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EXPLORING THE SYNTHESIS OF MASKED PHOSPHORAMIDO 6-VINYLCYTIDINE DERIVATIVES AS BUILDING BLOCKS FOR CROSS-LINKING OLIGONUCLEOTIDES

Marco Radi, Raffaella Spinosa, Maria Cristina Parlato, Federico Corelli, and Maurizio Botta*

Department of Medicinal Chemistry, University of Siena, Via Aldo Moro, 53100 Siena, Italy. *Corresponding author: botta@unisi.it

Abstract –In the present work the synthesis of building blocks (**2a,b)**, bearing a masked form of the reactive vinyl group bound to the C-6 position of a cytidine derivative, has been studied. The understanding of byproduct formation together with conformational considerations let us to plan a straightforward approach for the synthesis of the target compounds.

INTRODUCTION

After the first example of oligonucleotides application as Rous Sarcoma Virus (RSV) inhibitors in 1978,[1] a large number of antisense oligonucleotides are actually in preclinical and clinical trials mainly in the cancer, cardiovascular and infectious disease fields.[2] However, conventional, unmodified oligonucleotides have drawbacks for their practical use, due to their unsatisfactory binding affinity to the target sequence, the instability against cellular nucleases, the insufficient membrane penetration and the low bioavailability.[3] The function of oligonucleotides, either modified or unmodified, is passive, in that they can dissociate from the complex and so their inhibitory effect is transient. For that reason, many structural modifications have been brought so far to this class of compounds in order to improve their binding affinity to the target sequence and consequently their pharmacological profile.[4] In particular, oligonucleotides bearing a reactive group have been regarded as promising candidates for irreversible damage of the target sequence on account of their ability to cause an interstrand cross-linking reaction.[5-7] On the basis of the interesting Michael acceptor properties showed by some 6-vinylpirimidine derivatives synthesized in our group[8] and on the work developed by Sasaki,[9] the synthesis and evaluation of

alkylating properties of cytidine derivative (**1**) (Fig. 1) has been recently reported.[10] The vinyl group in C-6 position constrains this molecule into a non-natural *syn* conformation[11,12]:

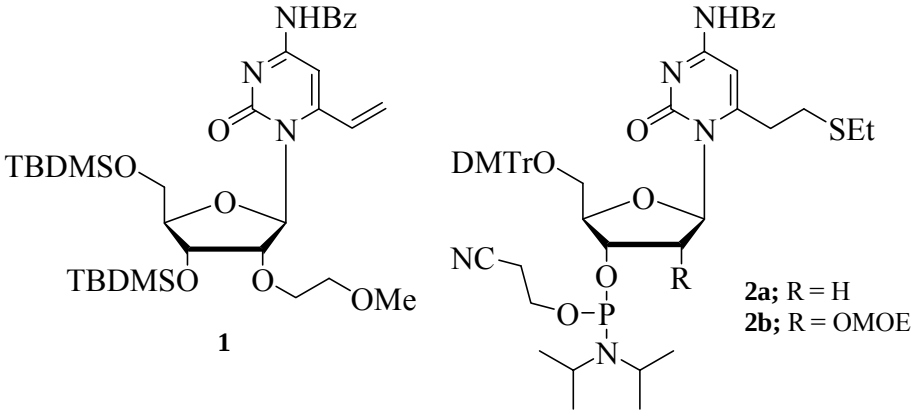


Figure 1

as a result a cross-linking reaction between the vinyl group of **1** and the amino group of a guanosine in the target sequence may likely occur, thus enhancing the stability of the resulting duplex (Fig. 2). Moreover, a masking strategy was developed in order to protect the reactive vinyl moiety of the monomer before its introduction into the oligonucleotide sequence and to release subsequently this reactive moiety.[10]Accordingly, the present work was focused on the synthesis of the active phosforamido derivatives (**2a,b**) in order to subsequently introduce these two monomers into a specific oligonucleotide sequence and to investigate the role played by the C-6 and C-2' substituents in the duplex stabilization and nuclease resistance.

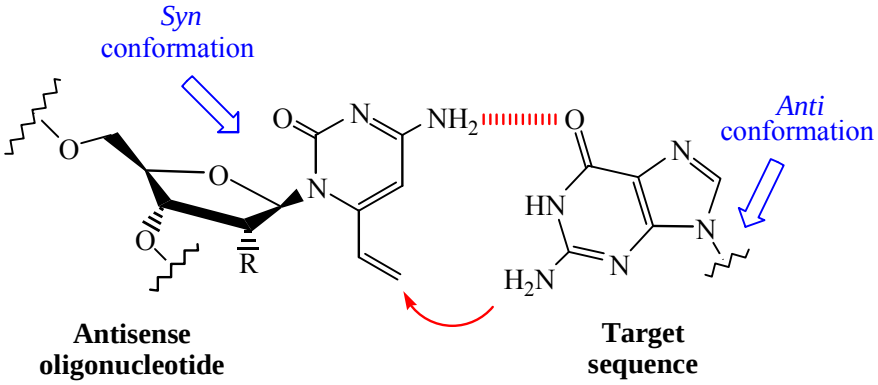
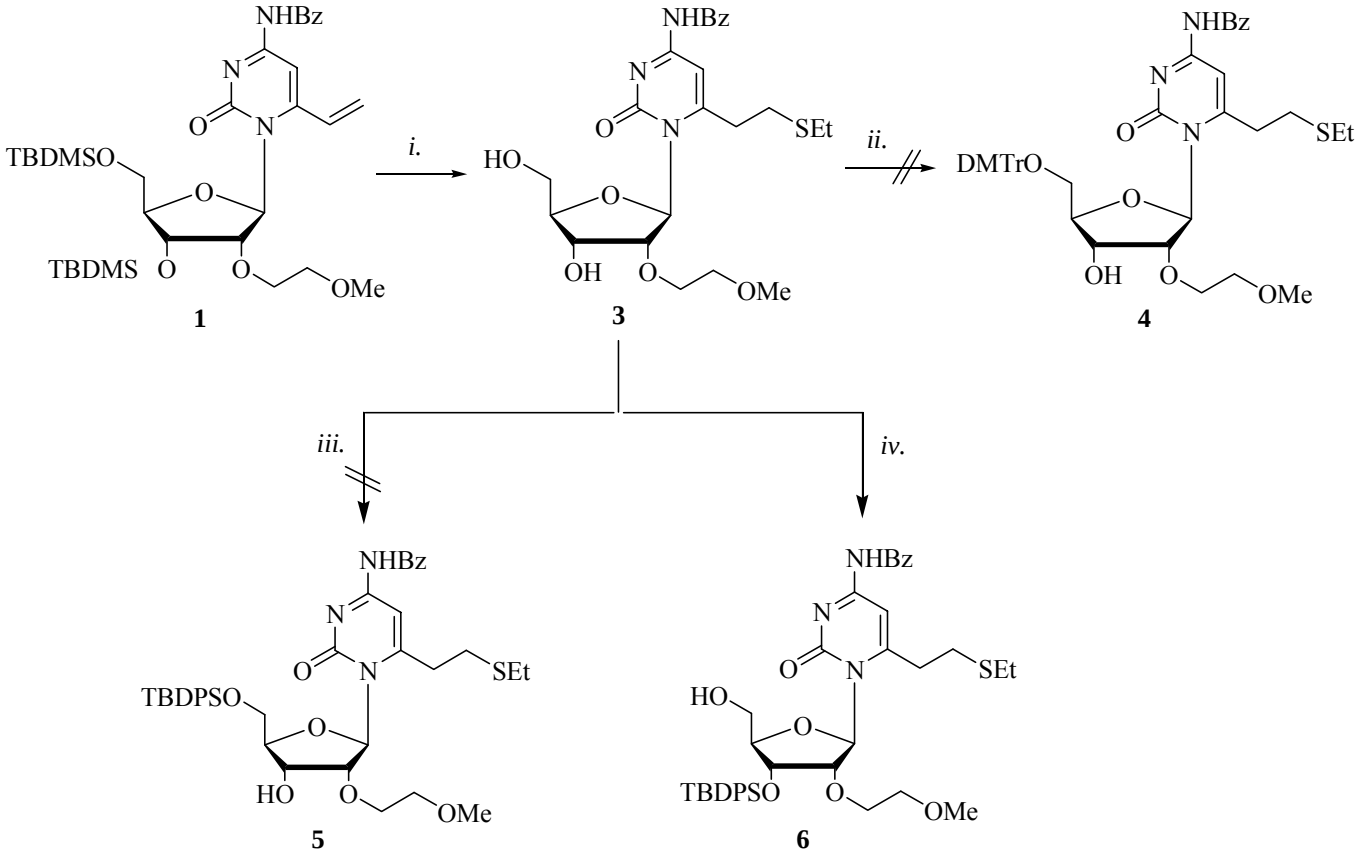


