysis using MALDI mass spectrometry of the two major peaks observed following the deprotection of ODN-T51 indicated that the minor peak (∼30%) contained the desired analogue **5a**, while the later-eluting major peak contains an oligonucleotide that is 18 mass units lower owing to the conversion of **5a** into the bicyclic analogue **8**. Owing to the complexity of the HPLC traces obtained for oligonucleotides containing analogue **5**, it was not possible to purify an oligonucleotide containing more than two such modifications.

Table 2. Sequences of oligonucleotides (ODNs) used in this study

Oligonucleotide	Sequence	Modification
ODN-D1	5′-CGC TAG AAG AAA GGA CG-3′	Unmodified
ODN-D2	3′-GCG ATC TTC TTT CCT GC-5′	
ODN-T31	5′-TCT TC**3** TTC CT-3′	Alkyl-
ODN-T32	5′-TCT **3**C**3** TTC CT-3′	
ODN-T33	5′-TCT **3**C**3** T**3**C CT-3′	
ODN-T36	5′-**3**C**3** **3**C**3** **33**C CT-3′	
ODN-T41	5′-TCT TC**4** TTC CT-3′	Alkynyl-
ODN-T42	5′-TCT **4**C**4** TTC CT-3′	
ODN-T43	5′-TCT **4**C**4** T**4**C CT-3′	
ODN-T46	5′-**4**C**4** **4**C**4** **44**C CT-3′	
ODN-T51	5′-TCT TC**5** TTC CT-3′	Z-Alkenyl-
ODN-T52	5′-TCT **5**C**5** TTC CT-3′	
ODN-T61	5′-TCT TC**6** TTC CT-3′	*E*-Alkenyl-
ODN-T62	5′-TCT **6**C**6** TTC CT-3′	
ODN-T63	5′-TCT **6**C**6** T**6**C CT-3′	
ODN-T66	5′-**6**C**6** **6**C**6** **66**C CT-3′	
ODN-T71	5′-TCT TC**7** TTC CT-3′	3-Hydroxy-alkynyl
ODN-T72	5′-TCT **7**C**7** TTC CT-3′	
ODN-pdU1	5′-TCT TC**P** TTC CT-3′	Propynyl
ODN-pdU2	5′-TCT **P**C**P** TTC CT-3′	
ODN-pdU3	5′-TCT **P**C**P** T**P**C CT-3′	
ODN-pdU6	5′-**P**C**P** **P**C**P** **PP**C CT-3′	
ODN-T	5′-TCT TCT TTC CT-3′	Unmodified

The suffix D or T refers to ODNs used in duplex formation or triplex formation, respectively. The first number refers to the analogue (see Figure 1) and the second number refers to the number of such modifications in the oligonucleotide. The sequence involved in triplex formation is underlined in ODN-D1. 'P' refers to C5-propynyl-2′-deoxyuridine.

Thermal stability studies of DNA triple helices containing C5-amino-modified 2′-deoxyuridines

The UV-melting curves for the triplex structures in which the third pyrimidine-rich strand contains one or more C5-amino-modified 2′-deoxyuridine were measured between pH 5.0 and 7.0 and compared with the melting of the unmodified triplex. The triplex structure and sequences of oligonucleotides are shown in Table 2. At low pH values cytosine becomes protonated (pK_a ~4.5) and the unmodified polypyrimidine strand participates in triplex formation resulting in two distinct melting transitions (triplex to duplex and duplex to single-stranded) with increased temperature. Figure 4 shows the first derivatives of the melting curves for the unmodified and modified triplexes measured at pH 5, in which a single thymidine in the third strand has been replaced by one of the analogues **3a–6a**, **7** or C5-propynyl-2′-deoxyuridine. In agreement with the previous work (4), the propargylamino-modified 2′-deoxyuridine has a dramatic effect on the stability of the triplex with an increase in T_m > 6°C with just a single substitution. When a single thymidine is replaced by the C5-aminoalkene analogue containing a *trans* (*E*) double bond in the linker (**6a**) the T_m is essentially unchanged (ODN-T61), while an aminopropyl-modified analogue significantly decreases the T_m (ODN-T31). Interestingly, an
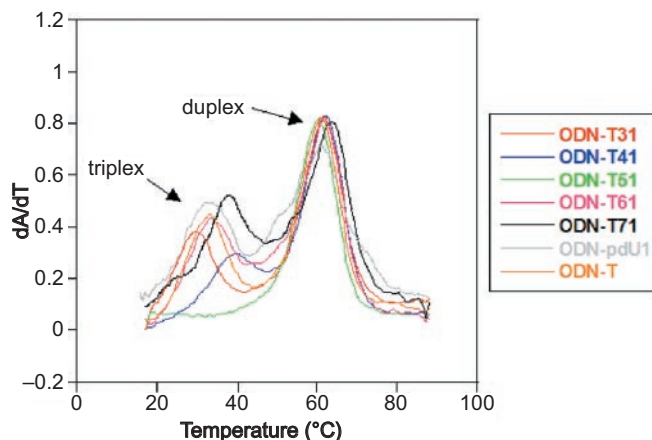

Figure 4. First derivative of melting curves for triplex structures containing a single C5-amino-modified dU analogue in the third strand. Spectra were recorded at 260 nm and at pH 5. For complete experimental details see Materials and Methods and for sequences see Table 2.

