Uridine 2′-Carbamates: Facile Tools for Oligonucleotide 2′-Functionalization

This unit contains procedures for synthesis of uridine 2′-carbamate phosphoramidites and oligonucleotides thereof. 3′,5′-Silyl-diprotected uridine can be converted into the corresponding 2′-carbamate by reaction with 1,1′-carbonyldiimidazole followed by treatment with an aliphatic amine. The 2′-carbamate can then be converted in several steps into a 3′-phosphoramidite suitable for machine-assisted oligonucleotide synthesis. The preparation of eleven different uridine 2′-carbamates and their phosphoramidites from several primary and secondary amines is described in the first two methods (see Basic Protocol 1 and Alternate Protocol). Their use in oligonucleotide synthesis is then described (see Basic Protocol 2).

2′-Carbamate modification is stable to conditions of standard phosphoramidite oligonucleotide synthesis. Although 2′-carbamate modification is somewhat destabilizing for DNA-DNA and DNA-RNA duplexes, it is suitable for the direction of ligands into the minor groove or into non-base-paired sites (e.g., loops, bulges) of oligo- and polynucleotides. Pyrene-modified oligonucleotide 2′-carbamates show a considerable increase in fluorescence intensity upon hybridization to a complementary RNA, and have interesting binding properties when hybridized to a mismatched DNA.

CAUTION: Carry out all operations involving organic solvents and reagents in a well-ventilated fume cupboard, and wear gloves and protective glasses.

PREPARATION OF URIDINE 2′-CARBAMATE PHOSPHORAMIDITES FROM PRIMARY AND SECONDARY AMINES

BASIC PROTOCOL 1

Nucleoside 2′-carbamates have been used previously in the syntheses of various modified nucleosides (McGee et al., 1996; Zhang et al., 2003) and oligonucleotides (Freier and Altmann, 1997; Seio et al., 1998; Dubey et al., 2000; Prhavc et al., 2001). The present protocol is based primarily on Korshun et al. (2002) and is illustrated in Figure 4.21.1. Stable 2′-*O*-(imidazol-1-ylcarbonyl)-3′,5′-*O*-(tetraisopropyldisiloxan-1,3-diyl)uridine (**S.3**) is first prepared from uridine (**S.1**). It is then treated with primary or secondary aliphatic amines to give uridine 2′-carbamates (**S.4**) in high yield. Preparation of 2′-carbamates bearing a variety of *N*-substituents is described (**S.4a-f**; see Fig. 4.21.2). After 3′,5′-*O*-deprotection with triethylamine trihydrofluoride, 5′-*O*-dimethoxytritylation, and 3′-*O*-phosphinylation with bis(*N,N*-diisopropylamino)-2-cyanoethoxyphosphine, the corresponding phosphoramidites (**S.7a-f**) are obtained. They are used in machine-assisted synthesis of modified oligodeoxynucleotides containing uridine-2′-carbamate residues bearing these *N*-substituents.

Strategic planning

Formation of 2′-carbamates. In many cases, the imidazolide **S.3** need not be isolated, but instead can be reacted in situ with an excess of the appropriate amine in dry dichloromethane (CH_2Cl_2). In the case of an amine hydrochloride (e.g., for **S.4d**), 1.1 eq triethylamine (TEA) or *N,N*-diisopropylethylamine (DIPEA) is added to the reaction mixture to liberate the free base. Reaction times differ vastly from one amine to another. Whilst propargylamine reacts rapidly in CH_2Cl_2 (<1 hr), *N*-methylpropargylamine, a secondary amine, requires overnight reaction. 2-Aminomethyl-15-crown-5 reacts very slowly in CH_2Cl_2. To accelerate the reaction, a change of solvent to acetonitrile (CH_3CN) and a temperature increase to 55°C is advised. Thus, two general procedures for the preparation of carbamates **S.4a-d** versus **S.4e-f** are presented below. 3′,5′-*O*-Silyl-protected uridine 2′-carbamates are then isolated by column chromatography.

Contributed by Vladimir A. Korshun, Dmitry A. Stetsenko, and Michael J. Gait

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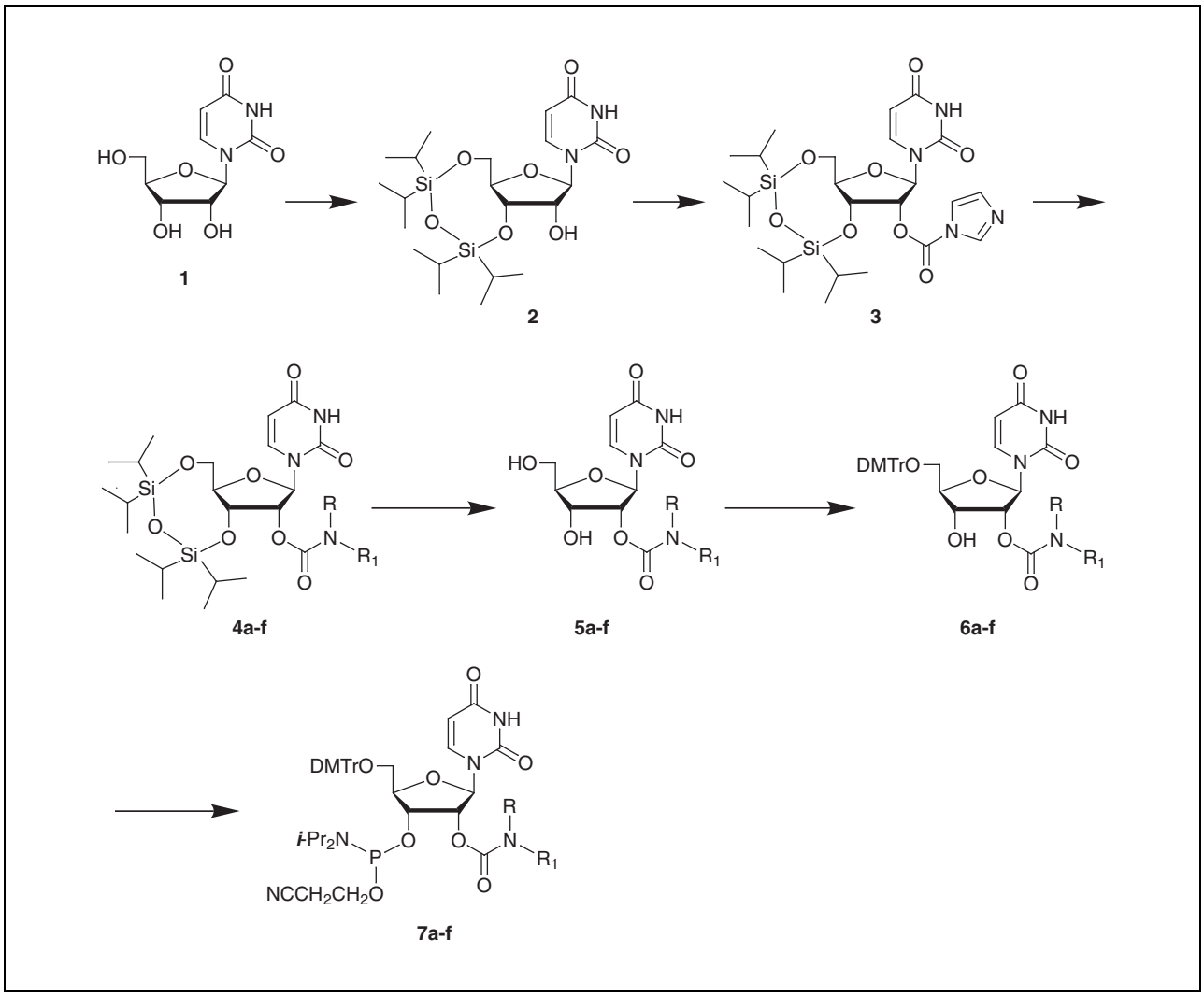


Figure 4.21.1 General scheme for conversion of uridine into 2′-carbamate 3′-phosphoramidites (see Basic Protocol 1). For R and R_1 groups, see Figure 4.21.2.

Desilylation. Tetrabutylammonium fluoride trihydrate (TBAF) in tetrahydrofuran (THF) was found unsuitable for removal of the Markiewicz protecting group from **S.4**, because of the observed 2′-3′ migration of the carbamate group under alkaline conditions (Korshun et al., 2002). Triethylamine trihydrofluoride, a nonbasic fluoride source, gave smooth silyl deprotection without any migration. Aromatic uridine 2′-carbamates **S.5c** and **S.5d** are crystalline solids. The other **S.5** compounds are viscous oils, but are nevertheless suitable for 5′-dimethoxytritylation without further purification.

Dimethoxytritylation. No 2′-3′ migration is detected when pyridine is used as a solvent for 5′-dimethoxytritylation or as an additive during column chromatography instead of TEA. The pyrene derivative **S.6d** and the dipeptide derivative **S.6f** show the highest propensity towards isomerization during column chromatography in the presence of TEA. Generally, 5′-*O*-DMTr-2′-carbamates **S.6** show fast 2′-3′ migration under strong basic conditions, but slow migration rates in the presence of protic solvents. The 2′- and 3′-isomers are usually easily distinguishable by TLC. Column-purified 5′-*O*-DMTr-derivatives (**S.6**) are stable as dry amorphous solids for at least 12 months. Usually,

co-evaporation with dry CH_2Cl_2 is a convenient way to remove traces of other solvents; however, it must be thoroughly vented off to give **S.6** as a foam.

Phosphinylation. 5′-*O*-DMTr-uridine 2′-carbamates **S.6** are phosphinylated with bis(*N,N*-diisopropylamino)-2-cyanoethoxyphosphine in dry CH_2Cl_2 in the presence of diisopropylammonium tetrazolide to give phosphoramidites **S.7**, which are then isolated by column chromatography. None of the starting carbamates gave rise to isomerization products during the phosphinylation reaction.

Compound characterization. Chemical characterization data are provided for all compounds. NMR spectra were recorded on a 300-MHz Bruker DRX300 NMR spectrometer in DMSO-d_6 unless otherwise stated. Chemical shifts (δ) are given in ppm and referenced to tetramethylsilane as an internal standard and 85% phosphoric acid (H_3PO_4) as an external standard. Coupling constants (*J*) are given in Hertz and refer to apparent multiplicities. D_2O exchange was carried out on all samples.

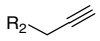
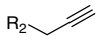
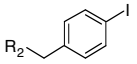
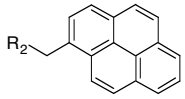
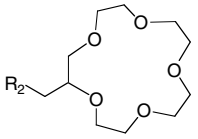
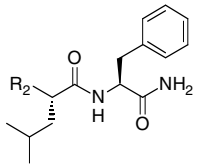
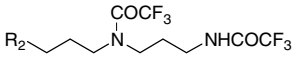
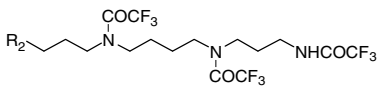
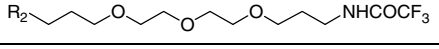
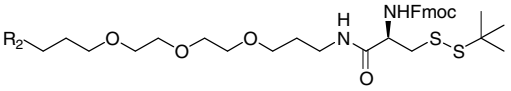
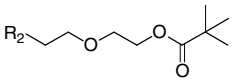
Compound	R	R_1
4a-7a	H	
4b-7b	CH_3	
4c-7c	H	
4d-7d	H	
4e-7e	H	
4f-7f	H	
4g-7g	H	
4h-7h	H	
4i-7i	H	
4j-7j	H	
4l-7l	H	

Figure 4.21.2 Side-chain substituents of uridine 2′-carbamates. R_2 stands for the rest of the molecule; see Figures 4.21.1 and 4.21.3.

Materials

Uridine (**S.1**), 99% pure
Anhydrous pyridine, 99.8% pure (Aldrich)
Nitrogen (or argon) gas
Markiewicz reagent: 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, 96% pure (Lancaster)
Ethyl acetate (EtOAc), HPLC grade
5% (w/v) sodium hydrogencarbonate ($NaHCO_3$)
Sodium sulfate (Na_2SO_4), anhydrous
Toluene, analytical grade
Silica gel: 0.040- to 0.063-mm Macherey-Nagel Kieselgel 60
Chloroform ($CHCl_3$), HPLC grade, ethanol free
Anhydrous dichloromethane (CH_2Cl_2), distilled from powdered CaH_2 (Fisher)
Hexane, HPLC grade
1,1′-Carbonyldiimidazole, 95% pure (Sigma)
Amine for **S.3** conversion (select one):
  Propargylamine (for **S.4a**)
  N-Methylpropargylamine (for **S.4b**)
  4-Iodobenzylamine (Lancaster; for **S.4c**)
  1-Pyrenemethylamine hydrochloride (Aldrich; for **S.4d**)
  2-Aminomethyl-15-crown-5 (for **S.4e**)
  H-Leu-Phe-NH_2 hydrochloride (for **S.4f**)
Triethylamine (TEA), ≥99% pure
Acetonitrile (CH_3CN), HPLC grade (optional; for **S.4e** and **S.4f**)
N,*N*-Diisopropylethylamine (DIPEA), ≥99% pure (Aldrich; optional; for **S.4f**)
5% (w/v) citric acid
Methanol (MeOH), analytical grade
Acetone, analytical grade
Dry tetrahydrofuran (THF), freshly distilled from $LiAlH_4$ (store over 4A molecular sieves under nitrogen)
Triethylamine trihydrofluoride
Absolute ethanol, analytical grade (optional; for **S.5c** and **S.5d**)
Diethyl ether, analytical grade (optional; for **S.5c** and **S.5d**)
4,4′-Dimethoxytrityl chloride (DMTr·Cl, 95% pure; Avocado Research Chemicals)
Diisopropylammonium tetrazolide
Bis(*N*,*N*-diisopropylamino)-2-cyanoethoxyphosphine, 98% pure (Fluka)
20% (w/v) sodium chloride (NaCl)

Rotary evaporator equipped with a water aspirator
4 × 20–cm sintered glass chromatography column, porosity 3
Vacuum oil pump
TLC plate: silica-coated aluminum plate with fluorescent indicator (Merck silica gel 60 F_{254})
254-nm UV lamp
30-mL screw-top Teflon bottle (Nalgene)

Additional reagents and equipment for thin-layer chromatography (TLC, *APPENDIX 3D*) and column chromatography (*APPENDIX 3E*)

Protect 3′- and 5′-hydroxy groups of uridine

1. Co-evaporate 3.663 g (15 mmol) uridine (**S.1**) twice with 30 mL anhydrous pyridine using a rotary evaporator equipped with a water aspirator, and then apply a dry nitrogen (or argon) atmosphere.
2. Dissolve the residue in 60 mL pyridine with magnetic stirring and rapidly add 5.0 g (15.8 mmol) Markiewicz reagent in one portion. Stopper the flask and continue stirring overnight.
3. Dilute the mixture with 300 mL EtOAc. Wash twice with 150 mL water and then once with 150 mL of 5% (w/v) $NaHCO_3$.
4. Dry over anhydrous Na_2SO_4 and filter off the drying agent. Evaporate the solution to dryness and then co-evaporate three times with 40 mL toluene.
5. Pack a 4 × 20–cm sintered glass column with silica gel in $CHCl_3$. Load the sample and elute minor side products with 1:9 (v/v) EtOAc/$CHCl_3$. Elute desired compound (**S.2**) with 1:2 (v/v) EtOAc/$CHCl_3$.
6. Evaporate fractions containing the product and co-evaporate three times with 40 mL anhydrous CH_2Cl_2.
7. Dry the resulting white foam 2 hr in vacuo (0.05 to 0.5 Torr).
8. Grind the foam with a stainless steel spatula and dry the resulting powder 12 to 16 hr using a vacuum oil pump.

