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Synthesis of the Phosphodiester of 3β(7β-hydroxycholesterol) and of 5'(3'deoxy, 3'azido-thymidine).

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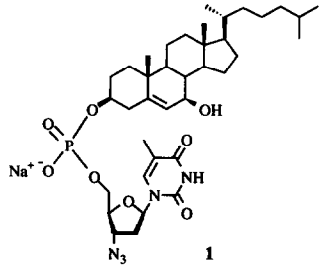
Abstract : In order to enhance the therapeutic efficacy of azido-thymidine (AZT) by improving its pharmacokinetic properties and to try to target its action to the lymphocytes, the phosphodiester of 3β(7β-hydroxycholesterol) and of 5'(3'deoxy, 3'azido-thymidine) **1** was synthesized using two different techniques of phosphorylation : the phosphoramidite and the hydrogen phosphonate methodologies. Preliminary results show an *in vitro* anti-HIV activity.

Introduction

3'Azido 3'deoxy thymidine (AZT) is a potent inhibitor of HIV replication and the first clinically successful drug for AIDS and AIDS related symptoms[1].

Pharmacokinetic studies in humans have shown that AZT has too short a plasma life[2], which is mainly ascribed to 5'-O-glucuronidation[3] resulting in rapid elimination. Thus, administration of higher dosages is required for maintaining adequate therapeutic drug levels in plasma, which leads to bone marrow toxicity[4]. Therefore, it was reasoned that 5'-O-conjugates of AZT in prodrug forms may significantly overcome the drawbacks of AZT and improve its efficacy.

Recently, several research groups have reported the synthesis of 5'-O-derivatives of AZT with amino acids[5-6], sugars[7], lipids[8] and steroids[9]. In these prodrugs, the conjugating moiety is linked to AZT through a 5'-O-ester or 5'-O-phosphate group. By comparison with AZT, some of the prodrugs show similar anti-HIV activity with much improved pharmacokinetic properties such as increased plasma half-life, lipophilicity and cell uptake, and decreased toxicity. These results clearly demonstrate the encouraging potential of a prodrug approach in improving the therapeutic profile of AZT. Prodrugs with phosphate linkages, deliver the parent compound as its monophosphate, thereby overcoming the problem of poor monophosphorylation[9]. These derivatives are also very stable in plasma[10].



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We therefore decided to synthesize the phosphodiester of 3β(7β-hydroxycholesterol) and of 5'(3'deoxy, 3'azido-thymidine) **1**. As a conjugating moiety, 7β-hydroxycholesterol was choosen for its lipophilic character, its lymphocyte membrane affinity[11] and its ability to reduce CD_4 receptor activities at the cell surface[12].

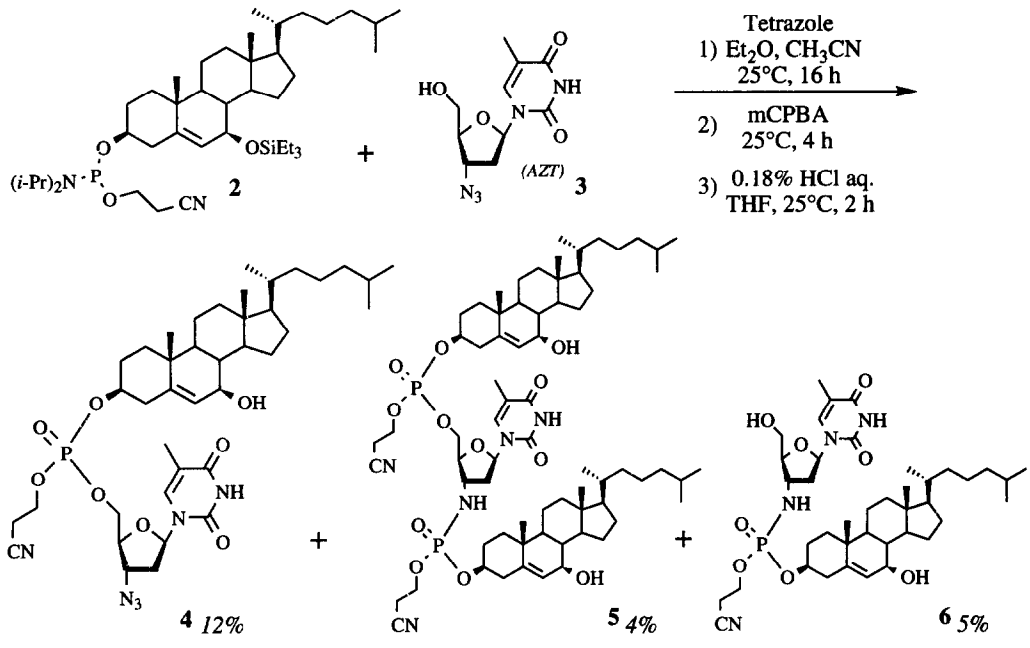
In principle, the phosphorus containing compounds could be synthesized based on P(III) or P(V) chemistry. Three common methodologies are generally used to make asymetric phosphodiesters of steroids : the phosphotriester methodology (1) introduced by Letsinger[13-15] seems now to be less useful than the phosphoramidite one (2), recommanded by Caruthers for oligonucleotide synthesis[16-17]. The latter was adapted to the oxysterol phosphorylation by Ji[18]. A new methodology, the hydrogen-phosphonate approach (3), appears to be in expansion[19-22].

