# Synthesis of oligodeoxynucleotides containing 5-aminouracil and its *N*-acetyl derivative

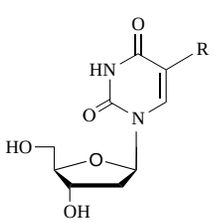
**Elisenda Ferrer, Gitte Neubauer, Matthias Mann and Ramon Eritja***

*European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany*

**The preparation of oligonucleotides containing 5-amino-2′-deoxyuridine is described. Three different protective groups for the amino function of 5-aminouracil including trifluoroacetyl, dimethylformamidine and 2-(4-nitrophenyl)ethoxycarbonyl are analysed in order to reduce the acetylation of this base observed during the assembly of oligonucleotides containing this base analogue. The side-reaction is avoided by using the base-labile 2-(4-nitrophenyl)ethoxy as protecting group and 2-(4-nitrophenyl)ethyl chloroformate during the capping step.**

## Introduction

5-Aminouridine, 5-amino-2′-deoxyuridine ($^{N}$dU, **1a**) and its N-aminoacyl derivatives have a wide range of biological effects, including antibacterial, antiviral, antifungal and antitumour activities.[1] Most of their biological properties come from interference in purine biosynthesis,[1] although a small incorporation into DNA and RNA has been found.[1c,2] Preparation of 5-aminouridine derivatives can be accomplished by reaction of 5-bromouridine with ammonia[3] or by reduction of the 5-nitrouridine.[4] Starting from 5-aminouridine, poly-(5-aminouridylic acids) have been prepared by enzymic phosphorylation and polymerisation.[5] Similarly to poly(U), poly-(5-aminouridylic acid) forms a triple-stranded complex with poly(A) and stimulates the synthesis of poly(Phe).[5] The p$K_a$-values of poly-(5-aminouridylic acid) are 4.1 and 9.4,[5] and 2.7 and 9.1 for 5-aminouridine,[5] indicating that this base is not ionised at physiological pH. Base-pairing properties of 5-amino-2′-deoxyuridine ($^{N}$dU) with 2′-deoxyadenosine (dA) have been measured by NMR spectroscopy.[6] Compared with thymidine (T), a small decrease in the association constants with dA is observed.




**1a;** R = $NH_2$, $^{N}$dU

**1b;** R = $NHCOCH_3$, $^{Ac}$dU

Chemical structures of 5-amino-2′-deoxyuridine **1a** and its *N*-acetyl derivative **1b**

The preparation of oligonucleotides containing 5-amino-2′-deoxyuridine **1a** has been described, using the trifluoroacetyl group as protective group for the 5-amino function.[7] A small decrease in the melting temperature ($T_m$) has been observed when 5-aminouracil is paired with A compared with the A:T base pair,[7] which is in agreement with the association constants measured by NMR spectroscopy at the monomer level.[6] The possibility of using the special reactivity of the amino function of 5-aminouracil to introduce fluorescent labels has also been described.[7] Recently, special triple-helix structures involving 5-aminouracil residues ($^{N}$U) have been found.[8] 5-Aminouracil:A base pairs can form the triad A:$^{N}$U:A if the third strand is parallel, and the triad G:$^{N}$U:A if the third strand is antiparallel.[8]

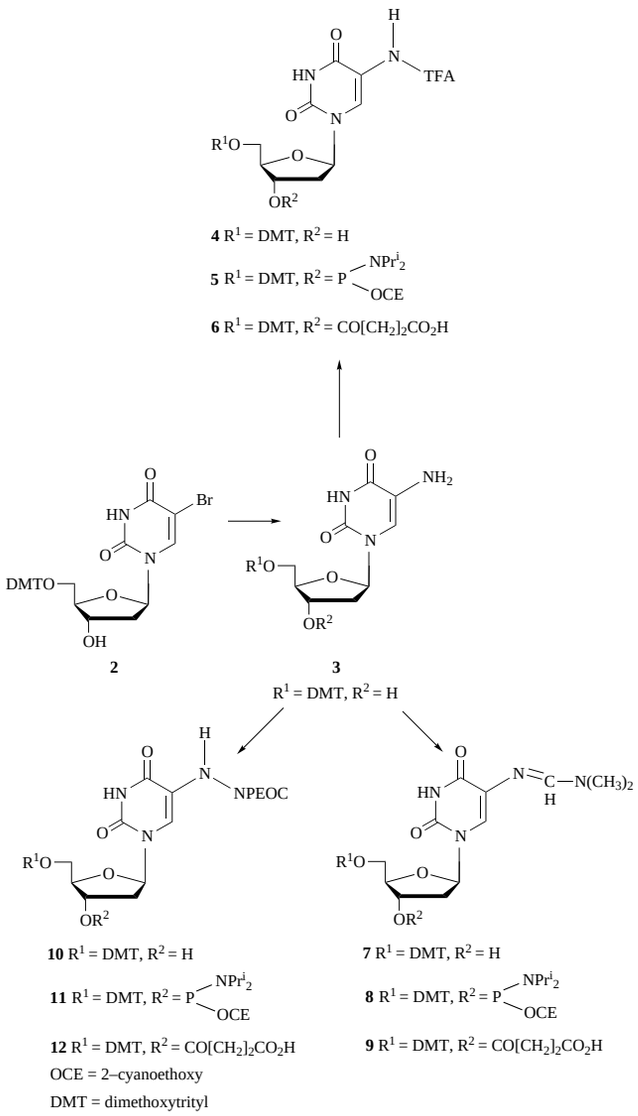
In this paper we show that protected 5-aminouracil ($^{N}$U) is prone to exchange with the acetyl group during capping reactions of the oligonucleotide assembly, yielding oligonucleotides containing 5-acetamidouracil ($^{Ac}$U). This side-reaction is avoided by selection of an appropriate protecting group and capping conditions. The method presented here yields pure 5-aminouracil oligonucleotides that could be used in non-radioactive labelling and structural studies.