   Make sure traces of chlorinated solvent are completely removed from the compound, otherwise some decomposition may occur, caused by the HCl traces generated.

9. Characterize the product by TLC (on silica gel; *APPENDIX 3D*) and ^{1}H NMR.

   The compound is stable at least 12 months during storage at ambient temperature.

   *3′,5′-O-(Tetraisopropyldisiloxan-1,3-diyl)uridine (**S.2**). Yield of white amorphous solid 6.897 g (94%). R_f: 0.35 (1:1 v/v EtOAc/hexane). ^{1}H NMR: 11.33 (s, 1H, H-3, exchangeable with D_2O), 7.68 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-6), 5.55–5.50 (m, 3H, H-5, H-1′, 2′-OH), 4.16 (m, 1H, ^2J = 8.6 Hz, $J_{4,5'a}$ = 4.9 Hz, H-5′a), 4.12 (m, 2H, H-4′, H-5′b), 3.97 (m, 1H, H-2′), 3.92 (dd, 1H, $J_{2',3'}$ = 2.5 Hz, $J_{3',4'}$ = 13.1 Hz, H-3′), 1.09–0.90 (m, 28H, Pr^i).*

Activate 2′-hydroxy group

10. Dissolve 2.434 g (5.0 mmol) **S.2** in 50 mL dry CH_2Cl_2 and add 852 mg (5.25 mmol) 1,1′-carbonyldiimidazole in one portion.

    This solution can be used directly for unhindered primary and secondary amines (step 16a). For hindered primary amines (step 16b), the product should be isolated first (steps 11 to 14).

11. Monitor reaction by TLC in 1:1 (v/v) EtOAc/hexane.

    *The starting compound **S.2** (R_f = 0.35) usually disappears after 0.5 to 2 hr.*

12. Wash the solution twice with 50 mL water and dry over Na_2SO_4.
13. Filter off the Na_2SO_4, evaporate the solvent in vacuo, and co-evaporate the residue three times with 40 mL dry CH_2Cl_2 using a rotary evaporator.
14. Dry the residue in vacuo overnight using an oil pump.

15. Characterize the compound by TLC and ^{1}H NMR.

*2′-O-(Imidazolylcarbonyl)-3′,5′-(tetraisopropyldisiloxan-1,3-diyl)uridine (**S.3**) is obtained as a white crystalline powder (2.872 g, 98.9%) that is pure according to TLC and ^{1}H NMR. R_f 0.18 (1:1 v/v EtOAc/hexane), m.p. 193°–195°C (recrystallized from EtOAc/$CHCl_3$). ^{1}H NMR: 11.44 (s, 1H, H-3), 8.39 (s, 1H, imidazole), 7.69 (s, 1H, imidazole), 7.64 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 7.09 (s, 1H, imidazole), 5.85 (s, 1H, H-1′), 5.79 (d, 1H, $J_{2',3'}$ = 5.4 Hz, H-2′), 5.61 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 4.74 (m, 1H, H-3′), 4.13–3.92 (m, 3H, H-4′, H-5′), 1.10–0.80 (m, 28H, Pr^i).*

*Imidazolide **S.3** is stable during aqueous extraction, but hydrolyzed under both acidic and alkaline conditions. Considerable decomposition was also observed during silica gel chromatography (1:1:1 to 1:1:2 v/v/v $CHCl_3$/EtOAc/acetone, 66% yield of impure product).*

Treat S.3 with primary or secondary aliphatic amine

For unhindered primary and secondary amines

16a. To the solution of **S.3** (prepared from 5 mmol **S.2** in step 10), add the appropriate amine and allow reaction to proceed for the appropriate amount of time at 25°C:

For **S.4a**: 0.514 mL (7.5 mmol) propargylamine for 1 hr
For **S.4b**: 0.633 mL (7.5 mmol) *N*-methylpropargylamine overnight
For **S.4c**: 1.630 g (7.0 mmol) 4-iodobenzylamine for 30 hr
For **S.4d**: 1.874 g (7.0 mmol) 1-pyrenemethylamine hydrochloride and 1.0 mL (7.2 mmol) TEA for 72 hr.

Monitor the completion of the reaction by TLC using EtOAc to monitor the disappearance of the imidazolide, or the appropriate solvents in step 23 to monitor the accumulation of product.

17a. Dilute the reaction mixture with 50 mL CH_2Cl_2. Proceed to step 18.

For hindered primary amines

16b. Prepare a solution of 1.162 g (2.0 mmol) imidazolide **S.3** in 25 mL CH_3CN. Add the appropriate amine and incubate at 55°C until all of the starting compound is consumed:

For **S.4e**: 514 mg (2.0 mmol) 2-aminomethyl-15-crown-5 for 96 hr
For **S.4f**: 942 mg (3.0 mmol) H-Leu-Phe-NH_2 hydrochloride and 0.61 mL (3.5 mmol) DIPEA for 72 hr.

Monitor progress by TLC using EtOAc to monitor the disappearance of the imidazolide, or the appropriate solvents in step 23 to monitor the accumulation of product.

17b. Cool the mixture to ambient temperature and evaporate to dryness. Dilute with 100 mL EtOAc. Proceed to step 18.

Work up S.4

18. Wash with 100 mL water, followed by 100 mL of 5% (w/v) citric acid, and then another 100 mL water.

19. Dry over Na_2SO_4, filter off the Na_2SO_4, and evaporate the solution.

20. Purify the residue by silica gel column chromatography (*APPENDIX 3E*) in the appropriate solvent system:

    For **S.4a**: 33% to 40% (v/v) EtOAc in $CHCl_3$
    For **S.4b**: 1:2 (v/v) EtOAc/$CHCl_3$
    For **S.4c**: 25% to 33% (v/v) EtOAc in $CHCl_3$
    For **S.4d**: 25% to 33% (v/v) EtOAc in $CHCl_3$
    For **S.4e**: 2% to 10% (v/v) MeOH in $CHCl_3$
    For **S.4f**: stepwise gradient of 33% to 50% (v/v) EtOAc in $CHCl_3$, then 33% to 50% acetone in 1:1 (v/v) $CHCl_3$/EtOAc.

    *Typical volumes are 1 to 1.5 L for **S.4a-e** and ~4 L for **S.4f**.*

21. Monitor fractions by TLC, combine fractions containing product, and evaporate.

22. Co-evaporate the residue three times with 20 to 50 mL CH_2Cl_2, dry in vacuo for 2 hr, and grind the foam as described above (step 8). Dry in vacuo again to afford the compounds listed below as white amorphous solids.

23. Characterize the compound by TLC and ^{1}H NMR.

    *2′-O-(Propargylaminocarbonyl)-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl)uridine (**S.4a**). Yield 2.780 g (97.9%). R_f: 0.49 (1:1 v/v $CHCl_3$/EtOAc). ^{1}H NMR: 11.42 (s, 1H, H-3), 7.84 (t, 1H, J = 5.4 Hz, OCONH), 7.69 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 5.64 (s, 1H, H-1′), 5.59 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.34 (d, 1H, $J_{2',3'}$ = 4.9 Hz, H-2′), 4.50 (m, 1H, H-3′), 4.34–3.88 (m, 2H, H-5′), 3.85–3.69 (m, 3H, CH_2N, H-4′), 3.06 (s, 1H, CH), 1.10–0.80 (m, 28H, Pr^i).*

    *2′-O-(N-Methyl-N-propargylaminocarbonyl)-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl)-uridine (**S.4b**). Yield 2.827 g (97.2%). R_f: 0.56 (1:1 v/v $CHCl_3$/EtOAc). ^{1}H NMR: 11.41(s, 1H, H-3), 7.66 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-6), 5.67 (s, 1H, H-1′), 5.59 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-5), 5.35 (m, 1H, H-2′), 4.53 (dd, 1H, $J_{2',3'}$ = 6.0 Hz, $J_{3',4'}$ = 7.9 Hz, H-3′), 4.17–3.83 (m, 5H, H-4′, H-5′, CH_2N), 3.21 (s, 1H, CH), 2.93 (s, 1.8H), 2.84 (s, 1.2H) (NCH_3, rotamers), 1.07–0.82 (m, 28H, Pr^i).*

    *2′-O-(4-Iodobenzylaminocarbonyl)-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl)uridine (**S.4c**). Yield 3.491 g (93.6%). R_f: 0.50 (1:1 v/v $CHCl_3$/EtOAc). ^{1}H NMR: 11.41 (s, 1H, H-3), 7.96 (br t, 1H, OCONH), 7.68 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 7.62 (d, 2H, J = 7.6 Hz, ArH), 7.05 (d, 2H, J = 7.6 Hz, ArH), 5.65 (s, 1H, H-1′), 5.58 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.35 (d, 1H, $J_{2',3'}$ = 5.4 Hz, H-2′), 4.48 (m, 1H, H-3′), 4.20–3.80 (m, 5H, CH_2Ar, H-4′, H-5′), 1.08–0.88 (m, 28H, Pr^i).*

    *2′-O-(Pyrene-1-ylmethylaminocarbonyl)-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl)uridine (**S.4d**). Yield 3.492 g (93.9%). R_f: 0.54 (1:1 v/v $CHCl_3$/EtOAc). ^{1}H NMR: 11.43 (s, 1H, H-3), 8.42 (d, 1H, $J_{9',10'}$ = 9.3 Hz, pyrene H-10′), 8.31–7.97 (m, 9H, ArH, OCONH), 7.69 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-6), 5.68 (s, 1H, H-1′), 5.59 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-5), 5.40 (d, 1H, $J_{2',3'}$ = 5.8 Hz, H-2′), 4.91 (m, 2H, CH_2Ar), 4.66 (m, 1H, H-3′), 4.04 (m, 1H, $^2J_{5'a,5'b}$ = 12.6 Hz, $J_{4',5'a}$ = 3.2 Hz, H-5′a), 3.93–3.77 (m, 2H, H-4′, H-5′b), 1.04–0.67 (m, 28H, Pr^i).*

    *2′-O-(1,4,7,10,13-Pentaoxacyclopentadecan-2-ylmethyl)-aminocarbonyl-3′,5′-O-(tetra-isopropyldisiloxan-1,3-diyl)uridine (**S.4e**). Yield 1.165 g (76.4%). R_f: 0.32 (17:3 v/v $CHCl_3$/MeOH). ^{1}H NMR: 11.41 (s, 1H, H-3), 7.68 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 7.40 (t, 1H, J = 5.7 Hz, OCONH), 5.64 (s, 1H, H-1′), 5.59 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.33 (d, 1H, $J_{2',3'}$ = 5.7 Hz, H-2′), 4.48 (dd, 1H, $J_{2',3'}$ = 5.7 Hz, $J_{3',4'}$ = 8.2 Hz, H-3′), 3.99 (m, 2H, $^2J_{5'a,5'b}$ = 12.9 Hz, $J_{4',5'a}$ = 2.7 Hz, $J_{4',5'b}$ = 4.1 Hz, H-5′), 3.84 (m, 1H, H-4′), 3.64–3.36 (m, 19H#, $CH(CH_2OCH_2)_4CH_2$), 3.00 (m, 2H, CH_2N), 1.06–0.82 (m, 28H, Pr^i).*

    (#Calculated value; the signal of water is also present in the region).

*Nα-[3′,5′-O-(Tetraisopropyldisiloxan-1,3-diyl)uridin-2′-O-ylcarbonyl]-L-leucyl-L-phenyl alaninamide (**S.4f**). Yield 610 mg (38.6%). R_f: 0.45 (EtOAc). ESI-MS: $[M+H]^+$ calcd. 790.39, found 790.43. ^{1}H NMR: 11.43 (s, 1H, H-3), 7.87 (d, 1H, J = 8.2 Hz, NHCHCH$_2$Ph), 7.70 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 7.44 (d, 1H, J = 8.4 Hz, OCONH), 7.39 (br s, 1H, NH$_2$), 7.19 (m, 5H, Ph), 7.06 (br s, 1H, NH$_2$), 5.64 (s, 1H, H-1′), 5.59 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.35 (d, 1H, $J_{2',3'}$ = 5.1 Hz, H-2′), 4.47 (m, 2H, H-3′, CHCH$_2$Ph), 4.15–3.83 (m, 4H, H-4′, H-5′, CHBu^i), 3.03–2.73 (m, 2H, ^2J = 13.4 Hz, J_{AX} = 4.7 Hz, J_{BX} = 9.1 Hz, CH$_2$Ph), 1.49 (m, 1H, CH$_2$CHMe$_2$), 1.29 (m, 2H, CH$_2$Pr^i), 1.10–0.72 (m, 34H, SiCH(CH$_3$)$_2$, CH$_2$CH(CH$_3$)$_2$).*

*When **S.4e** and **S.4f** are prepared according to steps 16a and 17a, yields are 44% and 23%, respectively, after 90 days.*

3′,5′-O-Desilylate uridine 2′-carbamates

24. Dissolve 2 mmol **S.4** in 5 mL freshly distilled THF in a 30-mL screw-top Teflon bottle. Add 0.814 mL (5 mmol) triethylamine trihydrofluoride and magnetically stir the mixture 6 hr or overnight at ambient temperature. Monitor deprotection by TLC using 15% (v/v) MeOH/$CHCl_3$.

25. Dilute the mixture with 25 mL hexane, shake the bottle well, and allow the two phases to separate.

26. Discard the upper layer and wash the residue three times by decantation with 25 mL of 1:1 (v/v) toluene/hexane.

*For uridine 2′-carbamates **S.5a**, **S.5b**, **S.5e**, and **S.5f**, the oily residues are co-evaporated first with toluene (step 27a) and then with pyridine (step 28) before dimethoxytritylation. For **S.5c** and **S.5d**, crystalline uridine 2′-carbamates are isolated by ethanol trituration (step 27b), and these are co-evaporated by pyridine only (step 28).*

27a. *For **S.5a**, **S.5b**, **S.5e**, and **S5.f**:* Co-evaporate the crude oily uridine-2′-carbamates three times with 20 mL toluene.