Figure 2

RESULTS AND DISCUSSION

In order to synthesize the target compound (**2b**), the reactive vinyl group in compound (**1**) was initially masked as ethylthio derivative by reaction with ethanethiol in dichloromethane and the intermediate obtained was then deprotected with tetrabutylammonium fluoride to give compound (**3**) in good yield (Scheme 1). However, selective protection of the primary hydroxy group in **3** proved to be a difficult task.

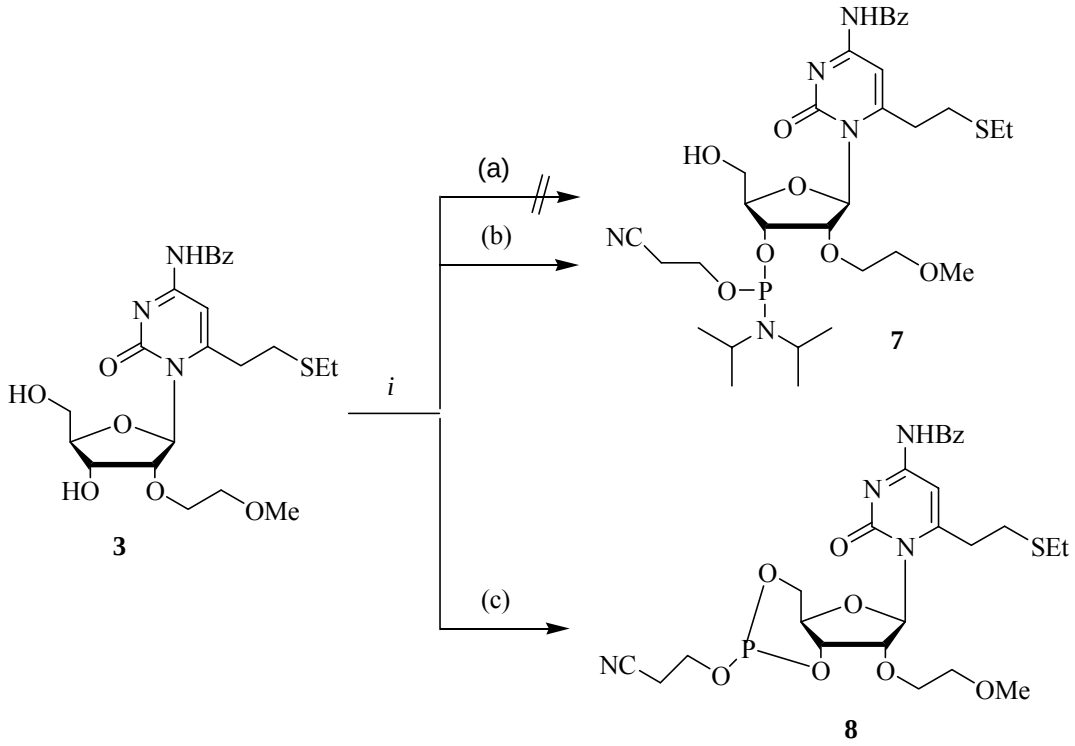
Reacting in fact compound (**3**) with dimethoxytrityl chloride either under classical, high temperature or microwave-assisted conditions, the expected derivative (**4**) was never isolated.



Scheme 1 Reagents and conditions: *i.* (a) EtSH, CH_2Cl_2, rt 2h; (b) TBAF, THF, rt 1h; *ii*. DMTrCl, pyridine, rt overnight; *iii*. TBDPSCl, CH_2Cl_2, DMAP, Et_3N, rt 48h; *iv*. TBDPSCl, CH_2Cl_2, DMAP, Et_3N, MW, 60 °C, 1h.

The introduction of a different selective protecting group in 5' position was then investigated by reacting compound (**3**) with *tert*-butyldiphenylsilyl chloride in the presence of dimethylaminopyridine and triethylamine at room temperature, but the expected compound (**5**) was never obtained. However, running the same reaction at 60°C under microwave assisted condition, the bulky *tert*-butyldiphenylsilyl moiety was introduced on the secondary hydroxy group of **3** and compound (**6**) was obtained as the only product. On the basis of the extensive literature reports[12,13] we assumed that the unsuccessful tritylation of **3** and the formation of compound (**6**) could be related to the formation of a strong hydrogen bond between the carbonyl group of the nucleobase and the 5'-hydroxy group of the ribose moiety .As a consequence of this hypothesis, we considered the formation of this strong hydrogen bond as an unconventional way to protect the 5'-hydrox y group and the direct introduction of the phosphoramidite moiety on the secondary hydroxy group was therefore investigated. Compound (**3**) was therefore directly reacted with 2-cyanoethyl-*N*,*N*,*N'*,*N'*-tetraisopropyl phosphoramidite in the presence of *N*,*N*-diisopropylammonium tetrazole but no reaction occurred (Scheme 2). We tried therefore to perform the same reaction increasing the temperature up to 70 °C under microwave irradiation obtaining a complex mixture with only traces of