## Results

### Preliminary studies using the trifluoroacetyl (TFA) protecting group

In order to synthesize oligonucleotides containing 5-amino-2′-deoxyuridine **1a**, a protected derivative should be obtained. The chemical properties of the amino group of 5-aminouracil are different from those of the amino groups of the natural bases (C, A and G). Previous work has shown that benzoyl and acetyl groups are not suitable for 5-aminouracil because they are not removed during standard ammonia deprotection conditions.[7] On the other hand, the more labile TFA group (which is too labile for the protection of the amino groups of natural bases) is stable on 5-aminouracil and it can be removed in 1 h at 60 °C. For these reasons, we prepared the phosphoramidite derivative of TFA-protected $^{N}$dU according to Barawkar and Ganesh[7] (Scheme 1). 5′-[4,4′-Dimethoxytrityl (DMT)]-5-amino-dU **3** was prepared by reaction of DMT-5-bromo-dU **2** with liquid ammonia in a steel bomb. Reaction of amine **3** with ethyl trifluoroacetate yielded the TFA-protected nucleoside **4** which was then treated with the appropriate chlorophosphine to yield the desired 2-cyanoethyl phosphoramidite **5**. Spectroscopic data for these products were in agreement with previously described data.[7]

Compound **5** was incorporated at defined positions into small synthetic oligonucleotides (6–7 bases) using standard oligonucleotide-synthesis protocols. Although the purified products were homogeneous as judged by reversed-phase high-performance liquid chromatography (HPLC), no satisfactory nucleoside compositions were observed (data not shown). First, the peak corresponding to $^{N}$dU was smaller than expected and second, an extra peak was observed near the peak corresponding to dG. To investigate the reason for the discrepancy in the nucleoside composition, the hemisuccinate of 5′-*O*-DMT-*N*-TFA-$^{N}$dU (compound **6**) was prepared and linked to controlled-pore glass.[9] The $^{5'}$A$^{N}$U$^{3'}$ dimer was prepared and analysed by reversed-phase HPLC. Two different peaks were




**Scheme 1** Preparation of the phosphoramidite derivatives of N-protected 5-amino-2′-deoxyuridines

obtained in a 40:60 ratio (Fig. 1) and were analysed by enzyme digestion. The first eluting peak contained $^{N}$dU and dA in a 1:1 ratio, indicating the expected product, and the second eluting peak contained dA and an extra peak eluting near dG. The new product had a maximum UV absorbance at 276 nm, while $^{N}$dU had a maximum at 294 nm. Mass spectrometric analysis of the products gave the expected molecular mass for the first eluting peak (M = 556) and 42 mass units more than expected for the second eluting peak (M = 598). The same two products were obtained when the dimer $^{5'N}$UA$^{3'}$ was prepared using the $^{N}$dU phosphoramidite protected with the TFA group, although in a different ratio (60:40 instead of 40:60).

The isolated side-product was characterised as a dimer containing the acetyl group attached to the 5-amino group of $^{N}$dU. The identity of the side-product was verified by synthesis of 5-acetamido-2′-deoxyuridine ($^{Ac}$dU **1b**, see below) and comparison of the spectroscopic and chromatographic data with data from the extra product obtained during enzyme digestion. The acetyl group was incorporated during the capping reaction used in the solid-phase oligonucleotide synthesis. Removal of the capping step eliminates the formation of this product. As has been described previously, once the acetyl group is incorporated the ammonia treatment is not able to remove it.[7] Finally, treatment of the side-product obtained during the synthesis of the dimer with 1 M NaOH at 60 °C gave the desired dimer without the acetyl group as described for the monomer.[7]

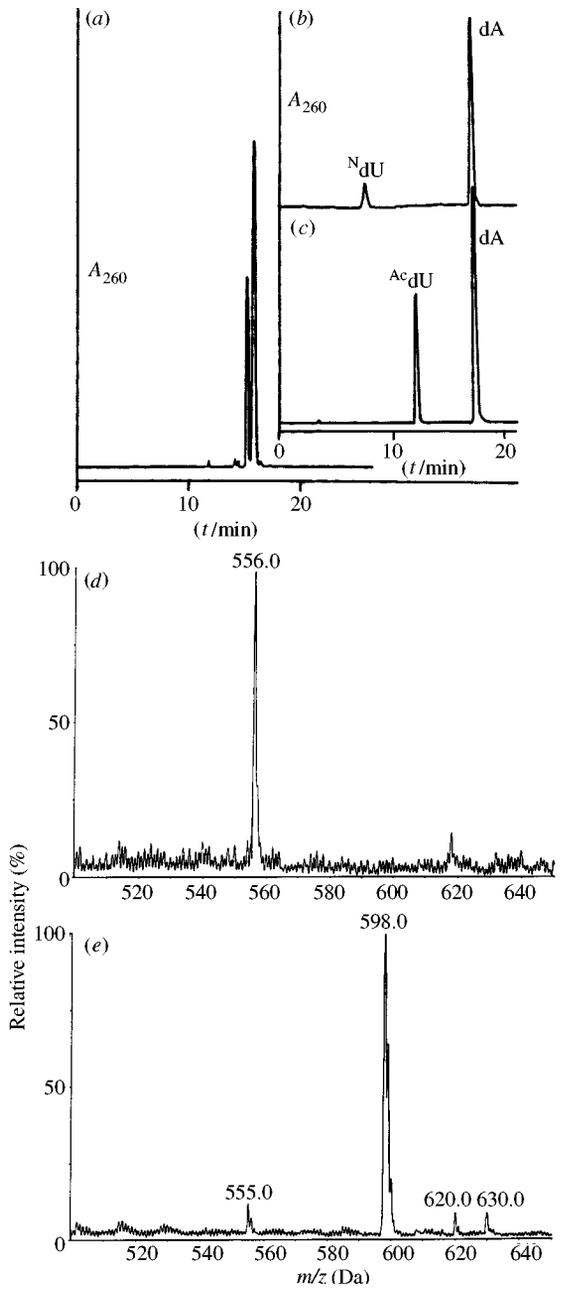



**Fig. 1** (*a*) Reversed-phase HPLC profile of dimer $^{5'}$A$^{N}$U$^{3'}$ prepared using $^{N}$U phosphoramidite protected with the TFA group (see conditions in Experimental section). (*b*) Enzyme digestion of first peak showing $^{N}$dU ($\lambda_{max}$ 293 nm) and dA. (*c*) Enzyme digestion of second peak showing $^{Ac}$dU ($\lambda_{max}$ 276 nm) and dA. (*d*) Mass spectrum of the product collected from the first peak. (*e*) Mass spectrum of the product collected from the second peak.

