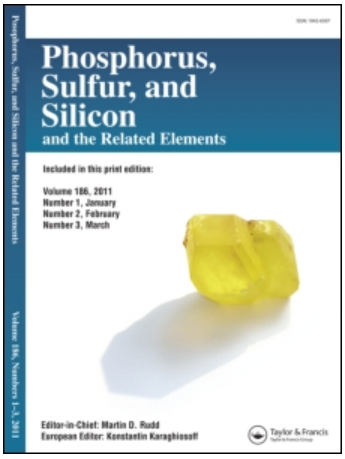

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Design and Synthesis of Nucleoside Phosphate Mono(Aminoalkyl)Ester

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DESIGN AND SYNTHESIS OF NUCLEOSIDE PHOSPHATE MONO(AMINOALKYL)ESTER

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This report describes the one step synthesis of nucleosides monophosphates alkylamines via direct condensation of N-protected phosphate alkylamines on nucleosides, providing a method for the preparation of nucleotide haptens.

Keywords: Immunogen; nucleoside; monophosphate alkylamine; nucleotide; phosphate; immunoassay; HIV

INTRODUCTION

ddNs (2′,3′-dideoxynucleosides) are potent inhibitors of HIV replication and are used clinically in polytherapy for the treatment of AIDS. Following their phosphorylation to triphosphates by cellular kinases, they compete with endogenous nucleosides to inhibit HIV-1 reverse transcriptase (RT) or act as chain terminators[1–4].

Several studies indicate that there was no correlation beetween plasma concentrations of ddNs and the intracellular concentrations of phosphorylated ddNs (ddNs monophosphates, ddNs diphosphates and ddNs triphosphates) which are related to drug responses and toxicities[5–10]. For example, the rate limiting step for the formation of the active metabolite of AZT (3′ azido-2′,3′ dideoxythymidine) is the conversion of AZT-MP (AZT monophosphate) to AZT-DP (AZT diphosphate). The toxicity of AZT is attributed to the cellular accumulation of AZT-MP which is not readily converted into AZTDP and AZTTP because its low affinity for thymidine kinase[11–12]. A lack of thymidine kinase activity[13–15] or an

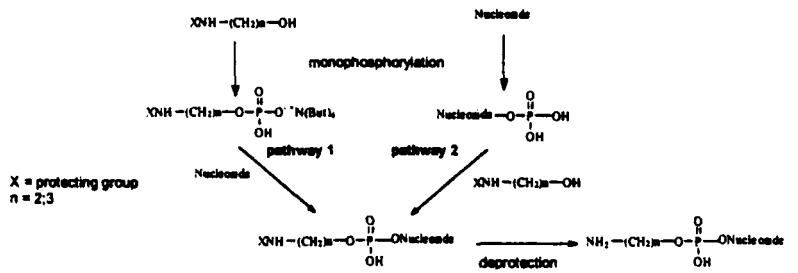
efflux of AZT-MP from cells[16] are thought to be potential sources for the cell resistance to AZT. It would be of interest to develop new methods for the determination of intracellular level of AZT-MP in order to improve treatment of HIV infection and to adjust individual antiretroviral drug doses.

We are interested in developing immunoassays for intracellular metabolites of anti-HIV nucleoside drugs. The development of specific immunoassays is restricted by two major difficulties: the synthesis of the hapten and its coupling to an antigenic carrier protein.

RESULTS AND DISCUSSIONS

Conjugation of phosphorylated ddNs to carrier proteins can be achieved either via the 5′ phosphate group or via the base of the nucleotides[17–18]. We previously reported that 5′-O-hemisuccinate ddN can be used to raise anti-ddN antibodies[19–23]. In this paper, we report the preparation of two N-protected alkylamine phosphate monoesters which allow syntheses of nucleoside mono, di or triphosphate haptens.

Scheme 1 shows the two possible strategies that can be used: (i) introduction of a phosphate moiety on a spacer followed by its coupling to the nucleoside (**pathway 1**), (ii) coupling the spacer on the nucleotide (**pathway 2**). Pathway 2 was choosen.

