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LARGE-SCALE OLIGONUCLEOTIDE SYNTHESIS BY THE H-PHOSPHONATE METHOD

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Abstract: The H-phosphonate method is capable of giving good yields, on scales in the area of 14 μmole, with as little as 2eq of monomer. Under these conditions, however, capping was found to be necessary. Cyanoethyl H-phosphonate has been introduced for this purpose and shown to be effective.

Oligonucleotide synthesis using nucleoside H-phosphonates has been reported,[1] but became practical only with the recent introduction of pivaloyl chloride as the condensing agent.[2] There since have been reports of successful use of this method in both the deoxy[3] and ribo[4] series as well as for the facile generation of phosphoramidate and phosphorthioate analogs.[5] We are interested in the synthesis, on a large scale, of oligonucleotides containing modified and/or isotopically labeled nucleosides.[6] The H-phosphonate method appeared to offer some advantages for this type of oligonucleotide synthesis. The potential recoverability of the unreacted excess deoxynucleoside H-phosphonates, especially if modified or isotopically labeled, and the use of inexpensive pivaloyl chloride rather than tetrazole, are particularly attractive features. The syntheses reported to date, however, typically have been on scales of 0.2 μmole or less, in contrast to the 10 - 20 μmole scales we prefer to use, at present, and the still larger scales which could be envisioned.

We report a series of experiments designed to explore large-scale synthesis by the H-phosphonate method. Two obvious areas of concern are the nature of the active intermediate and the extent of self-capping. It has been shown that reaction of pivaloyl chloride with an H-phosphonate produces an active intermediate, presumably a mixed anhydride,which reacts rapidly with the 5'-OH.[7] Alternatively, this intermediate can react further with pivaloyl chloride to generate a bis-acylphosphite, which can then undergo an Arbusov-type reaction.[8] On small scales, where only 5-10 mg of CPG are employed, reaction times may be short enough to avoid these competing reactions. This may no longer be true when hundreds of mgs of CPG are used, and the residence time in the column is therefore much longer. Hence our first concern was to the effect, if any, of this longer time on the coupling efficiency and on the ratio of coupling to capping.

As a model reaction we looked first at the reaction of 5'-DMT-thymidine-3'- H-phosphonate (**1**) with CPG bound thymidine. These experiments were carried out on a Biosearch 8600 synthesizer, on a scale of 14 μmole. The H-phosphonate, as its DBU salt (15 mM), and pivaloyl chloride (75 mM), were introduced in alternate pulses (0.5sec each, at ~80 μL/sec).[9] After detritylation, the products were cleaved from the CPG using aqueous ammonia and the mixture thus obtained was analyzed by hplc[10] to give the data presented in the Table. With ~4 eq of the H-phosphonate (**1**), in a 90 sec reaction, the yield of TT was 92% (expt 1). Note, however, that 8% of unreacted and uncapped thymidine remained. Dropping to ~2 eq of **1** in a 45 sec reaction (expt 2) gave, as expected, a lower yield, with a corresponding increase in the amount of T remaining. Doubling the amount of pivaloyl chloride (expt 3) increased the self-capping, at the expense of coupling. Premixing **1** and pivaloyl chloride for ~20 min (expt 4) significantly lowered the yield, indicative of the short lifetime of the active intermediate. The introduction of a 15 sec wait after each addition cycle was similarly counterproductive (expt 5), serving mainly to increase sharply the extent of

self-capping. Finally, addition of a 180 sec wait after completing the addition proved to be as effective at increasing the yield as was increasing the amount of **1** from 2 to 6 eq (expts 2, 6 & 7, respectively).

expt	eq **1**	eq piv-Cl	time (sec)	% T[a]	% TT[a]	% piv-T[a]
1	4	5 x **1**	90	7.6	92	0.8
2	2	5 x **1**	45	13	87	0.6
3	2	10 x **1**	45	15	82	3.0
4	2	10 x **1**	45[b]	49	48	2.5
5	2	10 x **1**	720[c]	12	71	17
6	2	5 x **1**	225[d]	6.5	93	1.2
7	6	5 x **1**	135	6.2	93	1.0

a: by hplc[10]; b: **1** & piv-Cl were mixed together ~20 min prior to the coupling reaction; c: after each cycle of **1** + piv-Cl (0.5 sec each) there was a 15 sec wait; d: after the 45 cycles were complete there was a 180 sec wait.

These results suggest that the species produced by further reaction of the initial intermediate, while clearly less reactive, may not pose additional problems. The amount of thymidine which remained after each of these experiments, however, is a problem. One possible source of this thymidine is from ammonolysis of the pivaloyl group from the piv-T produced by self-capping. Treatment of piv-T with aqueous ammonia indicated that ammonolysis under the conditions used is much too slow a reaction to be significant. Nevertheless, to rule out ammonolysis more definitively, and to determine whether this thymidine is in fact available for reaction, we carried out experiments in which a second round of coupling (without intervening detritylation) was performed using 5'-DMT-(6-*N*-benzoyl)-2'-deoxyadenosine-3'-H-phosphonate (**2**). For example, using the conditions of expt 6 above, coupling first with **1** and then with **2**, followed by detritylation and treatment with aqueous ammonia, gave a mixture containing: 5.6% T; 92% TT; 1.6% dAT; 0.7% piv-T. The dAT could have been produced only by coupling of **2** with free thymidine, indicating that these small residual amounts of thymidine are uncapped and available for further reaction.