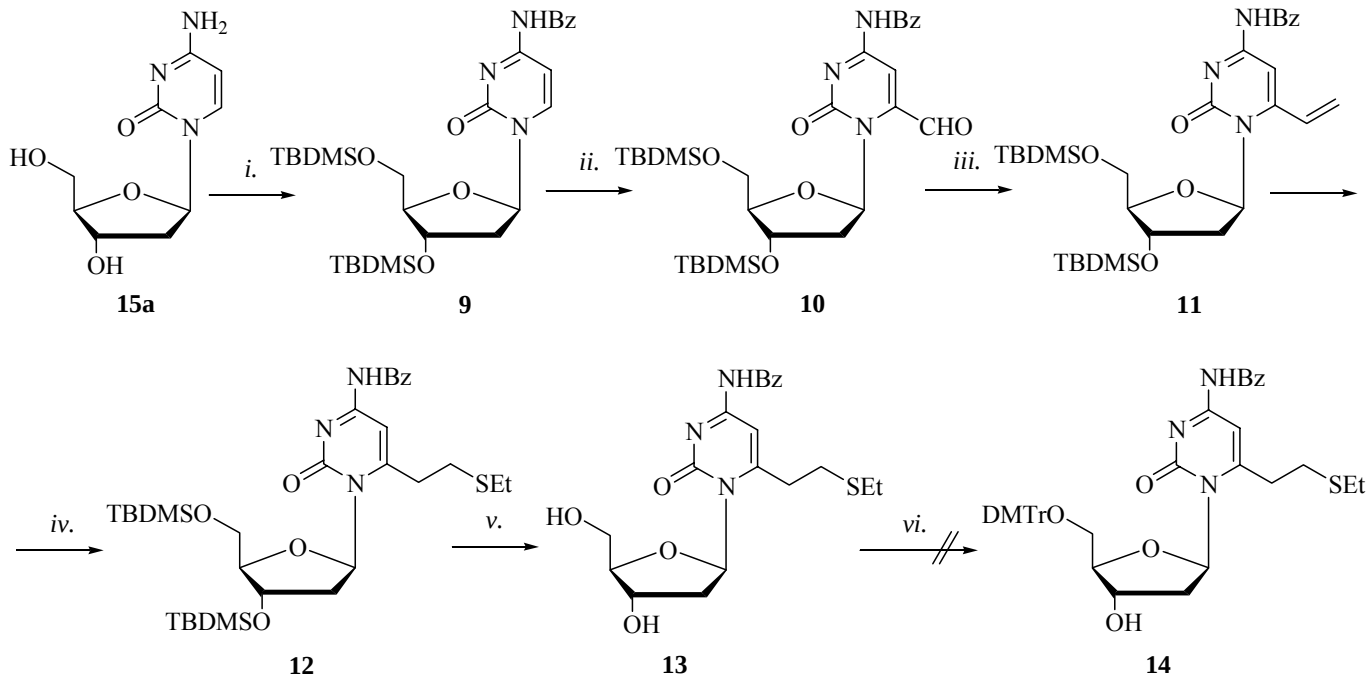
the expected phosphoramidite (**7)** while, increasing the temperature up to 100 °C in a sealed tube, the cyclic phosphite (**8)** was obtained as major reaction product.



Scheme 2. Reagents and conditions: *i.* 2-cyanoethyl-*N*,*N*,*N'*,*N'*-tetraisopropyl phosphoramidite, *N*,*N*-diisopropylammonium tetrazole, CH_3CN, (a: rt overnight; b: MW 70°C, 12 min; c: MW 100°C, sealed tube 12 min.)

It is well known how the complete definition of the conformation of a nucleoside is a complex subject. A simple modification of the 2'-position with an electronegative substituent is in fact able to confer a north conformation to the sugar moiety ($^3T_2$) while the 2'-deoxy derivatives possess a south conformation ($^2T_3$).[14] In order to investigate the relationship between the sugar conformation and the formation of the intramolecular hydrogen bond, the 2'-deoxy analogue (**13)** of compound (**3)** was synthesized starting from commercially available 2'-deoxycytidine (Scheme 3). Following the same synthetic approach used for the synthesis of **3**, compound (**13)** was easily obtained; however, the selective protection of the primary hydroxy group proved again to be problematic. Since the failure of the 5'-hydroxy protection seems to be exclusively related to the unnatural *syn* conformation imposed by the presence of a C-6 substituent, we planned to introduce the 5'-protecting group first and to functionalize the C-6 position in a late stage. A one pot procedure was used for the synthesis of the trityl derivative (**16a)** starting from 2'-deoxycytidine (**15a)** which was first submitted to N^4-benzoylation with benzoic anhydride in the presence of pyridine[15] and after 1h at 90 ºC, treated with dimethoxytrityl chloride for 10 h to give **16a** in good overall yield. Compound (**16a)** was then protected with *tert*-butyldimethylsilyl chloride to give the fully protected

derivative (**17a**) in quantitative yield. For the subsequent introduction of the 6-vinyl group, many procedures were investigated

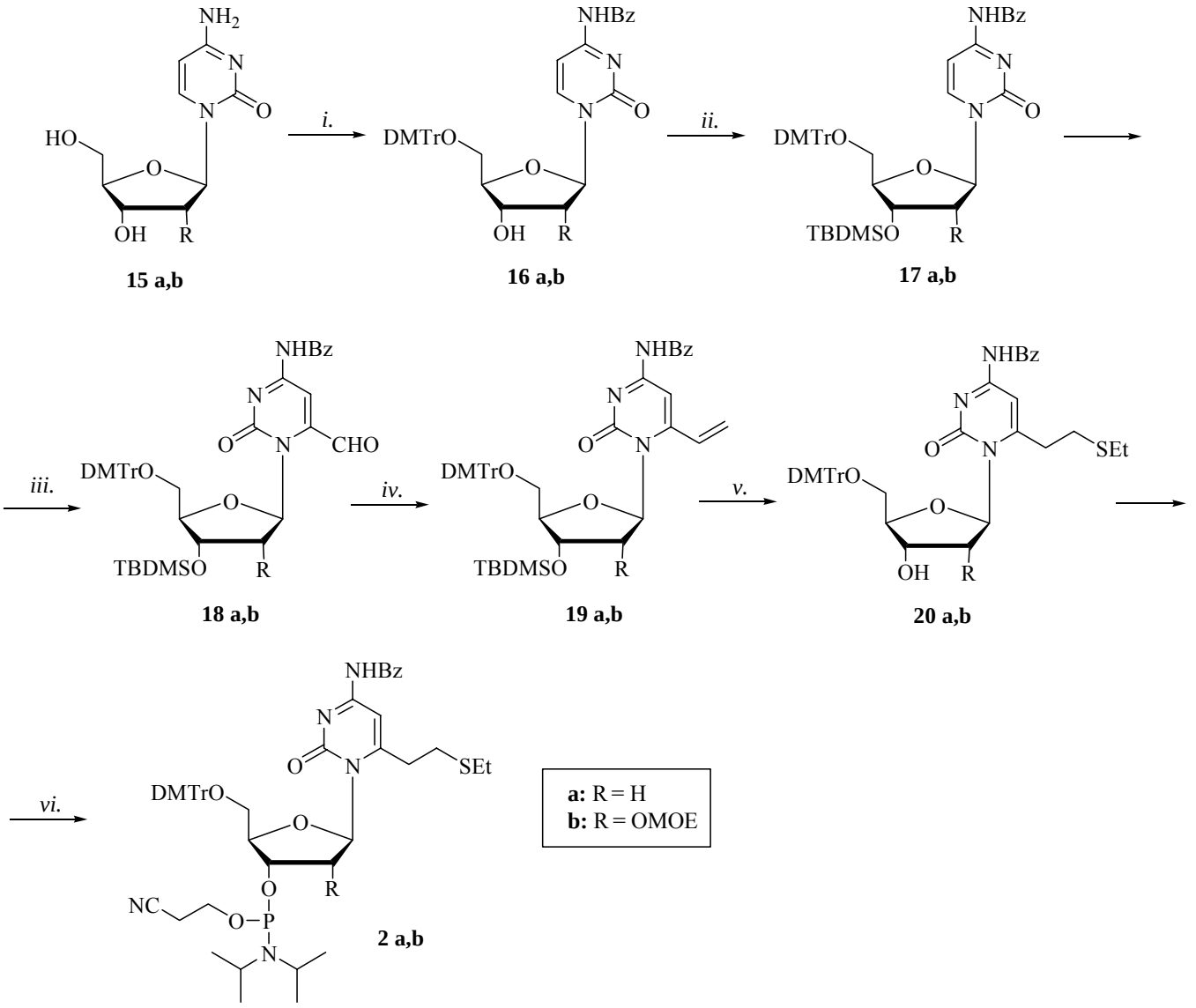


Scheme 3 Reagents and conditions: *i*. (a) TBDMSCl, imidazole, DMF, rt, 4h; (b) benzoic anhydride, DMF, MW, 160 °C. 15 min.; *ii*. (a) LDA, THF dry, -78 °C, 3h; (b) HCOOMe, -78 °C, 3h; *iii*. KHMDS, $CH_3P(C_6H_5)_3Br$, THF dry, from -78 to 0°C, 2h; *iv*. EtSH, CH_2Cl_2, rt, 2h; *v*. TBAF, THF dry, rt, 4h; *vi*. DMTrCl, Py, rt, overnight.

