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Synthesis of reactive cytidine derivatives as building blocks for cross-linking oligonucleotides

Marco Radi, Claudia Mugnaini, Elena Petricci, Federico Corelli* and Maurizio Botta*

Dip. Farmaco Chimico Tecnologico, Università degli Studi di Siena., Via A. Moro, 53100 Siena, Italy

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Abstract—6-Vinylcytidine derivative (**1**) possessing good Michael acceptor properties has been synthesized through C-6 formylation and subsequent Wittig reaction. In view of introducing the reactive nucleoside into the oligonucleotide sequence, protection of the vinyl group as ethylthio derivative was proved to be effective for the masking and subsequent regeneration of the reactive vinyl moiety.

After decades and hundreds of million of dollars spent on antisense research, currently a large number of antisense compounds are in preclinical and clinical trials, particularly in the cancer, cardiovascular and infectious disease fields.[1] Main drawbacks for the use of natural oligonucleotides as therapeutical agents are the unsatisfactory binding affinity, the instability against cellular nucleases, the insufficient membrane penetration and the low bioavailability.[2]

In order to overcome the problems associated with the binding affinity and the endonuclease digestion, our work was mainly focused on the synthesis of a modified monomer able to confer nuclease resistance and better binding properties once inserted into a specific oligonucleotide sequence. Based on the good Michael acceptor properties showed by some 6-vinylpyrimidine derivatives recently synthesized in our group,[3] we planned to synthesize the cytidine derivative **1** (Fig. 1): the 6-vinyl group could be able to confer good binding properties through Michael addition reaction while the 2′-*O*-MOE moiety is known to confer nuclease resistance properties.[4]

In order to provide efficient interaction with the target site, reactive groups within the oligonucleotide sequence should have high reactivity and the ability to be in proximity of the target site. While natural nucleosides exist predominantly in the *anti* conformation referred to the glycosyl bond, the introduction of a C-6 substituent on the nucleobase constrains the molecule into a non-natural *syn* conformation.[5] The introduction of the vinyl group in C-6 position of a cytidine derivative could therefore constrain the resulting molecule in an unnatural *syn* conformation, thus allowing a cross-linking reaction with the amino group of a guanosine in the target sequence (Fig. 2).

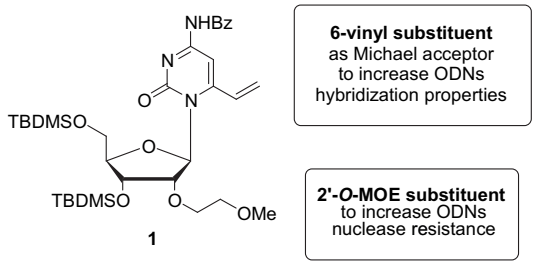

Figure 1. 2′-*O*-MOE-6-vinylcytidine derivative.

Even if 6-substituted pyrimidine oligonucleotides have proved to bring about duplex destabilization,[6] this class of compounds have not been thoroughly studied; moreover, the introduction of a reactive moiety in C-6 position could provide interesting insights into duplex base interactions.

In order to synthesize the target building block **1**, we initially envisaged to use the Stille reaction according to a procedure reported for the introduction of the 6-vinyl

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* Corresponding authors. Tel.: +39 (0)577234306; fax: +39 (0)577234333; e-mail: botta@unisi.it

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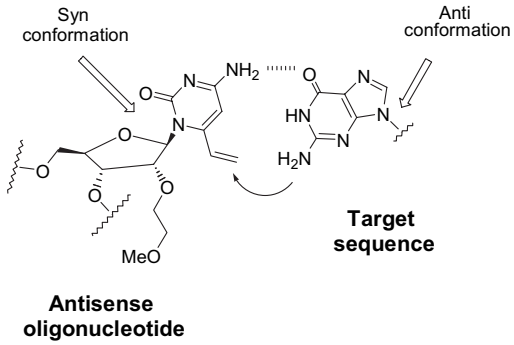


Figure 2. Hypothetical cross-linking reaction between the oligonucleotide bearing the Michael acceptor nucleoside and the guanosine of the target sequence.

moiety into uridine derivatives.[7] To this aim, compound **2** (Scheme 1), synthesized according to a literature procedure,[8] was initially protected in a two-step reaction obtaining **3** in quantitative yield.

