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Tetrahedron Letters 45 (2004) 509–513
Tetrahedron Letters

Synthesis of a formamidine-protected 5′-amino-2′,5′-dideoxyguanosine phosphoramidite and preparation of 5′-acylamidooligonucleotides ☆

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Received 2 October 2003; revised 28 October 2003; accepted 3 November 2003

Abstract—A 5′-amino-2′,5′-dideoxyguanosine phosphoramidite building block for DNA synthesis was prepared from 2-*N*-(dibutylformamidino)-2′-deoxyguanosine in four steps and 68% overall yield. Two of the three steps of the conversion from the 5′-alcohol to the monomethoxytritylamine were performed in the same flask.

Aminodeoxynucleosides have attracted attention as synthetic targets, since they can have biological activity,[1,2] act as probes of biochemical processes,[3] and are valuable intermediates in the synthesis of functional analogs of biomolecules.[4–8] The incorporation of 5′-amino-2′,5′-dideoxynucleosides into DNA strands featuring phosphoramidate linkages was reported as early as 1970.[9–11] Polydeoxynucleotides containing such linkages are susceptible to mild acidic hydrolysis, generating fragments useful for genotyping[12] and sequencing.[13] Enzymatic generation of oligophosphoramidates requires the conversion of aminodeoxynucleosides into triphosphates.[13,14] Alternatively, 5′-amino-2′,5′-dideoxynucleosides may be used to construct covalent hybrids of oligonucleotides and non-nucleosidic substituents enhancing the affinity for target strands, such as PNA strands,[15] or molecular caps for the termini of duplexes.[16,17] The latter may also enhance the fidelity of DNA chips.[18] Aminodeoxynucleosides have also been employed to prepare oligonucleotide analogs with an amide-linked backbone.[19]

The synthesis of oligonucleotides with 5′-acylamido caps requires suitably protected phosphoramidite building blocks of 5′-amino-2′,5′-dideoxynucleosides. One such phosphoramidite, namely that for thymidine, has been prepared by at least three different routes,[20–22] and is now commercially available.[23] For the phosphoramidites of the 5′-amino derivatives of the other three canonical nucleosides (deoxyadenosine, deoxycytidine, and deoxyguanosine), a route has been described that utilizes azides as intermediates.[21] For 2′-deoxyguanosine, the one-step conversion from 5′-alcohol to 5′-azide[24] has not been realized, making this aminonucleoside the most difficult to prepare. Perhaps characteristically, 5′-amino-5′-deoxythymidine **1** (Fig. 1) was reported as early as 1962,[25] but the first report on free 5′-amino-2′,5′-dideoxyguanosine **2** appeared only 40 years later.[13]

We were interested in an efficient synthesis of a phosphoramidite building block of 5′-amino-2′,5′-dideoxyguanosine in order to gain access to oligonucleotides with 5′-acylamido substituents. A synthesis of 5′-amino-2′,5′-dideoxy-*N*2-isobutyryl-5′-*N*-(monomethoxytrityl)-guanosine phosphoramidite **3** (Fig. 1) was known.[21] It proceeds via base protection, 5′-tosylation, substitution producing the azide, Staudinger reduction, and protection of the free amine with a monomethoxytrityl (MMT) group, followed by phosphitylation. Since the isobutyryl group can undergo transacylation reactions during the synthesis of aminoacylated oligonucleotides and requires forced deprotection conditions that can lead to racemization in chiral acylamido substituents, we wished to employ a formamidino group to protect the *N*2-position. Further, we were interested in a shorter

Keywords: Aminonucleosides; Oligodeoxyribonucleotides; Phosphoramidites; Mitsunobu reaction.
☆ Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2003.11.003
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doi:10.1016/j.tetlet.2003.11.003