27b. *For **S.5c** and **S.5d**:* Triturate the residue in 5 mL absolute ethanol, filter off the crystalline product, and wash it with 5 mL ethanol followed by 10 mL diethyl ether. Dry in vacuo. Characterize by TLC and ^{1}H NMR.

*2′-O-(4-Iodobenzylaminocarbonyl)uridine (**S.5c**). Yield 0.993 g (98.6%). R_f: 0.34 (17:3 v/v $CHCl_3$/MeOH), m.p. 192°–197°C (EtOH). ^{1}H NMR: 11.36 (s, 1H, H-3), 7.90 (m, 2H, H-6, OCONH), 7.65 (d, 2H, J = 7.4 Hz, ArH), 7.02 (d, 2H, J = 7.4 Hz, ArH), 5.99 (m, 1H, H-1′), 5.66 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.52 (d, 1H, J = 4.4 Hz, 3′-OH), 5.18 (m, 1H, 5′-OH), 5.05 (m, 1H, H-2′), 4.25–4.05 (m, 3H, H-3′, CH$_2$N), 3.88 (m, 1H, H-4′), 3.59 (m, 2H, H-5′).*

*2′-O-(Pyren-1-ylmethylaminocarbonyl)uridine (**S.5d**). Yield 0.467 g (93.5%). R_f: 0.37 (17:3 v/v $CHCl_3$/MeOH), m.p. 200°–208°C (EtOH). ^{1}H NMR: 11.38 (s, 1H, H-3), 8.42–7.90 (m, 11H, H-6, OCONH, ArH), 6.03 (d, 1H, $J_{1',2'}$ = 5.7 Hz, H-1′), 5.68 (d, 1H, $J_{5,6}$ = 7.8 Hz, H-5), 5.57 (m, 1H, 3′-OH), 5.21 (m, 1H, 5′-OH), 5.14 (m, 1H, H-2′), 4.91 (m, 2H, CH$_2$Ar), 4.24 (m, 1H, H-3′), 3.91 (m, 1H, H-4′), 3.60 (m, 2H, H-5′).*

5′-O-Dimethoxytritylate uridine 2′-carbamates

28. Co-evaporate residue from step 27a or 27b three times with 20 mL pyridine. Dissolve in 30 mL pyridine and half-evaporate, then apply a dry nitrogen atmosphere.

29. Cool the flask in an ice bath and add 0.85 g (2.5 mmol) DMTr·Cl in one portion. Monitor the reaction by TLC using 17:3 (v/v) $CHCl_3$/MeOH for **S.6e** and EtOAC for all others. Add 0.17-g (0.5-mmol) portions of DMTr·Cl every 4 hr until the starting nucleoside **S.5** disappears completely.

The total amount of DMTr·Cl needed is dependent on the residual fluoride content and usually varies from 3 to 4 mmol.

30. After completion of the reaction, quench excess DMTr·Cl with 1 mL MeOH, wait 10 min, and dilute the mixture with 100 mL $CHCl_3$.

31. Wash with 100 mL water, 100 mL of 5% $NaHCO_3$, and again with 100 mL water.

32. Dry over Na_2SO_4, filter off the Na_2SO_4, and then evaporate the mixture.

33. Co-evaporate three times with 25 mL toluene and purify the residue on a silica gel column in the appropriate solvent system:

    For **S.6a**: 1% to 5% (v/v) MeOH/0.5% (v/v) TEA in $CHCl_3$
    For **S.6b**: 1% to 2% (v/v) MeOH/0.5% (v/v) TEA in 1:1 (v/v) $CHCl_3$/EtOAc
    For **S.6c**: 0.5% to 2% (v/v) MeOH/0.5% (v/v) TEA in 1:1 (v/v) $CHCl_3$/EtOAc
    For **S.6d**: 0.5% to 1.5% (v/v) MeOH/0.5% (v/v) pyridine in $CHCl_3$
    For **S.6e**: 3% to 9% (v/v) MeOH/0.5% (v/v) TEA in 2:1 (v/v) $CHCl_3$/EtOAc
    For **S.6f**: 1% to 3% (v/v) MeOH/0.5% (v/v) pyridine in 2:1 (v/v) $CHCl_3$/EtOAc.

34. Combine the fractions containing product **S.6**, evaporate, and then co-evaporate three times with 25 mL CH_2Cl_2. Dry the residue in vacuo to afford the compounds as amorphous solids.

35. Characterize the compounds by TLC and ^{1}H NMR.

    *5′-O-(4,4′-Dimethoxytrityl)-2′-O-(propargylaminocarbonyl)uridine (**S.6a**). Yield 1.156 g (92.1%). R_f: 0.60 (EtOAc). ^{1}H NMR: 11.38 (br s, 1H, H-3), 7.85 (t, 1H, J = 5.7 Hz, OCONH), 7.66 (m, 1H, H-6), 7.40–7.19 (m, 9H, ArH), 6.89 (d, 4H, J = 8.7 Hz, ArH), 5.79 (d, 1H, J = 5.7 Hz, 3′-OH), 5.75 (s, 1H, H-1′), 5.39 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-5), 5.11 (m, 1H, H-2′), 4.35 (m, 1H, H-3′), 4.06 (m, 1H, H-4′), 3.79 (m, 2H, CH_2N), 3.73 (s, 6H, OCH_3), 3.40–3.15 (m, 3H$^{\#}$, H-5′, CH).*

    *5′-O-(4,4′-Dimethoxytrityl)-2′-O-(N-methyl-N-propargylaminocarbonyl)uridine (**S.6b**). Yield 1.097 g (85.5%). R_f: 0.73 (EtOAc). ^{1}H NMR: 11.39 (s, 1H, H-3), 7.70 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 7.41–7.19 (m, 9H, ArH), 6.89 (d, 4H, J = 8.6 Hz, ArH), 5.89 (d, 1H, $J_{1',2'}$ = 3.3 Hz, H-1′), 5.54 (m, 1H, 3′-OH), 5.39 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.15 (m, 1H, H-2′), 4.32 (m, 1H, H-3′), 4.12–3.93 (m, 3H, H-4′, CH_2N), 3.73 (s, 6H, OCH_3), 3.35–3.16 (m, 3H$^{\#}$, H-5′, CH), 2.92 (s, 1.8H), 2.85 (s, 1.2H), (NCH_3, rotamers).*

    *5′-O-(4,4′-Dimethoxytrityl)-2′-O-(4-iodobenzylaminocarbonyl)uridine (**S.6c**). Yield 1.378 g (85.5%). R_f: 0.75 (EtOAc). ^{1}H NMR: 11.42 (s, 1H, H-3), 7.95 (t, 1H, J = 6.0 Hz, OCONH), 7.70 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-6), 7.66 (d, 1H, J = 8.2 Hz, ArH), 7.40–7.18 (m, 9 H, ArH), 7.06 (d, 2H, J = 8.2 Hz, ArH), 6.88 (d, 4H, J = 8.8 Hz, ArH), 5.92 (d, 1H, $J_{1',2'}$ = 4.8 Hz, H-1′), 5.61 (d, 1H, $J_{3',OH}$ = 5.6 Hz, 3′-OH), 5.38 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-5), 5.17 (apparent t, 1H, $J_{1',2'} = J_{2',3'}$ = 4.8 Hz, H-2′), 4.33 (m, 1H, H-3′), 4.14 (d, 2H, J = 6.0 Hz, NCH_2), 3.98 (m, 1H, H-4′), 3.73 (s, 6H, OCH_3), 3.35–3.16 (m, 2H$^{\#}$, H-5′).*

    *5′-O-(4,4′-Dimethoxytrityl)-2′-O-(pyren-1-ylmethylaminocarbonyl)uridine (**S.6d**). Yield 1.482 g (92.2%). R_f: 0.75 (EtOAc). ^{1}H NMR: 11.44 (s, 1H, H-3), 8.41 (d, 1H, $J_{9',10'}$ = 9.3 Hz, H-10′), 8.32–8.00 (m, 9H, ArH, OCONH), 7.73 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 7.42–7.10 (m, 9H, ArH), 6.88 (d, 4H, J = 8.6 Hz, ArH), 5.97 (d, 1H, $J_{1',2'}$ = 4.6 Hz, H-1′), 5.65 (d, 1H, $J_{3',OH}$ = 5.6 Hz, 3′-OH), 5.39 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.26 (m, 1H, H-2′), 4.95 (d, 2H, J = 5.3 Hz, NCH_2), 4.38 (m, 1H, H-3′), 3.99 (m, 1H, H-4′), 3.72 (s, 6H, OCH_3), 3.35–3.15 (m, 2H$^{\#}$, H-5′).*

    *5′-O-(4,4′-Dimethoxytrityl)-2′-O-(1,4,7,10,13-pentaoxacyclopentadecan-2-ylmethyl-aminocarbonyl)uridine (**S.6e**). Yield 1.248 g (85.5%). R_f 0.29 (17:3 v/v $CHCl_3$/MeOH). ^{1}H NMR: 11.39 (s, 1H, H-3), 7.69 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 7.41–7.12 (m, 10H, ArH, OCONH), 6.89 (d, 4H, J = 8.8 Hz, ArH), 5.90 (d, 1H, $J_{1',2'}$ = 4.7 Hz, H-1′), 5.53 (m, 1H, 3′-OH), 5.38 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.15 (m, 1H, H-2′), 4.32 (m, 1H, H-3′), 3.97 (m, 1H, H-4′), 3.73 (s, 6H, OCH_3), 3.69–3.16 (m, 21H$^{\#}$, H-5′, $CH(CH_2OCH_2)_4CH_2$), 3.02 (m, 2H, CH_2N).*

*Nα-[5′-O-(4,4′-Dimethoxytrityl)uridin-2′-yloxycarbonyl]-L-leucyl-L-phenylalaninamide (**S.6f**). Yield 0.706 g (83.1%). R_f: 0.45 (EtOAc). ^{1}H NMR: 11.45 (s, 1H, H-3), 7.90 (d, 1H, J = 8.4 Hz, NHCHCH$_2$Ph), 7.72 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-6), 7.48–7.10 (m, 12H, ArH, NH$_2$,OCONH), 6.89 (d, 4H, J = 8.8 Hz, ArH), 5.90 (d, 1H, $J_{1',2'}$ = 4.4 Hz, H-1′), 5.52 (d, 1H, $J_{3',OH}$ = 5.9 Hz, 3′-OH), 5.43 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-5), 5.13 (m, 1H, H-2′), 4.47 (m, 2H, H-3′, CHCH$_2$Ph), 4.37 (m, 1H, H-3′), 4.17 (m, 1H, CHBu^i), 3.98 (m, 1H, H-4′), 3.73 (s, 6H, OCH$_3$), 3.52–3.10 (m, 2H$^\#$, H-5′), 3.05–2.70 (m, 2H, CH$_2$Ph), 1.57–1.22 (m, 3H, CH$_2$CHMe$_2$), 0.86–0.69 (m, 6H, CH(CH$_3$)$_2$).*

($^\#$Calculated value; the signal of water is also present in the region).

Phosphinylate 5′-O-DMTr-uridine 2′-carbamates

36. Co-evaporate 1.0 mmol **S.6** two times with 20 mL dry CH_2Cl_2, dissolve in 40 mL dry CH_2Cl_2, and then apply a dry nitrogen atmosphere.

37. Add 171 mg (1.0 mmol) diisopropylammonium tetrazolide and 0.38 mL (1.2 mmol) bis(*N,N*-diisopropylamino)-2-cyanoethoxyphosphine and half-evaporate on a rotary evaporator. Stir the mixture 2 hr under nitrogen. Monitor by TLC using 25% acetone/1% TEA in $CHCl_3$ (pair of diastereomers).

38. When conversion of **S.6** is complete, dilute the mixture with 100 mL $CHCl_3$ and wash with 100 mL of 5% $NaHCO_3$ followed by 100 mL of 20% (w/v) NaCl.

39. Dry over Na_2SO_4, filter off the Na_2SO_4, and evaporate the solution to dryness.

40. Purify the residue on silica gel in the appropriate solvent system.

    For **S.7a**: 33% to 100% (v/v) EtOAc/1% (v/v) TEA in $CHCl_3$
    For **S.7b**: 30% to 70% (v/v) EtOAc/1% (v/v) TEA in $CHCl_3$
    For **S.7c**: 20% to 25% (v/v) acetone/1% (v/v) TEA in $CHCl_3$
    For **S.7d**: 5% to 25% (v/v) acetone/1% (v/v) TEA in $CHCl_3$
    For **S.7e**: 20% to 66% (v/v) acetone/1% (v/v) TEA in $CHCl_3$
    For **S.7f**: 50% to 100% (v/v) acetone/1% (v/v) TEA in 1:1 (v/v) $CHCl_3$/EtOAc.

41. Combine fractions containing product **S.7**, evaporate, and dry in vacuo to afford the compounds as white amorphous solids.