This paper reports the synthesis of the sodium salt of 3β(7β-hydroxycholesteryl) 5'(3'deoxy, 3'azido-thymidinyl) monophosphate (**1**), using both the phosphoramidite and the hydrogen phosphonate methods.

Results and discussion

Synthesis of 1 by the phosphoramidite method

Compound **2** was synthesized using 7β-triethylsilyloxy-cholesterol[15,23] and (β-cyanoethoxy) bis (diisopropylamino) phosphine in the presence of diisopropylammonium tetrazolide as activating agent (90% yield)[18].

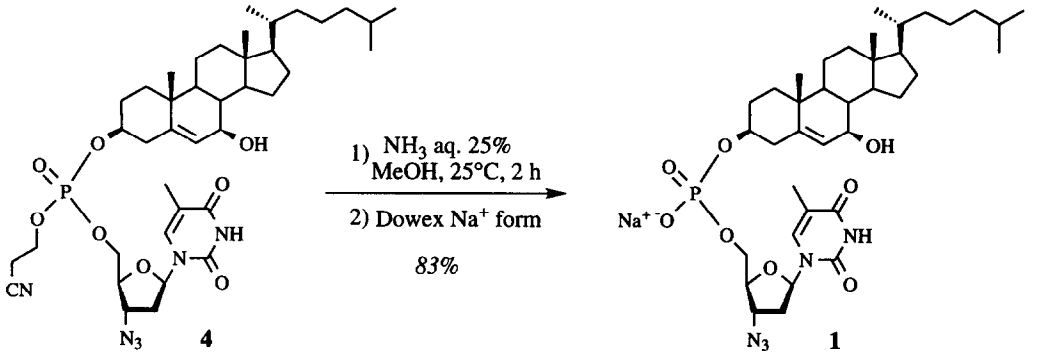


Scheme 1

The activation of the phosphoramidite **2** used a weak acid such as tetrazole in Et_2O.

Condensation with AZT (**3**) in CH_3CN gave a large number of products. Oxidation of the crude mixture with m-chloroperbenzoic acid, followed by the removal of the triethylsilyl protective group (0.18% HCl in THF) gave the desired phosphotriester **4** in only 12% yield (Scheme 1). Secondary products, such as **5** (4% yield) and **6** (5% yield) were difficultly isolated after cautious purification. Their postulated structures, based essentially on mass spectrometry analysis, could be explained by a nucleophilic attack of the free electron pair of the phosphorus atom in the phosphoramidite **2** on the α-nitrogen of the azide fonction of AZT (**3**).

