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2′-*C*-Methyluridine phosphoramidite: a new building block for the preparation of RNA analogues carrying the 2′-hydroxyl group

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Abstract—A new stereoselective synthesis of 2′-*C*-methyluridine was carried out. The preparation of the corresponding protected phosphoramidite suitable for the automated synthesis of oligonucleotides, including the regioselective protection of the 2′-hydroxyl group, is described here. This new modified nucleotide is expected to generate RNA analogues potentially useful in applications where proper RNA folding is required since the 2′-hydroxyl is conserved. © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

The improvements achieved in solid phase synthesis of oligonucleotides have enabled the development of new research areas in molecular and structural biology as well as the search of new therapeutic principles. Progress concerning RNA synthesis has been delayed due to problems related to the selective protection of the 2′-hydroxyl group, slower rate of coupling and the inherent fragility of RNA to hydrolytic and enzymatic degradation. However, recent advances[1,2] have allowed the preparation of proper quantities of RNA suitable for structural studies, the search of new aptamers and the generation and evaluation of synthetic ribozymes.

Since oligoribonucleotides are particularly prone to the attack of nucleases present both in serum and inside cells, the development of chemical modifications is essential for their biological applications. 2′-*O*-Alkyloligonucleotides[3,4] and other RNA mimics tried so far, such as 2′-amine[5] and 2′-fluoroligonucleotides,[6] lack the 2′-hydroxyl group, which has shown to be essential for RNA folding and, consequently, fundamental for the development of modified ribozymes, ribozyme inhibitors and RNA aptamers. In the particular case of ribozymes, the presence of the 2′-hydroxyl group in defined positions has proved to be indispensable for maintaining the activity. This requirement has been analysed through specific replacement of RNA moieties by either deoxynucleotides[7] or modified nucleotides without this group.[8,9] Such findings have been supported by X-ray diffraction analysis which evidenced that 2′-hydroxyls are involved in hydrogen bondings responsible for the stabilization of tertiary structures present in the catalytic core.[10,11]

Taking into account these facts, we propose a sugar modified analogue, namely 2′-*C*-methylnucleotide, which is expected to provide when substituting a particular ribonucleotide, a 2′-hydroxyl group able to make similar interactions than those displayed by the natural moiety. Previous preparations[12,13] of 2′-*C*-methyluridine involved the synthesis of the modified sugar and its subsequent coupling to the nucleobase which is often a lengthy and/or low yielding procedure. Another strategy dealt[14] with the addition of organometallic compounds to 2′-ketonucleosides but, since the reaction proceeded from the α-face of the sugar moiety, the major product obtained through this route was the 2′-*C*-methylarabinonucleoside. To overcome these limitations, we report in this paper the stereoselective preparation of 2′-*C*-methyluridine, the subsequent protection of the sterically hindered 2′-hydroxyl group and the final generation of the corresponding phosphoramidite suitable for solid phase synthesis.

The configuration at C-2′ of this nucleoside was established using NMR measurements. It is known that the configuration determined by NMR, is always highly correlated to the conformation,[15] and both issues must be resolved simultaneously. In the present case, the lack of a hydrogen attached to C-2′ reduced the available $^{1}H-^{1}H$ coupling constants to just one (J(H3′H4′)), and therefore we decided to measure also the $^{3}J(^{1}H-^{13}C)$ coupling constants. To this

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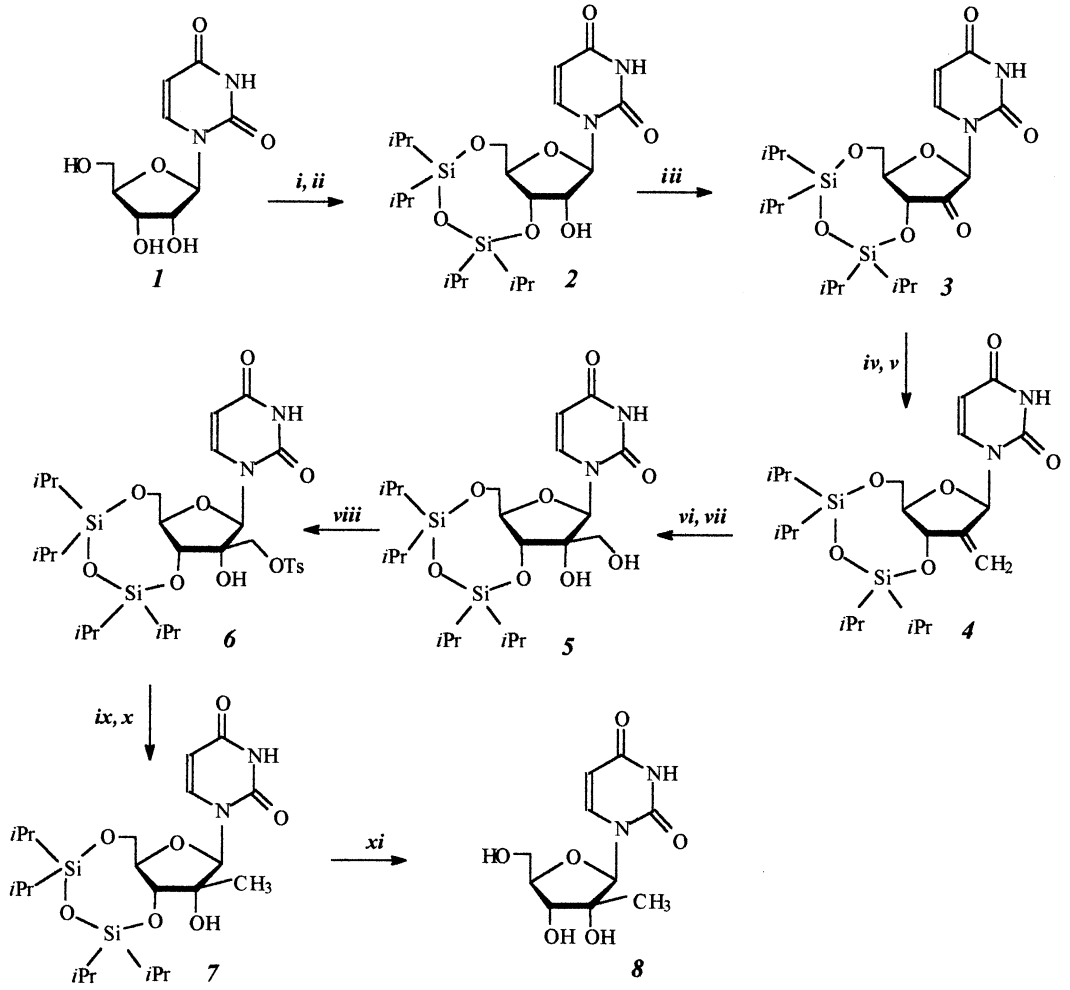