42. Characterize the compounds by TLC and ^{1}H and ^{31}P NMR.

    *3′-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-O-(propargylaminocarbonyl)uridine (**S.7a**). Yield 1.029 g (82.8%). Faster moving diastereomer: R_f: 0.56 (EtOAc). ^{1}H NMR: 11.45 (s, 1H, H-3), 7.95 (m, 1H, OCONH), 7.72 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-6), 7.41–7.18 (m, 9H, ArH), 6.88 (d, 4H, J = 8.5 Hz, ArH), 5.89 (d, 1H, $J_{1',2'}$ = 5.0 Hz, H-1′), 5.43 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-5), 5.33 (apparent t, 1H, $J_{1',2'} = J_{2',3'}$ = 5.0 Hz, H-2′), 4.56 (m, 1H, H-3′), 4.15 (m, 1H, H-4′), 3.85–3.68 (m, 8H, OCH$_3$, CH$_2$N), 3.63–3.44 (m, 4H, POCH$_2$, CHCH$_3$), 3.34–3.22 (m, 2H$^\#$, H-5′), 3.09 (t, 1H, ^4J = 2.3 Hz, CH), 2.58 (t, 2H, J = 6.1 Hz, CH$_2$CN), 1.19–0.95 (m, 12H, CHCH$_3$). ^{31}P NMR (CD$_3$CN): 150.8. Slower moving diastereomer: R_f: 0.41 (EtOAc). ^{1}H NMR: 11.44 (s, 1H, H-3), 7.90 (t, 1H, J = 5.7 Hz, OCONH), 7.73 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-6), 7.41–7.18 (m, 9H, ArH), 6.87 (m, 4H, ArH), 5.91 (d, 1H, $J_{1',2'}$ = 5.1 Hz, H-1′), 5.42 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-5), 5.36 (apparent t, 1H, $J_{1',2'} = J_{2',3'}$ = 5.1 Hz, H-2′), 4.55 (m, 1H, H-3′), 4.10 (m, 1H, H-4′), 3.82–3.68 (m, 10H, OCH$_3$, CH$_2$N, POCH$_2$), 3.46 (m, 2H, CHCH$_3$), 3.35–3.21 (m, 2H$^\#$, H-5′), 3.12 (m, 1H, CH), 2.74 (t, 2H, J = 5.8 Hz, CH$_2$CN), 1.19–0.89 (m, 12H, CHCH$_3$). ^{31}P NMR (CD$_3$CN): 150.7.*

*3′-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-O-(N-methyl-N-propargylaminocarbonyl)uridine (**S.7b**). Yield 1.004 g (79.5%). R_f: 0.58, 0.69 (EtOAc). ^{1}H NMR: 11.42 (s, 1H, H-3), 7.70 (m, 1H, H-6), 7.42–7.16 (m, 9H, ArH), 6.87 (m, 4H, ArH), 5.90 (m, 1H, H-1′), 5.47–5.18 (m, 2H, H-5, H-2′), 4.55 (m, 1H, H-3′), 4.20–3.92 (m, 3H, H-4′, CH_2N), 3.73 (s, 6H, OCH_3), 3.62–3.18 (m, 7H$^{\#}$, $POCH_2$, $CHCH_3$, H-5′, CH), 2.94–2.78 (m, 3H, NCH_3), 2.74, 2.56 (2m, 2H, CH_2CN), 1.20–0.88 (m, 12H, $CHCH_3$). ^{31}P NMR (CD_3CN): 150.9 (26%), 150.8 (30%), 150.4 (44%), diastereomers and rotamers.*

*3′-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-O-(4-iodobenzylaminocarbonyl)uridine (**S.7c**). Yield 0.674 g (67%). R_f: 0.47, 0.62 (EtOAc). ^{1}H NMR: 11.46 (s, 1H, H-3), 8.04 (m, 1H, OCONH), 7.73–7.61 (m, 3H, ArH, H-6), 7.42–7.18 (m, 9H, ArH), 7.07 (m, 2H, ArH), 6.87 (m, 4H, ArH), 5.93 (m, 1H, H-1′), 5.46–5.28 (m, 2H, H-5, H-2′), 4.53 (m, 1H, H-3′), 4.12 (m, 3H, H-4′, NCH_2), 3.78–3.19 (m, 12H$^{\#}$, $POCH_2$, CHN, H-5′, OCH_3), 2.68, 2.58 (2m, 2H, CH_2CN), 1.23–0.89 (m, 12H, $CHCH_3$). ^{31}P NMR (CD_3CN): 149.3.*

*3′-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-O-(pyren-1-ylmethylaminocarbonyl)uridine (**S.7d**). Yield 0.978 g (97.4%). R_f: 0.55, 0.68 (EtOAc). ^{1}H NMR: 11.47 (s, 1H, H-3), 8.41 (d, 1H, $J_{9',10'}$ = 9.3 Hz, H-10′), 8.36–7.98 (m, 9H, ArH, OCONH), 7.73 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 7.43–7.17 (m, 9H, ArH), 6.87 (d, 4H, J = 8.5 Hz, ArH), 5.95 (d, 1H, $J_{1',2'}$ = 5.1 Hz, H-1′), 5.42 (m, 2H, H-5, H-2′), 4.91 (d, 2H, J = 5.3 Hz, NCH_2), 4.57 (m, 1H, H-3′), 4.17 (m, 1H, H-4′), 3.71 (s, 6H, OCH_3), 3.42–3.35 (m, 6H, $POCH_2$, CHN, H-5′), 2.88, 2.58 (2t, 2H, J = 5.8 Hz, CH_2CN, diastereo- mers), 1.17, 1.02–0.89 (2m, 12H, $CHCH_3$). ^{31}P NMR (CD_3CN): 149.3.*

*3′-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-O-(1,4,7,10,13-pentaoxacyclopentadecyl-2-methylaminocarbonyl)uridine (**S.7e**). Yield 0.955 g (93.4%). R_f: 0.11, 0.24 (EtOAc). ^{1}H NMR: 11.44 (br s, 1H, H-3), 7.70 (m, 1H, H-6), 7.49–7.15 (m, 10H, ArH, OCONH), 6.87 (m, 4H, ArH), 5.91 (m, 1H, H-1′), 5.46–5.25 (m, 2H, H-5, H-2′), 4.53 (m, 1H, H-3′), 4.11 (m, 1H, H-4′), 3.80–3.19 (m, 31H$^{\#}$, $POCH_2$, CHN, H-5′, $CH(CH_2OCH_2)_4CH_2$, OCH_3), 3.00 (m, 2H, CH_2N), 2.75, 2.59 (2m, 2H, CH_2CN), 1.20–0.90 (m, 12H, $CHCH_3$). ^{31}P NMR (CD_3CN): 149.28, 149.27 (diastereomers).*

($^{\#}$Calculated value; the signal of water is also present in the region).

*Nα-[3′-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-5′-O-(4,4′-dimethoxytrityl) uridin-2′-yloxycarbonyl]-L-leucyl-L-phenylalaninamide (**S.7f**). Yield 0.271 g (51.6%). R_f: 0.13, 0.20 (EtOAc). ^{1}H NMR: 11.47 (s, 1H, H-3), 8.08 (d, 2H, J = 11.7 Hz, NH_2), 7.83 (d, 1H, J = 8.6 Hz, $NHCHCH_2Ph$), 7.70 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-6), 7.43–7.07 (m, 15H, ArH, OCONH), 6.88 (d, 4H, J = 8.8 Hz, ArH), 5.86 (d, 1H, $J_{1',2'}$ = 4.8 Hz, H-1′), 5.46 (d, 1H, $J_{5,6}$ = 7.8 Hz, H-5), 5.32 (t, 1H, J = 5.3 Hz, H-2′), 4.64 (m, 1H, $CHCH_2Ph$), 4.46 (m, 1H, H-3′), 4.17 (m, 1H, $CHBu^i$), 3.95 (m, 1H, H-4′), 3.73 (s, 6H, OCH_3), 3.58–3.46 (m, 4H, $POCH_2$, CHN), 3.04–2.99 (m, 2H, H-5′), 2.87–2.81 (m, 2H, CH_2Ph), 2.59 (m, 2H, CH_2CN), 1.23–0.98 (m, 12H, $NCHCH_3$), 0.83–0.72 (m, 3H, CH_2CHMe_2), 0.65–0.59 (m, 6H, $CH_2CH(CH_3)_2$). ^{31}P NMR (DMSO-d_6): 148.9.*

PREPARATION OF URIDINE 2′-CARBAMATE PHOSPHORAMIDITES FROM PRIMARY AMINES THAT REQUIRE ADDITIONAL SIDE-CHAIN PROTECTION

ALTERNATE PROTOCOL

With an excess of di- or polyamines, imidazolide **S.3** reacts mostly in a 1:1 ratio. The reaction with *N*-(3-aminopropyl)-1,3-propanediamine and spermine affords exclusively primary amino-substituted products (see Figure 4.21.3). This observation is in agreement with the recently reported good selectivity of imidazolecarbonyl derivatives towards primary versus secondary amines (Rannard and Davis, 2000). The remaining amino groups are either protected by trifluoroacetylation or used for further reaction (e.g., with protected cysteine derivative in **S.4j**). Trifluoroacetyl (Tfa) and 9-fluorenylmethoxycarbonyl (Fmoc) are the most common amino-protecting groups used in oligonucleotide synthesis. Aminoalcohols also show high selectivity towards *N*-substitution. The primary hydroxyl group of carbamate **S.4k** is further protected by a trimethylacetyl group to give

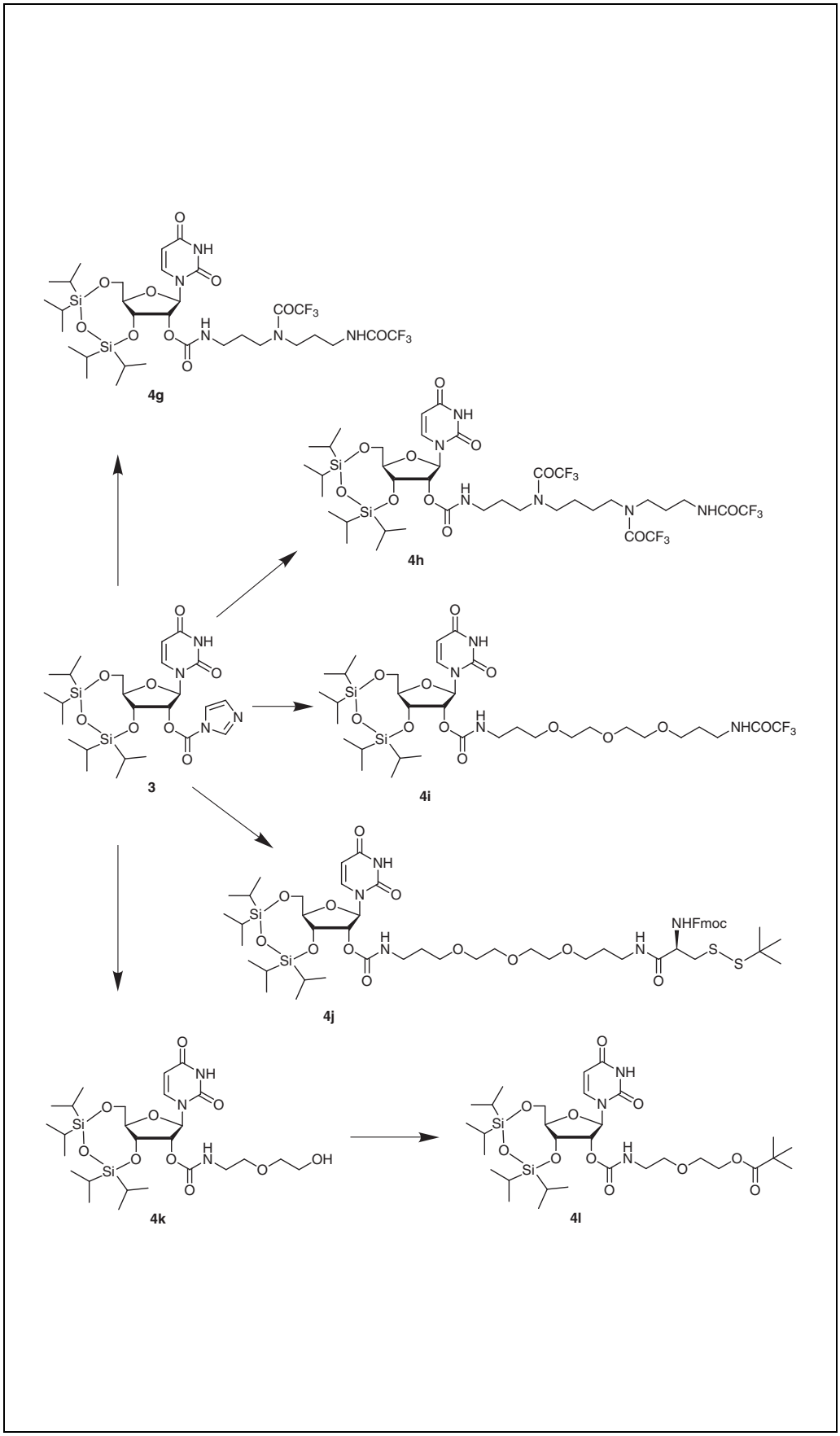


Figure 4.21.3 Preparation of side-chain-protected 3′,5′-*O*-silylated uridine 2′-carbamates (see Alternate Protocol).

S.4l. This protecting group was found to be rather stable under oligonucleotide deprotection conditions (see Basic Protocol 2).

NOTE: For Tfa 2′-carbamates (**S.4g**-**S.4i**), attempts to reduce the amine-to-imidazolide ratio to 1:1 give a decreased yield of the target compound and increased amounts of disubstituted byproducts.

NOTE: Although Tfa derivatives are stable in the presence of TEA, a premature Fmoc release may occur from Fmoc compounds. Therefore, use of pyridine is advised as an additive in the column chromatography of Fmoc-containing intermediates.

Additional Materials *(also see Basic Protocol 1)*

3′,5′-*O*-(Tetraisopropyldisiloxan-1,3-diyl)uridine (**S.2**; see Basic Protocol 1, step 9)
Amine for **S.3** conversion (select one):
N-(3-Aminopropyl)-1,3-propanediamine, 98% pure (Aldrich; for **S.4g**)
Spermine, 99% pure (Aldrich; for **S.4h**)
4,7,10-Trioxa-1,13-tridecanediamine, 97% pure (Sigma; for **S.4i**-**S.4j**)
2-(2-Aminoethoxy)ethanol (for **S.4k**)
Reagent for amine protection (select one):
S-Ethyl trifluorothioacetate, 97% pure (Aldrich; for **S.4g**-**S.4i**)
N^{α}-Fmoc-*S*-*tert*-butylthio-L-cysteine pentafluorophenyl ester, 99% pure (Novabiochem; for **S.4j**)
Trimethylacetyl chloride, 99% pure (Aldrich; for **S.4l**)

Prepare di- and polyamine 2′-carbamates (S.4g-i) and protect by trifluoroacetylation

For S.4g

1a. Prepare a solution of **S.3** from 2.434 g (5.0 mmol) **S.2** and 852 mg (5.25 mmol) 1,1′-carbonyldiimidazole in 50 mL dry CH_2Cl_2. Monitor the reaction by TLC (*APPENDIX 3D*) in 1:1 (v/v) EtOAc/hexane.

2a. Prepare a magnetically stirred, ice-cooled solution of 5.6 mL (40 mmol) *N*-(3-aminopropyl)-1,3-propanediamine in 100 mL CH_2Cl_2.

3a. Add the solution from step 1a dropwise to the solution in step 2a and incubate 2 hr.

4a. Remove the cooling bath and incubate the mixture overnight at room temperature.

5a. Wash the mixture with 200 mL water, 200 mL of 5% $NaHCO_3$, and then another 200 mL water.

6a. Dry over Na_2SO_4, filter to remove the solid, and evaporate the solution to dryness.

7a. Co-evaporate the residue with 30 mL THF, dissolve in 10 mL dry THF, and add 5.0 mL (39 mmol) *S*-ethyl trifluorothioacetate in one portion. Incubate 20 hr.

CAUTION: *S-Ethyl trifluorothioacetate generates ethanethiol, which has a strong stench.*

8a. Evaporate the mixture to dryness, co-evaporate the residue with $CHCl_3$, and purify on a silica gel column using 1:1 to 1:3 (v/v) $CHCl_3$/EtOAc (*APPENDIX 3E*).