Unfortunately, degradation of the dimer to monomers was observed and for this reason the hydrolytic treatment is not of practical use for the preparation of oligonucleotides.

### Selection of alternative protecting groups

In order to avoid the formation of the acetyl derivative, two alternative groups for the protection of the amino group were analysed. First, the dimethylformamidino (Dmf)[10] group (**7**, Scheme 1) and then the 2-(4-nitrophenyl)ethoxycarbonyl (NPEOC)[11] group. These groups were selected because the commonly used acetyl, benzoyl and trifluoroacetyl groups were proven to be unsuitable for the protection of 5-aminouracil.

Reaction of compound **3** with dimethylformamide dimethyl acetal gave the Dmf-protected nucleoside **7**, isolated in good yield. Deprotection studies showed that the Dmf group was completely removed after 6 h in conc. aq. ammonia at 60 °C.

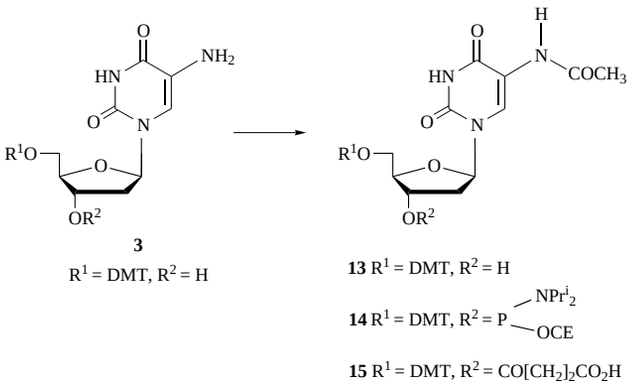
The phosphoramidite **8** and the hemisuccinate **9** derivatives were prepared following standard protocols. The hemisuccinate was linked to amino-LCAA-CPG (LCAA-CPG = long chain aminoalkyl-controlled-pore glass). The model dimer $A^{N}U$ was prepared, and analysed by HPLC. Also, two peaks were obtained (in a 1:1 ratio) that were characterised as the expected dimer and the acetylated dimer. As described for the TFA-protected derivative, the use of the phosphoramidite **8** gave lesser amounts of the acetyl derivative though still a large amount of acetylated side-product was observed, independent of the size of the oligonucleotide.

The introduction of the NPEOC group to 5′-DMT-$^{N}$dU **3** was performed using the NPEOC *N*-methylimidazolium chloride derivative.[11] First the reaction was performed without protection of the 3′-hydroxy group, but small amounts of a product containing two NPEOC groups were observed. Only transient protection of the 3′-hydroxy group with hexamethyldisilazane (HMDS) and subsequent reaction with the *N*-methylimidazolium derivative gave the desired NPEOC-protected nucleoside **10** in good yield. Deprotection of the NPEOC-protected amine was completed after treatment at room temperature with a 0.5 M solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile for 2 h. Compound **10** was linked to a sarcosyl[12] support after the preparation of the hemisuccinate **12**. The sarcosyl support was used to avoid premature loss of oligonucleotide during the DBU treatment needed for the removal of the NPEOC group.[12,13] Phosphoramidite **11** was prepared following standard phosphoramidite protocols. The model dimer $A^{N}U$ was prepared using standard protocols. Deprotection was performed as follows: treatment of the support with a 0.5 M DBU solution in acetonitrile for 2 h at room temperature, and then treatment with conc. ammonia for 6 h at 60 °C. A major peak corresponding to the desired product was obtained but small amounts of the acetylated dimer could also be observed (5–10%).

### Modification of the capping conditions

After these experiments, it was clear that it would be difficult to obtain pure oligonucleotides with 5-aminouracil by changing only the protecting group. Modifications on the capping conditions were also needed. First, we tried the replacement of acetic anhydride by trifluoroacetic anhydride (TFAA) together with the use of TFA-protected $^{N}$dU monomer. The dimer $A^{N}U$ was prepared, and analysed by HPLC. Pure dimer was obtained as the only product. However, when a longer sequence (15 bases) was prepared, a very broad peak was obtained containing a large number of products (data not shown). This negative result was obtained in spite of good incorporation of the monomers as judged by the DMT absorbance. It has been shown in peptide synthesis that trifluoroacetyl ester groups are labile and they can be transferred to amino groups, yielding truncated sequences.[14] This could be the cause of the heterogeneity observed when TFAA was used as a capping agent.

Second, we used a 2% solution of 2-(4-nitrophenyl)ethyl chloroformate instead of the acetic anhydride solution together with the NPEOC-protected $^{N}$dU monomer. The synthesis of the model dimer $A^{N}U$ gave only the desired product as expected. Afterwards, the following sequences were prepared: 15b-$^{N}$U: $^{5'}$GCA ATG GA$^{N}$U CCT CTA$^{3'}$, and $s_{11}$-$^{N}$U: $^{5'}$C$^{N}$U$^{N}$U CC$^{N}$U CC$^{N}$U CT$^{3'}$. Deprotection was performed in two steps. First the NPEOC groups were removed by a DBU solution in acetonitrile and then the standard ammonia deprotection was performed. Purification was performed using the DMT-on protocol and reversed-phase HPLC columns. In all cases a major peak was obtained that was collected and repurified without the DMT group. In the second HPLC purification one major peak was observed and collected. Analysis of purified products by enzyme digestion and mass spectrometry gave the correct nucleoside composition and the expected mass (see Table 1). During the second HPLC purification of the oligonucleotide containing more than one $^{N}$dU residue ($s_{11}$-$^{N}$U), small amounts of products eluting after the major peak were observed. These products could be produced by the addition of 4-nitrostyrene (formed during the β-elimination of NPEOC groups) to the bases.[15] The desired oligonucleotides were obtained in good yields (see Table 1) and they were well separated from the side-products. However, they could present a small problem if a longer oligonucleotide with several $^{N}$dU residues was prepared.