Scheme 1. (i) $TIPDSCl_2$, Py; (ii) MeOH; (iii) PCC, Ac_2O, CH_2Cl_2; (iv) $MePh_3PBr$, THF, BuLi; (v) Et_2O, NH_4Cl; (vi) OsO_4, *N*-methylmorpholine-*N*-oxide, *t*-BuOH, THF; (vii) $NaHSO_3$ 1 M, EtOAc; (viii) TsCl, Py; (ix) $NaBH_4$, DMF; (x) NH_4Cl; (xi) TBAF, THF.

purpose we used a newly developed pulse sequence,[16] which shows high sensitivity and is especially well suited for these measurements in natural abundance.

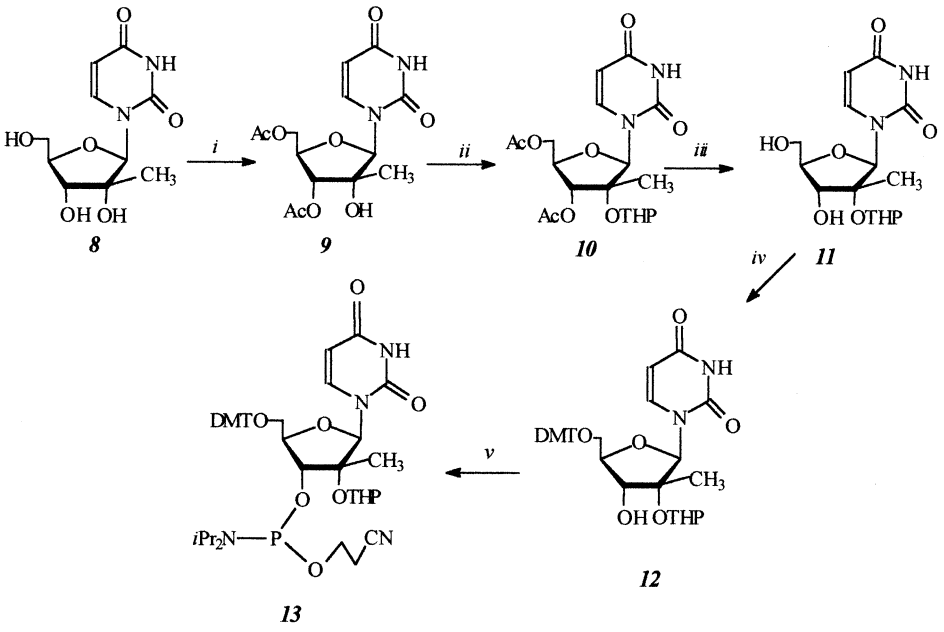
2. Results and discussion

Scheme 1 shows the synthetic route followed for the preparation of 2′-*C*-methyluridine (**8**). Starting from uridine the regioselective protection of the 3′ and 5′ hydroxyls was carried out by using 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane.[17] Intermediate **3** could be obtained from **2** by using either chromium trioxide/pyridine/acetic anhydride[18] or dimethyl sulfoxide/oxalyl chloride[19] affording similar yields (80%). Wittig reaction of **3** following standard procedures[20] gave the corresponding 2′-methylene derivative **4** (66%), which after catalytic oxidation with osmium tetroxide[21] produced the corresponding diol as a diastereomeric mixture (97:3 as determined by NMR). At this stage, the configuration at C-2′ of the major diasteromer was tentatively assigned as in compound **5**, based on the fact that the attack by osmium tetroxide is more likely to occur from the less hindered α-face of the sugar ring. This hypothesis was confirmed on a later step of the synthesis (see below). Subsequent treatment with an excess of *p*-toluenesulfonyl chloride yielded the primary tosylated derivative which could be resolved by column chromatography obtaining pure **6** (60%). Finally, reaction of **6** with sodium borohydride[22] gave the disiloxane bridged 2′-*C*-methyluridine (**7**).