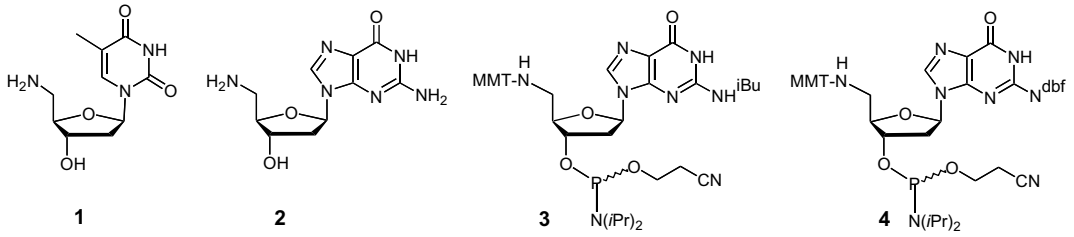
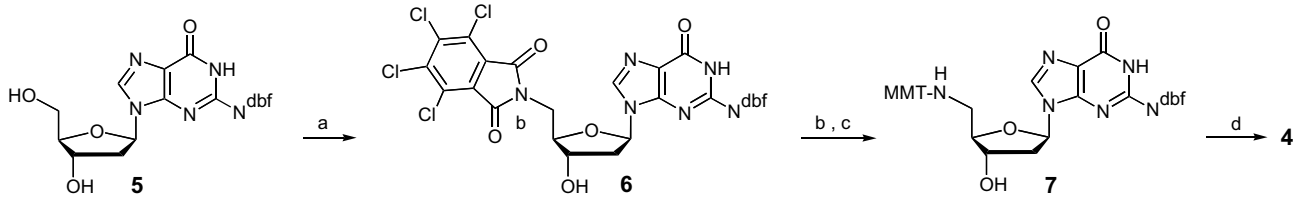


Figure 1. Structures of selected aminodeoxynucleosides.



Scheme 1. Reagents and conditions: (a) tetrachlorophthalimide, PPh_3, DIAD, 92%; (b) $H_2NC_2H_4NH_2$; (c) MMT-Cl, NEt_3, DMAP, 85% over two steps; (d) chloro-(2-cyanoethyl)-*N*,*N*-diisopropylaminophosphoramidite, DIEA, 87%.

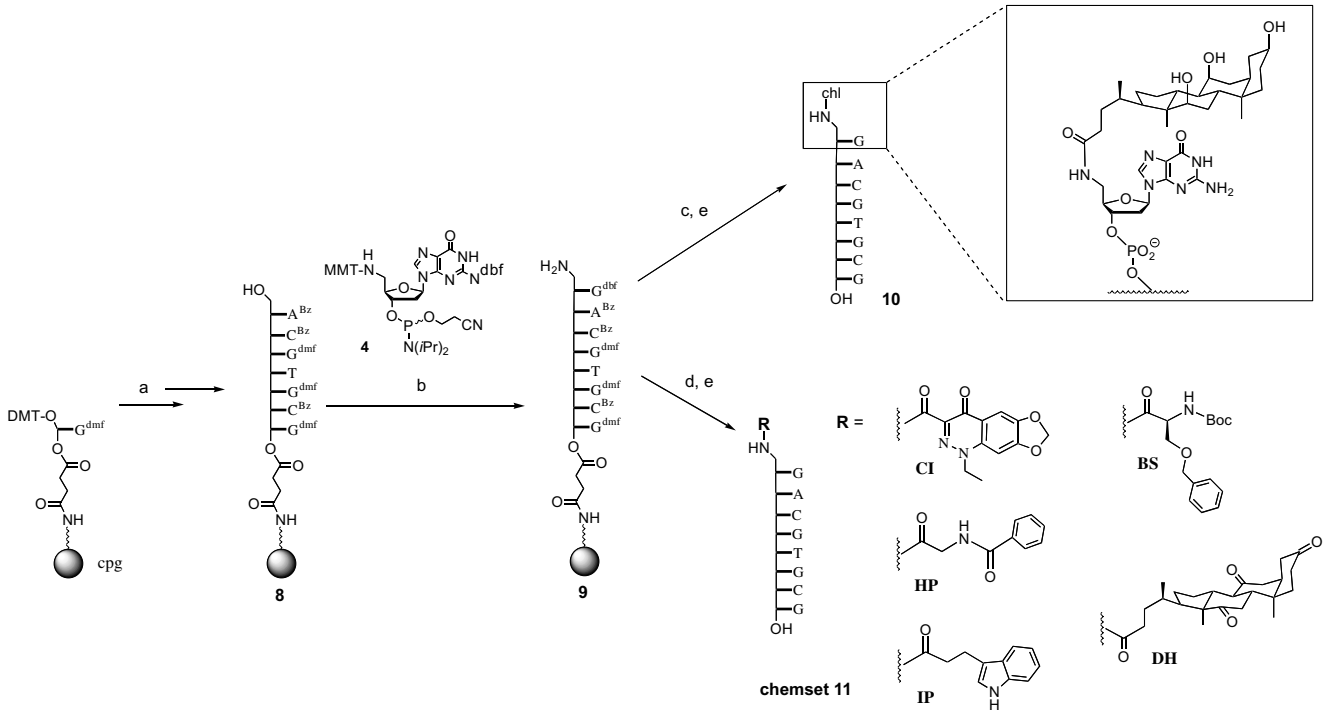
synthetic route that also avoided the use of azides. Here we describe the synthesis of **4** (Fig. 1), via a four step, three-pot route from 2′-deoxyguanosine.

