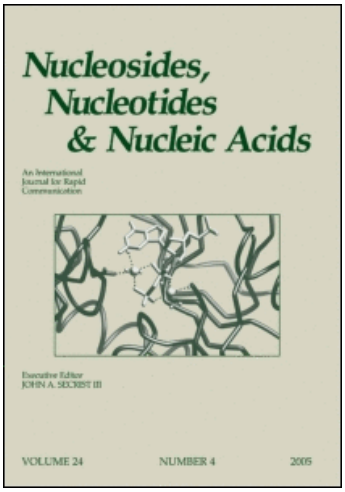

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A Simple and Efficient Procedure for the Synthesis of an Alendronate-Oligonucleotide Conjugate via a Carbamate Linker

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A SIMPLE AND EFFICIENT PROCEDURE FOR THE SYNTHESIS OF AN ALENDRONATE-OLIGONUCLEOTIDE CONJUGATE VIA A CARBAMATE LINKER

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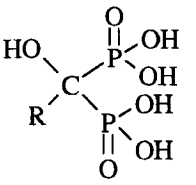
Abstract : In order to combine the biological properties of oligonucleotides, a synthetic chemical modelized reaction was performed and the procedure then applied to the preparation of an alendronate-deoxyoligonucleotide conjugate through a carbamate linker.

Introduction

Oligonucleotides and analogues are promising new therapeutics agents. One of the most studied field is their use in the antisens strategy [1]. A short nucleotide may be synthesized to be complementary to a target m-RNA transcript, thus permitting hybridization in an antiparallel orientation through Watson Crick base pairing. The formation of this DNA-RNA heteroduplex involves the m-RNA inactivation. Several synthetic oligonucleotides are in clinical evaluation now for a wide array of disease targets particularly in oncology [2]. In another approach, the use of gene therapy is the subject of intensive investigation [3]. The biological target is DNA. The synthetic oligonucleotide may bind to DNA to form a triple helix. This specific interaction is sequence dependent and can stop the gene transcription. Others targets can be aimed at such as ribozyme [4], protein receptor [5], enzyme inhibition [6].

Bisphosphonates **1** have a large therapeutic interest too. They are the stable analogues of

the endogenous inorganic metabolite of pyrophosphate in which the labile phosphoanhydride bond $(HO)_2P(O)$-O-$P(O)(OH)_2$ is replaced by a stable hydroxymethylene group $(HO)_2P(O)$-C(OH)R-$P(O)(OH)_2$.

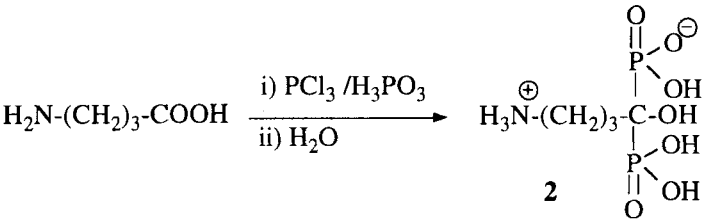


1

These compounds adsorb bone mineral and inhibit bone resorption [7]. Bisphosphonates are effective in others clinical disorders such as Paget's bone disease [8], myeloma [9], bone metastases [10] and osteoporosis [11]. Although the action mechanism is not completely clear, one of the hypothesis is that the bisphosphonates are involved in cellular mechanism and act by suppressing osteoclast activity [12]. However, all bisphosphonates are poorly (typically less than 1 %) absorbed from the gastrointestinal tract. Moreover, their oral administration is associated with gastrointestinal disturbance and other side effects [13].
The use of a peptidic carrier was shown to increase the drug absorption [14]. The results were very promising because the efficiency was improved by 100 %. Another method suggests encapsulating the drugs into liposomes to increase the therapeutic effects of bisphosphonates [15]. Bisphosphonates are used in nuclear medicine too. They form a stable complex with ^{99m}Tc and are routinely used in skeletal scintigraphy for delineation of bone metastases [16]. Furthermore, some studies indicate that bisphosphonate conjugates of a well-known antineoplasic agent behave like bone-seeking agents and exhibit an increased antineoplasic activity [17].