Table 1. Selected three-bond coupling constants (in Hz) of 2′-*C* methyluridine (**8**) in D_2O

	C1′	C2′	C3′	C4′	CH_3	H4′
H1′	–	–	3.8	1.0	<0.5	–
H3′	–	–	–	–	0.5	9.5
H4′	<0.5	<0.5	–	–	–	–

Compound **7** was desilylated with tetrabutyl ammonium fluoride, to yield **8**, which was used to perform the NMR measurements in order to assign unambiguously the configuration at C-2′. For this purpose we measured all the three-bond coupling constants that are sensitive to both configuration at C-2′ and the conformation of the molecule. This includes the coupling constant between H-3′ and H-4′, and a number of heteronuclear $^1H-^{13}C$ coupling constants. Table 1 shows the coupling constants measured for compound **8** in D_2O. The relatively high values measured for J(H3′H4′) (9.5 Hz) and for J(H1′C3′) (3.8 Hz), as well



Scheme 2. (i) Ac_2O, Py; (ii) DHP, *p*-TsOH, dioxane; (iii) NH_3, MeOH; (iv) DMTrCl, DMAP, TEA, Py; (v) iPr_2NEt, CH_2Cl_2, $[(CH_3)_2CH]_2NP(Cl)OCH_2CH_2CN$.

as the small value for $J(H4'C2')$ (<0.5 Hz), are all clear indications that **8** exists in solution mainly in the C-3′ *endo* conformation. The small value obtained for $J(H3'CH_3)$ (0.5 Hz) is only compatible with a *cis* orientation of the CH_3 group respect to H-3′, since a relatively high value (~4 Hz) would be expected in the case of these groups being *trans* diaxial, and thus establishing the configuration at C-2′.

2D NOESY experiments on nucleoside **8** also corroborate these results. In fact, a strong NOE was observed between H-6 and H-3′, indicating both a C-3′ *endo* conformation for the sugar and an *anti* orientation for the base. The NOE of the CH_3 group is stronger with the H-3′ than with the H-1′, a clear indication of a *cis* relationship between the 2′-*C*-methyl group and H-3′.

The C-3′ *endo* conformation, or RNA-like conformation, adopted by compound **8** confirms the fact that positioning the 2′-methyl group in a pseudo-equatorial orientation is the main driving force in defining the preferred conformation of this type of nucleosides,[23] as it was already demonstrated for 2′-*C*-alkyl-2′-deoxy analogues.[24] Additionally, compound **8** exhibits the most favourable spatial arrangement of the different substituents of the sugar ring: the bulky CH_3 group is positioned pseudo-equatorially, the 2′-OH group shows a positive *gauche* effect with the O-4′, the C-5′ side chain is pseudo-equatorial, and the base is pseudo-axial, satisfying the weak anomeric effect.

The next step in the route towards a suitable derivative for oligonucleotide solid phase synthesis involved the protection of the 2′-hydroxyl of **7**. This protecting group must fulfil general requirements such as stability to trichloroacetic acid treatment and towards oxidation conditions (iodine/water/pyridine) and quantitative removal at the end of the oligonucleotide synthesis. In order to derivatize regioselectively the tertiary 2′-hydroxyl group, 3′- and 5′-hydroxyls must be previously capped and therefore the 2′-protecting group of choice should be stable under the required conditions for deblocking the 3′ and 5′ positions. *tert*-Butyldimethylsilyl ether is the protecting group used in routine RNA synthesis but attempts to obtain the corresponding derivative of **7** using either standard (*tert*-butyldimethylsilyl chloride/triethylamine) or special conditions for steric hindered alcohols (*tert*-butyldimethylsilyl triflate/crown ether/potassium hydride)[25] were unsuccessful. In order to assess the influence of the bulky 3′,5′-disiloxane bridge, the same reaction was carried out on the crude 3′,5′-di-*O*-acetyl derivative **9** (Scheme 2). This compound was prepared by acetylation with acetic anhydride/pyridine (70% from **7**) of the free nucleoside **8**. Surprisingly, 2 equiv. of the acylating reagent had to be used in order to avoid peracetylation. 2′-*tert*-Butyldimethylsilylation of **9** did not proceed even after long reaction times (5 days) and using 3.5 equiv. of silylating agent.

Another suitable 2′-protecting group, due to its chemical properties and size, is tetrahydropyranyl ether. Reaction of **7** with 3,4-dihydro-2*H*-pyran/*p*-toluenesulphonic acid monoydrate[26] did not afford the expected ether even when a large excess of reagent and higher temperatures were employed. Finally, the desired product **10** was obtained when the diacetylated compound **9** was used as the starting material (80%). This reaction did not go to completion and degradation of the starting material was visible by TLC. Therefore, molar ratio of reagents and reaction time were optimised and the remaining compound **9** could be recycled after isolation by column chromatography. Expected compound **11** was obtained by treating **10** with methanolic ammonia (100%). In order to assess the removal of the THP group during the deprotection conditions used in RNA synthesis, compound **11** was treated with 0.01N HCl (pH 2.0) at rt. As expected, the behaviour of compound **11** is similar to that of the 2′-THP-protected uridine and the observed $t_{1/2}$ is around 30 min, monitored by TLC.

Finally, the appropriate building block **13** for solid phase synthesis was prepared following standard procedures.

We expect this building block to provide a 2′-hydroxyl group able of mimicking the interactions observed in natural RNA structures. This assumption is based on our NMR analysis which revealed that the preferred conformation adopted by this nucleoside (C-3′ *endo*) is similar to that found in RNA, suggesting that both structures position the 2′-hydroxyl group in an equivalent orientation.