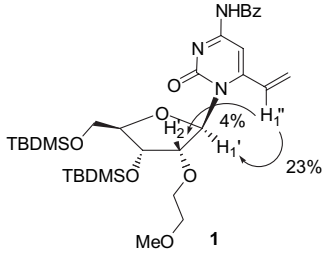
However, the next lithiation and electrophilic substitution never gave in our hands the 6-tributylstannyl or the 6-iodo derivatives for the subsequent Stille reaction. We ascribed the failure of the above described functionalization to the steric bulk of the electrophiles and therefore, the use of the Wittig reaction on the less hindered 6-formyl derivative was planned.[9] Compound **3** was first lithiated and then reacted with methyl formate thus affording the corresponding 6-formyl derivative **4**, whose formation was followed by HPLC–MS analysis.[10]

After a simple filtration on silica, the 40:60 mixture of compounds **3** and **4** was reacted with the suitable Wittig reagent to give the 6-vinyl derivative **1** in a good 60% yield.[11] Unreacted **3** was recovered from the reaction mixture and reused in the formylation reaction to increase the overall yield of **1**.

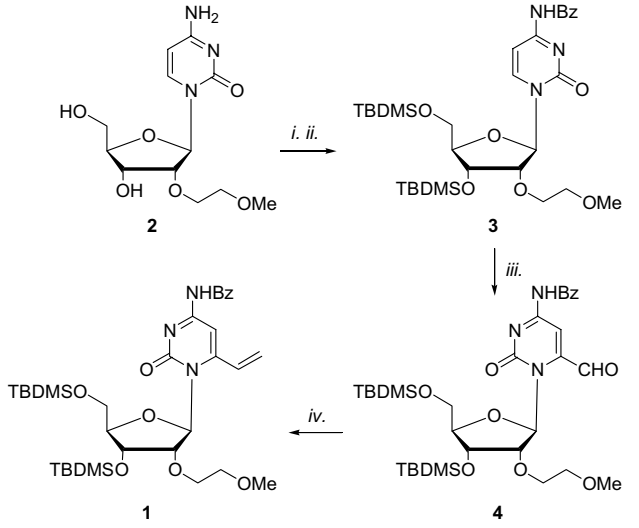
1D NOE difference spectrometry (NOEDS) experiments of **1** (Scheme 2) confirmed the expected *syn* conformation of the glycosyl bond since proton H_1' showed net NOE enhancement upon irradiation of proton H_1''. With this result in hand, we evaluated the ability of **1** to give a Michael addition reaction with a guanosine amino group (Scheme 3).

Guanosine monohydrate **5** was first protected as *t*-butyldimethylsilyl derivative **6** and then reacted in CH_2Cl_2 with compound **1** in a 1:1 stoichiometric ratio. Under these experimental conditions, the slow formation of the adduct **7** was detected after 12 h through MS analysis. However, compound **7** could be obtained in 35% yield after only 2 h carrying out the same reaction in the presence of an acidic catalyst, such as 10-camphorsulfonic acid. According to the work developed by Sasaki and co-workers,[12] we tried to enhance the alkylating properties of compound **1** towards the guanosine amino group by introducing an ethylsulfonyl moiety on the vinyl group (Scheme 4).

