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A new approach to oligonucleotide N3′ → P5′ phosphoramidate building blocks

Daria Zielinska, Krisztina Pongracz and Sergei M. Gryaznov*

Geron Corporation, 230 Constitution Drive, Menlo Park, CA 94025, USA

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Abstract—A new synthetic approach to 5′-phosphoramidites of 3′-aminonucleosides was developed. The methodology relies upon the use of 3′-amino-2′,3′-dideoxynucleosides as the key starting materials. The final products were obtained in high yields via 2–3-step processes using selective introduction of orthogonal protective groups to the 3′-aminonucleoside sugar and base moieties.

Oligonucleotide N3′ → P5′ thio-phosphoramidates (NPS) are currently under pre-clinical and clinical development as potential anticancer agents targeted to human telomerase.[1] Moreover, oligonucleotide N3′ → P5′ phosphoramidates (NP) have been successfully used as potent antisense and antigene agents, as well as diagnostic chromosomal DNA FISH probes.[2] These oligonucleotide analogues are currently prepared by an amidite transfer method, which utilizes the key 3′-amino-nucleoside–5′-phosphoramidite building blocks (**1a**,**g**,**c**,**t**; Fig. 1). Therefore, a readily accessible, economically viable synthetic route to these compounds may play an important role in the successful development of the oligonucleotide phosphoramidates as therapeutic and diagnostic agents.

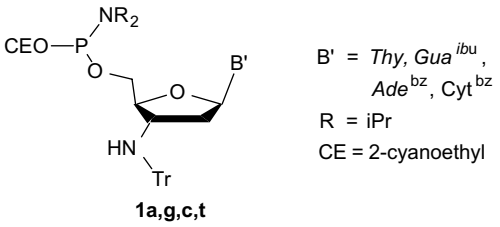
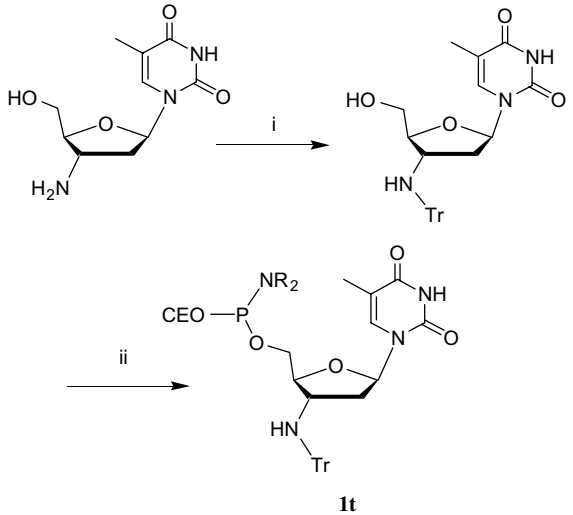


Figure 1. General structure of 3′-aminonucleoside 5′-phosphoramidites.

The earlier described synthesis of these monomers utilizes a highly complex, labor-intensive, low-yielding multi-step process: 10 steps for preparation of the purines and 7 steps for the pyrimidine compounds.[3] The total yield of the phosphoramidite products (on gram-to-kg scale) reached only approximately 4–5% for the purines and 15–20% for the pyrimidines. The natural 2′-deoxynucleosides with 3′-hydroxyl group were used as the chemistry defining starting materials for these reported procedures.



Scheme 1. Synthesis of thymidine phosphoramidite **1t**, where (i) TrCl, Py/EtN(*i*Pr)$_2$, (ii) (*i*Pr$_2$N)P(Cl)OCE, EtN(*i*Pr)$_2$ R = *i*Pr; CE = 2-cyanoethyl.