The product is obtained as a white amorphous solid. Trituration of ***S.4g*** *in CH_2Cl_2/hexane gives colorless crystals.*

9a. Analyze the product **S.4g** by TLC and ^{1}H NMR. Proceed to step 12.

*2′-O-[4-Trifluoroacetyl-7-(trifluoroacetylamino)-4-azaheptan-1-ylaminocarbonyl]-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl)uridine (**S.4g**). Yield 2.558 g (61.2%), m.p. 74°–77°C. R_f: 0.69 (EtOAc). ^{1}H NMR: 11.42 (s, 1H, H-3), 9.51, 9.43 (2 br t, 1H, NHTFA, rotamers), 7.69 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-6), 7.51, 7.45 (2m, 1H, OCONH, rotamers), 5.65 (d, 1H, $J_{1',2'}$ = 1.8 Hz, H-1′), 5.59 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-5), 5.32 (m, 1H, H-2′), 4.50 (m, 1H, H-3′), 3.97 (m, 2H, $^2J_{5'a,5'b}$ = 12.5 Hz, $J_{4',5'a}$ = 4.2 Hz, $J_{4',5'b}$ = 2.4 Hz, H-5′), 3.83 (m, 1H, H-4′), 3.33 (m, 4H#, CH_2NCH_2), 3.19 (m, 2H, CH_2NHTFA), 2.98 (m, 2H, $OCONHCH_2$), 1.74 (m, 4H, CH_2), 1.10–0.85 (m, 28H, Pr^i).*

(#Calculated value; the signal of water is also present in the region).

For S.4h

1b. Prepare a solution of **S.3** as in step 1a.

2b. Prepare a magnetically stirred, ice-cooled solution of 2.63 g (13 mmol) spermine in 200 mL CH_2Cl_2.

3b. Add the solution from step 1b dropwise to the solution in step 2b and incubate 2 hr.

4b. Remove the cooling bath and stir the mixture overnight at room temperature.

5b. Dilute with 150 mL $CHCl_3$ and wash two times with 200 mL of 20% NaCl.

6b. Dry over Na_2SO_4, filter, and evaporate the solution to dryness.

7b. Trifluoroacetylate the residue as in step 7a.

8b. Purify as in step 8a using 50% to 0% (v/v) $CHCl_3$ in EtOAc.

9b. Analyze the product **S.4h** by TLC and ^{1}H NMR. Proceed to step 12.

*2′-O-[4,9-bis(Trifluoroacetyl)-12-(trifluoroacetylamino)-4,9-diazadodecan-1-ylaminocarbonyl]-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl)uridine (**S.4h**). Yield 2.457 g (49.0%). R_f: 0.67 (EtOAc). ^{1}H NMR: 11.42 (s, 1H, H-3), 9.51, 9.44 (2 br t, 1H, NHTFA, rotamers), 7.69 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 7.51, 7.45 (2t, 1H, J = 5.2 Hz, OCONH, rotamers), 5.65 (s, 1H, H-1′), 5.59 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.32 (m, 1H, H-2′), 4.49 (m, 1H, H-3′), 3.98 (m, 2H, $^2J_{5'a,5'b}$ = 12.6 Hz, $J_{4',5'a}$ = 4.2 Hz, H-5′), 3.83 (m, 1H, H-4′), 3.40–3.28 (m, 8H#, $CH_2N(TFA)CH_2$), 3.20 (m, 2H, CH_2NHTFA), 2.98 (m, 2H, $OCONHCH_2$), 1.86–1.60 (m, 4H, CH_2CH_2NH), 1.51 (m, 4H, $CH_2CH_2CH_2CH_2$), 1.06–0.80 (m, 28H, Pr^i).*

(#Calculated value; the signal of water is also present in the region).

For S.4i

1c. Prepare a solution of **S.3** from 1.703 g (3.5 mmol) **S.2** and 592 mg (3.65 mmol) 1,1′-carbonyldiimidazole in 30 mL dry CH_2Cl_2. Monitor the reaction by TLC in EtOAc.

2c. Prepare a magnetically stirred, ice-cooled solution of 3.9 mL (17.5 mmol) 4,7,10-trioxa-1,13-tridecanediamine in 100 mL CH_2Cl_2.

3c. Add the solution from step 1c dropwise to the solution in step 2c and incubate 2 hr.

4c. Remove the cooling bath and incubate the mixture overnight at room temperature.

5c. Wash the mixture with 150 mL water, 150 mL of 5% $NaHCO_3$, and then another 150 mL water.

6c. Dry over Na_2SO_4, filter, and evaporate the solution to dryness.

7c. Dissolve the residue in 10 mL dry CH_2Cl_2 and add 3.0 mL (24 mmol) *S*-ethyl trifluorothioacetate in one portion. Incubate the mixture overnight.

CAUTION: *S-Ethyl trifluorothioacetate generates ethanethiol, which has a strong stench.*

8c. Evaporate the mixture to dryness and purify on a silica gel column using 1:1 to 1:4 (v/v) $CHCl_3$/EtOAc.

9c. Analyze the product **S.4i** by TLC and ^{1}H NMR. Proceed to step 12.

*2′-O-[13-(Trifluoroacetylamino)-4,7,10-trioxatridecan-1-ylaminocarbonyl]-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl)uridine (**S.4i**). Yield 1.888 g (65.1%) as colorless oil. R_f: 0.42 (EtOAc). ^{1}H NMR: 11.41 (s, 1H, H-3), 9.36 (br s, 1H, NHTFA), 7.69 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 7.36 (t, 1H, J = 5.7 Hz, OCONH), 5.64 (d, 1H, $J_{1',2'}$ = 1.8 Hz, H-1′), 5.59 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.31 (m, 1H, H-2′), 4.49 (dd, 1H, $J_{2',3'}$ = 5.0 Hz, $J_{3',4'}$ = 7.9 Hz, H-3′), 3.99 (m, 2H, $^2J_{5'a,5'b}$ = 13.0 Hz, $J_{4',5'a}$ = 2.7 Hz, $J_{4',5'b}$ = 4.4 Hz, H-5′), 3.82 (m, 1H, H-4′), 3.55–3.30 (m, 12H#, $(CH_2OCH_2)_3$), 3.22 (apparent q, 2H, J = 6.5 Hz, CH_2NHTFA), 3.00 (m, 2H, OCONHCH_2), 1.69 (apparent quintet, 2H, J = 6.8 Hz, CH_2), 1.60 (apparent quintet, 2H, J = 6.6 Hz, CH_2), 1.06–0.81 (m, 28H, Pr^i).*

(#Calculated value; the signal of water is also present in the region).

Prepare functionalized 2′-carbamate (S.4j) by acylation

1d. Prepare a solution of **S.3** as in step 1a.

2d. Prepare a magnetically stirred, ice-cooled solution of 5.5 mL (25 mmol) 4,7,10-trioxa-1,13-tridecanediamine in 100 mL CH_2Cl_2.

3d. Add the solution from step 1d dropwise to the solution in step 2d and incubate 2 hr.

4d. Remove the cooling bath and incubate the mixture overnight at room temperature.

5d. Wash the mixture with 200 mL water, 200 mL of 5% $NaHCO_3$, and then another 200 mL water.

6d. Dry over Na_2SO_4, filter, and evaporate the solution to dryness.

7d. Dissolve the crude amine in 20 mL dry CH_2Cl_2 and add 2.988 g (5.0 mmol) N^{α}-Fmoc-*S*-*tert*-butylthio-L-cysteine pentafluorophenyl ester. Incubate 2 hr.

8d. Dilute the mixture with 200 mL $CHCl_3$ and wash with 150 mL of 5% $NaHCO_3$, 150 mL of 5% citric acid, and 150 mL water.

9d. Dry as in step 6d and then purify the residue on a silica gel column using 1:1:0 to 1:3:0 to 1:2:1 (v/v/v) $CHCl_3$/EtOAc/acetone.

10d. Pool the appropriate fractions, evaporate, and co-evaporate three times with 20 mL CH_2Cl_2. Dry in vacuo.

11d. Analyze the product **S.4j** by TLC and ^{1}H NMR. Proceed to step 12.

*2′-{O-13-[S-(tert-Butylthio)-N-(9-fluorenylmethoxycarbonyl)-L-cysteinylamino]-4,7,10-trioxatridecan-1-ylaminocarbonyl}-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl)uridine (**S.4j**). Yield 3.732 g (65.2%), white amorphous solid. R_f: 0.41 (EtOAc). ^{1}H NMR: 11.41 (s, 1H, H-3), 8.01 (t, 1H, J = 5.2 Hz, NHCOCH), 7.88 (d, 2H, J = 7.4 Hz, ArH (fluorene)), 7.75–7.64 (m, 4H, ArH (fluorene H-1,4,5,8), H-6, NHFmoc), 7.43–7.27 (m, 5 H, ArH (fluorene H-2,3,6,7), OCONHCH_2), 5.64 (s, 1H, H-1′), 5.59 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.31 (d, 1H, $J_{2',3'}$ = 5.8 Hz, H-2′), 4.49 (m, 1H, H-3′), 4.32–4.13 (m, 4H, CHCH_2O (Fmoc), COCHN), 3.98 (m, 2H, $^2J_{5'a,5'b}$ = 13.0 Hz, $J_{4',5'a}$ = 4.2 Hz, $J_{4',5'b}$ = 2.5 Hz, H-5′), 3.82 (m, 1H, H-4′), 3.50–3.26 (m, 12H#, $(CH_2OCH_2)_3$), 3.15–2.82 (m, 6H, CH_2N, CH_2S), 1.60 (m, 4H, $CH_2CH_2CH_2$), 1.28 (s, 9H, Bu^t), 1.06–0.81 (m, 28H, Pr^i).*

(#Calculated value; the signal of water is also present in the region).

Prepare hydroxyalkyl 2′-carbamate (S.4k) and trimethylacetylate to give S.4l

1e. To obtain 2-(2-hydroxyethoxy)ethyl-3′,5′-silyl-2′-carbamate (**S.4k**), apply the procedure for unhindered primary amines (see Basic Protocol 1, steps 16a to 17a and 18 to 22), using 1.50 mL (15.0 mmol) 2-(2-aminoethoxy)ethanol in step 16a (~3 hr) and 2:1:1 to 1:1:1 to 1:1:2 (v/v/v) $CHCl_3$/EtOAc/acetone for chromatography in step 20.

*2′-O-[2-(2-Hydroxyethoxy)ethylaminocarbonyl]-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl)uridine (**S.4k**). Yield 2.269 g (73.5%). R_f: 0.28 (EtOAc). ^{1}H NMR: 11.41 (s, 1H, H-3), 7.68 (d, 1H, $J_{5,6}$ = 7.9 Hz, H-6), 7.40 (br s, 1H, OCONH), 5.64 (s, 1H, H-1′), 5.59 (d, 1H, $J_{5,6}$ = 7.9 Hz, H-5), 5.31 (d, 1H, $J_{2′,3′}$ = 5.4 Hz, H-2′), 4.55 (m, 2H, H-3′, OH), 4.12–3.79 (m, 3H, H-4′, H-5′), 3.53–3.27 (m, 6H$^{\#}$, $CH_2OCH_2CH_2$), 3.12 (m, 2H, CH_2N), 1.10–0.82 (m, 28H, Pr^i).*

($^{\#}$Calculated value; the signal of water is also present in the region).

2e. To a stirred solution of 2.162 g (3.5 mmol) **S.4k** in 20 mL dry CH_2Cl_2, add 1.0 mL (12.5 mmol) pyridine and 0.615 mL (5 mmol) trimethylacetyl chloride.

3e. Keep the reaction at ambient temperature until the starting compound disappears, as monitored by TLC using EtOAc (~20 hr).

4e. Dilute the mixture with 100 mL CH_2Cl_2.

5e. Wash with 100 mL water, 100 mL of 5% $NaHCO_3$, and another 100 mL water.

6e. Dry over Na_2SO_4, filter, and evaporate to dryness.

7e. Purify the residue on silica gel using 10% to 50% (v/v) EtOAc/$CHCl_3$.

8e. Combine the fractions, evaporate, and co-evaporate three times with 20 mL CH_2Cl_2. Dry in vacuo.

9e. Analyze the product **S.4l** by TLC and ^{1}H NMR. Proceed to step 12.

*2′-O-[2-(2-Pivaloyloxyethoxy)ethylaminocarbonyl]-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl)uridine (**S.4l**). Yield 1.977 g (80.5%). R_f: 0.34 (1:1 v/v $CHCl_3$/EtOAc). ^{1}H NMR: 11.41 (s, 1H, H-3), 7.69 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 7.37 (br t, 1H, J = 4.6 Hz, OCONH), 5.64 (s, 1H, H-1′), 5.59 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.31 (d, 1H, $J_{2′,3′}$ = 4.9 Hz, H-2′), 4.50 (m, 1H, H-3′), 4.17–3.78 (m, 5H, H-4′, H-5′, CH_2OPiv), 3.59 (m, 2H, CH_2OCH_2), 3.40 (m, 2H$^{\#}$, CH_2N), 3.10 (m, 2H, CH_2OCH_2), 1.12 (s, 9H, Bu^t), 1.07–0.83 (m, 28H, Pr^i).*

($^{\#}$Calculated value; the signal of water is also present in the region).

Prepare 5′-O-dimethoxytritylated side-chain-protected 2′-carbamates (S.6g-j and S.6l)

12. Deprotect the 3′- and 5′-hydroxyl groups of side-chain-protected 2′-carbamates (**S.4g-j** and **S.4l**) by treatment with triethylamine trihydrofluoride in THF (see Basic Protocol, steps 24 to 26). Coevaporate with toluene (step 27a).

13. 5′-*O*-Dimethoxytritylate crude 3′,5′-unprotected side-chain-protected 2′-carbamates (**S.5g-j** and **S.5l**) by treatment with DMTr·Cl in pyridine, and purify **S.6g-j** and **S.6l** by column chromatography (see Basic Protocol 1, steps 28 to 34).

For **S.6g**: stepwise gradient of 1% to 4% (v/v) MeOH/0.5% (v/v) TEA in 2:1 (v/v) $CHCl_3$/EtOAc
For **S.6h**: 1% to 5% (v/v) MeOH/0.5% (v/v) TEA in 2:1 (v/v) $CHCl_3$/EtOAc
For **S.6i**: 1% to 3.5% (v/v) MeOH/0.5% (v/v) TEA in 2:1 (v/v) $CHCl_3$/EtOAc
For **S.6j**: 0% to 6% (v/v) MeOH/1% (v/v) pyridine in 1:1 (v/v) $CHCl_3$/EtOAc
For **S.6l**: 0.5% to 2% (v/v) MeOH/0.5% (v/v) TEA in 2:1 (v/v) $CHCl_3$/EtOAc.