One commercial 2′-deoxyguanosine phosphoramidite building block for DNA synthesis designed for mild deprotection carries the dimethylformamidino (dmf) protecting group for the *N*2-position.[26] Earlier work suggested that this protecting group does not readily migrate when coupling carboxylic acids to a 5′-amino group of a support-bound oligonucleotide.[22] Our first attempt to prepare a 5′-amino-2′,5′-dideoxyguanosine phosphoramidite therefore started from 2-*N*-dimethylformamidino-2′-deoxyguanosine.[27,28] This compound proved too insoluble in common organic solvents for high yielding tosylations or Mitsunobu reactions. To improve solubility, 2-*N*-dibutylformamidino (dbf) protected guanosine[28] (**5**, Scheme 1) was employed, which is conveniently prepared by incubating 2′-deoxyguanosine with *N*,*N*-di-*n*-butylformamide diethyl acetal.[29] Attempts to convert **5** into an azide in one step, using either the pyridine adduct of $Zn(N_3)_2$ or $CBr_4/PPh_3/NaN_3$ gave little or no product. Tosylation proved unusually slow and not sufficiently regioselective. However, a Mitsunobu reaction with tetrachlorophthalimide[30] led to **6** in 92% yield.† Unlike the analogous tetrachlorophthalimide of thymidine,[22] **6** is sufficiently soluble for flash chromatography. Attempts to cleave selectively the tetrachlorophthalimide (TCP) group with ethylenediamine under the conditions established for the preparation of 5′-amino-5′-deoxythymidine[22] (15 equiv in THF) led to **2**. To avoid concurrent loss of the dbf group, the reaction conditions were carefully optimized under MALDI-TOF monitoring.‡

While employing stoichiometric amounts of ethylenediamine was expected to improve the chemoselectivity of the deprotection reaction, it also allowed for a one-pot conversion from **6** to **7**, since it would avoid excess amines at the end of the deprotection and lead to a solution suitable for tritylation. Interestingly, a one-pot conversion in DMF with 1.1 equiv of ethylenediamine gave incomplete deprotection even after 3 days, and no **7** was isolated after addition of MMT-Cl, DMAP, and pyridine. A deprotection with 1.1 equiv of hydrazine in DMF showed complete conversion of **6** to the amide intermediate, but the removal of the entire TCP moiety could not be induced, even when heating to 45 °C for 5 h. Heating to 60 °C led to decomposition. Treating **6** with 1.1 equiv of ethylenediamine in pyridine led to full liberation of the 5′-amine within 2 days at 4 °C, and tritylation to **7** in the same solution gave the protected amine in 50% yield after chromatography. However, scaling up proved difficult, presumably due to the presence of pyridinium salts that complicated purification.

