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An Improved Procedure for the Synthesis of Nucleoside Phosphoramidates

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Abstract:

Nucleoside phosphoramidates provide an elegant means to permit cellular absorption of masked phosphate esters, and, by doing so, remove any dependence of the biological response on nucleoside kinase activity. The present report details a means to synthesize nucleoside phophoramidates on a multipound scale with a high (>99%) degree of purity, suitable for use in human clinical trials.

Introduction

Nucleoside phosphoramidates were conceived as a means to improve cellular penetration of nucleotides.[1] Tempering the dianionic charge, via phosphoramidates, about the phosphate ester results in a markedly less polar molecule able to penetrate the nonpolar cellular membrane. Esterase cleavage of the amino acid ester[2] (Scheme 1) results in unmasking of a free carboxylate which is postulated[3] to cyclize with the phosphorus center, resulting in removal of the phenoxy group. Hydrolysis of the P—N bond is thought to be accomplished either by a phosphoramidase enzyme[4] or through phosphatase action.[5] Regardless of the activation mode, the result is Trojan Horse-like penetration of a phosphate ester through a nonpolar membrane, obviating nucleoside kinase dependence for activation.[6]

The initial report of phosphoramidate synthesis[1] involved stoichiometric treatment of phenyl dichlorophosphate with an amino acid HCl salt, typically L-alanine methyl ester,[7] in the presence of triethylamine. The resultant material was then treated with Et_2O and filtered, and the solvent was removed anew to achieve partial separation of Et_3NHCl. The phosphate mixture was used in the next step without further purification. Although this procedure was less than ideal, it still remains commonly used.[8] The interaction of the nucleophilic amine with the dielectrophilic phosphoric dichloride inevitably leads to a mixture of three possible products (Scheme 2), albeit with a preponderance of **4**. Phosphoric chlorides are not tremendously stable to silica gel, although a recent study reports the effective separation of **4** via flash chromatography.[9] Initial reports of nucleoside phosphoramidates were carried out on 2′-deoxynucleosides having substituents other than hydroxyl at C-3′.[1] In the present case we were interested in synthesizing a phosphoramidate of a nucleoside (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU, **6**, Scheme 3) having free hydroxyls at C-3′ and C-5′.[10] Given that the free deoxynucleoside employed in this reaction is a bis-nucleophile, and that the phenoxy group of the phosphoric chloride is a labile leaving group, the reaction between two nucleophiles and a myriad of electrophiles is bound to be capricious. While this may not be problematic on a small scale, on a larger scale it becomes a critical issue—particularly when a high degree of purity is required, for example, for use in human clinical trials.

Results and Discussion

NB1011 [(*E*)-5-(2-bromovinyl)-2′-deoxy-5′-phenyl-(L-methoxyalaninyl)phosphate-uridine (**7**, Scheme 3)][11] is a novel potential anticancer compound recently approved for human clinical trials by the FDA. Faced with the task of preparing sufficient quantities of NB1011 to enable a phase I/II clinical trial it was quickly realized that the current phosphoramidation process was insufficient to produce the requisite highly pure NB1011 at the multikilogram level.

In hopes of improving the synthesis of NB1011, we performed a series of test reactions varying reaction conditions. Specifically, reactions were performed on a 2 mmol