These experiments all deal with the first coupling reaction, performed on 500Å CPG without a spacer. To determine whether these results were limited to the first addition, or were more general, we carried out the following experiment: Nine additions of T (**1**) were followed by addition of dC {5'-DMT-(4-*N*-benzoyl)-2'-deoxycytidine-3'-H-phosphonate (**3**)} and (without detritylation) dA (**2**). The product mixture then was cleaved from the CPG using aqueous ammonia, and the trityl-containing material was isolated by hplc.[9, 11] After detritylation the mixture was degraded to deoxynucleosides using venom phosphodiesterase and alkaline phosphatase, so that the amount of dA, if any, could be determined by hplc (2 to 15% MeCN: 0.1M TEAA in 5 min at 4 mL/min). The ratio found was 93% dC: 7% dA, indicating once again that free 5'-OH remained after the addition of **3**. Thus, at least under these conditions of scale and coupling yield, self-capping by pivaloyl chloride is not sufficient. Presumably, given a sufficient excess of nucleoside phosphonate, the yields could be made to be high enough to overcome this problem. For example, for the dimer, using 8 eq of **1** the amount of dAT formed was reduced to 0.07%. But with larger scale work, and particularly if precious modified or labeled nucleosides are involved, large excesses are not desirable.

The alternative is to employ a capping step. The most convenient capping agent would be the pivaloyl chloride used as the coupling agent, if it were reactive enough to ensure complete capping. Trial experiments with CPG bound thymidine, however, indicated that the capping efficiency for pivaloyl chloride alone was under 50% for a 180 sec reaction. This seemed too slow to be useful. The next obvious choice, in view of the outstanding reactivity of the deoxynucleoside H-phosphonates, is to use some less valuable H-phosphonate as the capping agent, in a reaction similar to the coupling reaction.[12] We chose cyanoethyl H-phosphonate [$NCCH_2CH_2OP(H)O_2H$, **4**]. The failure sequences obtained using this compound, after deprotection with aqueous ammonia, will have a polar 5' phosphate group which should be helpful during the first hplc purification. Moreover, **4** is readily obtained by hydrolysis of the commercially available phosphoramidite.[13] To test the efficiency of this reagent the above

experiments were then repeated with an intervening 180 sec capping step consisting of alternate pulses of a 50mM solution of **4** and a 250 mM solution of pivaloyl choride in a 180 sec reaction. In the case of the dimer only traces of dAT could be detected (0.01%). In the CT_9 experiment a ratio of 99.8% dC: 0.2% dA was found. These dAT and dA peaks were so small that identification was somewhat tentative (the largest peak with approximately the correct retention was assumed) and therefore these amounts should be regarded as upper limits. Nevertheless, they correspond to capping efficiencies of >99% and >97%, respectively.

The octanucleotide d(CCTTAAGG) was then synthesized, on a scale of 13 μmole (350mg CPG), using the conditions of expt 6 and capping as above. In this case ~20 μmole of H-phosphonate was used per step and the average coupling yield, as determined both by an automated trityl assay[14] and by a manual assay, was 91%. The product was purified by reversed phase hplc before and again after detritylation,[11] to give 220 OD_{260} (2.9 μmole).[15] This corresponds to a real yield, for each deoxynucleoside addition step, of 81%. Note that this is a "real" yield, not a "coupling" yield. It is based on the amount of pure product actually obtained at the end of the synthesis, after two hplc purifications.

The 26-mer d(TTCCTTTTTGGAATTCCTTTTTGGAA) was synthesized on a 10 μmole scale (1000Å CPG), using a 90 sec coupling followed by a 90 sec wait and then the 180 sec capping cycle. The amount of H-phosphonate used per step was ~55 μmole and the average coupling yield, by automated trityl assay,[14] was 94%. After hplc purification[11] a total of 74 OD_{260} (0.32 μmole) was obtained.[15] This corresponds to a real yield, for each deoxynucleoside addition step, of 87%.