but, at last, the Wittig reaction on the 6-formyl derivative (**18a**) proved to give the best results.[8] Accordingly, compound (**17a**) was first lithiated[16] and then reacted with methyl formate to yield the 6-formyl derivative (**18a**) as a mixture (starting material still present) which was very difficult to purify using common chromatographic techniques. Therefore, after a simple filtration of the crude on silica gel, the 40:60 mixture of compounds (**17a** and **18a**) was reacted with the suitable Wittig reagent to give the 6-vinyl derivative (**19a**). Unreacted **17a** could be recovered at this stage from the reaction mixture and reused in the formylation reaction to increase the overall yield of **19a**. The vinyl group of compounds **19a** was then masked by reaction with ethanethiol in dichloromethane to give the corresponding ethanethioethyl derivative which was subsequently deprotected with tetrabutylammonium fluoride to give compound (**20a**)**.** The latter compound was finally transformed into the target phosphoramidite monomer (**2a**) by reaction with 2-cyanoethyl-*N*,*N*,*N'*,*N'*-tetraisopropyl phosphoramidite at room temperature. Following the same synthetic approach used for the synthesis of **2a**, the 2'-MOE derivative (**2b**) was obtained starting from **15b**. The incorporation of these active monomers into a specific oligonucleotide sequence is at present under study.

CONCLUSION

In the present work, the synthesis of masked phosphoramido 6-vinyl cytidine derivatives (**2a,b**) was studied. It was shown how the failure of the 5'-hydroxy protection in compound (**3**) seems to originate from the



Scheme 4. *i*. (a) Benzoic anhydride, Pyr, 90°C, 1h; (b) DMTrCl, rt, 10h; *ii*. TBDMSCl, imidazole, DMF, rt, 4h; *iii*. (a) LDA, THF dry, -78°C, 3h; (b) HCOOMe, -78°C, 3h; *iv*. KHMDS, $CH_3P(C_6H_5)_3Br$, THF dry, from -78 to 0°C, 2h; *v*. (a) EtSH, CH_2Cl_2, rt, 2h; (b) TBAF, THF dry, rt, 1h; *vi*. 2-cyanoethyl-*N,N,N',N'*-tetraisopropyl phosphoramidite, diisopropylammonium tetrazolide, CH_3CN, rt, 4h.

formation of a strong intramolecular hydrogen bond as a consequence of the unnatural *syn* conformation imposed by the presence of a C-6 substituent. In order to get round this stumbling block, the early introduction of the trityl protecting group on the sugar moiety and the C-6 functionalization of the nucleobase in a late stage allowed the obtainment of the activated monomers (**2a,b)** for the subsequent introduction into a specific oligonucleotide sequence.

ACKNOWLEDGEMENTS

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EXPERIMENTAL

General Considerations. Reagents were obtained from commercial suppliers and used without further purifications. Solvents were dried using standard procedures. ^{1}H NMR spectra were measured at 200 MHz with a Bruker AC 200F spectrometer. Chemical shifts are reported relative to TMS at 0.00 ppm. EI low-resolution MS spectra were recorded with an electron beam of 70 eV (GC-MS Varian Saturn 3 3400 CX). Elemental analyses were carried out on a Perkin-Elmer Elemental Analyzer 240L. Infrared spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrophotometer. Microwave reactions were conducted using a CEM Discover Synthesis Unit (CEM Corp., Matthews, NC) consisting of a continuous focused microwave power delivery system with operator-selectable power output from 0 to 300W. The reactions were performed in 10 mL sealed vessel. The temperature of the contents of the vessel was monitored using a calibrated infrared temperature control mounted under the reaction vessel. All experiments were performed using a stirring option whereby the contents of the vessel were stirred by means of a rotating magnetic plate located below the floor of the microwave cavity and a Teflon-coated magnetic stir bar in the vessel.

N^4-Benzoyl-2'-*O*-(2methoxyethyl)-6-{1-[2-(ethylthio)]ethyl}cytidine (**3**)**.**

To a solution of N^4-Benzoyl-3',5'-bis-*O*-(*tert*-butyldimethylsilyl)-2'-*O*-(2methoxyethyl)-6-vinylcytidine (**1**) (556 mg, 0.84 mmol) in CH_2Cl_2 (20 mL), ethanethiol (125 µL, 1.69 mmol) was added and the mixture was stirred at rt for 1h. After evaporation of the solvent under reduced pressure, the resulting residue was dried under high vacuum for 2 h, dissolved in anhydrous THF (20 mL) and treated with TBAF (2.85 mL, 2.84 mmol) at rt. After stirring for 4 h, the solvent was removed *in vacuo* and the residue was purified by flash chromatography ($CHCl_3$:MeOH 98:2 v/v) to give pure compound (**3**) (345 mg, 83%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.28 (t, J = 7.7 Hz, 3H,), 2.60 (q, J = 7.7 Hz, 2H), 2.80-3.12 (m, 4H), 3.37 (s, 3H), 3.40-3.95 (m, 6H), 4.20 (m, 1H), 4.52 (m, 1H), 5.08 (t, $J_{2'\text{-}3'}$ = 5.4 Hz), 5.72 (d, $J_{1'\text{-}2'}$ = 4.2 Hz), 7.40-7.70 (m, 4H), 7.82-7.97 (m, 2H); ^{13}C-NMR (200 MHz, $CDCl_3$): 14.7, 26.2, 34.8, 58.9, 63.2, 69.9, 70.3, 71.6, 78.2, 85.6, 89.8, 127.6, 129.1, 133.4; ESI-MS m/z: 516 $(M+Na)^+$; Anal. Calcd for $C_{23}H_{31}N_3O_7S$: C, 55.98; H, 6.29; N, 8.52. Found: C, 56.00; H, 6.20; N, 8.48.

N^4-Benzoyl-2'-*O*-(2-methoxyethyl)-3'-*O*-(tert-butyldiphenylsilyl)-6-{1-[2-(ethylthio)]ethyl}cytidine (6).

N^4-Benzoyl-2'-*O*-(2-methoxyethyl)-6-{1-[2-(ethylthio)]ethyl}cytidine (**3)** (124 mg, 0.25 mmol) was dried over P_2O_5 *in vacuo*, dissolved in anhydrous CH_2Cl_2 (3 mL) and transferred in a microwave sealed tube. DMAP (catalytic amount), triethylamine (241 µL, 1.5 mmol) and *tert*-butyldiphenylsilyl chloride (150 µL, 0.50 mmol) were added and the reaction was irradiated in the microwave at 60 °C for 10 min. The mixture was then diluted with CH_2Cl_2 (10 mL) and washed successively with water, brine and dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a crude oil, which was purified on a silica gel column ($CHCl_3$:MeOH, 98:2 v/v) to give pure compound (**6)** (119 mg, 65%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.04 (m, 9H), 1.24 (t, J = 7.7 Hz, 3H), 2.59 (q, J = 7.7 Hz, 2H), 2.80-3.15 (m, 4H), 3.35 (s, 3H), 3.40-4.30 (m, 7H), 4.83 (m, 1H), 5.28 (m, 1H), 5.62 (d, $J_{1'-2'}$ = 3.2 Hz, 1H), 7.25-8.10 (m, 16H); ESI-MS m/z: 732$(M+H)^+$; 754 $(M+Na)^+$; Anal. Calcd for $C_{39}H_{49}N_3O_7SiS$: C, 64.02; H, 6.70; N, 5.75 Found: C, 64.05; H, 6.81; N, 5.79.