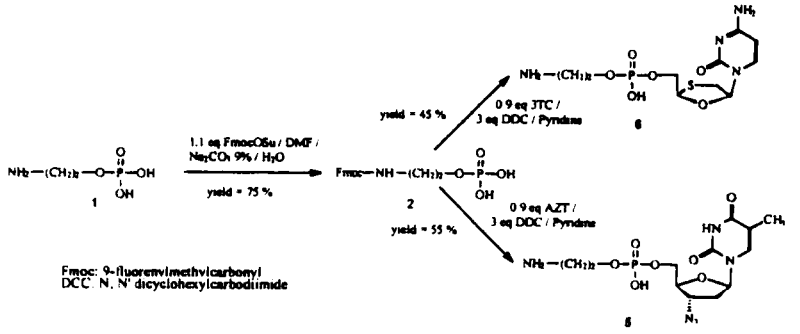


SCHEME I

For the preparation of the spacer, phosphate alkylamine is commercially available (2-aminoethyl phosphate **1**). In order to obtain two differents spacers (one with an basic-labile protecting group and another with an

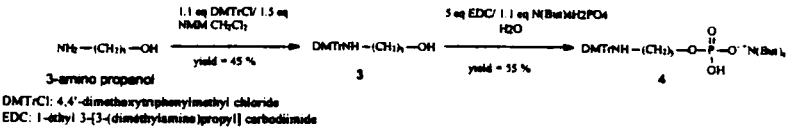
acid-labile protecting group), we fisrt attempted to protect the amine group with chorocarbonate reagents: Fmoc-Cl (9-Fluorenylmethyl chloroformate) and DMTr-Cl (4,4′-Dimethoxytriphenylmethyl choride). The reaction gave the 3-chloro-ethanolamine.

We then used the N-hydroxysuccinimide actived Fmoc reagent (9-Fluorenylmethyl-succinimidyl carbonate) and obtained the N-Fmoc-ethyl amine monophosphate **2** with a 75 % yield (scheme 2).



SCHEME II

The spacer with an acid-labile protecting group was obtained as follows. The reaction of 3-amino propanol with DMtr-Cl gave the corresponding protected alcohol **3** with a 45% yield. The coupling with tertiobutylammonium salt of monophosphate using EDC (1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; 5eq, RT) gave the desired N-DMtr-propylamine monophosphate **4** with a 55 % yield (Scheme 3).



SCHEME III

Both Fmoc-N-aminoethyl phosphate **2** and DMtr-N-aminopropyl phosphate **4** were coupled to AZT and 3TC. These compounds are currently used to prepare hapten of AZT monophosphate (5′-O-phosphate mono

(2-aminoethylamine)-AZT **5**) and 3TC monophate (2′-O-phosphate mono (2-aminoethylamine)-3TC **6**) with acceptable yield (45 and 35% respectively). Interestingly, condensation of Fmoc-N-ethylamine monophosphate to AZT or 3TC was achieved with the concomitant removal of the Fmoc group (Schema 2). This conclusion was supported by the appearance of DBF (dibenzofulvene) in the reaction mixture.

In conclusion, the synthesis of two N protected alkylamine phosphates is reported. The use of Fmoc-N-ethylphosphate is interesting because its condensation with nucleosides provides a direct method for the synthesis of nucleosides monophosphates alkylamines that do not require a deprotection step. We highlight the advantage of the Fmoc group in this approach to N-alkylamine nucleotides. Finally, preparation of hapten of diphosphates using the N protected alkyl phosphate is in progress in our laboratory.

EXPERIMENTAL

Materials

3TC and AZT was donated by Glaxo Wellcome (France). 2-aminoethyl dihydrogenphosphate, succinimidyl-9-fluorenylmethyl carbonate, 3-amino propanol, N-methyl morpholine, 4,4′-dimethoxytrityl chloride, 1-(3-dimethylaminopropyl)3-ethylcarbodiimide hydrochloride and 1,3-dicyclohexylcarbodiimide (DCC) were purchased by Sigma-Aldrich (Saint-Quentin, France). All other reagents were on analytical grade.

Anion-exchange resin used for separation of phosphorylated derivatives of 3TC and AZT was SEPHADEX-DEAE A-25 ion-exchange resin (40–120 micron) purchased from Aldrich. The HPLC system for purification of compounds synthesised consists on WATERS apparatus including 600 pump, 996 photodiode array detector and Millennium chromatographic manager. HPLC purification of compounds **5** and **6** were achieved on analytical/semi-preparative TSK-GEL DEAE-5PW column.

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded respectively at 200, 50.3 and 81 MHz by using a Brucker AC200 spectrometer. Mass spectra were determinated with Finnigan-Mat mass spectrometer.