The pharmacological potential of these two moieties encouraged us to bind a bisphosphonate at the 5' OH of an oligonucleotide. This oligonucleotide bisphosphonate conjugate is interesting for different reasons. The presence of the oligonucleotide moiety could allow the understanding of bone cellular mechanism and specific tissue targeting such as bone metastasis.

Different approaches may be considered to realize the coupling reaction between the oligonucleotide and the bisphosphonate. In this study, we will describe a procedure that makes possible the coupling of a fully deprotected bisphosphonic acid to the OH group of an oligonucleotide. This method uses the highly reactive N,N'-carbonyldiimidazole (CDI) to activate the unreactive 5'-hydroxyl group as its 5'-imidazolyl derivative which can undergo nucleophilic displacement reaction in the presence of an alkylamino compound [18]. This last one could be 4-amino-1-hydroxy-butylidene-1,1-bisphosphonic acid **2** (alendronate) already prepared in our laboratory (scheme 1) [19] and already commercially available from Merck .

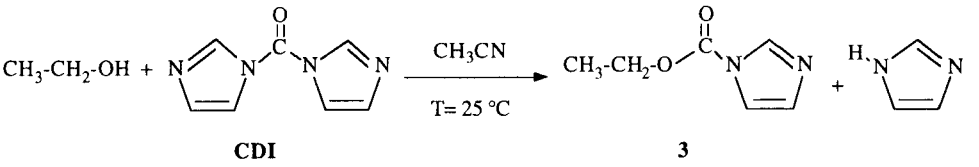


Scheme 1

Realizing this coupling directly on the oligonucleotide would lead to some limitation of cost and quantities. So we decided first to find the best operating conditions for the coupling reaction, studying the alendronate reactivity with an activated alcohol which looks like the 5' OH end of an oligonucleotide.

Results and discussion

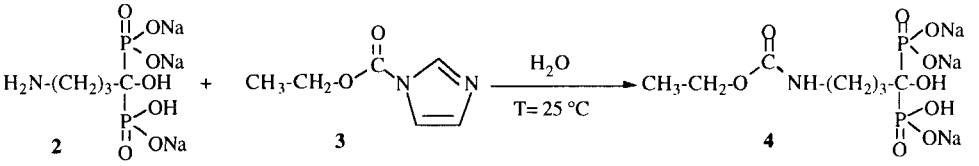
In order to get clear NMR spectra, a simple aliphatic alcohol, ethanol was first activated with CDI (scheme 2).



Scheme 2

In many cases, the activation with CDI has been made in dioxane as solvent [18, 20]. We preferred to carry out the reaction in acetonitrile because this solvent is well suited to the synthesis of oligonucleotides on the solid phase. In these conditions, the formation of the imidazolyl derivative **3** is very rapid and efficient (yield=80 %).

The coupling reaction with the alendronate is more difficult because we didn't want to protect the phosphonic acid groups so as to limit the number of synthesis steps (scheme 3).



Scheme 3

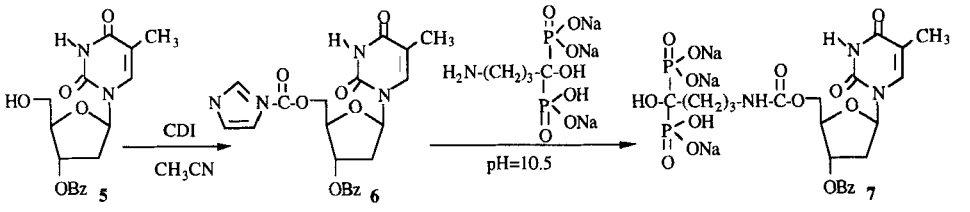
Table 1

Assay	pH	Time of the reaction	% of carbamate adduct **4**
1	8	24 h	30
2	9.5	24 h	50
3	10.5	24 h	90

In many of their papers [21], Bethell and coworkers have shown that the CDI-activated groups are reasonably stable to aqueous hydrolysis but are extremely susceptible to nucleophilic attack. Further they found that the formed carbamate is stable at room temperature over a wide pH range (2.5- 12). In our case, it was necessary that the amino function of the alendronate should not be protonated. We then investigated the pH influence on the course of the coupling reaction by ^{31}P NMR because the chemical shifts of the carbamate adduct **4** (19.3 ppm) and the unreacted alendronate **2** (18.9 ppm) are indeed different.