N^4-Benzoyl-2'-*O*-(2-methoxyethyl)-3'-*O*-(2-cyanoethyl-N,N-diisopropylphosphoramidite)-6-{1-[2-(ethylthio)]ethyl}cytidine (**7**)**.**

N^4-Benzoyl-2'-*O*-(2-methoxyethyl)-6-{1-[2-(ethylthio)]ethyl}cytidine (**3)** (15.5 mg, 0.031 mmol) was mixed with diisopropylamine tetrazolide (3.6 mg, 0.02 mmol) and dried over P_2O_5 *in vacuo* overnight. The resulting mixture was dissolved in anhydrous CH_3CN (5 mL) and 2-cyanoehtyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite (15 µL, 0.047 mmol) was added. The reaction mixture was irradiated into the microwave (open vessel condition) under an argon atmosphere at 70 ºC for 12 min, followed by removal of the solvent in a vacuum. EtOAc (10 mL) was added to the residue, and it was washed with 5% aqueous $NaHCO_3$ (10 mL) and brine (10 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The resulting residue was purified by preparative TLC ($CHCl_3$:MeOH, 9:1 v/v) to give trace of pure compound (**7)**. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.04-1.35 (m, 15H,), 2.58 (q, J = 7.7 Hz, 2H), 2.70 (t, J = 7.5 Hz, 2H), 2.85-3.18 (m, 4H), 3.32 (s, 3H), 3.43-3.98 (m, 10H), 4.20 (m, 1H), 4.51 (m, 1H), 5.08 (t, $J_{2'-3'}$ = 5.4 Hz, 1H), 5.72 (d, $J_{1'-2'}$ = 4.2 Hz, 1H), 7.40-7.70 (m, 4H), 7.82-7.97 (m, 2H); ESI-MS m/z: 732$(M+K)^+$; Anal. Calcd for $C_{32}H_{48}N_5O_8PS$: C, 55.40; H, 6.97; N, 10.09 Found: C, 55.55; H, 6.90; N, 10.15.

N^4-Benzoyl-2'-*O*-(2-methoxyethyl)-3',5'-*O*-(2-cyanoethyl phosphite)-6-{1-[2-(ethylthio)]ethyl}-cytidine (**8**).

N^4-Benzoyl-2'-*O*-(2-methoxyethyl)-6-{1-[2-(ethylthio)]ethyl}cytidine (**3)** (20 mg, 0.04 mmol) was mixed with diisopropylamine tetrazolide (4.5 mg, 0.03 mmol) and dried over P_2O_5 *in vacuo* overnight. The resulting mixture was dissolved in anhydrous CH_3CN (5 mL) and 2-cyanoehtyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite (19 µL, 0.06 mmol) was added. The reaction mixture was irradiated

into the microwave (sealed tube) under an argon atmosphere at 100 °C for 10 min, followed by removal of the solvent in a vacuum. EtOAc (10 mL) was added to the residue, and it was washed with 5% aqueous $NaHCO_3$ (10mL) and brine (10mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The resulting residue was purified by preparative TLC ($CHCl_3$:MeOH, 95:5 v/v) to give pure compound (**8)** (7 mg, 30%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.28 (t, *J* = 7.7 Hz, 3H,), 2.60 (q, *J* = 7.7 Hz, 2H), 2.71 (t, *J* = 7.5 Hz, 2H), 2.82-3.12 (m, 4H), 3.30 (s, 3H), 3.40-4.55 (m, 9H), 4.72 (m, 1H), 5.08 (m, 1H), 5.50 (s, 1H), 7.40-7.70 (m, 4H), 7.82-7.97 (m, 2H); ESI-MS m/z: 593 $(M+H)^+$, 615 $(M+Na)^+$; Anal. Calcd for $C_{26}H_{33}N_4O_8PS$: C, 52.70; H, 5.61; N, 9.45. Found: C, 52.78; H, 5.71; N, 9.46.

N^4-Benzoyl-3',5'-bis-*O*-(*tert*-butyldimethylsilyl)-2'-deoxycytidine (9).

A suspension of 2'-deoxycytidine monohydrate (660 mg, 2.69 mmol), *tert*-buthylchlorodimethylsilane (1.86 g, 12.37 mmol) and imidazole (842 mg, 12.37 mmol) in anhydrous DMF was stirred for 4 h at rt, then diluted with EtOAc (100 mL).The separated organic layer was washed successively with H_2O and brine then dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a crude oil, which was dried under vacuum for 2 h and then dissolved in anhydrous DMF (10 mL). To this solution, benzoic anhydride (1.22 g, 5.38 mmol) was added and the resulting mixture was irradiated into the microwave for 15 min at 160 °C, then diluted with EtOAc (10 mL) and washed successively with water, brine and finally dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a crude oil, which was purified on a silica gel column ($CHCl_3$:MeOH, 98:2 v/v) to give pure compound (**9)** (1.5 g, 99%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ 0.11, 0.13 (12H, each as s, 2×$SiMe_2$), 0.87, 0.93 (18H, each as s, 2×Si^{t}Bu), 2.02-2.25 (m, 1H), 2.42-2.68 (m, 1H), 3.65-4.10 (m, 3H), 4.31-4.50 (m, 1H), 6.25 (t, *J* = 5.6 Hz, 1H), 7.35-7.70 (m, 5H), 7.88 (d, *J* = 7.4 Hz, 1H), 8.41 (d, *J* = 7.4 Hz, 1H); ^{13}C-NMR (200 MHz, $CDCl_3$): -5.5, -5.4, -4.9, -4.6, 17.9, 18.4, 25.7, 25.9, 42.4, 61.8, 70.1, 86.9, 87.9, 96.3, 127.5, 129.0, 133.1, 144.8; HPLC reverse phase, zorbax C8 column, Eluent: CH_3CN 95%, H_2O 5%, flow 0.4 mL/min; retention time 9.29 min; ESI-MS m/z: 560 $(M+ H)^+$; Anal. Calcd for $C_{28}H_{45}N_3O_5Si_2$: C, 60.07; H, 8.10; N, 7.51. Found: C, 60.03; H, 8.11; N, 7.50.

N^4-Benzoyl-3',5'-bis-*O*-(*tert*-butyldimethylsilyl)-6-formyl-2'-deoxycytidine (10).

A solution of N^4-Benzoyl-3',5'-bis-*O*-(*tert*-butyldimethylsilyl)-2'-deoxycytidine (**9)** (1.03 g, 1.85 mmol) in anhydrous THF (20 mL) was added dropwise to a -78 °C solution of freshly prepared LDA (11.10 mmol) in anhydrous THF (20 mL) at -78 °C under a positive pressure of argon. After 4 h at -78 °C, HCOOMe (1.14 mL, 18.50 mmol) was added, and the reaction mixture was kept under magnetic stirring at -78°C for 3 h. The mixture was then allowed to warm to rt, diluted with EtOAc and quenched by the addition of

saturated NH_4Cl solution. The organic phase was washed successively with water and brine then dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a crude white foam which was used in the next Wittig reaction without further purification. HPLC reverse phase, zorbax C8 column, Eluent: CH_3CN 95%, H_2O 5%, flow 0.4 mL/min; retention time 6.65 min ESI-MS m/z 606 $(M+H_2O+H)^+$, 628 $(M+H_2O+Na)^+$; retention time 8.72 min ESI-MS m/z: 588 $(M+ H)^+$, 610 $(M+ Na)^+$.