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* Corresponding author. Tel.: +1 650 473 8611; fax: +1 650 473 7760; e-mail: sgryaznov@geron.com

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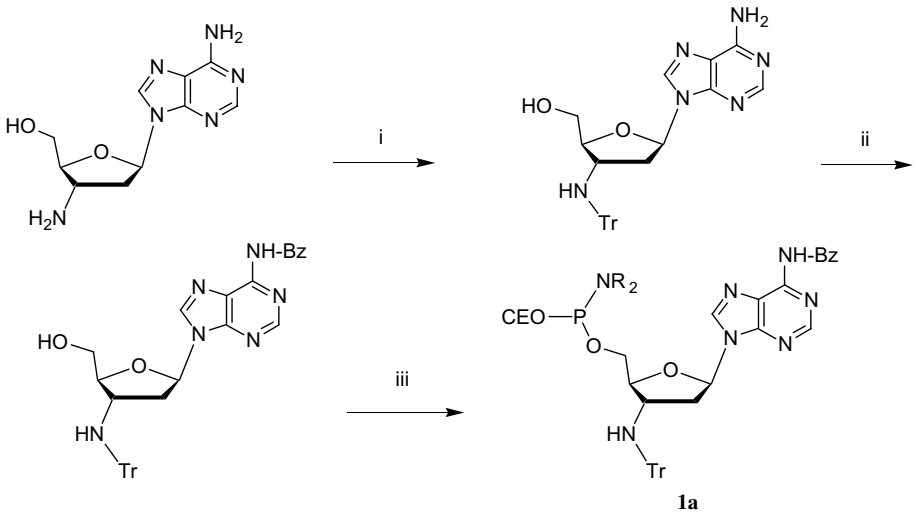


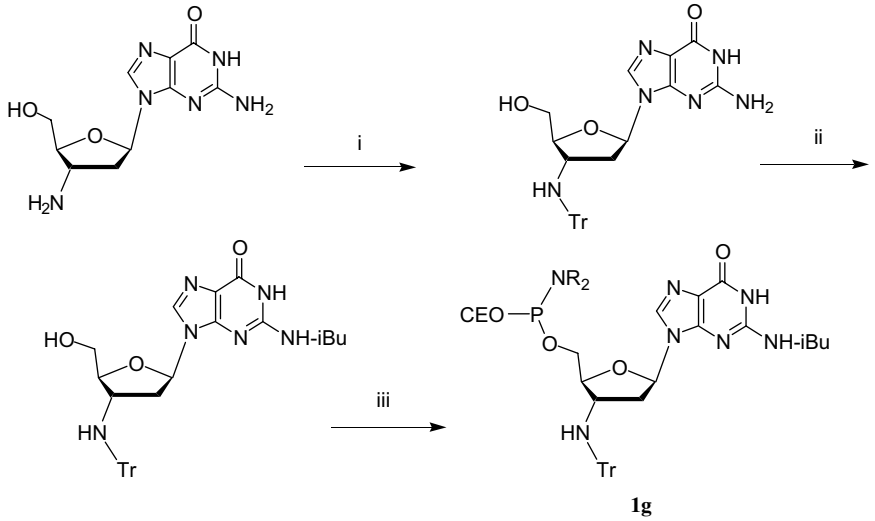
Scheme 2. Synthesis of adenosine phosphoramidite **1a**, where (i) TrCl, Py/DMF/Et_3N, (ii) BzCl, Py, (iii) (iPr_2)NP(Cl)OCE, EtN(iPr)$_2$ R = *i*Pr; CE = 2-cyanoethyl.

Here, we present a new synthetic approach to the purine (with Ade^{Bz} and Gua^{iBu} bases) and pyrimidine (Thy and Cyt^{Bz} bases) phosphoramidites (**1a**,**g**,**t**,**c**, respectively). The developed methodology relies upon the application of 3′-amino-2′,3′-dideoxynucleosides as the key new starting materials.[4] The final phosphoramidite products were obtained, in high yields, via a two- (for Thy), or three- (for Ade, Gua, Cyt)-step, fast and efficient chemical transformations using selective introduction of orthogonal protective groups to the 3′-aminonucleoside sugar and base moieties.

