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A Convenient Synthesis of 5'-Amino-5'-deoxythymidine and Preparation of Peptide-DNA Hybrids

Charles N. Tetzlaff, Ina Schwope, Colleen F. Bleczinski, Joshua A. Steinberg and Clemens Richert*

Department of Chemistry, Tufts University, 62 Talbot Avenue, Medford, MA 02155, USA

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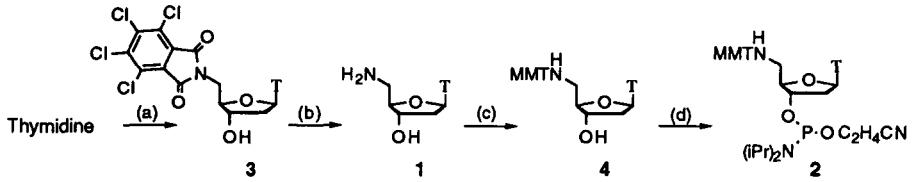
Abstract: 5'-Amino-5'-deoxythymidine was prepared from thymidine in two steps and converted to its known 5'-methoxytrityl-protected 3'-phosphoramidite building block for DNA assembly on solid supports. Using this building block, peptide-DNA hybrids were synthesized in stepwise manner or via fragment condensation, both as single compounds and as small combinatorial libraries.

Keywords: Nucleosides; Nucleic acid analogues; Mitsunobu reactions

5'-Amino-5'-deoxythymidine (**1**),[1] a nucleoside analog with antiviral activity,[2] has been employed for the synthesis of phosphoramidate-linked DNA analogs.[3,4] Further, oligodeoxyribonucleotides with a 5'-amino-5'-deoxythymidine terminus[5] are useful bioorganic model compounds.[6,7] Additionally, the aminothymidine building block allows a linker-free construction of 5'-peptide-DNA hybrids.[8,9] We became interested in developing an alternative to the currently favored synthetic route to 5'-amino-5'-deoxythymidine, which employs azides as the key intermediates.[1,2,4,10] Here we report such an alternative two-step synthesis of **1**, its conversion to the 3'-monomethoxytrityl-protected 3'-phosphoramidite **2**,[4] and the solid-phase synthesis of peptide-DNA hybrids. The synthesis established for **2** proceeds in 64% yield over four steps and requires a single chromatographic step.



3 **1** **4** **2**

(a) Tetrachlorophthalimide, PPh_3, DIAD, THF; (b) Ethylenediamine, THF; (c) MMT-Cl, TEA, DMAP, pyridine, 81% over 3 steps; (d) Diisopropylammonium tetrazolide, $NCCH_2CH_2OP(N\text{-}i\text{-}Pr_2)_2$, CH_3CN, 79%

Starting from thymidine, Fraser Reid's tetrachlorophthalimide group[11] was regioselectively introduced to the 5'-position under Mitsunobu conditions. Intramolecular formation of 2,5'-*O*-anhydrothymidine, a side product previously described for a Mitsunobu reaction with 2',3'-isopropylideneuridine,[12] appeared to compete successfully with formation of the desired product (**3**) when this reaction was performed with phthalimide. For the more reactive tetrachlorophthalimide, however, the desired *inter*molecular attack on the activated 5'-position strongly dominated, as long as 1.25 or more equivalents of the reagents were used. The low solubility of **3** in most organic solvents allowed its isolation via precipitation. Upon treatment with ethylenediamine in THF, **3** was smoothly converted to **1**, which could be isolated chromatographically. More convenient was direct conversion of the crude reaction product, however, which led to monomethoxytrityl-derivative **4** in 81% isolated