† To a stirred slurry of 2-*N*-(di-*n*-butylformamidino)-2′-deoxyguanosine (700 mg, 1.72 mmol), PPh_3 (575 mg, 2.58 mmol), and tetrachlorophthalimide (611 mg, 2.15 mmol) in THF (70 mL) was added DIAD (0.49 mL, 2.58 mmol) dropwise over 15 min. After 30 min, the slurry turned clear, and after 8 h, the solvent was removed in vacuo. Chromatography (silica, CH_2Cl_2/MeOH, 95:5) gave 1.06 g (1.58 mmol, 92%) of **6** as a slightly yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.6 (s, 1H), 8.5 (br s, 1H), 8.0 (s, 1H), 6.3 (t, $J = 6.4$ Hz, 1H), 4.8 (m, 1H), 4.4 (m, 1H), 4.0–3.8 (m, 2H), 3.4 (m, 4H), 3.1–2.6 (m, 2H), 1.6–1.3 (m, 8H), 0.9 (m, 6H); MALDI-TOF MS calcd for $C_{27}H_{30}Cl_4N_7O_5$([M+H]$^+$): 674.4, found 674.5.

‡ An aliquot (0.5 μL) of the reaction mixture was diluted 100-fold with $CHCl_3$/MeOH (9:1) and 0.5 μL of the resulting solution was placed on a MALDI target. A saturated solution of 6-aza-2-thiothymine (ATT) in MeCN (0.5 μL) was mixed with the analyte solution and the resulting mixture was dried in vacuo. MALDI-TOF spectra were recorded on a Bruker REFLEX IV spectrometer in positive linear mode.



Scheme 2. (a) DNA synthesis via phosphoramidite protocol; (b) extension/deprotection cycle with **4**; (c) cholic acid, HBTU, HOBT, DIEA; (d) carboxylic acid mixture, HBTU, HOBT, DIEA; (e) NH_4OH.

Improved yields were realized by switching to THF and CH_2Cl_2 as solvents. Ethylenediamine treatment of **6** in THF for 16 h at room temperature, followed by evaporation, re-dissolving in CH_2Cl_2 in the same flask, and addition of NEt_3, MMT-Cl and DMAP gave a clean conversion to **7**. The use of DMAP in the tritylation step and careful quenching with MeOH/CH_2Cl_2 at 0 °C proved essential for high yields. While the two solvents method gave 85% of **7**,[§] a single solvent method, where both steps were performed in dichloromethane, required refluxing for 24 h for the TCP removal to go to completion and gave 80% yield.[¶] Though two chromatographic steps have been used in the current protocol to remove residual by-products from a few mixed fractions, flash columns are much easier to run when employing stoichiometric amounts of ethylenediamine, compared to reactions with large excesses of the diamine that often produce poorly soluble TCP derivatives carrying MMT groups.

Phosphitylation of **7** using either 2-cyanoethyl-bis(*N*,*N*-diisopropylamino)phosphine or the related chloro-2-cyanoethyl-*N*,*N*-diisopropylaminophosphine gave **4** in 83% and 87% yield,[**] respectively. Phosphoramidite **4**