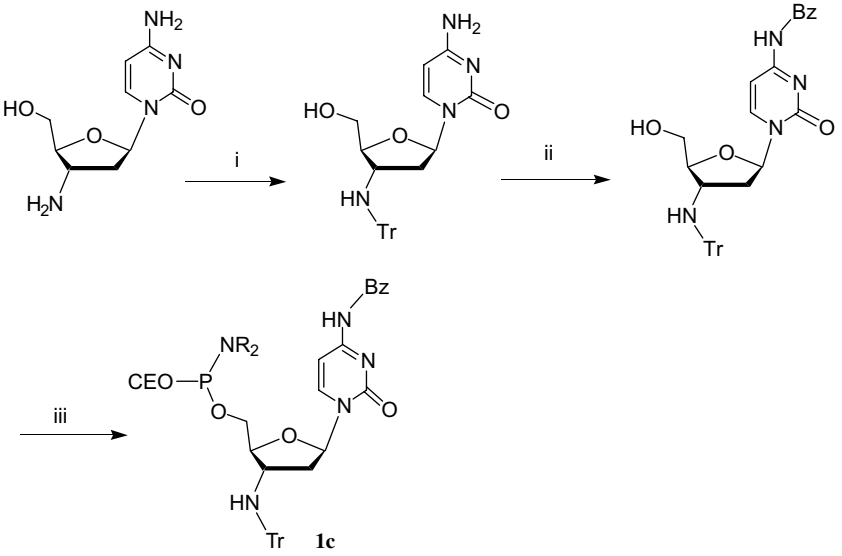
The performed chemical reactions are outlined in Schemes 1–4. In general, a regio-selective 3′-NH-tritylation is the key enabling step for this new approach to phosphoramidites **1a**,**g**,**t**,**c** through 3′-amino nucleosides.[5] Thus, preparation of the thymidine monomer **1t** involves only two chemical steps: 3′-NH-selective (3′-amino- vs 5′- hydroxyl group) tritylation in pyridine in presence of diisopropylethylamine of the nucleoside 3′-aminogroup (yield 85%). The tritylation reaction was subsequently followed by a standard 5′-O-phosphitylation of the 5′-OH-3′-NH-Tr–thymidine precursor, resulting in **1t**.[6,7] The overall yield of the phosphoramidite product **1t** was 70%, based on the starting 3′-amino-3′-deoxythymidine (Scheme 1).

The adenosine, guanosine and cytidine-based monomers **1a**,**g**,**c** were prepared in a similar manner (Schemes 2–4). However, presence of the second reactive amino group at the heterocyclic bases (N^6, N^2 and N^4, respectively), as well as the low solubility of these nucleosides in anhydrous organic solvents (particularly for 3′-aminoguanosine), required the use of different tritylation conditions.

Hence, adenosine phosphoramidite **1a** was prepared as follows: 3′-aminoadenosine was 3′-NH-tritylated in DMF in the presence of triethylamine. The 3′-NH-trityl-



Scheme 3. Synthesis of guanosine phosphoramidite **1g**, where (i) TrCl, Py/DMF/Et_3N, (ii) *i*BzCl, Py, (iii) (iPr_2)NP(Cl)OCE, EtN(iPr)$_2$ R = *i*Pr; CE = 2-cyanoethyl.



Scheme 4. Synthesis of cytidine phosphoramidite **1c**, where (i) TrCl, Py/Et_3N, (ii) Bz_2O, MeOH, (iii) (iPr_2)NP(Cl)OCE, EtN(iPr)$_2$ R = *i*Pr; CE = 2-cyanoethyl.

ated intermediate was isolated via aqueous wash and extraction procedure, with >90% yield, and it was taken to the next N^6-benzoylation step without any further purification (Scheme 2). Formation of small amounts (est. <5%) of the bis-5′-O-,3′-NH-trityl by-product was detected by ESI MS and TLC analysis. The subsequent N^6-benzoylation (using either a per-benzoylation process or TMS–Cl based transient protection procedure) resulted in 5′-OH-3′-NH-Tr-N^6-Bz–adenosine precursor (Scheme 2). This compound was purified by silica gel column chromatography and then 5′-O-phosphitylated, resulting in the desired phosphoramidite **1a** in 60% overall yield, based on the starting amino nucleoside.[6,7]