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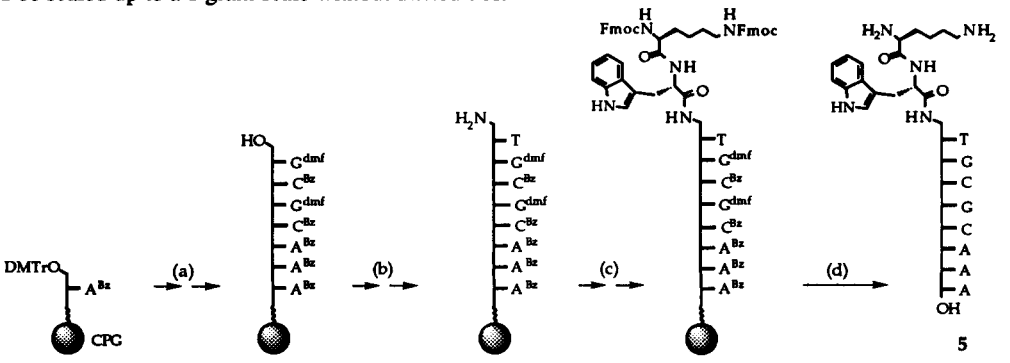
yield over three steps. 5'-Protected **4** was then phosphitylated to give **2**,[4] whose selective precipitation (79% isolated yield) concluded the preparation of the building block for automated oligonucleotide synthesis, which could be scaled up to a 1 gram scale without difficulties.[13]



(a) Standard phosphoramidite protocol; (b) coupling cycle with **2**; (c) 1. Fmoc-Trp-OH, HOBT, HBTU, DIEA, DMF; 2. Piperidine, DMF; 3. Fmoc-Lys(Fmoc)-OH, HOBT, HBTU, DIEA, DMF; (d) NH_4OH, *m*-cresol.

Assembly of peptide-DNA hybrids started from 5'-amino-terminal protected DNA hexamers and octamers, obtained when the last chain extension cycle was performed with **2**. While an oligothymidylate with an internal oligoalanine modification has been prepared on a sarcosine-modified controlled pore glass support (CPG) using both allyl and cyanoethyl phosphoramidites,[8] our synthesis uses standard CPG, commercially available cyanoethyl phosphoramidites, and allows preparation of mixed DNA sequences. Optimization relied heavily on MALDI-TOF mass spectrometry under conditions previously shown to give quantitative results.[14] Several side reactions had to be suppressed to achieve high yields in the coupling of the 5'-amino group with amino acid building blocks. First, migration of protecting groups from the exocyclic amino functionalities of the nucleobases to the terminal amino group was observed when *t*-butylphenoxyacetyl-protected phosphoramidite building blocks (EXPEDITE protocol) were employed. The use of the traditional benzoyl groups for A and C, and *i*-butyroyl or, preferably, dmf-protection groups for the amino groups of G solved this problem. Second, during standard ammonium hydroxide deprotection, the acrylonitrile released from the phosphotriester moieties alkylated amino groups of the terminus and lysine side chains. The same side reaction, which is particularly fast when deprotecting larger CPG batches at elevated temperature, has previously been observed when deprotecting 2'-*O*-aminopropyl ribonucleotides.[15] We were able to suppress this reaction without changes in the protecting group scheme[15] by adding 3% *m*-cresol scavenger to the deprotection mixture. Third, increased fragmentation of the phosphodiester backbone was observed, particularly when basic sequences were synthesized. This side reaction appeared to be absent when non-amino acid appendages were coupled to the amino terminus (not shown) and could not be induced by heating the purified conjugates in ammonium hydroxide, making an attack of nucleophilic groups from the peptide portion on phosphotriester intermediates the most likely cause. For the few sequences where decomposition of more than 20% of the product was observed, allyloxycarbonyl protecting groups gave improved yields.