The results reported above demonstrate that, despite the short lifetime of the active intermediate, yields in the area of 93% can be obtained with as little as 2 eq of deoxynucleoside H-phosphonate, on a scale of 14 μmole. Thus, large-scale oligonucleotide synthesis by the H-phosphonate method is feasible. However, since only a fraction of the remaining 7% is self-capped by pivaloyl chloride, a separate capping step is required. We have introduced cyanoethyl H-phosphonate for this purpose, and shown that it is effective, although other H-phosphonates presumably would be as well.[12] The need for capping here is in contrast to small-scale synthesis, where large excesses of the incoming deoxynucleoside H-phosphonates can be used to push the yield high enough that self-capping can be adequate. The use of such excesses on larger scales becomes prohibitive. The excess deoxynucleoside H-phosphonates are recoverable, but some effort is involved.[14] The inclusion of a capping step allows the number of equivalents used in a given synthesis to be a matter of choice, dependent on the yields required, which are mainly a function of the length of the oligomer to be synthesized, the value of the deoxynucleoside H-phosphonates involved, and the ease of their recovery. Moreover, it seems likely that for the H-phosphonate method to be successful for the synthesis of very long molecules, even on the smaller scales likely to be employed, a capping step will again prove to be required, simply because it will be that much more important to avoid absolutely the generation of deletion sequences. Finally, it is worth noting that, for the examples we have to date, the quality of the crude material obtained by this H-phosphonate method appears to be somewhat better than that we had obtained by the phosphoramidite method.

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9. The deoxynucleoside H-phosphonates were prepared by the procedure of Froehler et al.[3] with the following modifications: The amount of *N*-methylmorpholine used was 46 eq. The reaction mixture was quenched with 20% aqueous pyridine. Silica gel chromatography was performed using a gradient of CH_2Cl_2 to MeOH/ CH_2Cl_2 (2:8) each containing 3% pyridine. The combined product fractions were immediately washed with three portions of 0.1 M aqueous DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) bicarbonate, to generate the more stable DBU salts.
The synthetic cycle was:
   1. Wash, CH_2Cl_2: 20 sec wash, 10 sec wait, repeat 7 times.
   2. Deblock, 2.5% dichloroacetic acid, purines: 20 sec acid, 10 sec wait, 40 sec acid; pyrimidines: 20 sec acid, 20 sec wait, then 10 sec acid, 40 sec wait, repeat last two steps three times.
   3. Wash, CH_2Cl_2: 50 sec.
   4. Wash, pyridine/CH_3CN (1/1): 20 sec wash, 10 sec wait, repeat five times.
   5. Couple: 0.5 sec 15 mM H-phosphonate, 0.5 sec 75 mM or 0.3 sec 125 mM pivaloyl chloride, repeat as described.
   6. Wash, pyridine/CH_3CN (1/1): 20 sec wash, 10 sec wait, repeat five times.
   7. Deblock, or repeat from step 1 until completed, or cap: 0.5 sec 50 mM **4**, 0.5 sec 250 mM pivaloyl chloride, repeat 179 times, wash as step 4, then repeat from step 1 until completed.
   8. Oxidize: 0.5 sec 0.4 M I_2 in THF, 0.5 sec pyridine/*N*-methylimidazole/water/THF (10/2/10/78), 40 sec wait, repeat 30 times; 0.5 sec 0.4 M I_2 in THF, 0.5 sec triethylamine/water/THF (10/10/80), 40 sec wait, repeat 30 times.
   9. Deprotect: For the dimer experiments the CPG was treated with 7M aqueous ammonia for 1 hour at room temperature, filtered, and concentrated. For the oligomers concentrated aqueous ammonia was used for 2-3 days at room temperature.[11]

10. The hplc was carried out on a system consisting of a Waters M6000A pump, U6K injector and 440 absorbance detector along with an Autochrom CIM for generation of the gradient and a Baseline system for data collection and analysis. The column used was a Waters Nova Pak cartridge in an RCM 100. Gradients of acetonitrile and 0.1 M triethylammonium acetate (TEAA) at a flow rate of 4 mL/min were used in this work. The separations tabulated in the Table were obtained using a gradient of 2 to 20% acetonitrile in 5 min followed by 20 to 40% acetonitrile in 5 min.

11. Gaffney, B.L.; Marky, L.A.; Jones, R.A. *Biochemistry* **1984**, *23*, 5686.

12. Applied Biosystems, Inc. has recently reported using isopropyl H-phosphonate for this same purpose. Product Bulletin 7071333004-1187, November, 1987.

13. The hydrolysis of 5 g (21 mmol) of 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (Aldrich) was effected using 4 mL of water and 15 mL of acetonitrile. After 3 hours 15 mL of pyridine was added and the solution was concentrated. The residue was dissolved in pyridine and stored at -20°. 161 MHz ^{31}P nmr of a solution of **4** in pyridine showed a doublet of triplets at 0.61ppm (ref. 85% H_3PO_4/D_2O), $J_{H\text{-}P}$ 620 Hz, $J_{H\text{-}C\text{-}O\text{-}P}$ 8.8 Hz. The pyridine solution of **4** (which also contained 1 eq of *N,N*-diisopropylamine-HCl) was diluted to 50 mM in pyridine:acetonitrile (1:1) and used without further purification.

14. Gaffney, B.L. and Jones, R.A. Manuscript in preparation.

15. The purified oligomers were homogeneous by analytical hplc on both the Waters Nova Pak column and on a Beckman C_3 Ultrapure column using both 0.1 M TEAA and 0.1 M ammonium acetate as the aqueous buffer. In addition, samples degraded enzymatically[11] gave the expected ratios of the deoxynucleosides.

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