14. Analyze the products by TLC and ^{1}H NMR.

*5′-O-(4,4′-Dimethoxytrityl)-2′-O-[4-trifluoroacetyl-7-(trifluoroacetylamino)-4-azaheptan-1-ylaminocarbonyl]uridine (**S.6g**). Yield 1.556 g (86.8%), white amorphous solid. R_f: 0.49 (EtOAc). ^{1}H NMR: 11.37 (br s, 1 H, H-3) (exchangeable with D_2O), 9.51, 9.46 (2 br s, 1H, NHTFA, rotamers), 7.69 (m, 1H, H-6), 7.53–7.19 (m, 10H, ArH (DMTr), OCONH), 6.89 (m, 4H, ArH (DMTr)), 5.92 (m, 1H, H-1′), 5.56 (m, 1H, 3′-OH) (exchangeable with D_2O), 5.37 (m, 1H, H-5), 5.15 (m, 1H, H-2′), 4.34 (m, 1H, H-3′), 3.97 (m, 1H, H-4′), 3.73 (s, 6H, OCH_3), 3.40–3.14 (m, 8H$^{\#}$, H-5′, CH_2NTFA), 3.00 (m, 2H, $OCONHCH_2$), 1.73 (m, 4H, $CH_2CH_2CH_2$).*

*5′-O-(4,4′-Dimethoxytrityl)-2′-O-[4,9-bis(trifluoroacetyl)-12-(trifluoroacetylamino)- 4,9-diazadodecan-1-aminocarbonyl]uridine (**S.6h**). Yield 1.773 g (83.4%). R_f: 0.50 (EtOAc). ^{1}H NMR: 11.40 (s, 1H, H-3), 9.51, 9.44 (2m, 1H, NHTFA rotamers), 7.69 (m, 1H, H-6), 7.51–7.18 (m, 10H, ArH, OCONH), 6.88 (d, 4H, J = 8.8 Hz, ArH), 5.92 (m, 1H, H-1′), 5.56 (m, 1H, 3′-OH), 5.37 (m, 1H, H-5), 5.15 (m, 1H, H-2′), 4.34 (m, 1H, H-3′), 3.96 (m, 1H, H-4′), 3.73 (s, 6H, OCH_3), 3.44–3.14 (m, 12H$^{\#}$, H-5′, CH_2NTFA), 3.01 (m, 2H, $OCONHCH_2$), 1.73 (m, 4H, CH_2CH_2NH), 1.52 (m, 4H, $CH_2CH_2CH_2CH_2$).*

($^{\#}$Calculated value; the signal of water is also present in the region).

*5′-O-(4,4′-Dimethoxytrityl)-2′-O-[13-(trifluoroacetylamino)-4,7,10-trioxatridecan-1-ylaminocarbonyl]uridine (**S.6i**). Yield 1.427 g (80.3%). R_f: 0.22 (EtOAc). ^{1}H NMR: 11.39 (s, 1H, H-3), 9.36 (br s, 1H, NHTFA), 7.70 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-6), 7.41–7.19 (m, 10H, ArH, OCONH), 6.89 (d, 4H, J = 8.8 Hz, ArH), 5.89 (d, 1H, $J_{1′,2′}$ = 5.0 Hz, H-1′), 5.54 (d, 1H, $J_{3′,OH}$ = 5.6 Hz, 3′-OH), 5.38 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-5), 5.13 (apparent t, 1H, $J_{1′,2′}$ = $J_{2′,3′}$ = 5.0 Hz, H-2′), 4.32 (m, 1H, H-3′), 3.96 (m, 1H, H-4′), 3.73 (s, 6H, OCH_3), 3.53–3.36 (m, 12H, $(CH_2OCH_2)_3$), 3.28–3.16 (m, 4H, H-5′, CH_2NHTFA), 3.02 (m, 2H, $OCONHCH_2$), 1.75–1.56 (m, 4H, $CH_2CH_2CH_2$).*

*5′-O-(4,4′-Dimethoxytrityl)-2′-O-{13-[S-(tert-butylthio)-N-(9-fluorenylmethyloxycarbonyl) cysteinylamino]-4,7,10-trioxatridecane-1-ylaminocarbonyl}uridine (**S.6j**). Yield 2.304 g (95.5%). R_f: 0.21 (EtOAc). ^{1}H NMR: 11.39 (s, 1H, H-3), 8.02 (t, 1H, J = 5.5 Hz, NHCOCH), 7.88 (d, 2H, J = 7.5 Hz, ArH (fluorene)), 7.70 (m, 4H, ArH (fluorene), H-6, NHFmoc), 7.44–7.10 (m, 14H, ArH (fluorene H-2,3,6,7, DMTr), $OCONHCH_2$), 6.88 (d, 4H, J = 8.9 Hz, ArH (DMTr)), 5.90 (d, 1H, $J_{1′,2′}$ = 5.0 Hz, H-1′), 5.54 (d, 1H, $J_{3′,OH}$ = 5.6 Hz, 3′-OH), 5.38 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-5), 5.14 (apparent t, 1H, $J_{1′,2′}$ = $J_{2′,3′}$ = 5.0 Hz, H-2′), 4.37–4.15 (m, 5H, $CHCH_2O$ (Fmoc), COCHN, H-3′), 3.96 (m, 1H, H-4′), 3.72 (s, 6H, OCH_3), 3.50–3.33 (m, 12H, $(CH_2OCH_2)_3$), 3.28–2.88 (m, 8H, CH_2N, CH_2S, H-5′), 1.61 (apparent quintet, 4H, J = 6.6 Hz, $CH_2CH_2CH_2$), 1.28 (s, 9H, Bu^t).*

*5′-O-(4,4′-Dimethoxytrityl)-2′-O-[2-(2-trimethylacetoxyethoxy)ethylaminocarbonyl] uridine (**S.6l**). Yield 1.377 g (90.4%). R_f: 0.60 (EtOAc). ^{1}H NMR: 11.40 (s, 1H, H-3), 7.69 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-6), 7.41–7.19 (m, 10H, ArH, OCONH), 6.89 (d, 4H, J = 8.8 Hz, ArH), 5.90 (d, 1H, $J_{1′,2′}$ = 5.0 Hz, H-1′), 5.53 (d, 1H, $J_{3′,OH}$ = 5.7 Hz, 3′-OH), 5.38 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-5), 5.12 (apparent t, 1H, $J_{1′,2′}$ = $J_{2′,3′}$ = 5.0 Hz, H-2′), 4.32 (m, 1H, H-3′), 4.11 (m, 2H, CH_2OPiv), 3.97 (m, 1H, H-4′), 3.73 (s, 6H, OCH_3), 3.57, 3.43 (2m, 4H, CH_2OCH_2), 3.27–3.02 (m, 4H, H-5′, CH_2N), 1.11 (s, 9H, Bu^t).*

Prepare phosphoramidites S.7g-j and S.7l

15. Phosphinylate **S.6** compounds by treatment with bis(*N*,*N*-diisopropylamino)-2-cyanoethoxyphosphine and diisopropylammonium tetrazolide in dry CH_2Cl_2, and purify **S.7** by column chromatography (see Basic Protocol 1, steps 36 to 41).

For **S.7g**: 20% to 66% (v/v) acetone/1% (v/v) TEA in $CHCl_3$
For **S.7h**: 20% to 50% (v/v) acetone/1% (v/v) TEA in $CHCl_3$
For **S.7i**: 20% to 66% (v/v) acetone/1% (v/v) TEA in $CHCl_3$
For **S.7j**: 20% to 40% (v/v) acetone/1% (v/v) pyridine in $CHCl_3$
For **S.7l**: 1% (v/v) TEA in 1:1 (v/v) acetone/$CHCl_3$.

16. Analyze the products by TLC and ^{1}H and ^{31}P NMR.

*3′-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-O-[4-trifluoroacetyl-7-(trifluoroacetylamino)-4-azaheptan-1-ylaminocarbonyl]uridine (**S.7g**). Yield 1.396 g (93.8%). R_f: 0.14, 0.25 (EtOAc). ^{1}H NMR: 11.40 (br s, 1H, H-3), 9.52, 9.44 (2 br s, 1H, NHTFA, rotamers), 7.70 (m, 1H, H-6), 7.53 (m, 1H, OCONH), 7.41–7.15 (m, 9H, ArH), 6.87 (m, 4H, ArH), 5.92 (m, 1H, H-1′), 5.45–5.22 (m, 2H, H-5, H-2′), 4.54 (m, 1H, H-3′), 4.12 (m, 1H, H-4′), 3.80–3.09 (m, 18H$^{\#}$, OCH_3, $POCH_2$, CHN, H-5′, CH_2NTFA), 2.99 (m, 2H, $OCONHCH_2$), 2.74, 2.57 (2m, 2H, CH_2CN), 1.73 (m, 4H, $CH_2CH_2CH_2$), 1.20–0.87 (m, 12H, $CHCH_3$). ^{31}P NMR (CD_3CN): 150.74 (54%), 150.66 (22%), 150.58 (24%), diastereomers and rotamers.*

*3′-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-O-[4,9-bis(trifluoroacetyl)-12-(trifluoroacetylamino)-4,9-diazadodecyl-1-aminocarbonyl] uridine (**S.7h**). Yield 1.242 g (98.3%). R_f: 0.18, 0.32 (EtOAc). ^{1}H NMR: 11.36 (br s, 1H, H-3), 9.52, 9.45 (2 br s, 1H, NHTFA, rotamers), 7.69 (m, 1H, H-6), 7.53 (m, 1H, OCONH), 7.41–7.16 (m, 9H, ArH), 6.87 (m, 4H, ArH), 5.92 (m, 1H, H-1′), 5.44–5.21 (m, 2H, H-5, H-2′), 4.53 (m, 1H, H-3′), 4.12 (m, 1H, H-4′), 3.80–3.12 (m, 22H$^{\#}$, OCH_3, $POCH_2$, CHN, H-5′, CH_2NTFA), 2.99 (m, 2H, $OCONHCH_2$), 2.74, 2.57 (2m, 2H, CH_2CN), 1.74 (m, 4H, CH_2CH_2NH), 1.51 (m, 4H, $CH_2CH_2CH_2CH_2$), 1.19–0.86 (m, 12H, $CHCH_3$). ^{31}P NMR (CD_3CN): 150.8 (56%), 150.7 (20%), 150.6 (24%), diastereomers and rotamers.*

*3′-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-O-[13-(trifluoroacetylamino)-4,7,10-trioxatridecan-1-ylaminocarbonyl]uridine (**S.7i**). Yield 1.069 g (98.2%). R_f: 0.20, 0.34 (EtOAc). ^{1}H NMR: 11.44 (s, 1H, H-3), 9.37 (br s, 1H, NHTFA), 7.71 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 7.47–7.19 (m, 10H, ArH, OCONH), 6.87 (m, 4H, ArH), 5.91 (m, 1H, H-1′), 5.42 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.31 (m, 1H, H-2′), 4.55 (m, 1H, H-3′), 4.12 (m, 1H, H-4′), 3.80–3.16 (m, 24H$^{\#}$, $(CH_2OCH_2)_3$, $POCH_2$, CHN, H-5′, OCH_3), 3.01 (m, 2H, $OCONHCH_2$), 2.74, 2.57 (2m, 2H, CH_2CN), 1.74–1.55 (m, 4H, $CH_2CH_2CH_2$), 1.18–0.92 (m, 12H, $CHCH_3$). ^{31}P NMR (CD_3CN): 149.35, 149.28 (diastereomers).*

*3′-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-O-{13-[S-(tert-butylthio)-N-(9-fluorenylmethyloxycarbonyl)cysteinylamino]-4,7,10- trioxatridecane-1-ylaminocarbonyl}uridine (**S.7j**). Yield 1.130 g (81.4%). R_f: 0.30, 0.53 (EtOAc). Faster moving diastereomer: ^{1}H NMR: 11.44 (s, 1H, H-3), 8.02 (m, 1H, NHCOCH), 7.88 (d, 2H, J = 7.4 Hz, ArH (fluorene)), 7.70 (m, 4H, ArH (fluorene), H-6, NHFmoc), 7.47–7.08 (m, 14H, ArH (fluorene H-2,3,6,7, DMTr), $OCONHCH_2$), 6.88 (d, 4H, J = 8.6 Hz, ArH (DMTr)), 5.89 (d, 1H, $J_{1',2'}$ = 5.1 Hz, H-1′), 5.42 (d, 1H, $J_{5,6}$ = 7.9 Hz, H-5), 5.30 (m, 1H, H-2′), 4.55 (m, 1H, H-3′), 4.35–4.07 (m, 5H, $CHCH_2O$ (Fmoc), COCHN, H-4′), 3.72 (s, 6H, OCH_3), 3.60–3.21 (m, 24H$^{\#}$, $(CH_2OCH_2)_3$, $POCH_2$, $NCHCH_3$, H-5′), 3.15–2.85 (m, 6H, CH_2N, CH_2S), 2.57 (m, 2H, CH_2CN), 1.59 (m, 4H, $CH_2CH_2CH_2$), 1.28 (s, 9H, Bu^t), 1.13–0.93 (m, 12H, $CHCH_3$). Slower moving diastereomer: ^{1}H NMR: 11.44 (s, 1H, H-3), 8.02 (m, 1H, NHCOCH), 7.88 (d, 2H, J = 7.4 Hz, ArH (fluorene)), 7.70 (m, 4H, ArH (fluorene), H-6, NHFmoc), 7.42–7.12 (m, 14H, ArH (fluorene H-2,3,6,7, DMTr), $OCONHCH_2$), 6.87 (d, 4H, J = 8.2 Hz, ArH (DMTr)), 5.91 (m, 1H, H-1′), 5.42 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.34 (m, 1H, H-2′), 4.53 (m, 1H, H-3′), 4.33–4.03 (m, 5H, $CHCH_2O$ (Fmoc), COCHN, H-4′), 3.80–3.20 (m, 24H$^{\#}$, $(CH_2OCH_2)_3$, $POCH_2$, $NCHCH_3$, H-5′, OCH_3), 3.12–2.87 (m, 6H, CH_2N, CH_2S), 2.74 (t, 2H, J = 5.7 Hz, CH_2CN), 1.61 (m, 4H, $CH_2CH_2CH_2$), 1.27 (s, 9H, Bu^t), 1.12–0.88 (m, 12H, $CHCH_3$).*

*3′-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-O-[2-(2-trimethylacetoxyethoxy)ethylaminocarbonyl]uridine (**S.7l**). Yield 0.947g (98.4%). R_f: 0.43, 0.57 (EtOAc). ^{1}H NMR: 11.44 (s, 1H, H-3), 7.70 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 7.47–7.19 (m, 10H, ArH, OCONH), 6.88 (m, 4H, ArH), 5.90 (m, 1H, H-1′), 5.43 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-5), 5.30 (m, 1H, H-2′), 4.55 (m, 1H, H-3′), 4.10 (m, 3H, H-4′, CH_2OCO), 3.72 (s, 6H, OCH_3), 3.62–3.01 (m, 12H$^{\#}$, $POCH_2$, CHN, H-5′, $NCH_2CH_2OCH_2$), 2.74, 2.57 (2m, 2H, CH_2CN), 1.23–0.90 (m, 21H, $CHCH_3$, Bu^t). ^{31}P NMR (CD_3CN): 149.5, 149.4, 149.3 (diastereomers and rotamers).*

($^{\#}$Calculated value; the signal of water is also present in the region).