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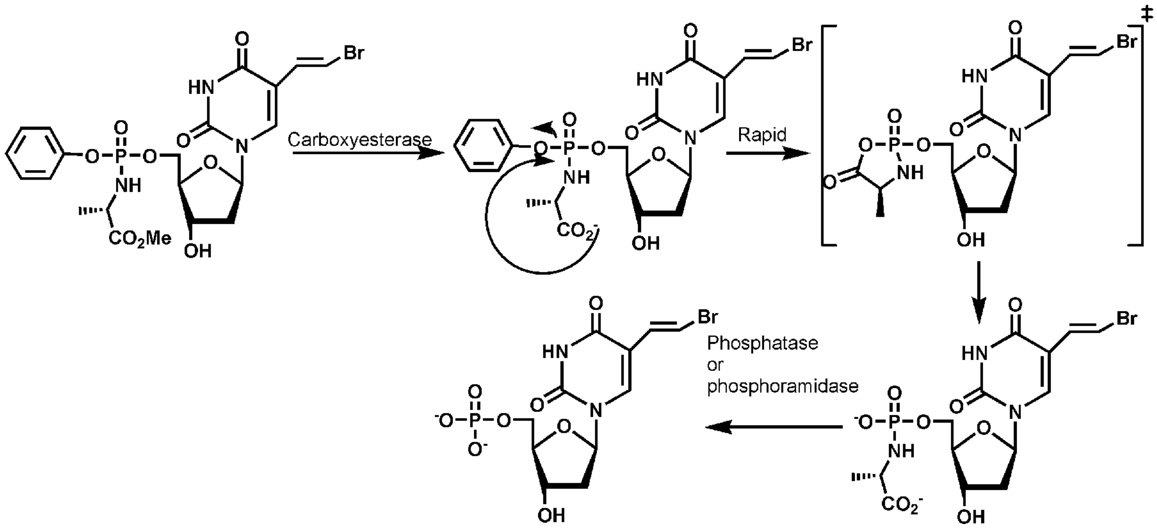
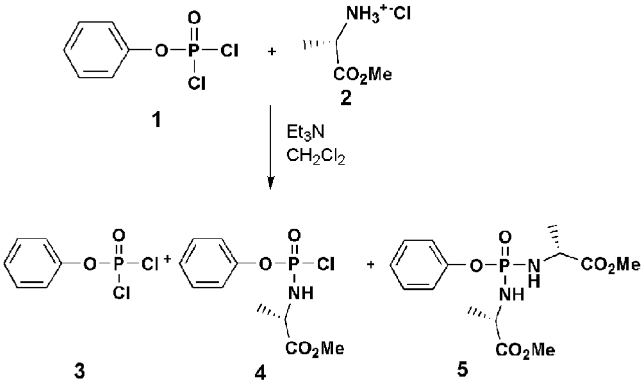
Scheme 1



Scheme 2



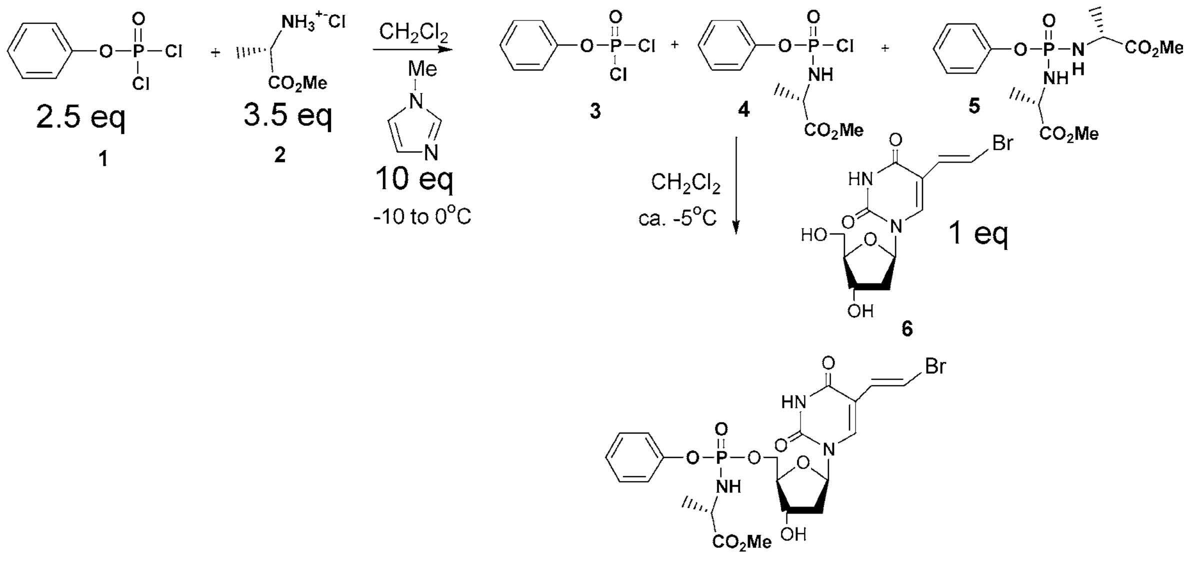
scale. Crude reaction mixtures were analyzed by HPLC. The endpoint, for the purpose of these exploratory studies, was disappearance of the starting nucleoside. Evaluation of reaction conditions by HPLC was focused on both chemical yield and purity. That is, higher proportions of NB1011 were discarded in favor of cleaner crude reaction mixtures. Solvents examined were toluene, ethyl ether, THF, and dichloromethane. Initial work was carried out at −78 °C using reaction conditions originally described.[1] Dichloromethane was by far the best solvent, and attempts at using others were discontinued. Next, the stoichiometries of L-alanine methyl ester HCl salt were varied with respect to phenyl phosphorodichloridate, again using Et_3N in the first step. It was determined that the use of 1.4 equiv of amino acid HCl salt was optimum. As this would be expected to increase the proportion of **5** present in the reaction mixture, the amount of $PhOP(O)Cl_2$ was also varied; 2.5 equiv, with respect to the nucleoside, was determined to be optimal.