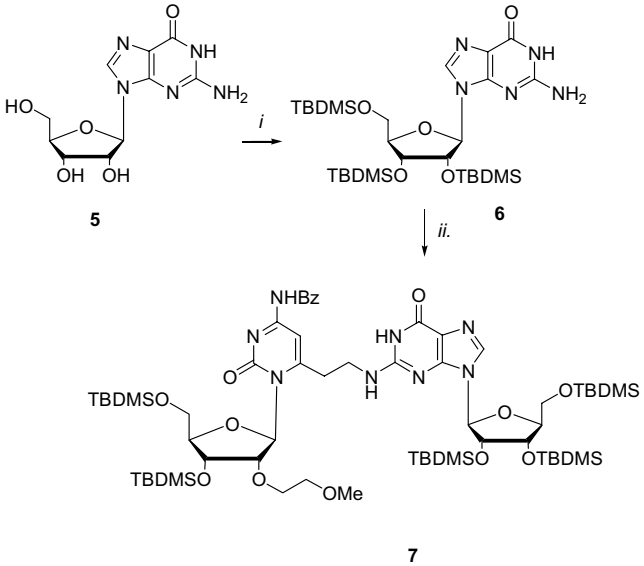
Retrosynthetically, the desired compound **8** could be obtained by reaction of **9** with a base and subsequent sulfur oxidation, while **9** could arise from bromine displacement in **10** obtained by bromination of the double bond



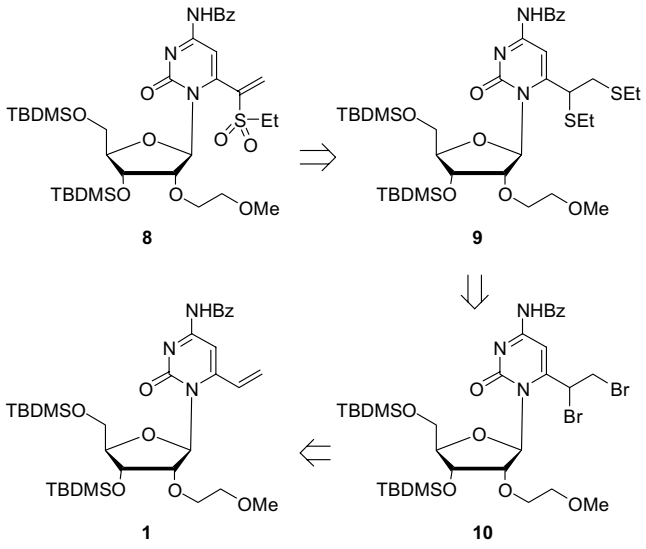
Scheme 2. Important NOE correlations.



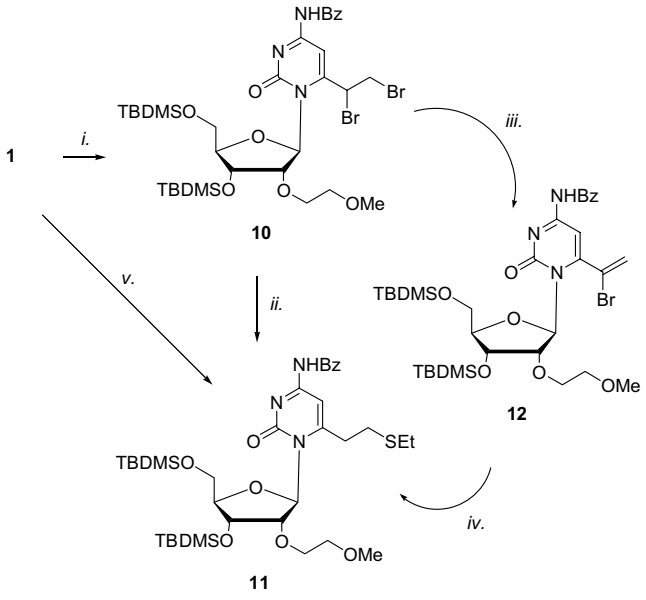
Scheme 1. Reagents and conditions: (i) TBDMSCl, imidazole, DMF, rt, 4 h, 99%; (ii) $(C_6H_5CO)_2O$, DMF, MW 160 °C, 15 min, 99%; (iii) LDA, THF, −78 °C, 3 h then HCOOMe, −78 °C, 3 h; (iv) N-BuLi, THF, $CH_3P(C_6H_5)_3Br$, rt, 3 h, 60%.