[§] To a solution of **6** (200 mg, 297 μmol) in THF (10 mL) was added ethylenediamine (22 μL, 327 μmol) at 0 °C, and the solution stirred for 60 min in the cold. The reaction was allowed to warm to room temperature and proceed for 16 h, during which time a white precipitate formed. The solvent was removed in vacuo, producing a crude product that was directly used for the subsequent conversion. (MALDI-TOF MS for the free amine gave calcd for $C_{19}H_{32}N_7O_3$ ($[M+H]^+$): 406.5, found 406.1) The crude product was taken up in CH_2Cl_2 (5 mL), followed by addition of NEt_3 (0.2 mL, 1.5 mmol), *p*-anisylchlorodiphenylmethane (MMT-Cl, 202 mg, 654 μmol), and DMAP (1.8 mg, 15 μmol). After 3 h, the solution was cooled to 0 °C and quenched dropwise with cold MeOH/CH_2Cl_2 (1:1, v/v, 20 mL). The solvents were removed in vacuo, followed by chromatography (silica, preconditioned with 2.5% of NEt_3, elution with a step gradient of MeOH in CH_2Cl_2 from 0% to 3%). Impure fractions were re-chromatographed to give a total of 171 mg (252 μmol, 85% over two steps) of **7** as an off-white foam. ^1H NMR (250 MHz, $CDCl_3$) δ (ppm): 8.7 (br s, 1H), 8.5 (s, 1H), 7.4 (s, 1H), 7.3–7.0 (m, 12H), 6.7 (m, 2H), 6.3 (t, $J = 6.4$ Hz, 1H), 4.5 (m, 1H), 4.1 (m, 1H), 3.6 (s, 3H), 3.4–3.2 (m, 2H), 2.5–2.1 (m, 4H), 1.6–1.2 (m, 8H), 0.9 (m, 6H); MALDI-TOF MS calcd for $C_{39}H_{48}N_7O_4$ ($[M+H]^+$): 678.8, found 677.8.

[¶] A sample of **6** (200 mg, 297 μmol) was taken up in CH_2Cl_2 (20 mL) and ethylenediamine (22 μL, 327 μmol) was added. The mixture was refluxed for 24 h. Subsequently, the solvent volume was reduced in vacuo to approximately 5 mL. Then, NEt_3 (0.2 mL, 1.5 mmol), MMT-Cl (202 mg, 654 μmol), and DMAP (1.8 mg, 15 μmol) were added. Reaction time, quenching, and chromatography were identical to those of the two-solvent method, above. Yield of **7**: 161 mg (238 μmol, 80% over two steps).

[**] To a solution of **7** (74 mg, 109 μmol) in anhydrous MeCN (2 mL) was added diisopropylethylamine (DIEA, 57 μL, 327 mmol) and chloro-2-cyanoethyl-*N*,*N*-(diisopropyl)chlorophosphoramidite (49 μL, 218 μmol). The solution was stirred for 4 h. Then, CH_2Cl_2 (50 mL) was added, and the solution was washed with satd $NaHCO_3$ sol (2×50 mL) and brine (2×50 mL). The organic phase was dried over Na_2SO_4 and concentrated to approximately 1 mL. The solution was added dropwise to pentane (50 mL) and cooled to −20 °C overnight. A white precipitate formed. The supernatant was aspired, and the precipitate dried at 0.1 Torr to yield 83 mg (94.8 μmol, 87%) of **4** (two diastereomers) as an off-white solid.[31] P NMR (202 MHz, $CDCl_3$, NEt_3) δ (ppm): 146.9, 146.4; MALDI-TOF MS calcd for $C_{48}H_{65}N_9O_5P$ ($[M+H]^+$): 879.1, found 879.4.