Alternatively, using a one-pot synthetic approach, 3′-aminoadenosine was 3′-NH-tritylated in solution of pyridine in the presence of triethylamine, and the crude reaction mixture was N^6-benzoylated without isolation of the 3′-tritylated intermediate using TMS–Cl-based transient protection method. The overall yield of the 5′-OH-3′-NH-Tr-N^6-Bz–adenosine intermediate was 70% after column chromatography. This synthetic route resulted in generation of a by-product (<10% by weight), which was readily separated from the desirable compound during silica gel chromatography. The proposed chemical structure of this unexpected by-product with an open imidazole ring is shown in Figure 2. The structure is consistent with the observed molecular weight, 1D and 2D COSY ^{1}H NMR spectra, and its chemical properties.[8] It appears that formation of this by-product is caused by the presence of triethylamine during the N^6-benzoylation step with benzoyl chloride, since removal of triethylamine (by aqueous washings prior to benzoylation) results in its elimination. Interestingly, this type of by-product was not detected during N^6-benzoylation of 2′-deoxyadenosine (with 3′-OH group) under similar experimental conditions. The difference is likely due to the electron-donating effect of the 3′-amino group to the adenine base.[9]

Synthesis of guanosine phosphoramidite **1g** was also performed in three steps as follows (Scheme 3). First, 3′-amino-2′,3′-dideoxyguanosine was selectively 3′-NH-tritylated in a mixture of DMF and pyridine at 50 °C. Formation of relatively small amounts of a single impurity bis-3′-NH,-N^2-trityl–guanosine (est. <5%) was detected. The desired product, 5′-hydroxy-3′-NH-trityl guanosine, was isolated by precipitation with water (or by crystallization from dichloromethane) with an yield of 93%. Subsequently, this compound was reacted with *iso*-butyryl chloride (*i*Bu–Cl) to form the phosphoramidite precursor N^2-*i*Bu-3′-NH-Tr–guanosine. Either N,O-per-acylation with *i*Bu–Cl, or transient protection with TMS–Cl followed by *i*Bu–Cl acylation was used for the N^2-*iso*-butyrylation step, resulting in product yields of 60% and 53%, respectively, (after crystallization from hot acetonitrile). Finally, the phosphoramidite **1g** was formed using typical phosphitylation procedure, resulting in the final product **1g** with an overall yield of 45%.[6,7]

Interestingly, if isobutyric anhydride rather than *i*Bu–Cl was used for N^2-amino group protection by per-acylation procedure, then the formation of primarily 5′-O-*i*Bu-3′-NH-Tr–guanosine with unacylated N^2-group was observed. This compound was isolated with 90%

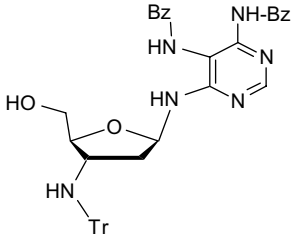


Figure 2. Proposed structure of the by-product formed during in situ N^6-benzoylation of 3′-NH-Tr–adenosine.

yield via crystallization from dichloromethane/acetonitrile. Moreover, it appears that the quality of *i*Bu–Cl plays an important role in achieving good yield of the product. The presence of products of hydrolysis of *i*Bu–Cl in the reaction mixture during the N^2-protection reaction led to formation of significant amounts (up to 50%) of a major by-product: 3′-NH-*i*Bu-N^2-Tr–guanosine.[10]

Preparation of phosphoramidite **1c** was conducted in a similar fashion (Scheme 4). First, 3′-amino group of 3′-amino-2′, 3′-dideoxycytidine was regio-selectively protected with trityl chloride in pyridine and DMF mixture, 1:4 (v/v), in presence of triethylamine (4 M equiv). Second, we sought to utilize the high nucleophilicity of the exocyclic N^4-amino group of 3′-amino cytidine, relative to the Ade and Gua counterparts. Hence, N^4-amino group was protected with benzoic anhydride in a mixture of acetonitrile and methanol, 9:1 (v/v), at 50 °C. This reaction results in the formation of N^4-Bz-3′-NH-Tr-5′-OH–cytidine precursor as the predominant product, with an isolated yield of ~70%, based on the starting 3′-amino nucleoside. Importantly, no significant 5′-O-benzoylation (est. <5%) was observed under the reaction conditions used. Finally, standard 5′-O-phosphitylation produced the desirable phosphoramidite **1c**.[6,7] This new 3′-amino nucleoside-based procedure for preparation of **1c** reduces the total number of chemical steps (from seven to three) with significant increase in the process efficiency and overall yield of final product (16% cf. 60%).[11]

In conclusion, we report an efficient and simple method for preparation of 3′-aminonucleoside-5′-phosphoramidites, the key building blocks used for assembly of oligonucleotide N3′ → P5′ phosphoramidates and thiophosphoramidates, which are currently under clinical development as potential therapeutic agents.