Scheme 3. Reagents and conditions: (i) TBDMSCl, imidazole, DMF, rt, 24 h, 58%; (ii) **1**, CH_2Cl_2, CSA, rt, 2 h, 45%.



Scheme 4. Retrosynthetic approach for the synthesis of the ethylsulfonyl derivative **8**.



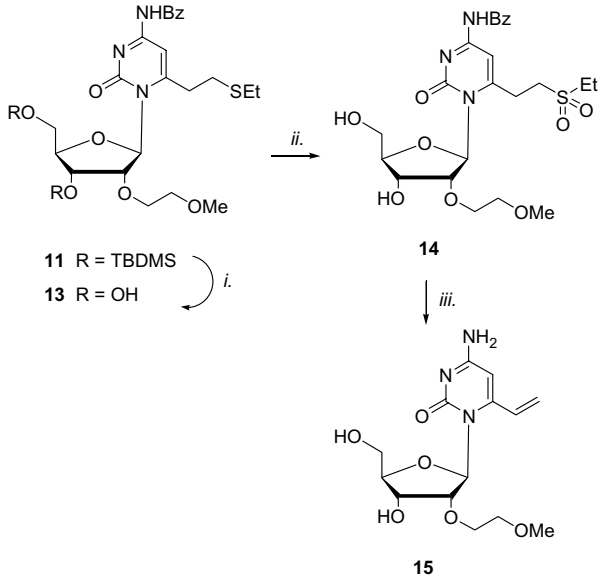
Scheme 5. Reagents and conditions: (i) Br_2, CCl_4, −10 °C, 1.5 h, 87%; (ii) DBU, EtSH, CH_3CN, −10 °C, 2 h, 80%; (iii) DBU, CH_3CN, −10 °C, 2 h, 48%; (iv) EtSH, CH_3CN, −10 °C, 1 h, 75%; (v) EtSH, CH_2Cl_2, rt, 2 h, 84%.

of **1**. Compound **1** was therefore first reacted with bromine in carbon tetrachloride at −10 °C to give the dibromide **10** (Scheme 5). Quite surprisingly, the subsequent bromine substitution to obtain the key intermediate **9** gave the monoethylthio derivative **11** as the only product which was fully characterized through ^1H, ^{13}C NMR, HPLC–MS and COSY experiments.[13]

To better understand the result thus obtained, the reaction progress was monitored by HPLC–MS immediately after the addition of ethanethiol. In this way, the presence in the reaction mixture of both **11** and a monobromo derivative **12**, probably arising from DBU promoted HBr elimination, was highlighted. In order to verify, if **12** is the precursor of **11**, compound **10** was reacted with DBU only, obtaining in this way the monobromo derivative **12**, which showed the same retention time as the hypothesized monobrominated intermediate and whose ^1H NMR spectra clearly showed the presence of two geminal vinyl protons.[14] Finally, compound **11** was obtained by reacting **12** with ethanethiol in CH_3CN at −10 °C.[15] According to these results and in the absence of more details regarding the reaction mechanism at the moment, we can only exclude the progress of the reaction through an initial elimination of bromine from **10** followed by Michael addition. Compound **11** could also be obtained in a comparable yield by simple reaction of **1** with ethanethiol at room temperature, thus highlighting the good Michael acceptor properties of **1** also towards thiols.

This result prompted us to consider **11** as a masked precursor of the reactive vinyl derivative **1**. Therefore, compound **11** was first deprotected to give **13**, which was then oxidized to the corresponding sulfone **14** by means of Oxone®[16] (Scheme 6). Subsequent treatment of the crude sulfone with 0.25 M NaOH gave a one-pot deprotection of the amino group and β-elimination affording **15** in 87% yield.