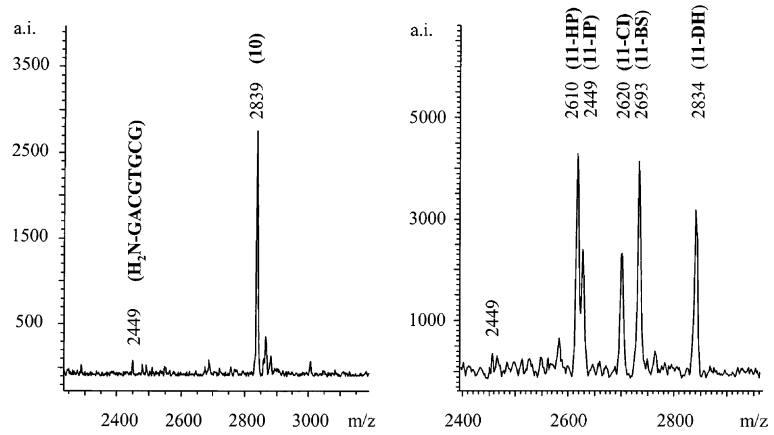


Figure 2. Molecular ion region of the MALDI-TOF mass spectra of crude **10** and **chemset 11**, acquired in negative, linear mode using a matrix of trihydroxyacetophenone and a co-matrix of diammonium citrate.

was coupled to the growing chain of oligonucleotide **8** on controlled pore glass (Scheme 2). Removal of the MMT group with the trichloroacetic acid solution routinely used for the deblocking step of automated chain assembly (3% in CH_2Cl_2) gave **9**, which was either coupled to cholic acid to give, after deprotection, 5′-capped **10**,[††] or treated with a reactivity-adjusted mixture of carboxylic acids[31] and deprotected to produce **chemset 11**.[‡‡] The cholic acid residue has been shown to enhance target affinity of hybridization probes and to improve base pairing fidelity at the terminus of duplexes.[16,17] Oligonucleotides bearing this bile acid residue have shown promise in DNA microarray applications.[18]

The MALDI-TOF spectra of the crude products of the solid phase syntheses (Fig. 2) show all the expected products and few impurities, making **chemset 11** suitable for spectrometrically monitored selection experiments (SMOSE).[32] The use of crude chemsets of oligonucleotides in SMOSE assays allows for a rapid search for new high affinity hybridization probes,[33] avoiding the time consuming synthesis and purification of individual compounds. Finally, the three-step/two-pot conversion from alcohol to MMT-protected amine, exemplified in the conversion of **5** to **7**, may prove useful in other synthetic work. Exploratory experiments with 2′-deoxy-6-*N*-(dimethylformamidino) adenosine[27,34] indicate that the route to a protected 5′-amino phosphoramidite presented in Scheme 1 can also be successfully employed for this substrate.

[††] A solution of cholic acid (40.9 mg, 100 μmol), HOBT (13.5 mg, 100 μmol), and HBTU (34.1 mg, 90 μmol) in DMF (600 μL) was treated with DIEA (40 μL, 230 μmol). After 10 min, the solution was added to support **8** (5 mg, approximately 0.19 μmol loading). After 45 min with occasional mixing, the solid support was rinsed with DMF (2 mL) and MeCN (3×2 mL). After drying in vacuo, the support was treated with ammonium hydroxide (30% aqueous NH_3, 1 mL) for 14 h. MALDI-TOF MS (linear, negative mode, THAP) calcd for $[M-H]^-$: 2839.2, found 2838.6.

[‡‡] A mixture of hippuric acid (3.26 mg, 18.1 μmol), 3-indolepropionic acid (3.75 mg, 19.8 μmol), Boc–Ser(Bzl)–OH (7.12 mg, 24.1 μmol), dehydrocholic acid (11.87 mg, 29.5 μmol), and cinoxacin (2.25 mg, 8.5 μmol) in DMF (500 μL) was prepared from stock solutions and activated with HBTU, HOBT, and DIEA in DMF (plus 100 μL) and coupled as described for **10**. MALDI-TOF MS calcd for **11-HP** ($[M-H]^-$): 2609.8, found 2610.3, calcd for **11-IP** ($[M-H]^-$): 2619.8, found 2620.2, calcd for **11-CI** ($[M-H]^-$): 2692.8, found 2693.4, calcd for **11-BS** ($[M-H]^-$): 2725.9, found 2726.3, calcd for **11-DH** ($[M-H]^-$): 2833.1, found 2833.

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