Preparation of Fmoc-N-aminoethyl-phosphate 2

300 mg of 2-aminoethyl dihydrogenphosphate (1.94 mmol) were dissolved in 9 % sodium carbonate solution (5 ml) and cooled in an ice bath. A solution of succinimidyl 9-fluorenylmethyl carbonate (576 mg; 1.1 eq) in dimethylformamide (4.7 ml) was added dropwise at 0°C. The mixture was stirred for 20 min and the solvents were concentrated. The obtained residue was dissolved in water (50 ml) and the aqueous solution was washed with ethyl acetate (3 × 20 ml). The aqueous phase was cooled, acidified to pH 5.5 with a cold solution of hydrochloric acid (0.1 N) and extracted with ethyl acetate (3 × 20 ml). The organic layer was dried with sodium sulphate and evaporated to give a solid product. On addition of mixture of methanol/ether (2/8), a solid product was obtained with a 75 % yield. ^{1}H NMR (D_2O) δ 7.75 (d, 2 H, J^3 = 7 Hz), 7.55 (d, 2H, J^3 = 7 Hz), 7.35 (m, 4H), 4.21 (d, 2H, J^3 = 6.6 Hz), 4.10 (t, 1H, J^3 = 6.6 Hz), 3.68 (t, 2H, J^3 = 4.2 Hz, CH_2N), 3.25 (t, 2H, J^3 = 4.2 Hz, CH_2O); ^{13}C NMR (D_2O) δ 162.12 (NH-C(O)O), 143.9, 140.9, 128.0, 127.48, 125.1, 120.2 (CH_{ar} Fmoc), 69.9 ($\underline{C}H_2$-OC(O)), 46.9 ($\underline{C}H$-CH_2-O-C(O)), 40.9 (CH_2N), 40.2 (CH_2O); ^{31}P-^{1}H NMR (D2O) δ 5.6 (t, 1P, J^3 = 8.1 Hz).

Preparation of N-DMTr-amino-propanol 3

To a solution of 100 mg (1.33 mmol) of 3-amino propanol and 0.2 ml (1.1 eq of N-methyl-morpholine) dissolved in 2.5 ml of dichoromethane, a solution of 451 mg (1.1 eq) of 4,4′-dimethoxytrityl chloride dissolved in 5 ml of dichoromethane was added dropwise. The mixture was stirred for 3h30 at room temperature and the solvents were evaporated. The DMTr-N-amino-propanol **3** was obtained with a 45 % yield after purification by coluum chromatography (silica gel, ethyl acetate/ methanol: 1/1 as eluent). ^{1}H NMR (CD_3OD) δ 7.43 (d, 2H, J^3 = 6.7 Hz), 7.24 (m, 7H), 6.74 (d, 4H, J^3 = 8.9 Hz), 3.61 (s, 6H, OCH_3), 3.15 (t, 2H, J^3 = 4.8 Hz, CH_2N); 2.82 (t, 2H, J^3 = 5.2 Hz, CH_2O), 1.72 (m, 2H, J^3 = 4.8 Hz, J^3 = 5.2 Hz, CH_2); ^{13}C NMR (CD_3OD) δ 158.6 (2C), 144.8 (3C), 135.9, 130.0 (2C), 128.1, 128.0 (8C), 126.9 (2C), 113.3 (2C), 61.2 (CNH), 55.3 (OCH_3), 50.7 (OCH_3), 38.9 (CH_2OH), 30.9 (CH_2NH), 28.5 (CH_2).

Preparation of N-DMTr-propylamine-monophosphate 4

100 mg (0.38 mmol) of N-DMTr-amino-propanol **3** and 76 mg (5 eq) of EDC (1-(3-dimethylaminopropyl)3-ethylcarbodiimide hydrochloride) are

dissolved in 100 ml of distillated water. 126 mg (1.1 eq) of tertiobutylammonium salt of monophosphate were added to this solution. The mixture was stirred overnight in dark at room temperature and lyophilised to give a white powder. The N-DMTr-propylamine-monophosphate **4** was purified by trituration in a mixture of methanol/ether (2/8) with a 55 % yield. ^{1}H NMR δ ^{1}H NMR (D_2O) δ 7.44 (d, 2H, J^3 = 6.7 Hz), 7.24 (m, 7H), 6.74 (d, 4H, J^3 = 8.9 Hz), 3.61 (s, 6H, OCH_3), 3.24 (t, 2H, J^3 = 4.8 Hz, CH_2N); 2.57 (t, 2H, J^3 = 5.2 Hz, CH_2O), 1.72 (m, 2H, J^3 = 4.8 Hz, J^3= 5.2 Hz, CH_2); ^{13}C NMR (D_2O) δ 158.6 (2C), 144.8 (3C), 135.9, 130.0 (2C), 128.1, 128.0 (8C), 126.9 (2C), 113.3 (2C), 61.2 (CNH), 55.3 (OCH_3), 50.7 (OCH_3), 39.1 (CH_2OH), 30.9 (CH_2NH), 28.5 (CH_2); ^{31}P-^{1}H (D_2O) δ 6.3 (t, P, J^3 = 9.4 Hz).