N^4-Benzoyl-3',5'-bis-*O*-(*tert*-butyldimethylsilyl)-6-vinyl-2'-deoxycytidine (11).

A hexane solution of BuLi (1.6M, 5.7 mL, 9.11 mmol) was added to a suspension of methyltriphenylphosphonium bromide (3.25g, 9.11 mmol) in THF (20 mL) at -78°C under a positive pressure of argon. The mixture was stirred for 30 min at 0 °C, followed by addition of a solution of N^4-benzoyl-3',5'-bis-*O*-(*tert*-butyldimethylsilyl)-6-formyl-2'-deoxycytidine (**10)** (1.07 g, 1.82 mmol) in THF (30 mL). The reaction mixture was stirred for 1 h at rt. A saturated NH_4Cl solution was added to the mixture, which was extracted with EtOAc (30mL). The organic phase was washed successively with water and brine then dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a crude oil, which was purified on a silica gel column ($CHCl_3$:MeOH, 98:2 v/v) to give pure compound (**11)** (798mg, 75%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ 0.05 and 0.07 (12H, each as s, 2×$SiMe_2$), 0.87 and 0.90 (18H, each as s, 2×Si^tBu), 1.95-2.20 (m, 1H), 2.60-2.85 (m, 1H), 3.70-4.05 (m, 3H), 4.52-4.70 (m, 1H), 5.63 (d, J_{cis} = 11.2 Hz, 1H), 5.98 (d, J_{trans} = 17.1 Hz, 1H), 6.51 (t, J = 7.3 Hz, 1H), 7.13 (dd, J_{cis} = 11.2 Hz, J_{trans} = 17.1 Hz, 1H), 7.32-7.70 (m, 5H), 7.82-8.00 (m, 1H); ^{13}C-NMR (200 MHz, $CDCl_3$): -5.4, -5.3, -4.8, -4.6, 17.9, 18.5, 25.7, 26.0, 38.7, 62.2, 70.9, 85.6, 79.8, 87.1, 124.3, 127.6, 129.0, 130.6, 133.1; HPLC reverse phase, zorbax C8 column, Eluent: MeOH 95%, H_2O 5%, flow 0.8 mL/min; retention time 4.67 min; ESI-MS m/z 608 $(M +Na)^+$; Anal. Calcd for $C_{30}H_{47}N_3O_5Si_2$: C, 61.50; H, 8.09; N, 7.17. Found: C, 61.42; H, 8.15; N, 7.20.

N^4-Benzoyl-3',5'-bis-*O*-(*tert*-butyldimethylsilyl)-6-{1-[2-(ethylthio)]ethyl}-2'-deoxycytidine (12).

To a solution of N^4-Benzoyl-3',5'-bis-*O*-(*tert*-butyldimethylsilyl)-6-vinyl-2'deoxycytidine (**11)** (171 mg, 0.292 mmol) in CH_2Cl_2 (10 mL), ethanethiol (44 µL, 0.585 mmol) was added and the mixture was stirred at rt for 1 h. After evaporation of the solvent under reduced pressure, the resulting residue was purified on a silica gel column ($CHCl_3$:MeOH, 98:2 v/v) to give pure compound (**12)** (161 mg, 85%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ 0.03 and 0.06 (12H, each as s, 2×$SiMe_2$), 0.87 (18H, each as s, 2×Si^tBu), 1.26 (t, J = 7.7 Hz, 3H), 1.92-2.25 (m, 2H), 2.57 (q, J = 7.7 Hz, 2H), 2.70-3.15 (m, 4H), 3.70-4.05 (m, 3H), 4.50-4.70 (m, 1H), 6.15 (t, J = 6.4 Hz, 1H), 7.38-7.70 (m, 5H), 7.80-7.95 (m, 1H); ^{13}C-NMR (200 MHz, $CDCl_3$): 14.6, 26.2, 34.4, 38.2, 62.0, 71.1, 86.8, 88.3, 127.8, 128.8, 132.8; ESI-MS m/z: 670 $(M+Na)^+$; Anal. Calcd for

$C_{32}H_{53}N_3O_5SSi_2$: C, 59.31; H, 8.24; N, 6.48. Found: C, 59.37; H, 8.16; N, 6.39.

N^4-Benzoyl-6-{1-[2-(ethylthio)]ethyl}-2'-deoxycytidine (13).

TBAF (3.84 mL, 3.84 mmol) was added to a solution of N^4-benzoyl- 3',5'-bis-*O*-(*tert*-butyldimethylsilyl-6-{1-[2-(ethylthio)]ethyl}-2'-deoxycytidine (**12)** (620 mg, 0.96 mmol) in THF (20 mL) at rt. After the solution was stirred for 4 h, the solvent was removed *in vacuo* and the residue was purified by flash chromatography ($CHCl_3$:MeOH 98:2 v/v) to give pure compound (**13)** (318 mg, 79%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.24 (t, *J* = 7.7 Hz, 3H), 1.55-1.85 (m, 1H), 2.08-2.32 (m, 1H), 2.56 (q, *J* = 7.7 Hz, 2H), 2.68-3.20 (m, 4H), 3.72-4.20 (m, 3H), 4.85-5.10 (m, 1H), 6.13 (t, *J* = 6.9 Hz, 1H), 7.35-7.70 (m, 5H), 7.82-8.05 (m, 1H); ^{13}C-NMR (200 MHz, $CDCl_3$): 14.6, 26.2, 34.4, 38.2, 62.0, 71.1, 86.8, 88.3, 127.8, 128.8, 132.8; ESI-MS m/z: 442 $(M+Na)^+$; Anal. Calcd for $C_{20}H_{25}N_3O_5S$: C, 57.26; H, 6.01; N, 10.02. Found: C, 57.26; H, 6.07; N, 10.00.

General procedure for 16 a,b.

Benzoic anhydride (166 mg, 0.73 mmol) was added to a solution of **15a** or **15b** (0.66 mmol) in anhydrous pyridine. The mixture was stirred at 90 °C; after 1 h, the reaction was cooled to rt and dimethoxytrityl chloride (248 mg, 0.73 mmol) was added. The reaction was stirred at rt for 10 h then methanol (10 mL) was added and the resulting solution evaporated to dryness under reduced. The residue was purified on a silica gel column ($CHCl_3$:MeOH, 97:3 v/v) to give pure compound (**16a** or **16b)**.

5'-Dimethoxytrityl-2'-deoxycytidine (**16a**).

^{1}H-NMR spectrum was identical with that of an authentic sample of **16a.**[17]

ESI-MS m/z: 634 $(M+H)^+$; Yield: 70%; Anal. Calcd for $C_{37}H_{35}N_3O_7$: C, 70.13; H, 5.57; N, 6.63. Found: C, 70.22; H, 5.64; N, 6.68.

5'-*O*-Dimetoxytrityl-2'-*O*-(2methoxyethyl)-cytidine (**16b**).