The generation of resistant oligonucleotides carrying 2′-hydroxyl groups can be a challenging task in the field of ribozymes,[27,28] since the presence of 2′-hydroxyl groups in specific positions has been shown to be essential for the activity of these molecules.[7–9] Alternatively, these modified nucleic acids fragments can be regarded as potential therapeutic agents based on short, resistant and high affinity inhibitors of ribozymes.[29,30] In this case, tertiary interactions between ribozymes and 2′-hydroxyl groups of the inhibitor are involved in the mechanism of inhibition. Combinatorial technologies is a new field in which oligonucleotides have proved their utility. RNA molecules have demonstrated to have strong affinity and specificity towards proteins and other targets.[31,32] Again, chemical modifications which confer enzymatic stability and provide the appropriate folding are essential for the development of RNA-based aptamers in therapeutics and diagnostics.

At present, the synthesis of chimeric oligonucleotides containing DNA, RNA or modified nucleotides and the modification here reported is being undertaken. Deprotection and purification conditions are also being evaluated. Further biochemical (activity of chemically modified ribozymes) and structural analysis (NMR) will be carried out in order to verify the hypothesis that 2′-*C*-methylnucleotides mimic the interactions occurring in natural RNA structures.

3. Experimental

3.1. General methods

Ethyl acetate (EtOAc), tetrahydrofuran (THF), methanol, petroleum ether, triethylamine (TEA), pyridine (Py), dimethylformamide (DMF), *t*-butanol (*t*-BuOH), diethyl ether (Et_2O) and HPLC grade methylene chloride (CH_2Cl_2), were supplied by J. T. Baker. THF was dried by refluxing over sodium metal using benzophenone as indicator of dryness and then distilled at atmospheric pressure. DMF was dried by heating at 100°C with calcium hydride and then distilled under reduced pressure. Pyridine, dioxane and triethylamine were dried by heating, under reflux, with calcium hydride and distilled at atmospheric pressure.

All other reagents were commercially available (Fluka, Aldrich) and uridine was of the best analytical grade (Pharma Waldhof). All moisture-sensitive reagents were transferred via a syringe under positive nitrogen pressure or argon atmosphere, moisture-sensitive reactions were carried out under positive pressure of nitrogen gas.

Column chromatography was performed by using Silica gel 20–45, 6–35 μm (Amicon) and Silica Gel 60F 230–400 mesh (Merck). Analytical thin layer chromatography (TLC) was conducted on precoated Merck Silica gel $60F_{254}$. Components were located by observation of the plates under UV light, and dimethoxytrityl products were detected as orange spots by treating the plates with H_2SO_4/EtOH 8:2 and heating.

^{13}C-, ^{1}H- and ^{31}P NMR spectra were recorded in Cl_3CD or DMSO-d_6 (Aldrich), on a 200 and 500 MHz AM Bruker equipment using tetramethylsilane (0.00 ppm, ^{1}H NMR) and Cl_3CD (76.95 ppm, ^{13}C NMR) as internal standards and H_3PO_4 (0.00 ppm, ^{31}P NMR) as external standard. Chemical shifts (δ, ppm) quoted in the case of multiplets are measured from the approximated centre.

^{1}H–^{13}C coupling constants were measured applying the phase-sensitive HMBC experiment described in Ref. 16, on a 20 mM sample of **8** in D_2O, recorded at 400 MHz and 25°C on a Bruker Avance spectrometer equipped with z-shielded gradient inverse detection probe. The acquisition times t_2 and t_1 were 1.3 s (spectral width 6410 Hz, 8K complex data points) and 3 ms (spectral width 20,100 Hz, 64 real data points), respectively. The relaxation delay was 2 s; 80 scans were accumulated per t_1 increment.

Compounds 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-uridine (**2**)[17], 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-oxouridine (**3**)[18] and 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-methyleneuridine (**4**)[20] were synthesized as previously described, affording satisfactory ^{1}H and ^{13}C NMR spectra.

3.1.1. 3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-hydroxymethyleneuridine (5). Compound **4** (48.3 g, 100.3 mmol, M_W: 482) was dissolved in a mixture of THF (250 mL), *t*-butanol (250 mL) and water (75 mL); *N*-methylmorpholine-*N*-oxide (15 g; 128 mmol, 1.2 equiv.) and OsO_4 (10 mL of a 2.5% w/v, solution in *t*-butanol; 1 mmol, 0.01 equiv.) were added under ice cooling. The mixture was stirred for 5 days at 4°C and then quenched with 1 M aqueous $NaHSO_3$ and extracted with ethyl acetate (2×500 mL). The organic layer was washed with water, dried (Na_2SO_4) and evaporated to obtain crude **5** (51.7 g), which was used for the next reaction without further purification (100%, R_f 0.34, petroleum ether/ethyl acetate, 1:2). ^{13}C NMR ($CDCl_3$) δ (ppm): 12.1, 12.7, 12.8, 13.3 (isopropyl CHs); 16.7–17.3 (isopropyl CH_3s); 59.8 (C-5′); 61.4 ($-CH_2OH$); 67.8 (C-3′); 80.1 (C-2′); 81.0 (C-4′); 90.3 (C-1′); 101.5 (C-5); 141.2 (C-6); 151.3 (C-2); 164.4 (C-4). ^{1}H NMR ($CDCl_3$) δ (ppm): 0.80–1.20 (m, $CH(CH_3)_2$); 3.45 (d, 1H, $J_{6′,6″}$=10.1 Hz, H-6′); 3.65 (m, 2H, OH-2′, OH-6′); 3.90 (d, 1H, $J_{6′,6″}$=10.1 Hz, H-6″); 3.95–4.40 (m, 4H, H-3′, H-4′, H-5′ and H-5″); 5.60 (d, 1H, $J_{5,6}$=7.8 Hz, H-5); 6.00 (s, 1H, H-1′); 7.81 (d, 1H, $J_{5,6}$=7.8 Hz, H-6); 10.35 (br s, 1H, NH). Anal. Calcd for $C_{22}H_{40}N_2O_8Si_2$: C, 51.14; H, 7.80; N, 5.42. Found: C, 51.21; H, 7.83; N, 5.39.