General procedure for condensation of N-Fmoc-aminoethyl phosphate 2 on nucleoside

1 eq of N-Fmoc-aminoethyl phosphate was dissolved in anhydrous pyridine (1 ml/ 0.1 mol of compound **2**) at 45°C. 1.5 eq of nucleoside (AZT or 3TC) and 1.2 eq of DCC (1,3-dicyclohexylcarbodiimide) were added. The mixture was stirred for 4 h at room temperature and the pyridine was removed by evaporation under reduced pressure. The residue was remained in methanol 1/ethyl ether 10 and cooled at 4°C. The white precipitate was evaporated to dryness at room temperature, remained in 5 ml of distilled water and loaded onto DEAE-SEPHADEX A-25 column which was equilibrated with 0.01 M TEAB. The column was eluted with a linear gradient of TEAB (pH = 7.6), [0.1 M- 0.3 M; 250 ml each]. The appropriate fractions were collected and lyophilised. The corresponding nucleoside phosphate mono(ethyl)ester were purified on analytical/semi-preparative ion-exchange HPLC with a linear gradient of triethylammonium buffer (TEAB) (pH = 7.6) [0.01 M-0.3 M for 25 min].

Analytical data of 5′-O-phosphate mono (2-aminoethylamine)-AZT (5)

^{1}H-NMR (D_2O 200 MHz): 7.73 (s, 1H, H6); 6.21 (t, 1H, J^3 = 6 Hz, H1′), 4.41 (m, 1H, H3′), 4.18 (m, 1H, H4′), 4.08 (m, 2H, H5′),), 3.87 (t, 2H, OCH_2, J^3 = 5.2 Hz), 2.92 (t, 2H, HCH_2, J^3 = 5.2 Hz), 2.63 (m, 2H, H2′), 1.85 (s, 3H, CH_3). ^{13}C-NMR (D_2O, 50.3 MHz): 165.3 (C2), 164.9 (C4), 137.1 (C6), 131.2 (C5), 86.1 (C1′), 82.9 (C4′), 64.5 (C5′), 61.0 (OCH_2), 60.8 (C3′), 42.5 (C2′), 15.0(CH_3), 40.8 (NCH_2). ^{31}P-^{1}H NMR (D_2O, 81 MHz): 4.2 (m); SM (ESI +): 412.9 [M + Na].$^+$.t_R (HPLC) = 12 min).

Analytical data of (2′-O-phosphate mono (2-aminoethylamine)-3TC (**6**)

^{1}H-NMR (D_2O, 200 MHz): 8.26 (d, 1H, H6, J^3 = 7.5 Hz), 6.38 (t, AA'X, 1H, H5′, J^3 = 5.5 Hz), 6.08 (d, 1H, H5, J^3 = 7.5 Hz), 5.33 (t, 1H, H2′, J^3= 4.4 Hz), 4.89 (d, 2H, CH_2O, J^3 = 4.4 Hz), 3.91 (t, 2H, OCH_2, J^3 = 5.2 Hz), 3.65 (dd, 1H, H4′a, J^2 = 12.1 Hz, J^3 = 4.6 Hz), 3.26 (dd, 1H, J^2 = 12.1 Hz, J^3 = 4.6 Hz), 3.15 (t, 2H, NCH_2 J^3 = 4.8 Hz). ^{13}C-NMR (D_2O, 50.3 MHz): 165.6 (C4), 154.5 (C2), 140.4 (C6), 94.3 (C5), 86.5 (C5′), 81.2 (C2′), 61.2 (OCH_2), 60.8 (OCH_2), 40,2 (NH_2), 35.9 (C4′). NMR ^{31}P-^{1}H(D_2O; 81 MHz): 5.2 (m); SM (ESI +): 373 $[M+ Na]^+$..t_R (HPLC) = 8.5 min.

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