The results in table 1 show that he pH is the factor which govern the efficiency of the reaction. If the pH is slightly basic (assay : 1, 2), the coupling reaction is less effective. The amino form of the alendronate is in equilibrium with the ammonium form as the pKa value (10.77) indicates [22]. All the amino forms react with the CDI-activated alcohol and the reaction stops itself for the hydrolysis of the carbamoyl derivative. When the pH is higher(i.e.>12), the amino group is deprotonated and becomes the only species able to react in the nucleophilic substitution. The pH value could not be increased further to make the process available without degradation of the oligonucleotides on the polymer support.

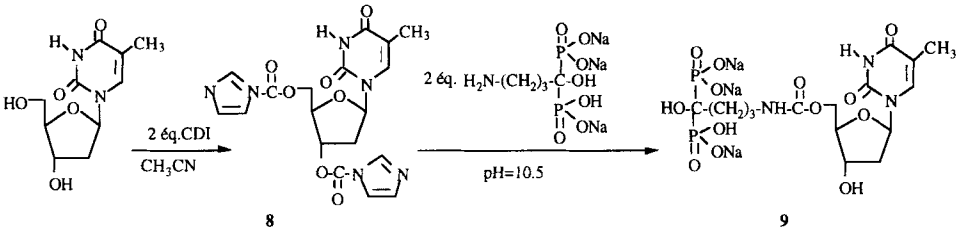
This method was next tested with a nucleoside, the thymidine. To begin with, the 3'-O-benzoyl thymidine **5** was synthesized [23] and we investigated the reactivity of the 5'-hydroxyl group (scheme 4).



Bz : benzoyl

Scheme 4

The 5'-hydroxyl group was activated with CDI to give carbamoyl derivative **6** which was coupled with alendronate to obtain **7**. The yield was poor because we observed a side reaction which is the benzoyl ester saponification. The investigation was therefore continued testing the reactivity with the two free hydroxyl groups of the thymidine (scheme 5).



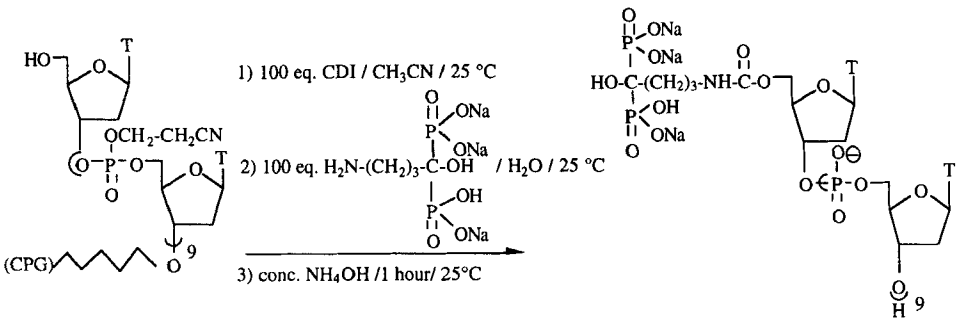
Scheme 5

After the usual activation process, the ^{1}H NMR spectrum indicated that only compound **8** was obtained. We observed two groups of two peaks characteristic of imidazolyl proton, showing clearly, using the NMR integration, that the two hydroxyl groups were activated. The addition of two equivalents of alendronate led to compound **9**. The ^{31}P NMR spectrum showed two peaks of equal intensity, the monocoupling product (19.4 ppm) and the alendronate (18.8 ppm). The electrospray ionization ion trap mass spectrometry experiment confirmed this hypothesis (M = 517 Da). The ^{1}H and ^{13}C NMR spectra analysis indicated that only the 5' CDI-activated group reacted with the amino group of the side chain of the alendronate. The 3' position seemed to be unreactive. This lack of reactivity can be attributed to steric hindrance which prevents the nucleophilic attack.

