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A NEW METHOD FOR INTRODUCING AMIDATE LINKAGES IN OLIGONUCLEOTIDES USING PHOSPHORAMIDITE CHEMISTRY

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Abstract: Cyanoethyl-protected phosphotriester links in oligonucleotides made with standard phosphoramidite chemistry were converted to phosphoramidate linkages during oligonucleotide synthesis on solid support. The cyanoethyl group was removed with piperidine, and the resulting phosphodiester was activated with *p*-tosyl chloride. An amine nucleophile displaced the tosyl to yield a phosphoramidate linkage.

Antisense oligonucleotides are finding a wide range of use from basic research to treating disease.[1] A great effort has been placed in creating modified oligomers either through modified bases, linkages, or conjugation chemistry.[2] Phosphoramidate links are phosphodiester analogs that have shown promise for improving duplex stability when used with other linkage types in the same oligonucleotide.[2,3] A variety of different approaches have been described, including oxidation of methyl phosphite triesters in the presence of an amine, condensation of phosphate triesters with an amine in the presence of triphenylphosphine-CCl_4, and oxidation of H-phosphonate diesters with iodine or CCl_4 in the presence of an amine.[4] Since most automated oligonucleotide synthesis utilizes cyanoethyl phosphoramidite chemistry, it is attractive to introduce amidate links using the same chemistry.

In the present work we demonstrate a new method that converts the cyanoethyl triester intermediate directly to an N-alkyl phosphoramidate by removing the cyanoethyl group, converting the phosphodiester to an activated arylsulfonyl mixed ester,[5] and displacing with the amine of choice. The result is a clean, high yielding conversion. This approach can be used to introduce a variety of functional groups to improve hybridization, or bifunctional amines to be used for conjugation. By using cyanoethyl amidites in conjunction with methyl-protected amidites, one can introduce site-selective amidate links within an otherwise phosphodiester (or thioate) oligonucleotide.

The model system was a TpT dimer synthesized via standard amidite chemistry[6] (Scheme 1). First piperidine was used to remove the cyanoethyl group on the support. The support was then treated with p-tosyl chloride in pyridine for 10 min to make the active ester,[5] followed by *n*-butylamine in pyridine for 10 min, with appropriate wash steps in between. The compound was cleaved in ammonia, and analyzed by HPLC, MS, and ^{31}P NMR.[7] After one piperidine/tosyl-Cl/amine cycle, the ratio of amidate to diester was about 1:1; the same result was achieved with triisopropylbenzenesulfonyl chloride. Repeating the cycle 6

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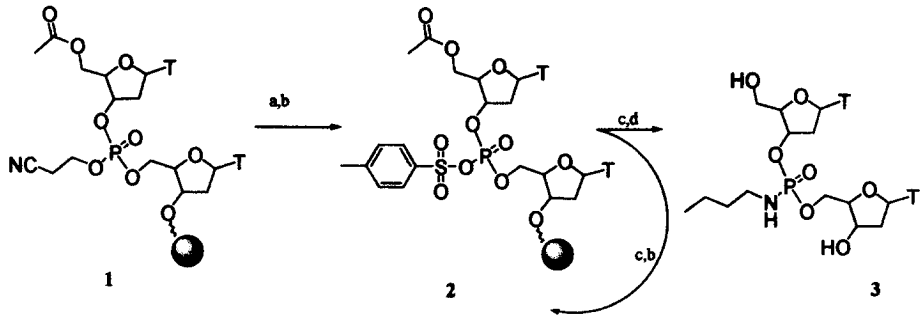
times in succession led to a 95% conversion of diester to amidate (Table 1.).



Scheme 1. Conversion of cyanoethyl phosphotriester to phosphoramidate. Conditions and reagents: (a) 10% piperidine in DMF, 0.2 mL, 10 min; (b) 0.2 M tosyl chloride, 0.4 M NMI, pyridine, 0.2 mL, 10 min; (c) 5% (v/v) *n*-butylamine in pyridine, 0.2 mL, 10 min; d) NH_4OH, 1.0 mL, 2 h.

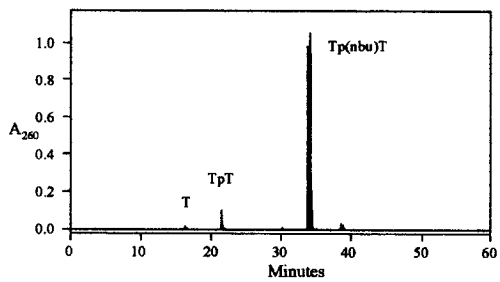


Figure 1. HPLC trace of crude **3** after six conversion cycles

Table 1. Conversion of phosphodiester to *n*-butylamine phosphoramidate

conversion cycles	TpT conversion	theoretical conversion[a]	HPLC purity
1	49%		98%
3	85%	87%	97%
6	95%	98%	97%

[a] based on a single-cycle conversion

References and Notes

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6. 1-μmole T-CPG (ABI, Foster City, CA) was coupled with T amidite (ABI), oxidized to the phosphotriester, and the DMT removed using standard reagents and conditions on an ABI 396 synthesizer. The support was treated with the capping reagents to acetylate the terminal hydroxyl. A custom cycle was used to first treat with 10% (v/v) piperidine in DMF, then alternate treatments of sulfonyl chloride solution (0.2 M chloride, 0.4 M N-methylimidazole, pyridine, 0.2 mL, 10 min) and butylamine (5% (v/v) in pyridine, 0.2 mL, 10 minutes) for the prescribed number of times, with appropriate wash steps in between. The compound was cleaved from the support with 1.0 mL conc NH_4OH, dried with a Speed Vac and reconstituted in H_2O.
7. Crude mixtures were analyzed by HPLC with a Waters 625 LC, 991 detector, Delta-pak C18(300A, 5 μ) 150 x 4.6 mm; a linear gradient of 0–100% acetonitrile in 100 mM ammonium acetate pH 6.5 over 50 minutes, 1 mL/min flow rate, and UV detection at 260 nM. The two diastereomer peaks were nearly equal in area. MS was collected on a Hewlett-Packard HP 5989 quadropole mass spectrometer; ESMS (M-H) 600.4 observed, 600.5 expected. ^{31}P NMR were collected on a Varian Gemini 200 NMR Spectrometer at 161.9 MHz; δ(**3**) 11.37(relative to conc H_3PO_4).