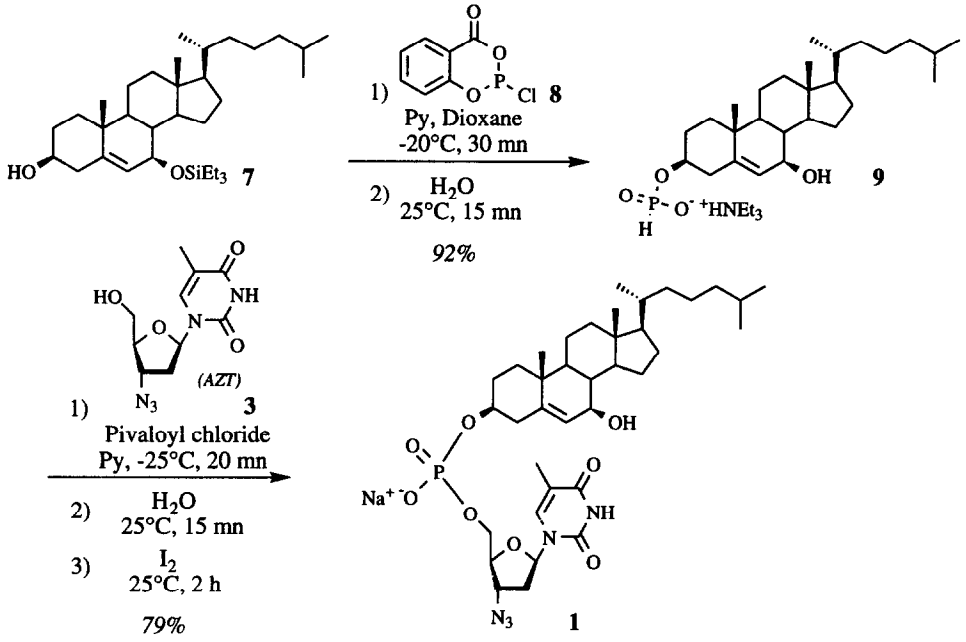
The base labile protective group, β-cyanoethoxy, was removed with NH_4OH in CH_3OH and gave the expected sodium salt of phosphodiester **1** in 83% yield (Scheme 2).



Scheme 2

*Synthesis of **1** by the hydrogen phosphonate method*

Treatment of 7β-triethylsilyloxy-cholesterol[15,23] (**7**) with commercially available phosphite **8** in a mixture of pyridine and dioxane gave after hydrolysis the triethylammonium salt **9** in 92% yield (Scheme 3).



Scheme 3

Activation with pivaloyl chloride in pyridine allowed at -25°C the selective coupling with the alcohol function of 3'azido 3'deoxy thymidine (AZT, **3**). Oxidation with iodine in the presence of water, afforded the corresponding desired phosphate **1** in 79% yield after chromatography.

Conclusion

The sodium salt of 3β(7β-hydroxycholesteryl) 5'(3'deoxy, 3'azido-thymidinyl) monophosphate (**1**) was synthesized using both the phosphoramidite and the hydrogen-phosphonate methods. The total yields were respectively 9% and 73% (starting from oxysterol **7**). In our case, according to the observed yields (and to the required purification steps), the hydrogen-phosphonate methodology appears to be more powerful than the phosphoramidite one.

Biological preliminary results show an *in vitro* anti-HIV activity; *in vivo* studies are underway (these results will be published later)[24].

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Experimental

Pyridine, dioxane and acetonitrile were dried by reflux over CaH_2 for several hours and distilled just before use. Ether was dried over Na. All the commercial reagents were purchased from Aldrich or Fluka. Tetrazole (Fluka) was purified by sublimation.

TLC were run on pre-coated silica gel plates (Merck, 60F254, 0.25 mm). The plates were dipped in a solution of vanillin (1g) in $EtOH/H_2SO_4$ (95/5, 1 l) and heated on a hot plate to reveal the compounds. The plates were also dipped in a Dittmer solution in order to visualise phosphorus-containing compounds. Short-column chromatography was carried out by using silica gel (40-63 µm, Merck G60 or Biosil A) columns. Dowex-50x8 resin (20-50 mesh, sodium salt) was used for ion-exchange chromatography.

IR spectra were recorded in $CHCl_3$ on a Perkin-Elmer 881 infrared spectrophotometer. Optical rotation ($[\alpha]_D$) were measured on a Perkin-Elmer 141 polarimeter in DMSO. NMR spectra were recorded with a Bruker SY (200 MHz) and AM (400 MHz) spectrometers using $CHCl_3$ (7.26 ppm) or CH_3OD (3.34 ppm) as internal standard for ^{1}H-NMR, $CDCl_3$ (77.0 ppm) or CD_3OD (49.0 ppm) as internal standard for ^{13}C-NMR and H_3PO_4 (0 ppm) as internal standard for ^{31}P-NMR; the chimical shifts are reported in ppm downfield from TMS (* : interchangeable assignment). FAB-MS were obtained on a VG analytical ZAB-HF double-focusing mass spectrometer using triethanolamine (TEA), 1-thioglycerol, or m-nitrobenzyl alcohol (m-NBA) as matrix. Microanalyses were performed by the Service Central de Microanalyse du CNRS (Vernaison) and the Strasbourg Local Section.