These previous results obtained on these models have shown that the conditions of the activation and the coupling reaction are controlled. We can therefore follow this work in oligonucleotide series. Further displacement reaction of the activated oligomer (still attached to the support by its 3'-end) will be performed prior to treatment with concentrated ammonia which is used for cleavage and deprotection. We also verified this carbamate linkage stability by treating compound **4** in concentrated ammonia at 55°C during 18h ; no degradation was observed.

The coupling reaction between the 10-mer T_{10} was realized according to scheme 6. The 5'-hydroxyl group was activated at room temperature with an excess CDI (100 eq.) in anhydrous acetonitrile for 12 h, without monitoring the progress of the reaction. The activated oligomer was then allowed to react with an aqueous solution of alendronate sodium salt (pH = 10.5) and the reaction was allowed to proceed for 12 h.

To cleave the oligomer from the support and to remove the phosphate-protecting groups, aqueous ammonia was added at room temperature for one hour.



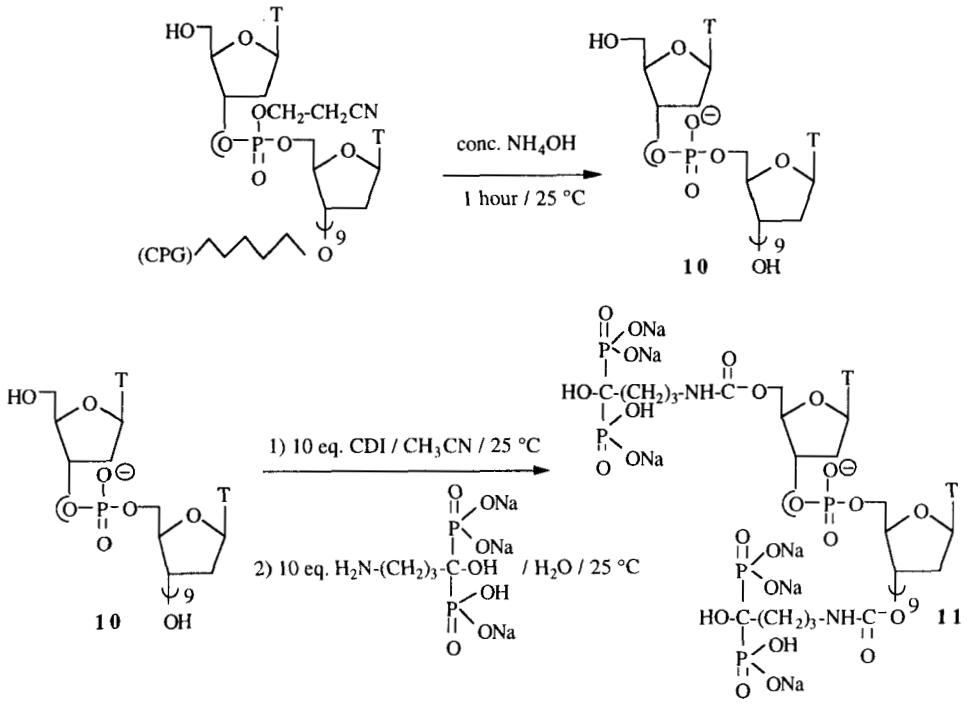
Scheme 6

The crude sample was then analyzed on an analytical HPLC reverse phase C18 column (TEAA pH = 7.00 / CH_3CN gradient ; detection λ=264 nm). The HPLC profile showed the presence of a single peak (retention time: 36.6 min). The ^{31}P NMR analysis indicated that the bisphosphonate was not coupled to the oligonucleotide. The presence of one peak (0.1 ppm) due to phosphate chemical shift was observed. Different operating conditions such as the time of the reaction and the alendronate concentration were tested but without success.