Peptide couplings employed commercially available N-α-Fmoc-protected amino acid building blocks, a uronium activating agent (HBTU),[16] hydroxybenzotriazole (HOBT), and Hünig's base in DMF. Coupling reactions with symmetrical anhydrides and an active ester (Fmoc-Gly-OPfp) were much less efficient. The HBTU/HOBT protocol typically gave 50-90% yield for the coupling to the 5'-aminothymidine residue, but much higher yields (>90%) for the subsequent chain elongation at the amino-terminus of the newly installed

amino acid residue. Repetitive coupling at the oligonucleotide-terminus improved the yields, but a fraction of uncoupled amino-terminal DNA was detectable in mass spectra, even after three reactions. Interestingly, this unreactive fraction appeared constant for a given CPG batch, largely independent of the amino acid coupled. To exclude intramolecular conjugate addition of the amino group, a reaction previously observed for a uridine derivative,[17] which would produce a compound isobaric to the unreacted amino-terminal DNA, we synthesized the dinucleotide 5'-amino-5'-deoxy-TpA on 10 μmolar scale, coupled it with Fmoc-Trp-OH, and analyzed the HPLC-purified uncoupled material by NMR. All spectroscopic data were consistent with the unmodified dinucleotide, indicating that the unreactive portion of the CPG-bound oligomers does probably not consist of covalently modified chains, unless the modification reaction is reversed under deprotection conditions. Possibly, steric factors decrease the reactivity of portions of the tightly packed CPG surface in the peptide coupling reactions, since, unlike typical peptide synthesis resins, CPG cannot swell in organic solvents. This assumption is corroborated by low yields obtained on high-loading CPG (90 μmol/g). When (30 μmol/g) CPG was employed and occasional unreactive batches were discarded, satisfactory coupling yields were obtained routinely, allowing the convenient assembly of a variety of peptide-DNA sequence combinations. All steps were performed in CPG cartridges for automated DNA synthesis, using standard Fmoc-deprotection conditions (20% piperidine in DMF) between couplings, and full deprotection with ammonium hydroxide, followed by purification via RP_{18} HPLC or ion exchange chromatography. While racemization cannot be rigorously excluded, HPLC traces and NMR spectra do not show signs of undesired diastereomers.

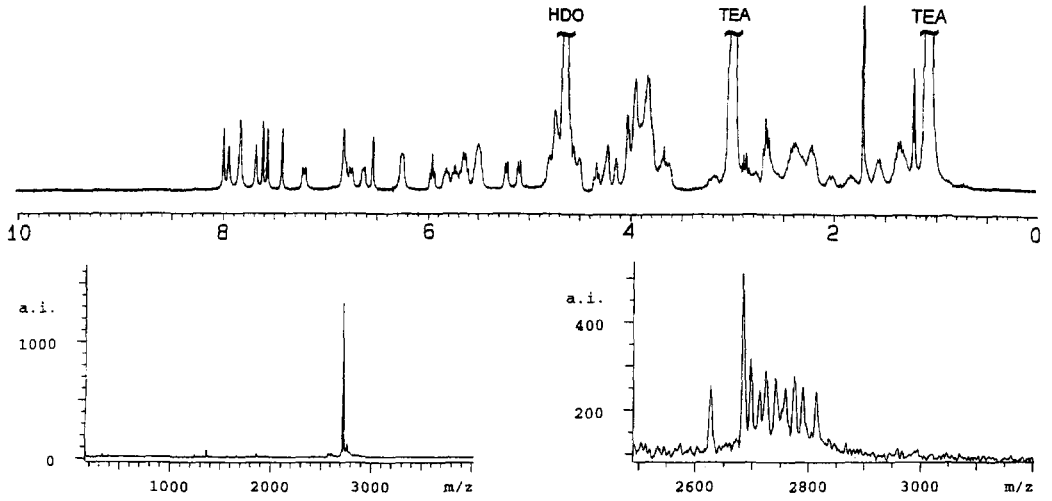


Figure 1. Top panel ^{1}H-NMR spectrum (D_2O, 300 MHz) of Lys-Trp-TGCGCAAA (**5**, $HNEt_3^+$ salt);[18] Lower left panel: MALDI-TOF mass spectrum of **5**; lower right panel: MALDI-TOF mass spectrum (molecular ion region) of the crude 10-component library XW-TGGTTGAC, where X = Ala, Asp, Gly, Met, Phe, Pro, Ser, Trp, Tyr, and uncoupled W-TGGTTGAC.