Table 3. Thermal melting midpoint (T_m) values for triplex melting at various pH values

Modification	Oligonucleotide	pH 5.0	5.5	6.0	6.5	7.0
Alkyl-	ODN-T31	29.6	25.0	X	X	X
	ODN-T32	24.9	19.7	X	X	X
	ODN-T33	19.0	X	X	X	X
	ODN-T36	X				
Alkynyl-	ODN-T41	39.7	32.0	22.9	X	X
	ODN-T42	43.3	35.0	25.0	X	X
	ODN-T43	47.5	39.1	29.0	X	X
	ODN-T46	63.8	52.7	38.2	27.4	X
Z-Alkenyl-	ODN-T51	X	X	X	X	
	ODN-T52	X	X	X	X	
E-Alkenyl-	ODN-T61	33.5	27.1	X	X	
	ODN-T62	33.1	26.8	X	X	
	ODN-T63	31.6	25.0	X	X	X
	ODN-T66	25.3	19.4	X		
3-Hydroxy-alkynyl	ODN-T71	37.6	30.0	X		
	ODN-T72	41.8	32.7	23.0		
Propynyl	ODN-pdU1	33.6	26.1	X	X	
	ODN-pdU2	37.6	30.6	22.2	X	
	ODN-pdU3	43.2	33.2	24.0	X	
	ODN-pdU6	48.9	37.2	26.7	X	
Unmodified	ODN-T	33.1	27.4	X	X	X

The oligonucleotide refers to the third strand of the triplex structure indicated in Table 1. 'X' indicates that no melting transition was observed. The shaded sections of the table indicate that no data was collected. For full experimental details see Materials and Methods.

oligonucleotide containing the aminoalkene analogue possessing a *cis* (*Z*) double bond (**5a**) (ODN-T51) is unable to form a triplex even at pH 5 despite the fact that under such conditions the amino group is essentially fully protonated. All triplex structures containing analogues **3a**, **5a** or **6a** become less stable as the number of amino-modified dU analogues is increased (see Table 3). This may result from intrinsic destabilizing properties of the analogues themselves or from a destabilization owing to repulsion between cationic side chains. In contrast, triple helices containing the

propargylamino-modified analogue **4a** exhibit a dramatic increase in stability that is proportional to the number of substitutions (compare triplexes containing ODN-T46 and ODN-T41).

The stabilities of all the triple-helical structures decrease with increasing pH and only when the third strand contains amino-modified dU analogues containing the alkyne linker triplexes are formed above pH 5.5 (see Table 3). Over the pH range studied, the relative stabilities of triplexes containing the amino-modified dU analogues are preserved; alkynyl > *trans*-alkenyl ∼ unmodified > alkyl > *cis*-alkenyl (Table 3). Below pH 7, all the amino-modified side chains of the dU analogues are largely protonated and it is quite clear that the relative abilities of such analogues to stabilize triple helices depend less on the ionization state of the base, but rather on the type of linker between the protonated amino group and the nucleobase. The ability of C5-propynyl-2′-deoxyuridine (i.e. with no appended cations to the nucleobase) to stabilize triple helices through enhanced base stacking is well-documented (36,37). In DNA duplexes and triplexes, a C5-propynyl-modified cytosine or uracil can enhance stability (7). Replacement with the propargylamino analogue **4a** is also known to increase stability further (6,8). When the amino group is protected as an amide, lower duplex stabilities result (8,38). These studies with duplexes clearly demonstrate the effect of an appended amino group on stability. However, the exact role it plays, in particular whether it enhances base stacking of the analogue or whether it forms favourable electrostatic interactions, e.g. with neighbouring phosphates is not clear.