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4. The 3′-amino purine nucleosides: 3′-amino-2′, 3′-dideoxyadenosine and 3′-amino-2′, 3′-dideoxyguanosine were acquired from Metkinen Oy (Finland). These compounds were prepared by an enzymatic trans-glycosylation process starting from 3′-amino-3′-deoxythymidine, which was obtained via reduction of readily available 3′-azido-3′-deoxythymidine (AZT). 3′-Amino-2′,3′-dideoxycytidine was prepared as described: Glinski, R. P.; Khan, S. M.; Kalamas, R. L.; Stevens, C. L. *J. Chem. Soc. D* **1970**, 915–916.
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6. Phosphoramidites **1a**,**g**,**c**,**t** were thoroughly characterized by an appropriate NMR spectroscopy methods, mass spectrometry and RP HPLC analysis; for **1t**: ESI MS, $M+H^+$ 684.54; ^{31}P NMR, ppm, (acetonitrile-d_3), 149.39, 149.26, Rp/Sp-isomers; for **1a**: ESI MS, $M+H^+$ 797.55; ^{31}P NMR, ppm, (acetonitrile-d_3), 149.03, 148.64, Rp/Sp-isomers; for **1g**: ESI MS, $M+H^+$ 779.33; ^{31}P NMR, ppm, (acetonitrile-d_3),149.30,148.60, Rp/Sp-isomers; for **1c**: ESI MS, $M+H^+$ 773.56; ^{31}P NMR, ppm, (acetonitrile-d_3), 149.48, 149.17, Rp/Sp-isomers.
7. Representative 3′-NH-tritylation and base-protecting procedures. 3′-NH-Tr-3′-dT: 3′-amino-3′-deoxythymidine (1.3 g, 5.4 mmol) was co-evaporated and then dissolved in 30 ml of anhydrous pyridine and 4.6 ml of diisopropylethylamine. After 10 min of stirring Tr–Cl (1.5 g, 5.4 mmol) was added and the reaction mixture was left overnight. Following the disappearance of the starting material (TLC control), the reaction was quenched with methanol and concentrated in vacuo. The obtained oil was dissolved in methylene chloride, washed with satd sodium bicarbonate, dried over sodium sulfate, concentrated in vacuo, and precipitated from methylene chloride-hexane as a white powder product (2.2 g , 85% yield). 3′-NH-Tr-2′,3′-ddG:3′-amino-2′,3′-ddG, 5 g (18.8 mmol), was co-evaporated (3×) with dry pyridine and suspended in 300 ml of anhydrous DMF; 2.6 ml (18.8 mmol) of TEA was added and the reaction mixture was heating at 50°C in an oil bath for 30 min while stirring. Then 5.24 g (18.8 mmol) of Tr–Cl was added and the reaction mixture was stirred for 1 h at rt. After the disappearance of the starting 3′-amino nucleoside (by TLC analysis; more Tr–Cl can be added if the starting material is still present) the reaction mixture was poured into ~300 ml of water and placed into the freezer (−18°C) for 2–3 h. The desired product was filtered-off as a white solid. This solid was re-crystallized from hot pyridine and acetonitrile (7:3, v/v,) to obtain essentially pure 3′-NH-Tr-2′,3′-ddG (93% yield). N^2-*i*Bu-3′-NH-Tr-2′,3′-ddG via N,O-peracylation: 5 g (9.8 mmol) 3′-NH-Tr-2′,3′-ddG was co-evaporated (2×) with pyridine, suspended in 200 ml of pyridine and cooled in an ice bath; 1.13 ml (10.8 mmol) *i*Bu–Cl was added to 8 ml of pyridine and the reaction mixture was stirred for 2 h (TLC control). After the disappearance of the starting material the reaction mixture was poured into 100 ml of ice cold aq Na-bicarbonate, and extracted with a 200 ml portion (2×) of ethyl acetate. The organic layer was washed with water and concentrated in vacuo to a yellow gum. The gum was dissolved in 50–100 ml of ethanol on an ice bath and an equal volume of ice-cold 1 M sodium hydroxide in water/ethanol, 1:1 (v/v) was added. The reaction mixture was stirred for 20–30 min and 1.2 equiv of ammonium chloride was added. The mixture was poured into 200 ml of aq sodium bicarbonate and extracted (2×) with 200 ml of ethyl acetate. The combined organic layers were dried over sodium sulfate and evaporated in vacuo to a yellow solid, which was then crystallized from hot acetonitrile to obtain a white solid product (3 g, 53% yield). N^2-*i*Bu-3′-NH–Tr-2′,3′-ddG via TMS-based transient protection: 5 g (9.8 mmol) 3′-NH-Tr-2′,3′-ddG was co-evaporated (2×) with pyridine, suspended in 200 ml of pyridine and cooled in an ice bath. 6.3 ml (49 mmol) of TMS–Cl was slowly added to the reaction mixture. After 30 min, 5.1 ml (49 mmol) of *i*Bu–Cl in pyridine was added and the reaction mixture was removed from the ice bath and stirred for 2 h (TLC control). The mixture was chilled in the ice bath and then 20 ml of cold water followed, in 15 min, by the addition of 20 ml of concentrated aqueous ammonia were added, and stirred for an additional 30 min. The reaction mixture was concentrated to oil