**Scheme 2** Preparation of the phosphoramidite derivative of 5-acetamido-2′-deoxyuridine

### Preparation of oligonucleotides containing 5-acetamido-2′-deoxyuridine

In order to confirm the stability of 5-acetamidouracil under ammonia deprotection conditions, the phosphoramidite derivative of this compound was prepared (Scheme 2). Transient protection of compound **3** with HMDS yielded the 3′-O-silylated nucleoside, which was treated with acetyl chloride. Compound **13** was phosphitylated with chloro-(2-cyanoethoxy)-(diisopropylamino)phosphine to yield the desired phosphoramidite **14** in 66% yield. Moreover, the DMT-protected acetamido-dU was attached to amino-CPG after the preparation of the 3′-*O*-hemisuccinate **15**. The synthesis of the dimer $A^{Ac}U$ gave a major product that was identical (by HPLC, mass spectrometry, UV spectroscopy and enzyme digestion) with the side-product observed during the preparation of 5-aminouracil. When ammonia deprotection was performed overnight at 60 °C, a small amount (5–10%) of $A^{N}U$ was observed, in agreement with previously reported data.[7] When ammonia deprotection was performed at room temperature, the hydrolysis product was not observed. Using these deprotection conditions the following oligonucleotide sequences were prepared and purified: 15b-$^{Ac}$U: $^{5'}$GCA ATG GA$^{Ac}$U CCT CTA$^{3'}$ and $s_{11}$-$^{Ac}$U: $^{5'}$C$^{Ac}$U$^{Ac}$U CC$^{Ac}$U CC$^{Ac}$U CT$^{3'}$. In both cases one homogenous product was obtained, and characterised by enzyme digestion and mass spectrometry (see Table 1).

### Melting experiments

Duplexes having 5-aminouracil and 5-acetamidouracil base pairs with the four natural bases were analysed. Melting temperatures ($T_m$) are shown in Table 2. Thymine base pairs were included as reference. As expected, the base pair is more stable when the uracil derivatives are paired with A. No big differences were observed between the different uracil derivatives. The small decrease observed in the $^{N}$U:A base pair by Barawkar and Ganesh[7] is not observed in our case but this could be due to differences in both the neighbouring bases and the length of the oligonucleotides. Mismatched base pairs have also very similar stability. 5-Acetamidouracil mispairs with G and T are slightly more stable than T:G and T:T mismatches, and $^{N}$U:G is also more stable than the T:G mispair. The small differences in the melting temperatures of the different base pairs are in agreement with the fact that the modification of the base occurs at position 5, which is not directly involved in base-pairing.

**Table 1** Oligonucleotide sequences containing 5-aminouracil and 5-acetamidouracil prepared in this work

| Sequence | Yield[a] (%) | *m/z* (Da)[b] | Expected mass (Da) | HPLC[c] |
|---|---|---|---|---|
| $^{5'}A^{N}U^{3'}$ | n.d. | 556.1 | 556.3 | 15.2[d] |
| $^{5'}A^{Ac}U^{3'}$ | n.d. | 598.3 | 598.3 | 15.7[d] |
| $s_{11}$-$^{N}$U: $^{5'}C^{N}U^{N}UCC^{N}UCC^{N}UCT^{3'}$ | 26 | 3199.2 | 3197.6 | 9.2 |
| $s_{11}$-$^{Ac}$U: $^{5'}C^{Ac}U^{Ac}UCC^{Ac}UCC^{Ac}UCT^{3'}$ | 30 | 3408.6 | 3408.8 | 10.2 |
| 15b-$^{N}$U: $^{5'}$GCAATGGA$^{N}$UCCTCTA$^{3'}$ | 35 | 4551.3 | 4552.4 | 11.2 |
| 15b-$^{Ac}$U: $^{5'}$GCAATGGA$^{Ac}$UCCTCTA$^{3'}$ | 22 | 4594.5 | 4594.4 | 11.2 |

[a] Overall yield after synthesis and HPLC purification. [b] Measured mass using electrospray mass spectrometry. [c] Retention time of the major peaks observed in HPLC in min ([d] dimers were run in conditions A and the rest in conditions B; see conditions in Experimental section). n.d., Not determined.

Moreover, the different electron-donating properties of the substituents (Me, NHCOMe, $NH_2$) have little influence in the base-pairing properties of these base analogues.

## Discussion

There is growing interest in modified oligonucleotides due to their application as oligonucleotide probes and antisense inhibitors of gene expression. The incorporation of modified heterocyclic bases opens the possibility of improving some of the biophysical properties of oligonucleotides at specific sites without introducing large changes in the oligonucleotide structure.[16] 5-Substituted pyrimidines are among the most studied compounds because of their interesting biological properties. For example, 5-propynylpyrimidines have higher hybridisation properties compared with the parent 5-H pyrimidines.[17] Also, the 5-position of pyrimidines is frequently used for the introduction of fluorescent groups, biotin and reactive probes in synthetic oligonucleotides.[18]

The aim of the present work is the preparation of oligonucleotides containing 5-aminouracil. Although the synthesis of oligonucleotides containing 5-aminouracil has been previously described,[7] in our hands the described method yielded variable amounts of oligonucleotides with an acetamido group instead of the amino group. The side-product was clearly identified and quantified at the dimer level where the differences in retention time are sufficient to distinguish both acetylated and non-acetylated products. This difference in elution time is not observed in larger oligonucleotides and, probably for this reason, the side-reaction was not previously detected.[7] Acetylation of protected nucleosides during the capping step has been previously observed in oligonucleotide synthesis. Acetylation of *tert*-butylphenoxyacetyl- and phenoxyacetyl-G has been reported by several groups.[19] This side-reaction was detected when labile, non-natural bases were incorporated into oligonucleotides and special deprotection conditions were used to avoid degradation of non-natural bases. Replacement of acetic anhydride for the corresponding *tert*-butylphenoxyacetic or phenoxyacetic anhydride avoided the side-reaction.[19] Conc. aq. ammonia at 60 °C could also be used for the removal of acetyl groups in natural nucleosides. However, in our case the stability of the acetamido group at position 5 made this removal impractical. The use of phenoxyacetyl groups for the protection of 5-aminouracil has not been attempted because of the difficulty in removing the acetyl group. However, this group remains an alternative protective group to be tested.