Another approach was to incorporate the bisphosphonate to the oligomer in solution. The oligonucleotide synthesis was performed as previously described and the oligomer was cleaved from the support with aqueous ammonia. At the same time, the phosphate protecting group was removed. The coupling reaction in solution between the oligonucleotide and the alendronate was realized as in scheme 7.

After the reaction, the analytical HPLC profile showed a retention time difference of 1 min. between the dT_{10} oligonucleotide **10** (retention time : 36.6 min) and modified oligonucleotide **11** (retention time : 35.6 min). After purification by semi-preparative C18 HPLC, the product was analyzed by ^{31}P NMR.

The ^{31}P NMR spectrum analysis shows the presence of two groups of signals. We observed one peak with a chemical shift of 0.1 ppm characteristic of phosphate and two others upfield peaks (19.1 and 19.4 ppm) due to two different bisphosphonate chemical shifts. This result was confirmed by electrospray ionization ion trap mass spectrometry analysis (M= 3528 Da). These data indicate that the activation and the coupling reactions are efficient in homogeneous phase with an oligonucleotide.



Scheme 7

Conclusion

In this work, we described a simple method for introducing a fully deprotected amino hydroxybisphosphonic acid to an imidazolyl activated alcohol via the amino function. In order to combine the biological properties of alendronate and oligonucleotide, we realized the binding of this bisphosphonic acid on the 3' and 5' positions of the oligonucleotide.

Experimental Part

Ethyl alcohol (99.95%, analytical reagent, PROLABO), $CHCl_3$ (HPLC grade, PROLABO) and CH_3CN (HPLC grade, PROLABO) were dried by means of 4 Å molecular sieves. Pyridine was distilled over KOH and was stored over 4 Å molecular sieves. N,N'-carbonyldiimidazole was purchased from AVOCADO and ACROS, aqueous ammonia (32%, extra pure) from MERCK. All the phosphoramidite nucleosides and reagents for oligonucleotides synthesis were purchased from EUROGENTEC. For column chromatography, silica gel 60 (0.04-0.063 mm) was used and TLC was carried out on silica gel 60 F 254 plates (0.25 mm, Merck, Darmstadt, FRG). NMR experiments were performed on a VARIAN Unity Inova 500 spectrometer at 500.6 MHz for proton,

200.7 MHz for phosphorous and 125.9 MHz for carbon. The ^{31}P NMR and ^{13}C NMR spectra were recorded using phosphoric acid and methanol as external reference respectively. Tetramethylsilane and HOD were used as internal standards in $CDCl_3$ and D_2O for the ^1H NMR spectra. IR spectra were performed on a PYE UNICAM SP3-300S Infrared Spectrometer. Oligonucleotide synthesis is realized on an automatic APPLIED BIOSYSTEMS-391 DNA synthesizer. HPLC analysis was achieved on a MERCK system : 655A-12 liquid chromatograph, L-5000 LC controller, L3000 multichannel photodetector, D2500 chromato integrator. The electrospray ionization ion trap mass spectrometry (ESI-ITMS) was performed with a ESQUIRE nanospray / ion trap mass spectrometer (Bruker-Franzen Analytic, GmbH, Bremen, Germany). Samples were dissolved in triethylammonium acetate solution (10 mM) at a final concentation of 20 pmol/µl. Data were aquired in the negative ionization mode from m/z = 100 to m/z=1000 in 200 ms.