and suspended in 50–100 ml water. The water phase was washed with ether. The organic layer was dried over sodium sulfate and evaporated in vacuo to a yellow solid, which was crystallized from hot acetonitrile to obtain a white solid product (3.4 g, 60% yield). N^4-Bz-3′-NH-Tr-2′,3′-ddC via N^4-selective benzoylation: 4.7 g (10 mmol) of dry 3′-NH-Tr-2′,3′-ddC was dissolved in a mixture of 150 ml acetonitrile and 15 ml methanol. To this solution benzoic anhydride (13.6 g, 60 mmol) and triethylamine (1 ml) were added, and the reaction mixture was stirred at 50 °C for 2 h, and then at ambient temperature overnight. After evaporation in vacuo the resultant oil was dissolved in a small amount of methylene chloride and precipitated with hexane. The precipitation was repeated, and after drying in vacuo, the product was obtained as a light yellow foam (4 g, 69.6% yield).

8. ESI MS, $M+H^+$ 690; ^1H NMR, ppm, (DMSO-d_6): 10.602 (1H, br s, D_2O exchangeable); 9.073 (1H, br s, D_2O exchangeable); 8.279 (1H, s C2); 7.819–7.106 (25H, 2m, Tr-, 2Bz-aromatic protons); 6.882 (1H, d, N9-H, D_2O exchangeable, coupled to H1′, $J = 8.8$ Hz; 2D COSY); 5.929 (1H, m, H-1′ coupled to N9-H and H-2′/2″, $J = 8.8$, 6.4 and 6.8 Hz, respectively; 2D COSY); 4.514 (1H, t, $J = 5.2$ Hz, 5′-OH, D_2O exchangeable); 3.725 (1H, d, H-3′); 3.447 (1H, m, H-4′); 3.306–3.066 (2H, m, H-5′); 1.100, 1.023 (2H, m, H-2′). Treatment with 80% aq acetic acid, at 50 °C overnight, resulted in cleavage of the C1′-N9 glycosidic bond and release of bis-N^6,N7-benzoylated pyrimidine heterocycle; ESI MS, $M+H^+$ 334.
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10. ESI MS: $M+H^+$ 579.30; this compound was distinguished from the desirable isomeric 3′-NH-Tr-N^2-*i*Bu product by ^1H NMR and TLC analyses.
11. Our attempts to selectively tritylate 3′-aminogroup of 3′-amino-2′,3′-dideoxy-2,6-diaminopurine, which contains more nucleophilic exocyclic amino groups than that in Gua, Ade or Cyt, resulted in the formation of a mixture of mono-(3′-NH-Tr-) and bis-(3′-NH-Tr-, N^2-Tr-) tritylated compounds with the molar ratio of 1:1.9, respectively.