Phosphotriester of 3β(7β-hydroxycholesterol) 5'-[3'-azido, 3'deoxy thymidine] 2-cyanoethyl (**4**).

3β-(7β-triethylsilyloxycholesteryl) 2-cyanoethyl N,N-diisopropylphosphoramidite (**2**) (570 mg, 0.8 mmol) was dissolved in minimum anhydrous Et_2O and coevaporated with CH_3CN. This procedure was repeated for three times, then compound **2** was taken up in 3 ml of dry Et_2O. Separately, 3'-azido, 3'-deoxy thymidine (AZT, **3**) (240 mg, 0.9 mmol) was dried three times by coevaporation with CH_3CN and was dissolved in CH_3CN (10 ml), in which the above Et_2O solution of **2** and tetrazole (59.5 mg, 0.85 mmol) were added. The reaction mixture was stirred at room temperature overnight, under Ar. When the TLC showed the disappearence of **2**, m-CPBA (90%) (160 mg, 0.93 mmol) was introduced and the solution was stirred for an additional 4 h. The mixture was poured into saturated NaCl solution (20 ml) and extracted several times with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4 and concentrated to dryness under reduced pressure. The oily residue was dissolved in THF (10 ml) and 10 ml of 0.36% HCl solution in THF was added. After stirring at room temperature for 2 h, the reaction mixture was poured into saturated NaCl solution (5 ml) and extracted 3 times with CH_2Cl_2 (30 ml). The organic layers were combined, dried over Na_2SO_4 and concentrated to dryness under reduced pressure. The residue was chromatographed on silica gel (Merck G60). Elution (CH_2Cl_2/CH_3OH : 100/0 to 96/4) gave pure **4** (72 mg, 12%) [Rf : 0.41; CH_3OH/CH_2Cl_2 : 1/9], relatively pure **5** (40 mg, 5%) [Rf : 0.28; CH_3OH/CH_2Cl_2 : 1/9] and relatively **6** (33 mg, 4%) [Rf : 0.23; CH_3OH/CH_2Cl_2 : 1/9].

(**4**) **IR** : azido typical absorption at 2100 cm^{-1}. **^{1}H-NMR** ($CDCl_3$) : 0.79 (s, 3H, CH_3-18); 0.87 (d, 6H, J = 6.5 Hz, CH_3-26 and 27); 0.94 (d, J = 6.5 Hz, 3H, CH_3-21); 1.04 (s, 3H, CH_3-19); 1.92 (s, 3H, CH_3nuc); 2.78 (t, 2H, J = 6.0 Hz, $OCH_2\underline{C}H_2CN$); 3.72 (d, 1H, J = 8.0 Hz, H-7); 4.00-4.07 (m, 4H, H-3, H-4'nuc, 2 H-5'nuc); 4.25 (m, 2H, $O\underline{C}H_2CH_2CN$); 4.52 (m, 1H, H-3'nuc); 5.25 (s, 1H, H-6); 6.25 (t, 1H, J = 6.6 Hz, H-1'nuc); 7.76 (s, 1H, H-6nuc). **^{13}C-NMR** ($CDCl_3$) : 11.6 (C-18); 18.6 (C-21); 18.9 (C-19); 22.5 (C-26*); 22.6 (C-27*); 56.5 (C-14); 57.1 (C-17); 61.1 (C-3'nuc); 63.6 ($\underline{C}H_2$-CH_2CN); 67.8 (C-5'nuc); 73.2 (C-7); 80.6 (C-3); 83.0 (C-4'nuc); 86.2 (C-1'nuc); 112 (C-5nuc); 118 ($CH_2CH_2\underline{C}N$); 128.6 (C-6); 137.2 (C-6nuc); 141.7 (C-5); 152 (C-2nuc); 165.5 (C-4nuc). **FAB-MS** negative (matrix: TEA) : 783.3 [M - H; 70]; 730.3 [M - CH_2-CH_2CN; 100]. **Microanalysis** : Found C : 61.53, H : 8.01; Calc. for $C_{40}H_{61}N_6O_8P$ (784.9) C : 61.21, H : 7.83.