3.1.2. 3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-*p*-toluenesulfonylmethyleneuridine (6). To a solution of compound **5** (51.7 g, 100.3 mmol, M_W: 516) in dry pyridine (130 L), *p*-toluenesulfonyl chloride (TsCl) (38 g, 200 mmol,

2.0 equiv.) was added and the mixture stirred over night. Then, a second portion of *p*-toluenesulfonyl chloride (56 g, 294 mmol, 3 equiv.) was added and the stirring was continued for 3 h. The solvent was removed in vacuo, the residue dissolved in ethyl acetate and washed with water, dried (Na_2SO_4) and stripped to obtain the crude. After purification by silica gel column chromatography eluting with petroleum ether/ethyl acetate (1:2) pure **6** was obtained as a foam, (60%, R_f 0.80, petroleum ether/ethyl acetate, 1:2). ^{13}C NMR ($CDCl_3$) δ (ppm): 12.6, 12.7, 12.9, 13.6 (isopropyl CHs); 16.7–17.3 (isopropyl CH_3s); 21.6 (tosyl CH_3); 60.9 (C-5′); 66.5 (–CH_2OTos); 72.5 (C-3′); 79.3 (C-2′); 81.8 (C-4′); 89.5 (C-1′); 102.4 (C-5); 127.9 (tosyl C-2); 129.7 (tosyl C-3); 131.6 (tosyl C-4); 144.8 (C-6); 145.2 (tosyl C-1); 151.8 (C-2); 163.1 (C-4). ^{1}H NMR. ($CDCl_3$) δ (ppm): 0.80–1.20 (m, $CH(CH_3)_2$); 2.35 (s, 3H, tosyl CH_3); 3.42 (s, 1H, OH); 3.82 (d, 1H, $J_{6',6''}$=10.8 Hz, H-6′); 3.90–4.50 (m, 5H, H-3′, H-4′, H-5′, H-5″ and H-6″); 5.62 (d, 1H, $J_{5,6}$=8.0 Hz, H-5), 5.98 (s, 1H, H-1′); 7,20 (d, 2H, J_O=7.4 Hz, phenyl); 7.60 (d, 1H, $J_{5,6}$=8.0 Hz, H-6); 7.70 (d, 2H, J_O=7.4 Hz, phenyl); 10.35 (br s, 1H, NH). Anal. Calcd for $C_{29}H_{46}N_2O_{10}SSi_2$: C, 51.92; H, 6.91; N, 4.18. Found: C, 51.97; H, 6.92; N, 4.21.

3.1.3. 3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-*C*-methyluridine (7). Compound **6** (40.4 g, 60 mmol, M_W: 670) was dissolved in 615 mL of anhydrous DMF, then $NaBH_4$ (11.75 g, 310 mmol, 5.0 equiv.) was added and the mixture kept at rt for 2 h. The reaction was quenched by careful dropwise addition of NH_4Cl (saturated solution) on the chilled mixture and extracted with ethyl acetate (2×800 mL). The combined organic layers were washed with brine (5×1.4 L), dried (Na_2SO_4) and evaporated to obtain the crude. The product was purified by flash chromatography, eluting with petroleum ether/ethyl acetate (1:1) yielding pure **7** as a foam. (93%, R_f 0.70, petroleum ether/ethyl acetate, 4:6). ^{13}C NMR ($CDCl_3$) δ (ppm): 12.5, 12.8, 13.4, 13.6 (isopropyl CHs); 16.8–17.3 (isopropyl CH_3s); 20.4 (–CH_3); 59.8 (C-5′); 72.7 (C-3′); 78.9 (C-2′), 81.6 (C-4′); 90.4 (C-1′); 102.3 (C-5); 139.6 (C-6); 150.5 (C-2); 163.4 (C-4). ^{1}H NMR ($CDCl_3$) δ (ppm): 0.80–1.20 (m, $CH(CH_3)_2$); 1.22 (s, 3H, –CH_3); 2.53 (s, 1H, OH-2′); 3.90–4.15 (m, 3H, H-4, 5′ and 5″); 4.21 (d, 1H, $J_{3',4'}$=8.1 Hz, H-3′); 5.70 (d, 1H, $J_{5,6}$=8.1 Hz, H-5); 6.00 (s, 1H, H-1′); 7.73 (d, 1H, $J_{5,6}$=8.1 Hz, H-6); 8.40 (br s, 1H, NH). Anal. Calcd for $C_{22}H_{40}N_2O_7Si_2$: C, 52.77; H, 8.05; N, 5.59. Found: C, 52.74; H, 8.02; N, 5.51.