SYNTHESIS, ISOLATION, AND CHARACTERIZATION OF OLIGONUCLEOTIDES CONTAINING URIDINE 2′-CARBAMATES

3′-Phosphoramidites of 5′-dimethoxytritylated uridine 2′-carbamates are stable during prolonged storage at –20°C. The compounds are less reactive compared to standard 2′-deoxyribonucleoside phosphoramidites due to the steric bulk of the 2′-carbamate group. Therefore, the coupling time should be increased to 10 to 15 min to achieve a 97% to 98% yield, which is acceptable for introduction of one or a few modifications into an oligonucleotide chain. In all cases, a 2′-carbamate function is completely stable to the conditions of oligonucleotide synthesis as well as to final deprotection with concentrated aqueous ammonia (55°C, 8 to 16 hr). In general, an increased coupling time is the only change in oligonucleotide synthesis and isolation that is necessary for preparation of uridine-2′-carbamate-containing oligonucleotides. However, there are two important details that should be noted. First, the 2′-trimethylacetate ester obtained from phosphoramidite **S.7l** is unexpectedly stable to ammonia treatment. Only after 96 hr of standard deprotection does the MALDI-TOF spectrum show complete ester hydrolysis in a doubly modified oligo-2′-deoxyribonucleotide. Second, the phosphoramidite **S.7f** is sparingly soluble in acetonitrile and *N*,*N*-dimethylformamide and is highly prone to gel formation, which makes it difficult to use for standard oligonucleotide synthesis. All the other phosphoramidites can be used as 0.1 M solutions in anhydrous acetonitrile.

Oligonucleotide synthesis can be carried out on any automated DNA/RNA synthesizer. The authors have used Applied Biosystems 380B and 394 on both a 0.2- and 1-µmol scale using 2′-deoxyribonucleoside and 2′-*O*-methyl-ribonucleoside phosphoramidites (Transgenomics), as well as 2′-TOM-ribonucleoside phosphoramidites (Glen Research; also see *UNITS 2.9 & 3.8*). A number of techniques may be used for isolation and purification of oligonucleotides with uridine 2′-carbamate modifications, such as reversed-phase or ion-exchange HPLC, on cartridges (e.g., PolyPak, Glen Research), or by denaturing PAGE. A standard vertical gel electrophoresis apparatus is suitable for PAGE of modified oligonucleotides. The molecular mass of the oligonucleotides was checked by MALDI-TOF mass spectrometry on an Applied Biosystems Voyager DE workstation in positive ion mode. Thermal denaturation experiments with oligonucleotide duplexes were performed on a Perkin Elmer Lambda 40 UV/Vis spectrophotometer with Peltier temperature programmer in the hybridization buffer. Fluorescence spectra were recorded using a Perkin Elmer LS 50B Luminescence Spectrometer in the same buffer. For evaporation of small volumes, a Speedvac evaporator is ideal.

Materials

Uridine 2′-carbamate phosphoramidite(s) (**S.7a-j,l**; see Basic Protocol 1 and Alternate Protocol)
Acetonitrile, anhydrous
Phosphoramidites:
  2′-Deoxyribonucleoside phosphoramidites (Transgenomics)
  2′-*O*-Methyl-ribonucleoside phosphoramidites (2′-OMe; Transgenomics)
  2′-*O*-[(Triisopropylsilyl)oxy]methyl-ribonucleoside phosphoramidites (2′-*O*-TOM; Glen Research; also see *UNITS 2.9 & 3.8*)
30% ammonia
Nitrogen
Mobile phase A: 5% CH_3CN in 0.1 M triethylammonium acetate (TEAA), pH 7.0 (DMTr-ON, HPLC)
Mobile phase B: 100% CH_3CN (DMTr-ON, HPLC)
1 to 400 mM sodium perchlorate in 20 mM Tris·Cl (pH 6.8; *APPENDIX 2A*)/25% formamide (DMTr-OFF, HPLC)

15% (w/v) polyacrylamide gel (*APPENDIX 3D*) containing 2 M urea in 0.5× TBE electrophoresis buffer (*APPENDIX 2A*) (DMTr-OFF, PAGE)
0.5 M $LiClO_4$ (DMTr-OFF, PAGE)
Matrix solution I: 40 mg/mL 2,6-dihydroxyacetophenone in methanol
Matrix solution II: 80 mg/mL diammonium hydrogen citrate in water

Water aspirator
Screw-capped tube (Sarstedt) or vial
Speedvac evaporator (Savant)
Spin-X tube (Costar)
Reversed-phase cartridges for DNA isolation (e.g., PolyPak, Glen Research; DMTr-ON)
HPLC system (optional) with:
Column: 3.9 × 300–mm Phenomenex Bondclone 10 C18 column (DMTr-ON) *or* 9 × 250–mm Dionex NucleoPac PA-100 column (DMTr-OFF)
Detector: 254 nm (DMTr-ON) or 280 nm (DMTr-OFF)
Lyophilizer
Microcon tube (Millipore) or NAP-10 column (DMTr-OFF, PAGE)

Additional reagents and equipment for automated solid-phase oligonucleotide synthesis (*APPENDIX 3C*) and purification of oligonucleotides (*UNITS 10.1, 10.4, 10.5, 10.7 & APPENDIX 3B*)

1. Dissolve the appropriate uridine 2′-carbamate phosphoramidite(s) in anhydrous CH_3CN at up to 1.0 M.
2. Start the automated solid-phase oligonucleotide synthesis (*APPENDIX 3C*) from an appropriate solid support–filled column (e.g., on a 0.2- or 1-µmol scale).
3. Elongate the desired oligonucleotide chain using modified 2′-carbamate phosphoramidites (**S.7a-j** and **S.7l**) and standard 2′-deoxy-, 2′-OMe-, or 2′-*O*-TOM-ribonucleoside phosphoramidites in either DMTr-ON or -OFF mode. Allow at least 10 to 15 min for each 2′-carbamate phosphoramidite coupling.
4. After completion of the assembly, remove the column, wash with CH_3CN (e.g., two times with 10 mL for 1-µmol synthesis), and quickly dry using a water aspirator.
5. Transfer the support into a screw-capped tube or vial and add 1 mL of 30% ammonia.

6a. *For oligonucleotides prepared from phosphoramidites **S.7a-j**:* Vortex the mixture and incubate at least 8 hr at 55°C or 24 hr at ambient temperature.

6b. *For oligonucleotides prepared from phosphoramidite **S.7l**:* Vortex the mixture and incubate at least 48 hr at 55°C per modified residue to ensure complete trimethylacetate ester deprotection.

7. Allow the solution to cool to ambient temperature, evaporate most of the ammonia under a stream of nitrogen, and dry the rest in vacuo using a Speedvac evaporator.

   For DMTr-ON oligonucleotides, it may be necessary to add 5 µL TEA to completely preserve the DMTr group.

8. Add 0.5 mL deionized water, transfer the solution to the upper part of a Spin-X tube, and microcentrifuge 5 min at 13,000 rpm.
9. Add 0.25 mL deionized water to the upper part of the tube, centrifuge again to wash, and then repeat. Concentrate the combined filtrates (sample and two washes) in vacuo.

10a. *For DMTr-ON oligonucleotides:* Purify on a reversed-phase cartridge following manufacturer's recommendations (also see *UNIT 10.7*) or by RP-HPLC (*UNIT 10.5*) using the following recommended conditions:

3.9 × 300–mm Phenomenex Bondclone 10 C18 column
Buffer A: 5% CH_3CN in 0.1 M TEAA, pH 7.0
Buffer B: 100% CH_3CN
Gradient: 0% to 60% buffer B in 45 min
Flow rate: 1 mL/min
Detection: 254 nm.

Pool the appropriate fractions, re-evaporate twice with water, and lyophilize.

10b. *For HPLC of DMTr-OFF oligonucleotides:* Purify by ion-exchange HPLC (*UNIT 10.5*) using the following recommended conditions:

9 × 250–mm Dionex NucleoPac PA-100 column
Gradient: 1 to 400 mM sodium perchlorate in 20 mM Tris·Cl (pH 6.8)/25% formamide
Flow rate: 1 mL/min
Detection: 280 nm.

Pool the appropriate fractions, desalt on a NAP-10 column, and lyophilize.

10c. *For PAGE of DMTr-OFF oligonucleotides:* Use denaturing gel electrophoresis (*UNIT 10.4 & APPENDIX 3B*) in 15% polyacrylamide containing 2 M urea in 0.5× TBE electrophoresis buffer. Cut the band(s) and elute the product with 0.5 M $LiClO_4$. Desalt using a Microcon tube or a NAP-10 column, and lyophilize.

11. Dissolve the oligonucleotide in deionized water and quantitate by measuring UV absorbance at 260 nm. Store the solution up to 2 years frozen (i.e., –20°C).

12. Check the molecular mass of the oligonucleotides by MALDI-TOF-MS (*UNIT 10.1*) using a 1:1 (v/v) mixture of matrix solutions I and II. Prepare matrix just before loading the samples onto a plate.

For a discussion of hybridization (duplex stability) studies and fluorescence measurements using 2′-carbamate-modified oligonucleotides, see Background Information. The optimal hybridization buffer for these experiments is 10 mM sodium phosphate, pH 7.0 (APPENDIX 2A) containing 0.1 mM EDTA and 100 mM NaCl.

See Figure 4.21.4 for sample spectra and Table 4.21.1 for melting temperatures in a duplex stability study.

COMMENTARY

Background Information

Application of nucleoside carbamates

The studies that are described here were originally driven by a desire to develop a convenient method for the introduction of various ligands into ribozymes. Use of ligands such as polyamines and amino acids together with the hairpin ribozyme was investigated as a possible way to enhance the ribozyme's RNA cleavage potential under low metal ion concentration (Stolze et al., 2001). Since nucleobases in the core region are often involved in noncovalent interactions important for maintaining structure and function, the sugar moiety of nucleosides, particularly the 2′-hydroxyl groups, seem to be attractive for ligand attachment, especially because 2′-functionalization already has proven value in various applications (Zatsepin et al., 2002).

There are numerous examples of 2′-*O*-alkylnucleoside derivatives and their introduction into oligonucleotides. An irritating shortcoming is a sometimes poor yield of 2′-*O*-alkylation. An interesting opportunity for 2′-functionalization is via a carbamate function, easily

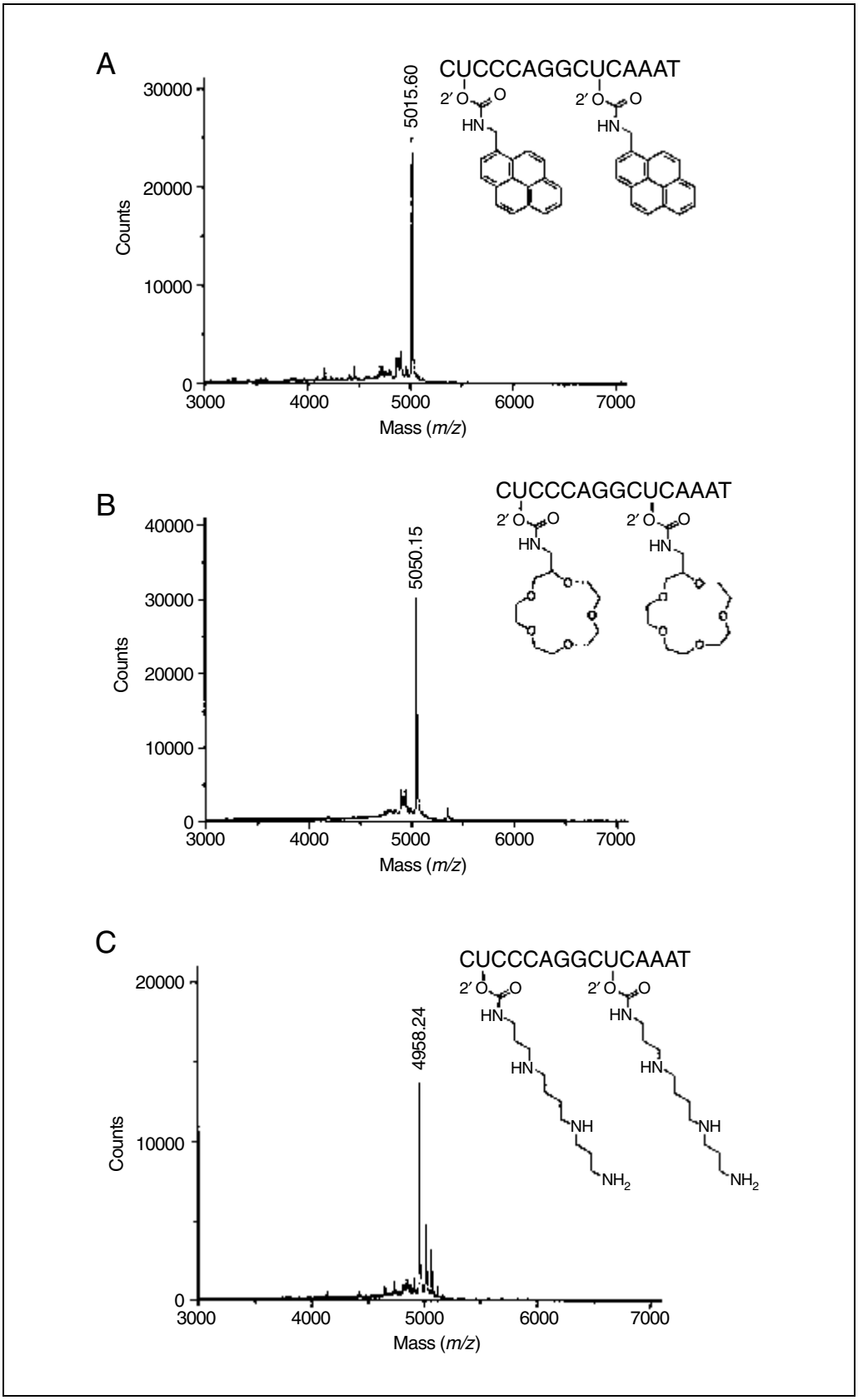


Figure 4.21.4 MALDI-TOF mass spectra of 2′-carbamate oligodeoxyribonucleotides containing two modified uridines CU*CCCAGGCU*CAAAT, where U* is from phosphoramidites **S.7d** (**A**), **S.7e** (**B**), and **S.7h** (**C**) (see Figures 4.21.1 and 4.21.2 and Table 4.21.1).