(**5**) **IR** : no absorption at 2100 cm^{-1}. **^{1}H-NMR** ($CDCl_3$) : 0.70 (s, 6H, 2 CH_3-18); 1.9 (s, 3H, CH_3nuc); 2.84 (b, 4H, 2 $OCH_2\underline{C}H_2CN$); 3.73 (d, 2H, J = 6.0 Hz, 2 H-7); 3.90-3.98 and 4.1-4.5 (m, 10H, 2 H-3; 2 H-5'nuc; H-3'nuc; H-4'nuc; 2 $O\underline{C}H_2CH_2CN$); 5.34 (s, 2H, 2 H-6); 6.13 (b, 1H, H-1'nuc); 7.44 (s, 1H, H-6nuc). **^{13}C-NMR** ($CDCl_3$) : 11.8 (C-18); 18.6 (C-21); 18.8 (C-19); 22.4 (C-26*); 22.5 (C-27*); 56.7 (2 C-14); 57.1 (2 C-17); 85.2 (C-1'nuc); 112.0 (C-5nuc); 118.0 (2-$\underline{C}N$); 128.3 and 128.5 (2 C-6); 137.0 (C-6nuc); 141.7 and 142.0 (2 C-5); 152 (C-2nuc). **FAB-MS** negative (matrix: NBA) : 1274.7 [M - H], see reference[25].

(**6**) **IR** : no absorption at 2100 cm^{-1}. **FAB-MS** negative (matrix: NBA): 757.4 [M - H; 4], 704.4 [M - CH_2CH_2CN; 100], 373.1 [M - steroide; 14], 320.0 [M - steroide - CH_2CH_2CN; 15].

Triethylammonium salt of 3β(7β-hydroxycholesteryl) hydrogen phosphonate (**9**)

2-Chloro-1,3,2-benzodioxaphosphorin-4-one (**8**) (10 mmol, 10 ml of stock solution of 1M in anhydrous dioxane) was added to a -20°C solution of 7β-triethylsilyloxycholesteryl (**7**) (1.75 g, 3.39 mmol) in anhydrous pyridine (7.5 ml) and dioxane (20 ml). The reaction was kept at -20°C for 20 min under Ar, then hydrolysed with 0.5 ml of water. The reaction mixture was stirred for another 15 min and concentrated to dryness under reduced pressure. The residue was chromatographed on silica gel (Biosil A, desactivated with NEt_3). Elution (CH_2Cl_2/CH_3OH : 95/5 to 85/15) gave the triethylammonium salt of 3β(7β-hydroxycholesteryl) hydrogen phosphonate (**9**), 1.76 g (92% yield).

(**9**) **^{1}H-NMR** ($CDCl_3$) : 0.60 (s, 3H, CH_3-18); 0.77 (d, 3H, J = 6.4 Hz, CH_3-26*); 0.78 (d, 3H, J = 6.4 Hz, CH_3-27*); 0.83 (d, 3H, J = 6.4 Hz, CH_3-21); 0.95 (s, 3H, CH_3-19); 1.31 (t, $HN^+(CH_2\underline{C}H_3)_3$); 3.02 (q, $HN^+(\underline{C}H_2CH_3)_3$); 3.74 (d, 1H, J = 8.0 Hz, H-7); 3.95 (b, 1H, H-3); 5.19 (s, 1H, H-6); 6.81 (d, 1H, J = 616.0 Hz, P-$\underline{H}$). **^{13}C-NMR** ($CDCl_3$) : 8.5 ($HN^+(CH_2\underline{C}H_3)_3$); 11.7 (C-18); 18.6 (C-21); 18.9 (C-19); 22.4 (C-26*); 22.6 (C-27*); 45.6 ($HN^+(\underline{C}H_2CH_3)_3$); 55.3 (C-14); 55.8 (C-17); 72.9 (C-7); 73.3 (C-3); 125.8 (C-6); 142.8 (C-5). **^{31}P-NMR** ($CDCl_3$) : 2.46 (d, J = 616.0 Hz). **Microanalysis** : Found C : 69.26, H : 11.22; Calc. for $C_{33}H_{62}NO_4P$ (567.8) C : 69.80, H : 11.00.