In order to assess the contribution towards stability imparted by the combination of the amino group and the propynyl group of **4a**, we decided to investigate the ability of oligonucleotides containing the novel dU analogue **7** and those containing C5-propynyl-2′-deoxyuridine to form triple-helical structures. Compound **7** retains the propynyl side chain that is present in analogue **4a**, but also bears a tethered electronegative hydroxyl group. Thus, it is analogous to the protonated amino group of **4a** but lacking a positive charge. Interestingly, incorporation of analogue **7** into the third strand of the triplex results in an increase in stability that is only slightly lower than that observed for triplexes that contain the propargylamino modification (compare ODN T41 and 71 and ODN T42 and 72). Furthermore, essentially the same pH-dependence of triple-helix stability is observed for structures containing either analogue **4a** or **7** (Supplementary Figure 2), which presumably results from the requirement to protonate cytosines within the triple-helix forming oligonucleotide (TFO). The analogous triplex structures containing C5-propynyl-2′-deoxyuridine display enhanced stabilities relative to the unmodified sequence but these are much less than those containing **4a** or **7**. The results suggest that a major factor in the ability of the propargylamino-modified dU analogue to stabilize triple helices was derived mainly from the nature of the alkynyl group itself with a lesser contribution being derived from the possible favourable electrostatic interactions. Indeed, modelling studies (8) suggest that the amino group of the propargylamino analogue **4a** displays a preference for location within the major groove of DNA rather than for salt bridge formation with nearby phosphate groups. Therefore, it seems most probable that other electron-withdrawing groups appended to the alkyne would also result in a similar stability increase.

In the previous studies, the effects of ionic strength on the stabilities of DNA duplexes containing C5-aminoalkyl-modified dU analogues have been undertaken (2,3,6). At lower ionic strengths, unmodified duplexes show a greater relative decrease in stability when compared with the same sequences containing such appended cationic residues. In their study of triple-helix stabilization by the analogue **6a**, Brown and Fox (4) obtained data at 100 mM NaCl concentration using a TFO containing the analogue within an oligothymidine sequence. In this study, we used a mixed sequence TFO (i.e. containing cytosine and thymine bases) and did not observe triplex formation at 100 mM NaCl. We were also unable to measure T_m values at 200 mM NaCl for the doubly modified sequences, ODN-T72 and ODN-T42, which contain the hydroxypropynyl and aminopropynyl-modified analogues, respectively. Therefore, a comparison of the ionic strength dependence on the stability of TFOs containing the two analogues was not possible. Therefore, in comparison with the previous studies using the same triplex sequence (35), we measured the triplex melting at 300 mM NaCl. At this ionic strength, it might be argued that the differences in stabilities of TFOs containing these two analogues might be masked to some extent. While this cannot be entirely ruled out, the fact that TFOs containing propynyl dU are much less stable (compare ODN-T42 and ODN-T72 with ODN-pdU2 in Table 3) suggests that the combination of amino or hydroxyl group and the propynyl substituent are particularly favourable.

The reason for the low stabilities of triplexes containing C5-amino-modified dU analogues that have alkyl or *Z*-alkenyl linker arms is unclear. However, for the propyl analogue **3a** interaction of the amino group with the O4-carbonyl group of the nucleobase is possible, which would severely disrupt the ability of these analogues to form Hoogsteen base pairs required for triplex formation. Indeed it is clear from PAGE, modelling and NMR studies (12,39–41) that the side chain of this analogue prefers to interact with the major groove of a DNA duplex rather than the phosphate diester backbone. For the Z-alkenyl analogue, the *transoid* conformation would be required for such an interaction with the carbonyl group. Although NMR experiments indicate the presence of both the *transoid* and *cisoid* conformations, it is quite possible that for the latter conformation the charged amino group lies outside the plane of the base in order to minimize unfavourable steric interactions with the H6 proton of the base. Such a conformation would also likely be highly disruptive to DNA secondary structures. In the previous studies, we have noted that the triphosphates of analogues **3a** and **5a** are not incorporated by a variety of DNA polymerases (13) while those of analogues **4a** and **6a** are good substrates. Furthermore, we have been unable to form duplex structures that contain the *Z*-alkene **5a** (Brazier,J.A. and Williams,D.M., unpublished data). We attribute these findings to the positively charged amino group, which for each analogue is placed in a position that is highly destabilizing to base pair formation. In contrast, for the *E*-alkene analogue **6a** (*cisoid* conformation) and alkyne analogue **4a** such destabilizing interactions cannot take place since the amino group is located much further away from the base and is able to reside in the same plane. The

results of the current study imply that this latter feature is the most important in terms of stability and that the effects of a charged amino group are to enhance the intrinsic stabilizing properties of C5-propynyl group in nucleic acid structures.

CONCLUSIONS

We have reported on the conformational properties and pK_a values of four different C5-amino-modified 2′-deoxyuridines that differ in the nature of the linker between the amino group and the nucleobase. We have prepared oligonucleotides containing these analogues and have shown that the relative stabilities of DNA triplexes containing these analogues are in the following order: alkyne > *E*-alkene > alkane > *Z*-alkene. We have also shown that the stabilizing effects of the propargylamino modifications are likely to arise principally from favourable base stacking interactions since we have found a high stability for triplex structures containing 5-(3-hydroxy-prop-1-ynyl)-2′-deoxyuridine. The results are relevant to the design of both triplex and duplex structures that contain analogues designed to enhance their stabilities. In addition, the physicochemical properties of the analogues will be relevant to understanding the properties of nucleic acid structures and catalysts designed using the SELEX technique.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

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