Recently, acetylation of oligonucleotides carrying 3-aminopropane-1,2-diol groups at the 3′-end have been reported.[20] Protection of the amino group with TFA and fluorenylmethoxycarbonyl (Fmoc) groups yielded variable amounts of 3′-acetamido oligonucleotides; this could be avoided by using the phthaloyl group for the protection of the amino group.[20] Those authors suggested partial removal of Fmoc and TFA groups during the synthesis cycle to explain the formation of the acetylated products. In our case, NPEOC groups are very stable to synthesis conditions and small amounts of acetyl groups were still found. Therefore, at least in the case of the NPEOC-protected $^{N}$dU, acetylation of the urethane to form an *N*-acetyl-NPEOC intermediate is more likely. Selective removal of NPEOC group by treatment with DBU will yield $^{Ac}$dU residues that are stable to ammonia. Also the higher acetylation found in TFA-protected $^{N}$dU is in agreement with an increased acidity of the NH due to the electron-withdrawing properties of the TFA group. On the other hand, acetylation of Dmf-protected $^{N}$dU needs partial removal of the Dmf group, but we were unable to determine the conditions under which this process was happening. Overnight treatment of 5′-*O*-DMT-*N*$^{5}$-Dmf-protected $^{N}$dU with the capping reagents yielded only 3′-acetyl derivative (data not shown). The Dmf group was stable to capping conditions in solution. However, under the solid-phase conditions with a larger excess of reagents and in the presence of the silicate matrix, partial removal of the Dmf could occur.

Among the three different protective groups that have been evaluated for the protection of the amino group of $^{N}$dU, NPEOC was found to be the most appropriate. As described by Pfleiderer,[11] this group is very stable to synthesis conditions but can be removed under mild and selective conditions by β-elimination. Moreover, unwanted acetylation of protected $^{N}$dU is greatly reduced and it can be eliminated by using NPEOC-Cl as capping agent. Although in this work pure products have been obtained, the method could be limited to short–medium size oligonucleotides, since some side-products were detected with the 11-mer containing four $^{N}$dU residues. Diethoxy *N*,*N*-diisopropylphosphoramidite could be an interesting alternative but this possibility has not been explored.[21] The results obtained in this work may be of interest for the preparation of oligonucleotides carrying alkylamino groups such as 5′- and 3′-aminooligonucleotides,[22] 2′-*O*-(alkylamino)-ribonucleosides,[22] aminoalkyl derivatives on the nucleobases[16,18,22] and peptide nucleic acid (PNA) synthesis.[23] In these cases an alkylamino group is introduced and often this group is protected using TFA or Fmoc groups. Acetylation of the protected amino groups will yield acetyl derivatives that would not be deprotected by ammonia. On acetylation, the special reactivity of the amino groups is lost and functionalisation of oligonucleotides with active esters could be more inefficient.

**Table 2** Melting temperatures ($T_m$/°C) of 5-amino-2′-deoxyuridine and 5-acetamido-2′-deoxyuridine duplexes[a]

$^{5'}$TAG AGG XTC CAT TGC$^{3'}$
$^{3'}$ATC TCC YAG GTA ACG$^{5'}$

| | Y | | |
|---|---|---|---|
| X | T | $^{N}$U | $^{Ac}$U |
| A | 56.0 | 56.1 | 56.2 |
| G | 51.3 | 51.6 | 53.0 |
| T | 47.0 | 48.3 | 48.1 |
| C | 45.5 | 43.2 | 45.3 |

[a] From duplexes prepared in a solution containing 0.15 M NaCl, 0.05 M Tris-HCl buffer (pH 7.4) (see Experimental section).

## Experimental

*Abbreviations*: Ac: acetyl, ACN: acetonitrile, CE: 2-cyanoethyl, CPG: controlled-pore glass, DBU: 1,8-diazabicyclo[5.4.0]-undec-7-ene, DCM: dichloromethane, DMAP: 4-(dimethylamino)pyridine, Dmf: dimethylformamidine, DMF: dimethylformamide, DMT: 4,4′-dimethoxytrityl, $^{Ac}$dU: 5-acetamido-2′-deoxyuridine, $^{N}$dU: 5-amino-2′-deoxyuridine, LCAA: long-chain aminoalkyl, NPEOC: 2-(4-nitrophenyl)ethoxycarbonyl, TFA: trifluoroacetyl, THF: tetrahydrofuran, $^{Ac}$U: 5-acetamidouracil, $^{N}$U: 5-aminouracil.

### General methods

All reactions were carried out in oven-dried glassware, under nitrogen or argon, unless specified otherwise. Before use, starting materials were dried by evaporation with the dry solvent which was later used for the reaction. *N*-Methyl-3-[(4-nitrophenyl)ethoxycarbonyl]imidazolium chloride[11] was prepared according to previously described protocols. Reagents for oligonucleotide synthesis were from Glen Research and PE Applied Biosystems. Dry solvents were from SDS and Romil. HPLC-grade solvents were from Romil and E. Merck. Snake venom phosphodiesterase (from *Crotalus durissus*) and alkaline phosphatase were from Boehringer Mannheim. The rest of the reagents were from Aldrich and Fluka and were used without further purification. Analytical TLC was run on aluminium sheets coated with silica gel 60 F254 from E. Merck. Silica gel column chromatography was performed with Chromatogel 60 A C.C. (40–60 microns, 230–400 mesh). Solutions were dried over anhydrous sodium sulfate.