Preparation of alendronate **2** : see reference 19

Preparation of -3'-O-benzoylthymidine **5** : see reference 23

General procedure for the activating reaction

CDI (1 mmol) in 15 ml of dried CH_3CN is placed in a three-necked flask equipped with a mechanical stirrer, an addition funnel, a thermometer and a nitrogen inlet tube. Hydroxylated compound (1 mmol) in 5 ml of CH_3CN was slowly added to the stirred solution (exothermic reaction). The mixture is stirred 12 hours at room temperature. The solvent was evaporated under vacuum. The residue was dissolved in $CHCl_3$ (50 ml), the organic layer was washed twice with H_2O (2 x 20 ml) and dried over Na_2SO_4. The solvent was evaporated and the product was used without purification.

Imidazolyl activated ethanol **3**

Yield = 87%. Light yellow oil .

IR (film) : ν (CO) = 1760 cm^{-1}

^1H NMR ($CDCl_3$) : 8.14 (s, 1H, imidazolyl) ; 7.43 (s, 1H, imidazolyl) ; 7.06 (s, 1H, imidazolyl) 4.48 (q, 2H, $^3J_{H-H}$ = 7 Hz, OCH_2- CH_3) ; 1.44 (t, 3H, $^3J_{H-H}$ = 7 Hz, OCH_2-CH_3).

$^{13}C\{^1H\}$ NMR ($CDCl_3$) : 148.7 (*C*=O) ; 136.6, 129.9, 116.7 (imidazolyl) ; 64 (0-CH_2) ; 13.5 (OCH_2-CH_3).

Imidazolyl activated 3'-O-benzoylthymidine **6**

Yield = 86 %. White powder .

IR (pellet) : ν (CO carbamate) = 1760 cm^{-1} ; ν (CO ester) = 1710 cm^{-1} ; ν (CO thymidine) = 1690 cm^{-1}.

^1H NMR ($CDCl_3$) : 8.73 (s, 1H, N*H*) ; 8.14 (s, 1H, imidazolyl) ; 7.98 (d, 2H, $^3J_{H-H}$=8 Hz, C_6H_5) ; 7,56 (t, 1H, $^3J_{H-H}$ = 8Hz, C_6H_5) ; 7.52 (s, 1H, *H*6) ; 7.41 (t, 2H, $^3J_{H-H}$ = 8 Hz, C_6H_5) ; 7.39 (s, 1H, imidazolyl) ; 7,07 (s, 1H, imidazolyl) ; 6.27 (dd, 1H, $^3J_{H-H}$ = 7.5 Hz, $^3J_{H-H}$ = 6 Hz, *H*1') ; 5.52-5.48 (m, 1H, *H*3') ; 4.78-4.70 (m, 2H, *H*5') ; 4.42-4.38 (m, 1H, *H*4'); 2.63 (ddd, 1H, $^2J_{H-H}$ = 14.5 Hz, $^3J_{H-H}$ = 6Hz, $^3J_{H-H}$ = 2 Hz, *H*2a') ; 2.40-2.63 (m,1H, *H*2b') 1.82 (s, 3H, CH_3).

$^{13}C\{^1H\}$ NMR ($CDCl_3$) : 166.3 (*C*O-C_6H_5) ; 163.5 (NH*C*O thymidine) ; 150.4 (NH*C*O-N thymidine) ; 149.2 (O*C*O-imidazolyl) ; 137.5 (*C*6); 135.1 (imidazolyl) 134.2, 134.1, 130.1, 129.0 (C_6H_5), 117.3 (imidazolyl) ; 112.3 (*C*7) ; 86.0 (*C*1') ; 82.1 (*C*3') ; 74.4 (*C*4') ; 68.3 (*C*5') ; 37.3 (*C*2') ; 12.8 (C7- CH_3).

Imidazolyl activated thymidine **8**

Yield = 82 %. White powder .

IR (pellet) : ν (CO carbamate) = 1770 cm^{-1} ; ν (CO thymidine) = 1690 cm^{-1}.