Hybrids up to the tetrapeptide level have so far been synthesized with *N*-α-Fmoc building blocks of Ala, Asn, Gln, Gly, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val, as well as with Fmoc-Asp(OBzl)-OH, Fmoc-Cys(*t*-Buthio)-OH, Fmoc-Glu(OBzl)-OH, and Fmoc-Lys(Fmoc)-OH, allowing hybrid syntheses with all but two naturally occurring amino acids. To explore the feasibility of combinatorial syntheses with peptide libraries prepared via "split and combine" methodology, we coupled *N*-acetylated pentapeptide Ac-Ala-Tyr-Gln-Val-Phe to amino-terminal tri- and tetranucleotides. While this synthesis succeeded uneventfully,[19] coupling with sidechain Fmoc-protected peptides suffered from their low solubility in DMF, making a stepwise assembly

of hybrid libraries advisable. An exploratory experiment with a mixture of equimolar amounts of Fmoc-protected amino acids and two equivalents of Fmoc-Pro-OH gave comparable incorporation rates for a nine component mixture, except for Gly, which appears to be approximately twice as reactive as the competing building blocks (Figure 1). Combinatorial libraries prepared in this fashion allow for spectrometrically-monitored *in vitro* selection of peptide-DNA hybrids, facilitating the efficient identification of antisense inhibitors with improved bioavailability and target selectivity. Progress in this area will be reported in due course.

Acknowledgments

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13. To a suspension of thymidine (1.04g, 4.29mmol), PPh_3 (1.69g, 6.44mmol), and tetrachlorophthalimide (1.53g, 5.36mmol) in THF (100mL) was added diisopropyl azodicarboxylate (1.25mL, 6.35mmol) over 15 min. A clear solution resulted, from which a white precipitate formed. The precipitate was filtered off, the filtrate was reduced to 5mL, and additional product precipitated with ethyl acetate/petroleum ether, (1:1). Precipiates were combined, washed and dried. ^1H NMR of **3** (300MHz, DMSO-d_6, δ) 1.80 (s, 3H), 2.06 (m, 1H), 2.26 (m, 1H), 3.81 (m, 2H), 3.98 (m, 1H), 4.24 (quin., 1H, J=3.2), 6.12 (dd. 1H, J=8.1, 6.0 Hz), 7.54 (s, 2H). LD-TOF MS *m/z* 509.6 (base peak of 4xCl isotope pattern). Crude **3** was suspended in THF (35mL) and treated with ethylenediamine (4.8mL, 72mmol). After 4h, the clear solution was evaporated and dried to give a glass, which could be chromatographed to give pure **1**, or directly converted to **4** by addition of *p*-anisylchlorodiphenylmethane (2.85g, 9.22 mmol), dimethylaminopyridine (1mg, 8 μmol), and pyridine (20 mL). Methanol (5mL) was added after overnight stirring, followed by evaporation, drying and chromatogtaphy (silica, CH_2Cl_2, NEt_3, 99:1; MeOH 2% to 5% to 10%), yielding **4** (1.88g, 3.48mmol, 81%), whose spectroscopic data were in agreement with the literature (ref. 4).
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18. ^1H NMR, **5** (D_2O, 300MHz, low field region only): δ 7.96, 7.91, 7.65, 7.58, 7.53, 7.39 (6s, 6x1H) and 7.80 (s, 2H) (H8, H2 purines); 7.17, 6.72 (2d, 2x1H, H-6 C); 6.79 (m, 2H), 6.59 (m, 1H), 6.50 (s, 1H) and 6.22 (m 2H) (Trp ring H, H-6 T); 5.92, 5.79, 5.70 (3t, 3x1H), 5.60 (m, 2H) and 5.48 (m, 3H) (H-1'); 5.19, 5.06 (2d, 2x1H, H-5 C).
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