In conclusion, a facile synthesis of the 6-vinylcytidine derivative **1** as well as its Michael acceptor properties have been described. Moreover, an ethylthio moiety was proved to be effective for the protection of the vinyl group and easily removable under mild alkaline conditions after selective oxidation with Oxone®. This strategy could be favourably used during the subsequent oligonucleotide synthesis thus avoiding unwanted side reactions on the reactive vinyl moiety. Further studies on the introduction of compound **13** into a specific oligonucleotide sequence are underway.



Scheme 6. Reagents and conditions: (i) TBAF, THF, rt, 1 h, 87%; (ii) Oxone®, MeOH/H_2O, rt, overnight; (iii) NaOH 0.25 M, rt, 2 h, 80%.

Acknowledgements

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10. Procedure for the preparation of **4**: A solution of **3** (708 mg, 1.12 mmol) in anhydrous THF (20 mL) was added dropwise to a freshly prepared solution of LDA (14.54 mmol) in anhydrous THF (20 mL) kept at −78 °C. After 4 h, HCOOMe (896 μL, 29.08 mmol) was added and the reaction mixture was stirred for 3 h at −78 °C, then allowed to warm to room temperature, diluted with EtOAc and quenched by the addition of NH_4Cl saturated solution. The organic phase was washed successively with water and brine then dried over anhydrous Na_2SO_4. Evaporation of the solvent gave **4** as white foam which was used without further purification.
11. Procedure for the preparation of **1**: A solution of BuLi (1.6 M in hexane, 1.28 mL, 2.04 mmol) was added to a suspension of methyltriphenylphosphonium bromide (729 mg, 2.04 mmol) in THF (10 mL) kept at −78 °C. The mixture was stirred at 0 °C for 30 min, followed by the addition of a solution of **4** (270 mg, 0.41 mmol) in THF (10 mL). The reaction mixture was stirred for 1 h at room temperature, then a saturated NH_4Cl solution was added followed by EtOAc. 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 chromatographed on a silica gel column (eluent: $CHCl_3$/MeOH, 98/2) to give pure **1** (169 mg, 60%).
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13. Procedure for the preparation of **11** starting from **10**: A solution of **10** in CH_3CN (5 mL) was added dropwise to a solution of ethanethiol (6.5 μL, 0.086 mmol) and DBU (13 μL, 0.086 mmol) in CH_3CN (5 mL) kept at −10 °C. The reaction mixture was stirred at −10 °C for 2 h then CH_3CN was removed under reduced pressure and the residue was dissolved in $CHCl_3$. The organic phase was washed successively with 5% HCl, water and brine then it was dried over anhydrous Na_2SO_4. Evaporation of the solvent gave an oil, which was chromatographed on a silica gel column (eluent: $CHCl_3$/MeOH, 98/2) to give **11** (23 mg, 80%) as a pure compound.
14. Procedure for the preparation of **12**: To a stirred solution of **10** (25 mg, 0.038 mmol) in THF (5 mL), DBU (13 μL, 0.084 mmol) was added at −10 °C. Stirring was continued at −10 °C for 30 min and at room temperature for 5 h. DBU hydrobromide was filtered off and the solution was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 and then washed successively with water, brine and dried over anhydrous Na_2SO_4. Evaporation of the solvent gave an oil, which was chromatographed on a silica gel column (eluent: $CHCl_3$/MeOH, 98/2) to give pure **12** (13.5 mg, 48%).
15. Procedure for the preparation of **11** starting from **12**: To a solution of **12** (556 mg, 0.84 mmol) in CH_2Cl_2 (20 mL), ethanethiol (125 μL, 1.69 mmol) was added and the reaction mixture was stirred for 1 h at room temperature. After evaporation of the solvent under reduced pressure, the resulting residue was chromatographed on a silica gel column (eluent: $CHCl_3$/MeOH, 98/2) to give **11** (456 mg, 75%) as a pure compound.
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