### Instrumental

$^{1}$H NMR (200 and 250 MHz) $^{13}$C NMR (63 MHz) and $^{31}$P NMR (101 MHz) spectra were recorded on a Varian Gemini 200 MHz NMR and a Brüker AM-250 spectrometer. *J*-Values are given in Hz. HPLC chromatography was performed on an HPLC System Gold (Beckman) and a Waters instrument. The steel bomb was an HR 700 model from Gerghof GmbH. Mass spectra were obtained on a Perkin-Elmer API III SCIEX equipped with a triple-quadrupole detector.

### General protocol for the preparation of phosphoramidites

DMT-Protected nucleosides were dried by evaporation of dry ACN. The residue was dissolved in dry ACN, dry DCM or dry 1,4-dioxane, and 4 mole equivalents of ethyldiisopropylamine were added. The mixture was cooled with an ice-bath and 2 mole equivalents of chloro-(2-cyanoethoxy)-(diisopropylamino)phosphine were added with a syringe. After 1 h of magnetic stirring, the reaction mixture was stopped by the addition of 1 cm$^3$ of MeOH, and solvents were evaporated. The residue was dissolved in DCM and the solution was washed successively with saturated aq. $NaHCO_3$ and saturated aq. NaCl. The organic phase was dried, concentrated to dryness and the residue was purified by column chromatography on silica gel.

### 5-Amino-2′-deoxy-5′-*O*-(dimethoxytrityl)uridine 3

Compound **2**[7] (4.42 g, 7.25 mmol) was dissolved in 30 cm$^3$ of 1,4-dioxane. The solution was treated with an excess of liquid ammonia in a steel bomb. The stirred (500 rpm) mixture was heated to 60 °C for 5 days. The mixture was filtered and concentrated to dryness. The residue was purified by silica gel chromatography (0–10% MeOH gradient in DCM) to afford compound **3** (2.02 g, 51%). Spectroscopic data are in agreement with previously described data:[7] $\lambda_{max}$(EtOH)/nm 280 ($\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$ 8000) and 298 (7400); $\delta_H$($CDCl_3$) 8.10 (1 H, s), 6.7–7.4 (13 H, m), 6.4 (1 H, t, *J* 6.6), 4.6 (1 H, m), 3.9 (1 H, m), 3.7 (6 H, s), 3.6 (2 H, m) and 2.6 (2 H, m); $\delta_C$($CDCl_3$) 160.8, 158.5, 158.3, 149.3, 144.4, 135.4, 135.2, 131.9, 129.9, 128.9, 127.8, 127.0, 126.9, 116.6, 113.2, 86.9, 86.1, 84.5, 72.1, 63.6, 55.1 and 40.4.

### 2′-Deoxy-5′-*O*-(dimethoxytrityl)-5-trifluoroacetamidouridine 4

Compound **3** (0.65 g, 1.2 mmol) was dissolved in 14 cm$^3$ of MeOH, and 12 mmol of $Et_3N$ and 12 mmol of ethyl trifluoroacetate were added. The solution was stirred overnight at room temperature. The solvent was evaporated off and the residue was dissolved in DCM. The solution was washed with 1 M aq. $NaHCO_3$ (2×) and the organic phase was dried and concentrated to dryness. The residue was purified by column chromatography on silica gel eluted with ethyl acetate–hexane (2:1) to give compound **4** (0.27 g, 35%), $R_f$ 0.3 [ethyl acetate–hexane (2:1)]. Spectroscopic data are in agreement with previously described data:[7] $\delta_C$($CDCl_3$) 159.1, 158.5, 154.8, 154.6, 154.2, 154.0, 148.5, 144.5, 135.9, 135.8, 130.0, 129.0, 128.1, 127.7, 126.8, 113.1, 112.3, 86.8, 85.9, 72.2, 63.8, 55.0 and 39.9.

### 3′-*O*-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-2′-deoxy-5′-*O*-(dimethoxytrityl)-5-trifluoroacetamidouridine 5

Compound **4** (0.21 g, 0.33 mmol) was dissolved in ACN (8 cm$^3$) and the solution was treated with 0.12 cm$^3$ of chloro-(2-cyanoethoxy)(diisopropylamino)phosphine and 0.23 cm$^3$ (1.32 mmol) of ethyldiisopropylamine as described above. The residue was purified on silica gel eluted with ethyl acetate–hexane–$Et_3N$ (66:33:1) to afford compound **5** (0.11 g, 40%), $R_f$ 0.33 [ethyl acetate–hexane–$Et_3N$ (66:33:1)]. Spectroscopic data are in agreement with previously described data:[7] $\delta_P$($CDCl_3$) 148.0.

### 3′-*O*-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-2′-deoxy-5′-*O*-(dimethoxytrityl)-5-[(dimethylamino)methyleneamino]-uridine 8

Compound **3** (2.02 g, 3.70 mmol) was dissolved in 17 cm$^3$ of DMF, and 1.63 g (13.8 mmol) of dimethylformamide dimethyl acetal were added. After being stirred overnight the mixture was concentrated to dryness. The residue was purified on silica gel eluted with 0–10% MeOH gradient in DCM to afford compound **7** (1.28 g, 58%), $R_f$ 0.22 (5% MeOH–DCM); $\lambda_{max}$(EtOH)/nm 274 (12 000) and 302 (8600); $\delta_H$($CDCl_3$) 7.9 (1 H, s), 7.6 (1 H, s), 6.79–7.47 (13 H, m), 6.4 (1 H, t), 4.5 (1 H, m), 4.0 (1 H, m), 3.7 (6 H, s), 3.3 (2 H, m), 2.9 (3 H, s), 2.8 (3 H, s) and 2.3 (1 H, m); $\delta_C$($CDCl_3$) 158.5, 158.4, 144.7, 135.8, 135.7, 129.9, 128.0, 127.8, 126.8, 126.7, 126.0, 113.1, 85.8, 85.6, 84.1, 71.5, 63.6, 54.6, 40.1, 38.9 and 33.2.