^1H NMR ($CDCl_3$) : 9.98 (s, 1H, N*H*) ; 8.26 (s, 1H, imidazolyl) ; 8.14 (s, 1H, imidazolyl) ; 7.45 (s, 1H, *H*6) ; 7.44 (s, 2H, imidazolyl) ; 7,12 (s, 1H, imidazolyl) ; 7,09 (s, 1H, imidazolyl) ; 6.17 (t, 1H, $^3J_{H-H}$=6.5 Hz, *H*1') ; 5.71-5.68 (m, 1H, *H*3') ; 4.83-4.73 (m, 2H, *H*5') ; 4.54-4.50 (m, 1H, *H*4'); 2.79-2.73 (m, 1H, *H*2a') ; 2.70-2.63 (m,1H, *H*2b') 1.88 (s, 3H, CH_3).

$^{13}C\{^1H\}$ NMR ($CDCl_3$) : 164 (NH*C*O thymidine) ; 150.4 (NH*C*O-N thymidine) ; 147.8 (O*C*O-imidazolyl) ; 147.1 (O*C*O-imidazolyl) ; 137.2, 136,8 (imidazolyl) ; 135.6 (*C*6) ; 135.1, 134,8 (imidazolyl); 117.0, 116.8 (imidazolyl) ; 111.5 (*C*7) ; 86.6 (*C*1') ; 81.1 (*C*3') ; 77.2 (*C*4') ; 66.7 (*C*5') ; 36.2 (*C*2') ; 12.7 (C7- CH_3).

General procedure for the coupling reaction

The CDI-activated compound (1 mmol) in 1 ml of CH_3CN is placed in a three-necked flask (50 ml) equipped with a mechanical stirrer, an addition funnel, a thermometer and a nitrogen inlet tube. The pH of the alendronate solution (1 mmol in 5 ml of water) is raised to 10.5 by addition of a concentrated sodium hydroxide solution. This solution is added dropwise. The mixture is stirred 24 hours at room temperature. The solution is washed twice with chloroform (2 x 20 ml). The aqueous layer is concentrated under vacuum and lyophilized.

Compound **4**

Yield = 82%. White powder .

$^{31}P\{^1H\}$ NMR (D_2O) : 19.3 ppm

^1H NMR (D_2O) : 4.05 (q, 2H, $^3J_{H-H}$=7.5 Hz, OCH_2- CH_3) ; 3.11 (t, 2H, $^3J_{H-H}$ = 6.5 Hz, NH-CH_2) ; 1.90-1.86 (m, 2H, CH_2-CH_2) ; 1.78-1.74 (m, 2H, CH_2-COH) ; 1.20 (t, 3H, $^3J_{H-H}$=7.5 Hz, OCH_2- CH_3).

$^{13}C\{^1H\}$ NMR (D_2O) : 159 (*C*=O) ; 74 (t, $^2J_{C-P}$ = 128 Hz, *C*-OH) ; 64 (0-CH_2) ; 41.2 (NH*C*H_2) ; 31.3 (*C*H_2-CH_2) ; 24.3 (t, $^3J_{C-P}$=9 Hz, *C*H_2-C-OH) ; 13.5 (OCH_2-*C*H_3).

Compound **7**

Yield = 32%. White powder.

$^{31}P\{^1H\}$ NMR (D_2O) : 21.0 ppm

^1H NMR (D_2O) : 8.07 (d, 2H, $^3J_{H-H}$=8 Hz, C_6H_5) ; 7.68 (t, 1H, $^3J_{H-H}$= 8Hz, C_6H_5) ; 7.55 (s, 1H, *H*6) ; 7.52 (t, 2H, $^3J_{H-H}$=8 Hz, C_6H_5) ; 6.42 (dd, 1H, $^3J_{H-H}$=9 Hz, $^3J_{H-H}$ = 5 Hz, *H*1') ; 5.60-5.58 (m, 1H, *H*3') ; 4.30-4.20 (m, 2H, *H*5') ; 4.42-4.38 (m, 1H, *H*4') ; 3.23-3.11 (m, 2H, NH-CH_2) ; 2.64-2.55 (m, 2H, H_{2a}) ; 2.55-2.41 (m, 2H, H_{2b}) ; 1.94-1.89 (m, 2H, CH_2-CH_2) ; 1.20 (s, 3H, CH_3) ; 1.80-1.70 (m, 2H, CH_2-COH).