Activation of the amino acid phosphorus chloride is achieved by interaction with *N*-methyl imidazole (NMI) which reacts to form, from an already reactive acyl chloride, an even more reactive acyl imidazolium salt. Thus, the amount of NMI was varied. However, NMI is in itself a base. No clear reason was seen for using two different bases in the course of the reaction. Again, the amount of NMI was varied; however, the NMI was added in the first step (to reveal the amino acid ester) in place of the Et_3N, and no further NMI was added in the second step. Use of 10 equiv of NMI (with respect to nucleoside) was found to be optimum. That such an amount of NMI was required is unexpected. As a base to neutralize the amino acid HCl salt 3.5 equiv (with respect to nucleoside) are consumed in the formation of **3**, **4**, and **5**. In the formation of NB1011 another equivalent of NMI scavenges HCl produced during the course of the reaction. This still leaves 5.5 equiv of NMI to enhance the electrophilicity of the phosphoric chloride, surprising as the NMI acting as a “catalytic” nucleophile ought to be regenerated. However, experimental evidence clearly depicted a lower purity of NB1011 when less than 10 equiv were used. Using more than 10 equiv of NMI offered no benefit. The use of so much NMI had the added advantage that a homogeneous mixture was transferred from the first flask to the next (in one attempt, using the original procedure at scale, a filtration line became clogged). Finally, reaction temperature was varied. Keeping the temperature for both reactions between −10 and 0 °C was found to afford as good a result as going to lower temperatures. The use of higher temperatures led to substantial impurities.

On the basis of the above experiments, the following reaction conditions (Scheme 3) were found to be optimum on a small (2 mmol) scale: 2.5 equiv of $PhOP(O)Cl_2$ in conjunction with 3.5 equiv of L-alanine methyl ester hydrochloride (1.4 equiv with respect to $PhOP(O)Cl_2$) and 10 equiv of *N*-methylimidazole in CH_2Cl_2. The temperature for the first step, formation of **3**, **4**, and **5,** was kept near −5 °C. Addition of the phosphoric chloride mixture to the nucleoside was done between −10 and 0 °C. The reaction was left to stir at room temperature until completion of the reaction as judged by TLC, typically 2−3 h. The reaction scaled well from 2 mmol of BVDU to 3 mol of BVDU.

To assess the change brought about by this procedure, blank reactions were performed wherein the active amino acid phosphoramidating species were quantified by ^{31}P NMR. Specifically, reactions between $PhOP(O)Cl_2$ and L-alanine methyl ester HCl salt were carried out as originally described[1]

Scheme 3



7, NB1011, 53% yield

and as described in the present report. Reactions were quenched by addition of MeOH. By examination of integration of the ^{31}P NMR spectra it was noted that following the original procedure the ratio of **3**:**4**:**5** was 55:100:3. When using the present reaction conditions the ratio of **3**:**4**:**5** was 40:100:77. Thus, the effect of increasing the amino acid ester HCl salt was to favor production of the unreactive **5** at the expense of the highly reactive **3**, as was expected. That, proportionally, more **5** than **3** (with concomitant increase in the proportion of **4**) is present is advantageous as **3** is more electrophilic than **4**, lessening undesired side reactions. The increase in **5** is not problematic as it is relatively inert. The phosphoramidating mixture was added dropwise to a flask containing BVDU in CH_2Cl_2. To achieve selectivity of the 5′- over the 3′-hydroxyl substitution, it is desirable to control both the temperature and the rate of addition of the electrophile. A recent report on the synthesis of a phosphoramidate of 6-thio-7-deaza-2′-deoxyguanosine, having hydroxyl groups at both C-3′ and C-5′, describes formation of complex mixtures of products when the reaction was carried out at 0 °C.[12] As the BVDU is only sparingly soluble in CH_2Cl_2 (but dissolves in the presence of NMI), slow addition of an NMI solution in CH_2Cl_2 serves to further control of the reaction by slowly solubilizing the nucleoside to the electrophile. Following this procedure we were able to obtain 907 g (53% yield) of highly pure (>99% by HPLC, Figure 1) NB1011 starting from 1 kg of BVDU. The overall sequence is depicted in Scheme 3.