Sodium salt of 3β(7β-hydroxycholesteryl) 5'(3'deoxy, 3'azido-thymidinyl) monophosphate (**1**)

*Synthesis of **1** by the phosphoramidite methodology.*

Concentrated 25% aqueous ammonia (2 ml) was added to a solution of 3β(7β-hydroxycholesteryl) 5'-[3'-azido, 3'deoxy thymidinyl] 2-cyanoethyl phosphotriester (**4**) (67 mg, 0.085mmol) in CH_3OH (8 ml). After 2 h, TLC indicated the complete conversion of **5** and the mixture was concentrated to dryness under reduced pressure. The residue was then taken up in water (1 ml) and was applied to a ion-exchange chromatography over Dowex-50x8 resin eluted with bidistilled water. The combined fraction was lyophilized to give pure sodium salt of 3β(7β-hydroxycholesteryl) 5'(3'deoxy, 3'azido-thymidinyl) monophosphate (**1**), 53 mg as a white powder (83% yield).

*Synthesis of **1** using the hydrogen phosphonate methodology.*

Pivaloyl chloride (246 µl, 2 mmol) in 3 ml anhydrous pyridine was added dropwise over 20 min to a -25°C, light-protected, solution of pyridinium salt of 3(7β-triethylsilyloxycholesteryl) hydrogen-phosphonate (**9**) (401 mg, 0.71 mmol) and AZT (**3**) (280 mg, 1.04 mmol) in anhydrous pyridine (15 ml). The reaction was kept at -25°C for 20 min under Ar, then quenched by introducing 1 ml of water. The cooling bath was removed and the solution stirred for 15 mn. Iodine (200 mg, 0.79 mmol) was added and the resulting dark-brown mixture was stirred for another 2 h. 10% aqueous solution of Na_2SO_3 was added dropwise to destroy the excess of iodine (disappearance of brown coloration). After addition of $NaHCO_3$ (50 mg), the reaction mixture was concentrated to dryness

under reduced pressure. The residue was adsorbed on Celite and applied to a short-column chromatography over silica gel (Biosil A, desactivated with NEt_3). Elution (CH_2Cl_2/CH_3OH : 95/5 to 85/15) gave pure sodium salt of 3β(7β-hydroxycholesteryl) 5'(3'deoxy, 3'azido-thymidilyl) monophosphate (1), 424 mg (79% yield).

(1) **IR** : azido typical absorption at 2100 cm^{-1}. $[\alpha]_D$ = + 7 (c =1, DMSO). **^{1}H-NMR** (CD_3OD) : 0.79 (s, 3H, CH_3-18); 0.87 (d, 6H, J = 6.5 Hz, CH_3-26 and 27); 0.94 (d, J = 6.5 Hz, 3H, CH_3-21); 1.04 (s, 3H, CH_3-19); 1.92 (s, 3H, CH_3nuc); 3.72 (d, 1H, J = 8.0 Hz, H-7); 4.00-4.07 (m, 4H, H-3, H-4'nuc, 2 H-5'nuc); 4.52 (m, 1H, H-3'nuc); 5.25 (s, 1H, H-6); 6.25 (t, 1H, J = 6.6 Hz, H-1'nuc); 7.76 (s, 1H, H-6nuc). **^{13}C-NMR** (CD_3OD) : 13.2 (C-18); 20.1 (C-21); 20.2 (C-19); 23.8 (C-26*); 24.0 (C-27*); 56.5 (C-14); 57.1 (C-17); 61.1 (C-3'nuc); 67.8 (C-5'nuc); 73.2 (C-7); 80.6 (C-3); 83.0 (C-4'nuc); 86.2 (C-1'nuc); 112.0 (C-5nuc); 128.6 (C-6); 137.2 (C-6nuc); 141.7 (C-5); 152 (C-2nuc); 165.5 (C-4nuc). **^{31}P-NMR** (CD_3OD) : - 1.08 (s). **FAB-MS** negative (matrix: NBA): 752.4 [M-H ; 24]; 730.4 [M - Na^+; 100]; 481. 3 [M - nucleotide - Na^+ + H; 10]; 346 [M - Steroid - Na^+ + H; 8], see reference[25]. **Microanalysis** : found C : 54.01, H : 7.46, N : 8.15; calc for C_{37} H_{57} O_8 N_5 P Na, 3.5 H_2O (816.4) C : 54.40, H : 7.80, N : 8.57.

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