Compound **7** (0.77 g, 0.96 mmol) was dissolved in DCM (40 cm$^3$) and was treated with 0.42 cm$^3$ (1.92 mmol) of chloro-(2-cyanoethoxy)(diisopropylamino)phosphine and 0.67 cm$^3$ (5.13 mmol) of ethyldiisopropylamine. The residue was purified on silica gel eluted with ethyl acetate–$CHCl_3$ (1:1) containing 1% $Et_3N$ to give compound **8** (0.73 g, 71%) (Found: C, 62.5; H, 7.0; N, 10.5. $C_{42}H_{53}N_6O_8P$ requires C, 62.9; H, 6.7; N, 10.5%); $\lambda_{max}$(EtOH)/nm 274 (7500) and 299 (5800); $R_f$ 0.34 [ethyl acetate–$CHCl_3$ (1:1) containing 1% $Et_3N$]; $\delta_P$($CDCl_3$) 145.3.

### 3′-*O*-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-2′-deoxy-5′-*O*-(dimethoxytrityl)-5-[2-(4-nitrophenyl)ethoxycarbonyl-amino]uridine 11

Compound **3** (1 g, 1.83 mmol) was dissolved in 4 cm$^3$ of DMF and 0.39 cm$^3$ (1 mol equiv.) of HMDS was added. After 20 min of magnetic stirring at room temperature, solvents were evaporated off. The residue was coevaporated with toluene and dissolved in 10 cm$^3$ of DCM. The solution was cooled with ice and 0.5 g (2.2 mmol) of *N*-methyl-3-[(4-nitrophenyl)ethoxycarbonyl]imidazolium chloride[11] was added dropwise to the solution. After 30 min of magnetic stirring, solvents were evaporated off and the residue was dissolved in DCM and washed with 1 M aq. $NaHCO_3$. The organic phase was dried and concentrated to dryness. The residue was dissolved in 4 cm$^3$ of a solution of MeOH–1,4-dioxane (1:1) and 1 cm$^3$ of conc. aq. ammonia was added. After 1 h of magnetic stirring the solvent was evaporated off. The residue was purified on silica gel eluted with 0–8% MeOH gradient in DCM to give compound **10**

(0.66 g, 55%), $R_f$ 0.15 (5% MeOH–DCM); $\delta_H(CDCl_3)$ 8.18 (1 H, s), 8.0–6.7 (17 H, m, ArH), 6.2 (1 H, t, *J* 7), 4.3 (1 H, m), 4.1 (2 H, t, *J* 6.4), 3.9 (1 H, m), 3.6 (6 H, s), 3.3 (2 H, m), 2.8 (2 H, t, *J* 6.4) and 2.2 (2 H, m).

Compound **10** (0.66 g, 0.89 mmol) was dissolved in ACN (4 $cm^3$) and treated with 0.32 $cm^3$ of chloro-(2-cyanoethoxy)-(diisopropylamino)phosphine and 0.64 $cm^3$ (3.57 mmol) of ethyldiisopropylamine as described above. The residue was purified on silica gel eluted with ethyl acetate–hexane–$Et_3N$ (66:33:1) to give compound **11** (0.63 g, 75%) (Found: C, 61.0; H, 6.3; N, 8.9. $C_{48}H_{55}N_6O_{12}P$ requires C, 61.4; H, 5.9; N, 8.9%); $\lambda_{max}$(EtOH)/nm 273 (17 000); $R_f$ 0.21, 0.34 (ethyl acetate–hexane–$Et_3N$ 66:33:1); $\delta_P(CDCl_3)$ 148.7.

### 5-Acetamido-3′-*O*-[(2-cyanoethoxy)(diisopropylamino)phosphino]-2′-deoxy-5′-*O*-(dimethoxytrityl)uridine 14

Compound **3** (1 g, 1.83 mmol) was dissolved in 4 $cm^3$ of DMF and 0.39 $cm^3$ (1 mol equiv.) of HMDS was added. The mixture was stirred at room temperature for 30 min. The solvent was evaporated off and the residue was dissolved in 4 ml of pyridine. The solution was cooled with ice and 1.1 mol equiv. of acetyl chloride was added. After 1 h of magnetic stirring, the reaction was stopped by addition of 2 $cm^3$ of MeOH. The solvent was evaporated off and the residue was dissolved in DCM and washed with 1 M aq. $NaHCO_3$. The organic phase was dried and concentrated to dryness. The residue was dissolved in 6 $cm^3$ of a MeOH–1,4-dioxane (1:1) solution, and 1 $cm^3$ of conc. aq. ammonia was added. After 1 h of magnetic stirring, the solvent was evaporated off and the residue was purified on silica gel eluted with 2–10% MeOH gradient in DCM to give compound **13** (0.70 g, 66%), $R_f$ 0.17 (5% MeOH–DCM); $\delta_H(CDCl_3)$ 8.4 (1 H, s), 6.7–7.5 (13 H, m), 6.2 (1 H, t, *J* 6.4), 4.2 (1 H, m), 3.9 (1 H, m), 3.7 (6 H, s), 3.6 (2 H, m), 2.3 (2 H, m) and 2.0 (3 H, s); $\delta_C(CDCl_3)$ 168.5, 159.8, 158.5, 149.2, 148.7, 144.7, 136.1, 135.8, 130.1, 128.2, 128.0, 127.8, 127.2, 126.7, 114.6, 113.0, 86.4, 85.4, 85.2, 71.5, 63.9, 55.0, 39.5 and 23.6.

Compound **13** (0.6 g, 1.03 mmol) was dissolved in 4 $cm^3$ of 1,4-dioxane and the solution was treated with 0.36 $cm^3$ (1.54 mmol) of chloro-(2-cyanoethoxy)(diisopropylamino)-phosphine and 0.74 ml (4.12 mmol) of ethyldiisopropylamine as described above. The product was purified on silica gel eluted with ethyl acetate–hexane (3:1) containing 1% $Et_3N$ to give compound **14** (0.54 g, 66%) (Found: C, 62.5; H, 6.8; N, 8.7. $C_{41}H_{50}N_5O_9P$ requires C, 62.5; H, 6.4; N, 8.9%); $R_f$ 0.57 [ethyl acetate–hexane (3:1) containing 1% $Et_3N$]; $\lambda_{max}$(EtOH)/nm 277 (7500); $\delta_P(CDCl_3)$ 148.8.