Table 4.21.1 Melting Temperatures of Duplexes of 2′-Carbamate Oligonucleotides with Complementary DNA and RNA

Sequence, 5′ to 3′	**U*** = U-2′-OCONHCH$_2$**R** where **R** =	DNA		RNA	
		T_m (°C)	ΔT_m/mod (°C)	T_m (°C)	ΔT_m/mod (°C)
CTCCCAGGC**U***C-AAAT	Control, **U*** = 2′-deoxyuridine	56.4	—	59.1	—
	C≡CH	50.0	–6.4	54.5	–4.7
	4-Iodophenyl	49.9	–6.5	54.7	–4.5
	1-Pyrenyl	54.7	–1.7	57.8	–1.4
	1,4,7,10,13-Pentaoxacyclo-pentadecan-2-yl	49.8	–6.6	53.9	–5.3
	$CH_2OCH_2CH_2OH$	50.1	–6.3	53.7	–5.5
	$(CH_2)_2[O(CH_2)_2]_2O(CH_2)_3NH_2$	49.8	–6.6	53.9	–5.3
	$(CH_2)_2NH(CH_2)_3NH_2$	51.5	–4.9	53.4	–5.8
	$(CH_2)_2NH(CH_2)_4NH(CH_2)_3NH_2$	51.4	–5.0	55.1	–4.0
C**U***CCCAGGCTC-AAAT	Control, **U*** = 2′-deoxyuridine	56.2	—	58.8	—
	4-Iodophenyl	53.4	–2.8	54.4	–4.4
	1-Pyrenyl	57.5	+1.3	55.5	–3.3
	1,4,7,10,13-Pentaoxacyclo-pentadecan-2-yl	53.5	–2.8	54.8	–4.0
	$CH_2OCH_2CH_2OH$	52.8	–3.4	56.2	–2.6
	$(CH_2)_2[O(CH_2)_2]_2O(CH_2)_3NH_2$	52.6	–3.6	55.5	–3.3
	$(CH_2)_2NH(CH_2)_3NH_2$	53.1	–3.1	55.9	–2.9
	$(CH_2)_2NH(CH_2)_4NH(CH_2)_3NH_2$	54.1	–2.3	59.0	+0.2

generated from primary or secondary alcohols by successive treatment with 1,1′-carbonyldiimidazole (CDI) and an aliphatic amine. This chemistry has been used successfully for 5′-modification of oligonucleotides (Wachter et al., 1986). Aliphatic 5′-carbamates are known to be stable to ammonia deprotection at 55°C, in contrast to aromatic nucleoside 3′-carbamates (Sproat and Brown, 1983).

Oligonucleotides containing nucleoside 2′-carbamates have been reported previously. Cytidine 2′-carbamate was obtained by CDI activation followed by amine treatment (Dubey et al., 2000). In general, 2′-carbamate modifications are detrimental to the stability of DNA-RNA duplexes (Freier and Altmann, 1997; Prhavc et al., 2001). Recently we have described the preparation of a number of uridine 2′-carbamates and their introduction into oligo-2′-deoxy- and 2′-OMe-ribonucleotides, and studied their hybridization with complementary DNA and RNA. It was found that 2′-pyrene carbamate-modified oligonucleotides show interesting binding properties and remarkable DNA mismatch affinity (Korshun et al., 2002), and exhibit enhanced fluorescence when bound to complementary RNA, but not DNA (see Fig. 4.21.5).

Synthesis of uridine 2′-carbamates and their phosphoramidites

The authors developed a general approach for the preparation of uridine 2′-carbamates using 1,1′-carbonyldiimidazole (CDI) activation of the 2′-hydroxy group of 3′,5′-Markiewicz-protected uridine (**S.2**; see Basic Protocol 1). The reaction with CDI in dry dichloromethane gives the corresponding activated imidazolide in nearly quantitative yield. Dichloromethane proved to be the best solvent for the transformation, whereas use of the more polar tetrahydrofuran and acetonitrile led to a much longer reaction time. Other investigators have described a successful preparation of 2′-carbamates using disuccinimidyl carbonate (DSC) and triethylamine in acetonitrile (Prhavc et al., 2001). In the authors' hands, however,

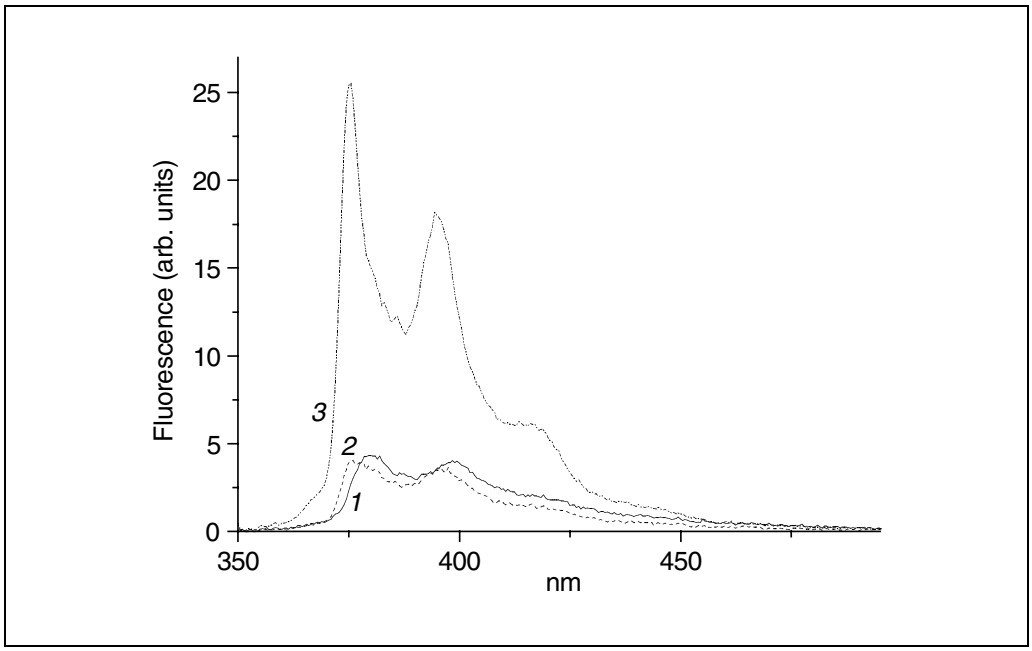


Figure 4.21.5 Fluorescence spectra of 2′-pyrene carbamate oligodeoxyribonucleotide CTCCCAGGCU*CAAAT (1) and its duplex with complementary DNA (2) and RNA (3) (see Table 4.21.1).

DSC gave poorer results, requiring a higher excess of reagent and longer reaction times, while giving lower conversion yields. An additional advantage of CDI is that in many cases the imidazolide **S.3** need not be isolated, but can be reacted in situ with an excess of amine.

The corresponding Markiewicz-protected 2′-carbamates (**S.4**) are stable compounds that can be easily purified by column chromatography. A facile technique was developed to protect side-chain amino groups of di- and polyamine 2′-carbamate derivatives, which involved trifluoroacetylation without isolation of the unprotected intermediate (see Alternate Protocol).

Initial attempts to remove the Markiewicz group from 2′-carbamates by tetrabutylammonium fluoride trihydrate (TBAF) in THF showed smooth silyl deprotection, but also the appearance of a by-product with similar mobility. After several hours of treatment, an equilibrium was reached where an ~1:1 ratio of the two products was obtained. The side-product proved to be the corresponding 3′-carbamate, generated by carbamoyl migration in the 1,2-diol system (Korshun et al., 2002). The possibility of carbamoyl migration has been discussed in a similar case, but the 3′-carbamate itself was neither detected nor isolated (Seio et al., 1998). Interestingly, those authors used a safer reagent by buffering TBAF solution with acetic acid. In other cases, 0.1 M TBAF in THF was used (Dubey et al., 2000; Prhavc et al., 2001). In our hands, however, TBAF in THF *always* gave a mixture of regioisomers. However, using the alternative desilylating reagent triethylamine trihydrofluoride, only pure 2′-carbamate product **S.5** was obtained, even after overnight treatment.

The following step of 5′-dimethoxytritylation required some modification. Although care was taken to get rid of most of the fluoride by repeated toluene/hexane washings, some of the latter apparently remained in the crude mixture, which thus consumed additional amounts of 4,4′-dimethoxytrityl chloride (DMTr·Cl; up to 3 eq). In one case (**S.6e**), this led to an increased formation of 3′,5′-ditritylated product and lower yield of the target compound. A new work-up procedure was recently adopted to obviate the need for higher excess of DMTr·Cl. It uses an excess of inexpensive ethoxytrimethylsilane to scavenge the remaining fluoride, with corresponding formation of only volatile products (Stetsenko, pers. comm.). Use of pyridine (0.5% v/v) instead of TEA as an additive for column chromatography of 5′-DMTr-protected 2′-carbamates is strongly advised to prevent 2′,3′-carbamate migration on silica gel. The final 3′-phosphinylation went smoothly and uneventfully according to the published procedure (Caruthers et al., 1987).

Synthesis of oligonucleotides containing uridine 2′-carbamate residues

Automated solid-phase oligonucleotide synthesis was accomplished using a manufacturer's protocol that has been modified only by increasing the coupling time of the 2′-carbamate phosphoramidites to 10 to 15 min (see Basic Protocol 2). Uridine-2′-carbamate-modified oligonucleotides were routinely synthesized in DMTr-ON mode and isolated on a reversed-phase cartridge (PolyPak) following the supplier's standard technique. The results were consistently good as judged from the MALDI-TOF mass spectra of doubly modified oligomers (see Fig. 4.21.4).

Effect of 2′-carbamate modifications on duplex stability

Melting studies with uridine-2′-carbamate-modified oligonucleotides (see Table 4.21.1) confirmed the overall destabilizing effect of the 2′-carbamate group in both the DNA-DNA and DNA-RNA series. Most carbamate modifications result in a considerable decrease in T_m values of the duplexes, but there was almost no correlation between the steric bulk of the carbamate group and the T_m of the corresponding duplexes. The least destabilizing is pyrene carbamate, especially in the case of DNA-DNA duplexes. As expected, a single modification close to the 5′-terminal position of the oligonucleotide caused less destabilization than one in the middle. A number of studies of pyrene attached to the 2′-position of individual nucleosides within duplexes have been published recently (Silverman and Cech, 1999; Yamana et al., 2001). Pyrene as well as other planar polyaromatic hydrocarbons often show a greater stabilizing effect in DNA-DNA duplexes than in DNA-RNA duplexes (Yamana et al., 2001). The pyrene 2′-carbamate modification is not an exception, giving a clear stabilizing effect of pyrene superimposed on the destabilizing influence of the 2′-carbamate group, while even the spermine carbamate, which should carry three positive charges under physiological pH, is only negligibly stabilizing in just one most favorable situation.

That pyrene is more stabilizing in DNA-DNA duplexes than DNA-RNA duplexes might suggest the better fit of pyrene into the shallower DNA-DNA minor groove. This notion is strengthened by interesting fluorescent properties of pyrene 2′-carbamate oligonucleotides. The total fluorescence intensity upon duplex formation of single pyrene-labeled oligomers with a complementary DNA does not change very much (see Fig. 4.21.5). By contrast, the binding to a complementary RNA gives a 5- to 30-fold increase in fluorescence for single-pyrene oligonucleotides and a 30-fold increase for double-pyrene oligonucleotides. This gives evidence that the excited pyrene 2′-carbamate is effectively quenched in single-stranded oligodeoxyribonucleotides and in DNA-DNA duplexes, but not in DNA-RNA duplexes. Similar examples of fluorescence increases upon hybridization with a complementary RNA are demonstrated for probes containing 2′-attached pyrene (Yamana et al., 2001).

Thus, uridine 2′-carbamate modification in a DNA strand is usually destabilizing for both DNA-DNA and DNA-RNA duplexes, and therefore might be less suitable for those cases where higher stability of a duplex is preferential (e.g., antisense applications). However, the 2′-carbamate modification affords a convenient way to place various functional groups into the minor groove of a DNA-DNA duplex (e.g., crown ether for metal ion binding, or an aliphatic amino group for postsynthetic labeling with activated derivatives of fluorescent dyes). Other possible applications include the specific minor groove delivery of reactive functionalities (e.g., for site-directed protein cross-linking or conjugation with peptides or other biomolecules), as well as modifications of flexible parts of highly structured RNA molecules for studies of ribozyme catalysis or RNA folding.

Critical Parameters and Troubleshooting

The synthesis of 2′-carbamate compounds (see Basic Protocol 1 and Alternate Protocol) is relatively short, straightforward, and fairly efficient. However, careful attention to details of basic organic synthesis procedures is required. Preparation of the various compounds requires prior experience with routine chemical laboratory techniques such as solvent evaporation, extraction, TLC, and column chromatography. HPLC and gel electrophoresis are the most widely used methods of modified oligonucleotide isolation. Characterization of the products demands knowledge of ^{1}H and ^{31}P NMR, UV and MALDI-TOF mass spectroscopy. General laboratory safety is also of primary concern when hazardous materials are involved. Strict adherence to the outlined methods is therefore highly recommended.

Anticipated Results

Good to moderate yields of the final uridine 2′-carbamate 3′-phosphoramidites (**S.7**) from the parent Markiewicz-protected nucleoside (**S.2**) are expected following these procedures (see Basic Protocol 1 and Alternate Protocol). Analogously, others have reported good yields of 2′-carbamate nucleosides obtained by both similar (Dubey et al., 2000) and different chemistries (Prhavc et al., 2001). Basic Protocol 2 is intended to make use of the 2′-carbamate phosphoramidites to synthesize, purify, and characterize 2′-carbamate-containing oligonucleotides.

Time Considerations

The synthesis of the 2′-carbamate 3′-phosphoramidites starting from Markiewicz-protected uridine (**S.2**) can be accomplished in 2 to 3 weeks per amine derivative, depending on the nature of the side chain and whether an additional protecting group is needed. The authors suggest, therefore, the preparation of a large batch of **S.2** (from ~30 mmol upwards) to use in portions as needed. The time for oligonucleotide synthesis varies from standard phosphoramidite methods only by the increased 2′-carbamate coupling time of 10 to 15 min per residue. The times needed for deprotection, isolation, purification, and analysis of modified oligonucleotides are the same as with standard techniques.

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Contributed by Vladimir A. Korshun
Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry
Moscow, Russia

Dmitry A. Stetsenko and Michael J. Gait
Medical Research Council
Laboratory of Molecular Biology
Cambridge, United Kingdom