3.1.4. 2′-*C*-Methyluridine (8). A solution of compound **7** (28 g, 56 mmol, M_W: 500) in 900 mL of anhydrous THF was treated with 1 M tetrabutylammonium fluoride solution in THF (TBAF) (123 mL, 123 mmol, 2.2 equiv.) at rt. Five minutes later the reaction mixture was diluted with 0.4 mL of pyridine/methanol/water (3:3:1) and the solution was poured into stirred pyridinium form Dowex 50Wx4-200 resin (5 g) suspended in pyridine/methanol/water (3:3:1, 33.6 mL). The mixture was stirred for 10 min, the resin filtered off and washed with methanol (3×56 mL). Combined filtrate and washings were evaporated to dryness in vacuo to produce a crude syrup (R_f 0.40, CH_2Cl_2:methanol, 9:1). ^{13}C NMR (DMSO-d_6) δ (ppm): 19.3 (–CH_3); 59.9 (C-5′); 72.7 (C-3′); 79.4 (C-2′); 82.1 (C-4′); 92.0 (C-1′); 102.6 (C-5); 141.8 (C-6); 151.0 (C-2); 166.4 (C-4). ^{1}H NMR (DMSO-d_6) δ (ppm): 1.00 (s, 3H, –CH_3); 3.79 (dd, 1H, $J_{5',5''}$=11.8 Hz, $J_{5',4'}$=2Hz, H-5′); 3.84 (d, 1H, $J_{3',4'}$=9.5 Hz, H-3′); 3.98 (m, 2H, H-4′ and H-5″); 5.05 (br s, 2H, OH-3′ and OH-2′); 5.19 (br s, 1H, OH-5′); 5.84 (d, 1H, $J_{5,6}$=8.2 Hz, H-5); 5.95 (s, 1H, H-1′); 7.86 (d, 1H, $J_{5,6}$=8.2 Hz, H-6). Anal. Calcd for $C_{10}H_{14}N_2O_6$: C, 46.51; H, 5.47; N, 10.85. Found: C, 46.54; H, 5.44; N, 10.86.

3.1.5. 3′,5′-Di-*O*-acetyl-2′-*C*-methyluridine (9). The syrup obtained in the previous reaction was suspended in 280 mL of pyridine and acetic anhydride (Ac_2O) (9.5 mL, 100 mmol, 1.8 equiv.) was added. The mixture was stirred overnight and the then 1 mL (0.2 equiv.) of acylating reagent were added. After an hour the mixture was cooled (0°C), quenched with 28 mL of methanol and concentrated in vacuo. The colourless oil obtained was dissolved in CH_2Cl_2 (280 mL) and poured into $NaHCO_3$ (5×56 mL). The organic phase dried (Na_2SO_4) was evaporated and the product was purified by silica gel column chromatography, eluting with CH_2Cl_2:methanol (98:2). Pure **9** was obtained as a foam (70% from **7**, R_f 0.40, CH_2Cl_2:methanol, 95:5). ^{13}C NMR ($CDCl_3$) δ (ppm): 20.6[a] (–CH_3); 20.7[a] (acetyl –CH_3s); 62.1 (C-5′); 73.9 (C-3′); 78.1 (C-2′); 78.3 (C-4′); 91.6 (C-1′); 102.7 (C-5); 139.4 (C-6); 151.0 (C-2); 163.3 (C-4); 170.1 (CO); 170.2 (CO). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.20 (s, 3H, –CH_3); 2.12 (s, 3H, CH_3CO); 2.17 (s, 3H, CH_3CO); 3.25 (s, 1H, OH-2′); 4.35–4.45 (m, 3H, H-4′, 5′ and 5″); 4.92 (d, 1H, $J_{3',4'}$=6.7 Hz, H-3′); 5.78 (d, 1H, $J_{5,6}$=8.2 Hz, H-5); 6.01 (s, 1H, H-1′); 7.63 (d, 1H, $J_{5,6}$=8.2 Hz, H-6); 9.45 (br s, 1H, NH). Anal. Calcd for $C_{14}H_{18}N_2O_8$: C, 49.12; H, 5.30; N, 8.18. Found: C, 49.15; H, 5.34; N, 8.25.