In summation, we have described a means to synthesize nucleoside phosphoramidates on a kilogram scale. The method described affords material of very high purity and offers advantages in reaction conditions including the ability to perform the reaction at a temperature substantially higher than −78 °C and omission of a filtration step. The present method has successfully been applied to an array of phosphoramidates.

Experimental Section

General Methods. NMR spectra were obtained using a Varian VXR 4000 (^{1}H 400 MHz, ^{13}C 100 MHz, ^{31}P 162 MHz). Proton and carbon chemical shifts are reported relative to tetramethylsilane ($\delta = 0$); phosphorus chemical shifts are reported relative to 85% phosphoric acid ($\delta = 0$). The multiplicities reported are those observed within the resolving power of the spectrometer and are not necessarily true multiplicities. Mass spectra were obtained using an Agilent 1100 HPLC system with a LC/MS detector. Elemental analysis was performed using a Perkin-Elmer 2400 series II CHNS/O analyzer. Reagents were reagent grade and were used as supplied. Silica gel was ICN 63-200, 60 Å. BVDU was prepared as previously described.[13]

NB1011. A 50-L flask was charged with CH_2Cl_2 (24 L) and L-alanine methyl ester hydrochloride (1.48 kg, 10.6 mol). The mixture was cooled to −5 °C, and phenyl phosphorodichloridate (1.587 kg, 7.6 mol) was added slowly. The reaction mixture was equilibrated for 10 min, maintaining the temperature between 0 and −10 °C. Keeping the temperature near −10 °C a solution of *N*-methylimidazole (2.472 kg, 29.4 mol) in CH_2Cl_2 (4.0 L) was added dropwise. The mixture was left to stir 60 min. A 50-L flask was charged with CH_2Cl_2 (12.0 L) and BVDU (1.0 kg, 3.0 mol). The reaction mixture was cooled to −5 °C. With vigorous stirring, the solution prepared above was transferred over the course of 40 min to the flask, keeping the temperature ∼−5 °C. The cooling bath was removed, and the reaction was left to stir at room temperature until TLC indicated disappearance of the starting nucleoside, typically 2−2.5 h. The reaction mixture was cooled on an ice bath and then quenched by slow addition of MeOH (2.0 L). The volume of solvent was reduced to about half under reduced pressure, and the concentrated mixture was washed with 1 N HCl (2 × 8 L), saturated $NaHCO_3$ (8 L), and water (8 L), dried over Na_2SO_4 (∼500 g), and filtered, and the solvent was removed under

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(12) The report (ref 9) describes the major impurity as being the 3′,5′-di-acylated phosphoramidate.