^{1}H-NMR (200 MHz, $CDCl_3$): δ 3.35 (s, 3H), 3.43-3.68 (m, 3H), 3.72-4.02 (m, 7H), 4.02-4.32 (m, 3H), 4.35-4.53 (m, 2H), 5.95 (m, 1H), 6.75-6.98 (m, 3H), 7.15-7.68 (m, 15H), 7.88 (d, *J* = 7.4 Hz, 1H), 8.55 (d, *J* = 7.4 Hz, 1H); ^{13}C-NMR (200 MHz, $CDCl_3$): 55.19, 58.81, 60.91, 68.01, 70.28, 71.51, 83.11, 86.97, 89.42, 96.53, 113.2, 127.0, 127.5, 127.7, 128.0, 128.1, 128.8, 129.1, 129.9, 130.0, 132.9, 135.4, 135.6, 144.96; ESI-MS m/z: 730 $(M+Na)^+$; 1437 $(2M+Na)^+$; Yield: 77%; Anal. Calcd for $C_{40}H_{41}N_3O_9$: C, 67.89; H, 5.80; N, 5.94 Found: C, 67.99; H, 5.98; N, 5.76.

General procedure for 17 a,b.

A suspension of **16a** or **16b** (1,47 mmol), *tert*-butylchlorodimethylsilane (665 mg, 4.42 mmol) and imidazole (600 mg, 8.83 mmol) in dry DMF (8 mL) was stirred for 4 h at rt, then diluted with EtOAc (100 mL), washed successively with H_2O and brine, then dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a crude oil, which was purified on a silica gel column ($CHCl_3$:MeOH, 95:5 v/v) to give a white solid. This intermediate was dissolved in anhydrous CH_2Cl_2 (10 mL), then benzoyl chloride (100 μL, 0.85 mmol) and triethylamine (140 μL, 1.01 mmol) were added, and the mixture was stirred overnight at rt. After dilution with CH_2Cl_2, the mixture was washed successively with 0.01M HCl, H_2O, saturated solution of $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a crude oil, which was purified on a silica gel column ($CHCl_3$:MeOH, 95:5 v/v) to give pure compound (**17a** or **17b).**

***N*^{4}-Benzoyl-3'-*O*-(*tert*-butyldimethylsilyl)-5'-dimethoxytrityl-2'-deoxycytidine** (**17a**).
^{1}H-NMR (200 MHz, $CDCl_3$): δ 0.1 (s, 6H), 0.88 (s, 9H), 2.15-2.30 (m, 1H), 2.50-2.65 (m, 1H), 3.35 (m, 1H), 3.52 (m, 1H), 3.78 (s, 6H), 4.02 (m, 1H), 4.55 (q, *J* = 5.66 Hz, 1H,), 6.25 (t, *J* = 5.68 Hz, 1H), 6.85 (d, *J* = 8.17 Hz, 4H), 7.24-7.42 (m, 12H), 7.46 (d, *J* = 8.17 Hz, 1H), 7.96 (d, *J* = 7.4 Hz, 2H), 8.42 (d, *J* = 7.4 Hz, 1H); ESI-MS m/z: 749 $(M+H)^+$, 771 $(M+Na)^+$; Yield: 99%; Anal. Calcd for $C_{43}H_{49}N_3O_7Si$: C, 69.05; H, 6.60; N, 5.62 Found: C, 69.18; H, 6.76; N, 5.64.

***N*^{4}-Benzoyl-2'-*O*-(2methoxyethyl)-3'-*O*-(*tert*-butyldimethylsilyl)-5'-*O*-dimetoxytritylcytidine** (**17b**).
^{1}H-NMR (200 MHz, $CDCl_3$): δ 0.14 (s, 6H), 0.76 (s, 9H), 3.35 (s, 3H), 3.50-3.65 (m, 4H), 3.68-3.93 (m, 9H), 4.10-4.42 (m, 2H), 6.01 (s, 1H), 6.85 (d, *J* = 8.17 Hz, 4H), 7.24-7.42 (m, 12H), 7.46 (d, *J* = 8.17 Hz, 1H), 7.86 (d, *J* = 7.4 Hz, 2H), 8.73 (d, *J* = 7.4 Hz, 1H); ESI-MS m/z: 823 $(M+H)^+$, 845 $(M+Na)^+$; Yield: 99%; Anal. Calcd for $C_{46}H_{55}N_3O_9Si$: C, 67.21; H, 6.74; N, 5.11 Found: C, 67.11; H, 6.77; N, 5.11.

General procedure for 18 a,b.

A solution of **17a** or **17b** (0.16 mmol) in dry THF (3 mL) was added dropwise to a -78 °C solution of freshly prepared LDA (2.1 mmol) in dry THF (3 mL) at -78 °C under a positive pressure of argon. After 4 h at -78 °C, methyl formate (60 μL, 0.96 mmol) was added, and the reaction mixture was kept under magnetic stirring at -78 °C for 3 h. The mixture was then allowed to warm to rt, diluted with EtOAc and quenched by the addition of saturated NH_4Cl solution. The organic phase was washed successively with water and brine then dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a crude white foam which was used in the next Wittig reaction without further purification.

***N*^{4}-Benzoyl-3'-*O*-(*tert*-butyldimethylsilyl)-5'-dimethoxytrityl-2'-deoxy-6-formylcytidine** (**18a**).

HPLC reverse phase, zorbax C8 column, Eluent: CH_3CN 95%, H_2O 5%, flow 0.4 mL/min; retention time 4.39 min ESI-MS m/z 794 (M+ H_2O +H)$^+$; retention time 4.91 min ESI-MS m/z 808 (M+ CH_3OH +H)$^+$, 830 (M+ CH_3OH +Na)$^+$; retention time 6.74 min ESI-MS m/z 776 (M+H)$^+$; retention time 7.98 min

N^4-Benzoyl-2'-*O*-(2methoxyethyl)-3'-*O*-(*tert*-butyldimethylsilyl)-5'-dimethoxytrityl-6-formylcytidine (**18b**).

HPLC reverse phase, zorbax C8 column, Eluent: CH_3CN 95%, H_2O 5%, flow 0.4 mL/min; retention time 4.71 min ESI-MS m/z 869 (M+ H_2O +H)$^+$; retention time 5.01 min ESI-MS m/z 883 (M+ CH_3OH +H)$^+$, 905 (M+ CH_3OH +Na)$^+$; retention time 6.74 min ESI-MS m/z 851 (M+H)$^+$; retention time 8.51 min

General procedure for 19 a,b.

Methyltriphenylphosphonium bromide (140 mg, 0.390 mmol) and KHMDS 0.5 M (780 μL, 0.390 mmol) were stirred together in dry THF (10 mL) at –78°C under a positive pressure of argon; after 15 min **18a** or **18b** (0.13 mmol) was added, and the mixture was stirred at –78 °C per 15 min and then at 0 °C for 2 h. The mixture was allowed to warm to rt, diluted with EtOAc and quenched by the addition of saturated NH_4Cl solution. The organic phase was washed successively with water and brine then dried over anhydrous Na_2SO_4. After evaporation of the solvent, the crude product was purified on a silica gel column ($CHCl_3$:MeOH, 98:2 v/v) to give pure compound (**19a** or **19b).**

N^4-Benzoyl-3'-*O*-(*tert*-butyldimethylsilyl)-5'-dimethoxytrityl-2'-deoxy 6-vinylcytidine (**19a**).

^{1}H-NMR (200 MHz, $CDCl_3$): δ 0.05 (s, 6H), 0.86 (s, 9H), 1.99-2.12 (m, 1H), 2.59-2.73 (m, 1H), 3.70-4.05 (m, 9H), 4.56 (m, 1H), 5.63 (d, J_{cis} = 11.2 Hz, 1H), 5.98 (d, J_{trans} = 17.1 Hz, 1H); 6.51 (t, J = 7.3 Hz, 1H), 6.68-7.03 (m, 5H), 7.07-7.98 (m, 15H); ESI-MS m/z 775 (M+H)$^+$; Yield: 45% (from **17a**); Anal. Calcd for $C_{45}H_{51}N_3O_7Si$: C, 69.83; H, 6.64; N, 5.43 Found: C, 69.68; H, 6.75; N, 5.40.