3.1.6. 3′,5′-Di-*O*-acetyl-2′-*O*-tetrahydropyranyl-2′-*C*-methyluridine (10). 13.40 g of compound **9** (39.2 mmol, M_W: 342) were added to a solution of 187 mg (0.1 mmol) of *p*-toluenesulfonic acid monohydrate (*p*-TsOH) in 133 mL of 1,4-dioxane and then, 3,4-dihydro-2*H*-pyran (DHP) (52 mL, 572 mmol, 14.6 equiv.) was dropped into the mixture. After six hours the reaction was completed and quenched with 133 mL of triethylamine. The mixture was concentrated and poured into Na_2CO_3. The product was purified by silica gel column chromatography, eluting with CH_2Cl_2/methanol (98:2) yielding **10** as an epimeric mixture (80%, R_f 0.65, CH_2Cl_2/methanol, 97:3). ^{13}C NMR ($CDCl_3$) δ (ppm): 16.6/17.1 (–CH_3); 19.2/19.3, 19.7/20.3, 20.4 (acetyl –CH_3s and *C*-3, THP); 24.9/25.2 (*C*-4, THP); 31.3/31.5 (*C*-2, THP); 61.3/61.5 (C-5′); 62.0/63.1 (*C*-5, THP); 73.2/73.5 (C-3′); 76.8/77.0 (C-4′); 83.3/83.5 (C-2′); 89.7/89.9 (C-1′); 94.5/95.4 (*C*-1, THP); 102.1/102.3 (C-5); 138.5/139.2 (C-6); 150.1/150.2 (C-2); 162.9/163.0 (C-4); 169.8 (CO); 169.9 (CO). ^{1}H NMR ($CDCl_3$) δ (ppm): 0.85 (m, 1H, *H*-3 THP); 1.11 (m, 1H, *H*-3′, THP); 1.19/1.20 (s, 3H, –CH_3); 1.20–1.90 (m, 4H, *H*-4, *H*-4′, *H*-2 and *H*-2′, THP); 2.06/2.09 (s, 3H, CH_3CO); 2.10/2.11 (s, 3H, CH_3CO); 3.20–4.40 (m, 5H, H-4′, H-5′, H-5″ and, *H*-5 and *H*-5′, THP); 4.32 (m, 1H, H-3′); 4.95 (m, 1H, *H*-1, THP); 5.72 (m, 1H, H-5); 5.98/6.44 (s, 1H, H-1′); 7.62 (m, 1H, H-6); 9.97/10.03 (br s, 1H, NH). Anal. Calcd for $C_{19}H_{26}N_2O_9$: C, 53.52; H, 6.15; N, 6.57. Found: C, 53.48; H, 6.45; N, 6.75.

3.1.7. 2′-*O*-Tetrahydropyranyl-2′-*C*-methyluridine (11).

13.63 g of 3′,5′-di-*O*-acetyl-2′-*O*-tetrahydropyranyl-2′-*C*-methyluridine (**10**) (39 mmol, MW: 426) were dissolved in 140 mL of methanolic ammonia and the mixture was stirred overnight at rt. The diasteromeric mixture was isolated after the evaporation of volatiles (100%, R_f 0.5 in dichloromethane/methanol, 95:5). ^{13}C NMR ($CDCl_3$) δ (ppm): 15.7/16.5 (–CH_3); 20.4/20.7 (*C*-3, THP); 24.8/25.3 (*C*-4, THP); 31.8/31.9 (*C*-2, THP); 61.9/62.1 (C-5′); 64.1/64.6 (*C*-5, THP); 72.5/73.1 (C-3′); 82.5/82.7 (C-4′); 85.2/84.4 (C-2′); 90.2 (C-1′); 95.1/95.5 (*C*-1, THP); 102.0/102.1 (C-5); 140.2 (C-6); 150.8 (C-2); 164.7 (C-4). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.28 (m, 1H, *H*-3′, THP); 1.27/1.30 (s, 3H, –CH_3); 1.57 (m, 3H, *H*-3′, *H*-4 and *H*-4′, THP); 1.85 (m, 2H, *H*-2 and *H*-2′, THP); 3.37–4.05 (m, 5H, H-4′, H-5′, H-5″ and, *H*-5 and *H*-5′, THP); 4.07/4.15 (m, 1H, H-3′); 4.94/5.12 (m, 1H, *H*-1, THP); 5.73/5.74 (d, 1H, $J_{5,6}$=9.0 Hz, H-5); 6.03/6.51 (s, 1H, H-1′); 7.93/8.02 (d, 1H, $J_{5,6}$=9.0 Hz, H-6). Anal. Calcd for $C_{15}H_{22}N_2O_7$: C, 52.63; H, 4.48; N, 8.18. Found: C, 52.66; H, 4.49; N, 8.20.

3.1.8. 5′-*O*-Dimethoxytrityl-2′-*O*-tetrahydropyranyl-2′-*C*-methyluridine (12). Dried compound **11** (10.95 g, 32 mmol, M_W: 342) was dissolved in anhydrous pyridine (250 mL). Triethylamine (6.75 mL, 48.4 mmol, 1.5 equiv.), 4-dimethylaminopyridine (DMAP) (213 mg, 1.7 mmol, 0.05 equiv.) and 4,4′-dimethoxytrityl chloride (DMTrCl) (14.58 g, 43 mmol, 1.3 equiv.) were added with stirring and exclusion of moisture, and the reaction mixture was stirred at rt. After 2 h the reaction was not completed, therefore more DMTrCl (24.30 g, 71 mmol, 2.2 equiv.) and triethylamine (11.2 mL, 80 mmol, 2.5 equiv.) were added and the stirring was continued for 5 h. The reaction was quenched with an equivalent volume of water and extracted with ethyl ether (2×1 L), dried (Na_2SO_4) and evaporated. The crude product (R_f 0.45, methanol/CH_2Cl_2, 97:3) was purified by column chromatography eluting with petroleum ether/ethyl acetate/triethylamine (66:33:1) to yield pure **12** as a foam (78%). ^{13}C NMR ($CDCl_3$) δ (ppm): 15.9/16.9 (–CH_3); 20.7/21.0 (*C*-3, THP); 24.9/25.0 (*C*-4, THP); 29.7/32.0 (*C*-2, THP); 55.3 (–OCH_3, DMTr); 60.6 (C-5′); 64.4/64.6 (*C*-5, THP); 73.4/74.1 (C-3′); 81.4/82.1 (C-4′); 84.2/84.3 (C-2′); 87.2/87.8 (C–Ar_3, DMTr); 90.4 (C-1′); 95.3/95.7 (*C*-1, THP); 102.0/102.1 (C-5); 113.3/113.2 (Ar, DMTr); 127.1, 128.0/128.3, 130.2/130.3, 135.2, 135.4/135.5 (Ar, DMTr); 140.2/140.4 (C-6); 144.4/144.5 (Ar, DMTr); 150.7 (C-2); 158.7 (C_{Ar}–OCH_3, DMTr), 163.4/163.5 (C-4). ^{1}H NMR ($CDCl_3$) δ (ppm): 0.88 (m, 1H, *H*-3, THP); 1.32 (s, 3H, –CH_3); 1.52 (m, 3H, *H*-3′, *H*-4 and *H*-4′, THP); 1.70/1.85 (m, 2H, *H*-2 and *H*-2′, THP); 3.54–4.17 (m, 9H, H-3′, H-4′, H-5′, H-5″, –OCH_3, DMTr and, *H*-5 and *H*-5′, THP); 5.02/5.06 (d, 1H, $J_{5,6}$=9.0 Hz, H-5); 5.21/5.33 (m, 1H, *H*-1, THP); 6.04/6.50 (s, 1H, H-1′); 6.85 (m, 4H, DMTr), 7.20–7.45 (m, 9H, DMTr), 8.18/8.21 (d, 1H, $J_{5,6}$=9.0 Hz, H-6). Anal. Calcd for $C_{36}H_{40}N_2O_9$: C, 67.07; H, 6.25; N, 4.35. Found: C, 67.47; H, 5.53; N, 4.39.