Compound **9**

Yield = 74%. White powder.

IR (pellet) : ν (OH) = 3600-2400 cm^{-1} ; ν (CO carbamate) = 1760 cm^{-1} ; ν (P=O) = 1260 cm^{-1}; ν (P-O) = 1050 cm^{-1}.

ESI-ITMS : M = 578 Da

$^{31}P\{^1H\}$ NMR (D_2O) : 19.5 ppm

^1H NMR (D_2O) : 7.36 (s, 1H, *H*6) ; 6.12 (dd, 1H, $^3J_{H-H}$= 7.5 Hz, $^3J_{H-H}$= 12.5 Hz; *H*1') ; 5.10-4.98 (m, 1H, *H*3') ; 4.29-4.20 (m, 2H, *H*5a') ; 4.13-4.00 (m, 1H, *H*5'b) ; 3.73-3.60 (m, 1H, *H*4) ; 3.11-2.97 (m, 2H, NH-CH_2) ; 2.40-2.73 (m, 1H, *H*2) ; 1.94-1.89 (m, 2H, CH_2-CH_2) ; 1.74 (s, 3H, CH_3) ; 1.69-1.62 (m, 2H, CH_2-COH).

$^{13}C\{^1H\}$ NMR (D_2O) : 167.7 (NH*C*O thymidine) ; 158.8 (O*C*O carbamate) ; 152.8 (NH*C*O-N thymidine) ; 138.5 (*C*6) ; 112.7 (*C*7) ; 86.1 (*C*1') ; 83.9 (*C*3') ; 74.5 (t, $^2J_{C-P}$= 128 Hz, *C*-OH) ; 73.5 (*C*4') ; 62.4 (*C*5') ; 41.1 (NH*C*H_2) ; 37.6 (*C*2') ; 31.3 (*C*H_2-CH_2) ; 24.3 (t, $^3J_{C-P}$=7 Hz, *C*H_2-C-OH) ; 12.7 (C7- *C*H_3).

Synthesis of bisphosphonate oligonucleotide conjugate

- *Oligodeoxynucleotide synthesis*

The decamer T_{10} was synthesized using the solid-phase phosphoramidite method [24] on the 10 µmol scale. The synthesizer was programmed Dmt-off.

- *Cleavage and phosphate deprotection*

The oligomer was cleaved from the support with concentrated ammonia (6 ml) progressively injected and collected for 1h15. The ammonia solution was removed under vacuum leaving a white residue .

- Activation reaction

CDI (0.1 mmole) was dissolved in dry acetonitrile (1 ml) and oligonucleotide (10 µmol) in 1ml of dry acetonitrile was added. The mixture was stirred 12 hours at room temperature.

- Coupling reaction

Alendronate (0.1 mmol) was dissolved in 1 ml of water and the pH of the solution was raised to 10.5 by a concentrated sodium hydroxide solution. This solution is added dropwise to the solution of activated oligonucleotide. The mixture is stirred 12 hours at room temperature. The solution was concentrated under vacuum and lyophilized.

- Purification of alendronate-oligonucleotide conjugate II

The compound **11** was purified by reverse phase HPLC on a semi-preparative Nucleosil C18 5 µm column (250 mm x10mm ID) using a linear gradient from solvent A (Triethylammonium acetate 0.1M, pH=7.00) to solvent B(Acetonitrile). The gradient was run from 0 % to 25 % B for 60 min. with a flow rate of 3 ml/min.. The modified oligonucleotide is eluted at 35.6 min. The fractions containing the product were concentrated under vacuum and lyophilized. ^{31}P NMR and ES-MS spectroscopy were used for the characterization of this modified oligonucleotide.

$^{31}P\{^{1}H\}$ NMR (D_2O) : 19.5 ; 19.2 ; 0.1 ppm.

ESI-ITMS : M = 3528 Da

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