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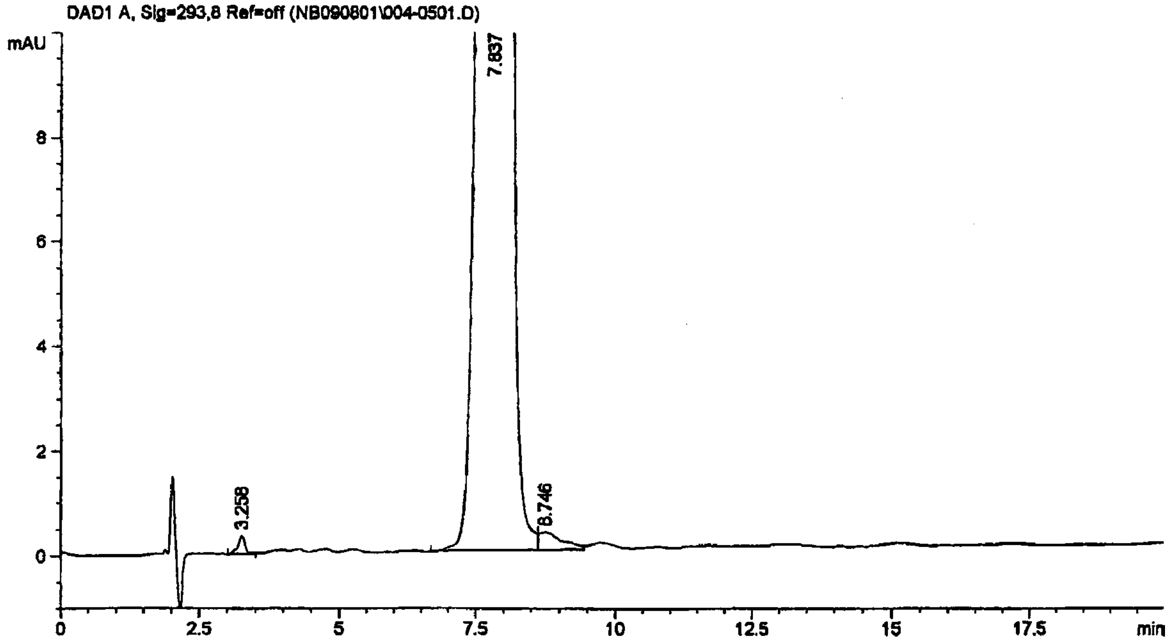

Figure 1. **HPLC trace of NB1011 prepared as described. HPLC analysis was performed on a HP 1100 chromatograph using a Nucleosil ODS (5 μm, 150 mm × 4.6 mm) C-18 column eluting with a 60:40 (v:v) methanol:0.01 M potassium phosphate buffer with a flow rate of 0.8 mL/min and a column temperature of 30 °C. Observation was performed at 293 nm. Note: the scale has been tailored to depict the low level (cf. peak areas) of impurity in the sample. On the basis of peak areas (mAU × s) the material is >99.5% pure.**

reduced pressure. The crude material was purified on SiO_2[14] (77.5 kg) eluting with 97:3 (v:v) CH_2Cl_2:MeOH to 94:6 (v:v) CH_2Cl_2:MeOH. A total of 907 g (53%) of pure NB1011 was collected. ^{1}H NMR ($CDCl_3$) δ 1.35 (dd, 3 H, J = 7.0, 2.9 Hz, CH_3-ala), 2.02 (m, 1 H, H-2′), 2.40 (m, 1 H, H-2″), 3.67 (s, 3H, OCH_3), 4.10−4.52 (a series of complex multiplets, 5 H, H-α-ala, H-3′, H-4′, H-5′, H-5″), 6.24 (t, 1/2H, J = 6.4 Hz, H-1′), 6.27 (t, 1/2H, J = 6.4 Hz, H-1′), 6.67 (d, 1/2H, J = 13.5 Hz, CH=C*H*Br), 6.70 (d, 1/2H, J = 13.5 Hz, CH=C*H*Br), 6.86 (s, 1H, H-6), 7.03 (s, 1 H, H-2), 7.13−7.31 (a series of multiplets, 5 H, OPh), 7.37 (d, 1/2H, J = 13.5 Hz, C*H*=CHBr), 7.40 (d, 1/2H, J = 13.5 Hz, C*H*=CHBr), 7.44 (br s, 1 H, NH). ^{13}C NMR ($CDCl_3$) δ 20.95 (CH_3-ala), 33.52 (C-2′), 40.60, 50.43, 52.84, 65.93, 66.30, 70.29, 70.69, 85.39, and 85.44 (C-1′), 110.03, 111.65, 120.33, 125.53, 128.73, 129.43, 130.05, 137.815, 137.97, 149.52, 150.52, 161.78, 174.27. ^{31}P NMR ($CDCl_3$) δ 3.67, 4.00. MS (ESI$^-$) Expected for $C_{21}H_{25}BrN_3O_9P$ $[M - H]^-$ 574.1. Found 574.2. Anal. Calcd for $C_{21}H_{25}BrN_3O_9P$: C, 43.92; H, 4.39; N, 7.32. Found: C, 43.70; H, 4.18; N, 7.10.

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(14) To achieve highly pure NB1011 the SiO_2 was first washed with a column volume of 9:1 (v:v) CH_2Cl_2:MeOH and then reequilibrated by washing with two column volumes of 97:3 (v:v) CH_2Cl_2:MeOH.