N^4-Benzoyl-2'-*O*-(2methoxyethyl)-3'-*O*-(*tert*-butyldimethylsilyl)-5'-dimethoxytrityl-6-vinylcytidine (**19b**).

^{1}H-NMR (200 MHz, $CDCl_3$): δ 0.05 (s, 6H), 0.85 (s, 9H), 3.26 (s, 3H), 3.37-4.05 (m, 13H), 4.48-4.68 (m, 2H), 5.69 (d, J_{cis} = 11 Hz, 1H), 5.86 (d, J = 3 Hz, 1H), 6.02 (d, J_{trans} = 17 Hz, 1H), 6.68-7.03 (m, 5H), 7.07-7.98 (m, 15H); ESI-MS m/z 849 (M+H)$^+$; Yield: 40% (from **17b**); Anal. Calcd for $C_{48}H_{57}N_3O_9Si$: C, 67.98; H, 6.77; N, 4.95 Found: C, 67.99; H, 6.78; N, 4.90.

General procedure for 20 a,b.

To a solution of **19** or **19b** (0.84 mmol) in CH_2Cl_2 (20 mL), ethanethiol (125 μL, 1.69 mmol) was added and the mixture was stirred at rt for 1h. After evaporation of the solvent under reduced pressure, the resulting residue was dried under high vacuum for 2 h, dissolved in anhydrous THF (20 mL) and treated with TBAF (2.85 mL, 2.84 mmol). After stirring at room temperature for 4 h, the solvent was removed *in vacuo* and the residue was purified by flash chromatography ($CHCl_3$:MeOH 98:2) to give pure compound (**20a** or **20b**).

***N*^{4}-Benzoyl-5'-dimethoxytrityl-2'-deoxy-6-{1-[2-(ethylthio)]ethyl}cytidine** (**20a**).
^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.24 (t, J = 7.7 Hz, 3H), 1.55-1.85 (m, 1H), 2.08-2.32 (m, 1H), 2.56 (q, J = 7.7 Hz, 2H), 2.68-3.20 (m, 4H), 3.72-4.20 (m, 3H), 4.85-5.10 (m, 1H), 6.13 (t, J = 6.9 Hz, 1H), 6.75-6.98 (m, 3H), 7.15-7.68 (m, 16H); ^{13}C-NMR (200 MHz, $CDCl_3$): 14.6, 26.2, 34.4, 38.2, 62.0, 71.1, 86.8, 88.3, 113.2, 127.0, 127.5, 127.7, 128.0, 128.1, 128.8, 129.1, 129.9, 130.0, 132.8, 132.9, 135.4, 135.6, 145.0; ESI-MS m/z: 723 $(M+H)^+$; Yield: 64%; Anal. Calcd for $C_{41}H_{43}N_3O_7S$: C, 68.22; H, 6.00; N, 5.82 Found: C, 68.17; H, 6.08; N, 5.80.

***N*^{4}-Benzoyl-5'-dimethoxytrityl-2'-*O*-(2methoxyethyl)-6-{1-[2-(ethylthio)]ethyl}cytidine** (**20b**).
^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.27 (t, J = 7.7 Hz, 3H), 2.60 (q, J = 7.7 Hz, 2H), 2.78-3.12 (m, 4H), 3.27 (s, 3H), 3.33-3.62 (m, 4H), 3.65-3.84 (m, 8H), 3.84-4.01 (m, 1H), 4.54 (dd, $J_{2'\text{-}3'}$ = 5.4 Hz, $J_{3'\text{-}4'}$ = 4.3 Hz, 1H), 4.85 (dd, $J_{1'\text{-}2'}$ = 4.2 Hz, $J_{2'\text{-}3'}$ = 5.4 Hz, 1H), 5.67 (d, $J_{1'\text{-}2'}$ = 4.2 Hz, 1H), 6.74-7.03 (m, 3H), 7.15-7.66 (m, 16H); ^{13}C-NMR (200 MHz, $CDCl_3$): 14.7, 29.7, 29,8, 34.7, 58.8, 63.5, 70.4, 71.5, 72.0, 79.1, 85.7, 91.2, 113.0, 127.1, 127.5, 127.7, 129.8, 128.1, 128.7, 129.1, 129.9, 130.3, 133.0, 133.4, 135.5, 135.8, 144.9; ESI-MS m/z: 797 $(M+H)^+$; Yield: 60%; Anal. Calcd for $C_{44}H_{49}N_3O_9S$: C, 66.40; H, 6.21; N, 5.28 Found: C, 66.41; H, 6.32; N, 5.20.

General procedure for 2 a,b.

Compound (**20a** or **20b** (0.142 mmol) was mixed with diisopropylamine tetrazolide (25 mg, 0.142 mmol) and dried over P_2O_5 *in vacuo* overnight. The resulting mixture was dissolved in anhydrous CH_3CN (5 mL) and 2-cyanoehtyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (180 μL, 0.568 mmol) was added. The reaction mixture was stirred at rt for 4 h under an argon atmosphere, followed by removal of the solvent *in vacuo*. EtOAc (10 mL) was added to the residue, and it was washed with 5% aqueous $NaHCO_3$ (20mL) and brine (20 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The resulting residue was purified by flash silica gel column ($CHCl_3$:MeOH, 98:2 v/v) to give pure compound (**2a** or **2b**)**.**

N^4-Benzoyl-5'-dimethoxytrityl-2'-deoxy-6-{1-[2-(ethylthio)]ethyl}cytidine 3'-*N*,*N*-Diisopropyl (cyanoethyl) phosphoramidite (**2a**).

^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.06-1.40 (m, 15H), 1.55-1.85 (m, 1H), 2.08-2.32 (m, 1H), 2.43-3.20 (m, 8H), 3.70-4.20 (m, 12H), 4.35-4.48 (m, 2H), 6.09 (t, *J* = 6.9 Hz, 1H), 6.75-6.98 (m, 3H), 7.15-7.68 (m, 16H); ^{31}P NMR (600 MHz, CDCl3): δ 151.8, 149.3; ESI-MS m/z: 923 $(M+H)^+$; Yield: 53%; Anal. Calcd for $C_{50}H_{60}N_5O_8PS$: C, 65.13; H, 6.56; N, 7.60 Found: C, 65.28; H, 6.50; N, 7.77.

N^4-Benzoyl-5'-dimethoxytrityl-2'-*O*-(2methoxyethyl)-6-{1-[2-(ethylthio)]ethyl}cytidine 3'-*N*,*N*-Diisopropyl (cyanoethyl) phosphoramidite (**2b**).

^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.04-1.39 (m, 15H), 1.55-1.80 (m, 1H), 2.05-2.29 (m, 1H), 2.42-3.25 (m, 8H), 3.33 (s, 3H), 3.65-4.22 (m, 16H), 4.35-4.48 (m, 2H), 6.10 (t, *J* = 6.9 Hz, 1H), 6.73-7.03 (m, 3H), 7.15-7.68 (m, 16H); ^{31}P NMR (600 MHz, CDCl3): δ 150.5, 149.2; ESI-MS m/z: 997 $(M+H)^+$. Yield: 35%; Anal. Calcd for $C_{53}H_{66}N_5O_{10}PS$: C, 63.90; H, 6.68; N, 7.03 Found: C, 63.95; H, 6.77; N, 7.05.

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