3.1.9. 5′-*O*-Dimethoxytrityl-2′-*C*-methyluridine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl phosphoramidite) (13). Dried compound **12** (16.07 g, 25 mmol, M_W: 644) was dissolved in dry 1,2-dichloroethane (60 mL) containing *N*,*N*-diisopropylethylamine (*i*-Pr_2NEt) (23 mL, 132 mmol, 5.2 equiv.) under argon and the solution was cooled in an ice bath. β-Cyanoethoxy-*N*,*N*-diisopropylaminochlorophosphine (6.1 mL, 27.3 mmol, 1.1 equiv.) was added dropwise. The mixture was stirred at rt for 15 min. And then, since the reaction was not completed, more β-cyanoethoxy *N*,*N*-diisopropylaminochlorophosphine (1.1 mL, 5 mmol, 0.2 equiv.) was added and the stirring continued for 30 min. Methanol (0.36 mL), ethyl acetate (550 mL) and triethylamine (28 mL) were subsequently added. And the resulting solution washed twice with aqueous Na_2CO_3 (10%) and twice with brine. The organic layer was dried (Na_2SO_4) and concentrated in vacuo. The crude product was purified by column chromatography eluting with petrol ether/ethyl acetate/triethylamine (33:66:1) to yield pure **13** as a foam (72%, R_f 0.6, petrol ether/ethyl acetate, 3:4). ^{13}C NMR. ($CDCl_3$) δ (ppm): 17.1/17.8/18.0 (–CH_3); 18.8/18.9/19.7 (CH_2–CN, phosp); 20.1/20.2/20.3/21.0 (*C*-3, THP); 24.6 (CH_3, phosp); 25.3/25.5 (*C*-4, THP); 29.7/31.7/31.9/32.0 (*C*-2, THP); 43.4/43.7 (CHs, phosp); 55.2/55.3 (–OCH_3, DMTr); 57.9/58.0 (–OCH_2, phosph); 60.2/60.4/60.5 (C-5′); 61.5/62.7 (*C*-5, THP); 75.8/75.9/76.0/76.2 (C-3′); 80.6/80.7 (C-4′); 83.8/84.0 (C-2′); 87.2 (C–Ar_3, DMTr); 89.2/89.3/89.5/89.6 (C-1′); 93.6/93.8/94.9 (*C*-1, THP); 102.1/102.3 (C-5); 113.2/113.3 (Ar, DMTr); 117.4 (CN, phops);127.2/127.3, 128.0, 128.6/128.7, 130.2/130.5, 135.2/135.3 (Ar, DMTr); 140.2/140.5 (C-6); 144.3 (Ar, DMTr); 150.6 (C-2); 158.8 (C_{Ar}–OCH_3, DMTr), 163.1/163.2 (C-4). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.10 (m, 1H, *H*-3′, THP); 1.18 (m, 12H, CH_3, phosp), 1.26 (s, 3H, –CH_3); 1.55 (m, 3H, *H*-3′, *H*-4and *H*-4′, THP); 2.28/2.34 (m, 2H, *H*-2 and *H*-2′, THP); 2.63 (m, 2H, CH_2–CN, phosp), 3.41–4.42 (m, 16H, H-3′, H-4′, H-5′, H-5″, –OCH_3, DMTr, OCH_2 and CHs phosp and, *H*-5 and *H*-5′, THP) 3.37–4.05 (m, 5H,); 4.07/4.15 (m, 1H, H-3′); 4.94/5.12(m, 1H, *H*-1, THP); 5.73/5.74 (d, 1H, $J_{5,6}$=9.0 Hz, H-5); 6.03/6.51 (s, 1H, H-1′); 7.93/8.02 (d, 1H, $J_{5,6}$=9.0 Hz, H-6). ^{31}P NMR ($CDCl_3$) δ (ppm): 151.09/151.13/151.18/151.22. Anal. Calcd for $C_{45}H_{57}N_4O_{10}P$: C, 63.97; H, 6.80; N, 6.63. Found: C, 64.25; H, 6.95; N, 6.75.

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