### General procedure to prepare 5′-*O*-DMT-deoxynucleoside-3′-*O*-hemisuccinate supports

First, 3′-*O*-hemisuccinates were prepared as follows: 5′-*O*-(dimethoxytrityl)nucleosides **4**, **7**, **10** and **13** were dissolved in dry DCM and the solution was cooled with an ice-bath. Succinic anhydride (1.5 mol equiv.) and DMAP (1.5 mol equiv.) were added to the mixture and the solution was stirred overnight at room temperature. The solvent was evaporated off, the residue was dissolved in DCM, and the solution was washed successively with pH 5 aq. sodium phosphate and saturated aq. NaCl. The organic phase was dried and concentrated to dryness. The residue was dissolved in the minimum volume of DCM and precipitated by addition of the DCM solution dropwise to a mixture of diethyl ether–hexane (1:1). The product was recovered by filtration. Compound **6**: yield 58%, $R_f$ 0.52 (10% MeOH–DCM). Compound **9**: yield 61%, $R_f$ 0.60 (10% MeOH–DCM). Compound **12**: yield 42%, $R_f$ 0.52 (10% MeOH–DCM). Compound **15**: yield 57.2%, $R_f$ 0.31 (10% MeOH–DCM).

The incorporation of nucleosides 3′-*O*-hemisuccinates onto amino-LCAA-CPG (500 Å, 95 μmol amino $g^{-1}$) was carried out using triphenylphosphine and 2,2′-dithiobis(5-nitropyridine) as described by Gupta *et al.*[9] The resulting loading expressed in μmol $g^{-1}$ CPG were as follows: compound **6**: 53; compound **9**: 25; compound **12**: 38; compound **15**: 46. For the incorporation of compound **12**, sarcosyl-LCAA-CPG (75 μmol $g^{-1}$) was used instead of amino-LCAA-CPG. The preparation of the sarcosyl support has been previously described.[12,13]

### Oligonucleotide synthesis

The following sequences have been synthesized: dimers $^{5'}A^{N}U^{3'}$ and $^{5'}A^{Ac}U^{3'}$; 15b-Y: $^{5'}$GCAATGGAYCCTCTA$^{3'}$, where Y = $^{N}$U, $^{Ac}$U and T; and $s_{11}$-Y: $^{5'}$CYYCCYCCYCT$^{3'}$, where Y = $^{N}$U and $^{Ac}$U. Oligonucleotides were prepared on an Applied Biosystems DNA synthesizer using standard 2-cyanoethyl phosphoramidites and the modified phosphoramidites. For the preparation of the dimers the solid supports prepared above were used. For the preparation of the sequences 15b-$^{N}$U and $s_{11}$-$^{N}$U, using compound **11**, the usual capping A solution (acetic anhydride) was substituted by a solution of 2% NPEOC chloroformate[11] in ACN. Capping B solution was a 16% *N*-methylimidazole solution in THF as usual. Complementary pentadecamers containing natural bases were prepared using commercially available chemicals and following standard protocols.

Oligonucleotide supports of dimer $A^{N}U$, 15b-$^{N}$U and $s_{11}$-$^{N}$U were treated for 2 h with a solution of 0.5 M DBU in ACN, and then with 32% aq. ammonia at 50 °C for 16 h. The remaining oligonucleotide supports were treated with 32% aq. ammonia at 50 °C for 16 h. Ammonia solutions were concentrated to dryness and the products were purified either by cartridge purification or by reversed-phase HPLC. Oligonucleotides and dimers were prepared on a 1 μmol scale. Dimers were prepared without the last DMT group (DMT-off protocol). The remaining oligonucleotides were synthesized with the last DMT group at the 5′ end (DMT-on protocol) to help reversed-phase purification. All purified products presented a major peak that was collected, and analysed by snake venom phosphodiesterase and alkaline phosphatase digestion followed by HPLC analysis of the nucleosides (HPLC conditions B). The retention time of the nucleosides obtained after enzyme digestion were: dC 2.8 min, dG 5 min, T 5.9 min, dA 9 min, $^{N}$dU 2.8 min, $^{Ac}$dU 4.7 min. Yield (absorbance *A* units at 260 nm): $s_{11}$-$^{N}$U: 23 *A*, $s_{11}$-$^{Ac}$U: 28 *A*, 15b-$^{N}$U: 46 *A*, 15b-$^{AcN}$U: 27 *A*. HPLC solutions were as follows. Solvent A: 5% ACN in 100 mM triethylammonium acetate (pH 7.8) and solvent B: 70% ACN in 100 mM triethylammonium acetate (pH 7.8). For analytical runs the following conditions were used. Column: Nucleosil 120C18, 250 × 4 mm; flow rate: 1 ml $min^{-1}$. (Conditions A) a 40 min linear gradient from 0 to 75% B. (Conditions B) a 20 min linear gradient from 0 to 20% B. For semipreparative runs the following conditions were used: Columns: Nucleosil 120C18, 250 × 10 mm. Flow rate: 3 ml $min^{-1}$. A 20 min linear gradient from 15 to 60% B (DMT-on), or a 30 min linear gradient from 0 to 40% B (DMT-off).

### Melting experiments

Melting experiments of pentadecamer (15b-Y) duplexes were made by mixing equimolar amounts of two pentadecamer strands dissolved in a solution that contained 0.15 M NaCl and 0.05 M Tris-HCl buffer (pH 7.4). Duplexes were annealed by slow cooling from 80 to 4 °C. UV absorption spectra and melting curves (absorbance *vs.* temperature) were recorded in 1 cm path-length cells using a Varian Cary 13 spectrophotometer with a temperature controller and a programmed temperature increase of 0.5 °C $min^{-1}$. Melts were run on duplex concentrations of 4 μM at 260 nm.

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