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SIMPLIFICATION OF DNA SYNTHESIS BY THE PHOSPHOTRIESTER METHOD

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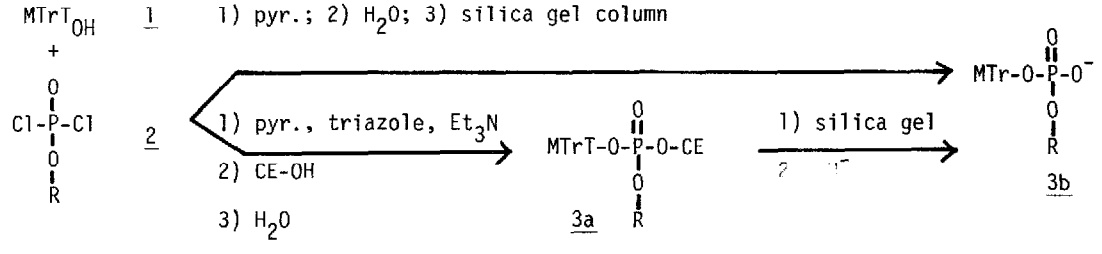
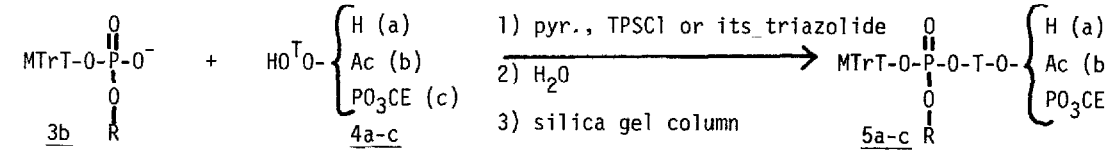
The present phosphotriester strategy for the (solution-phase) synthesis of the 3'-5' internucleotide bond of DNA is basically a two-step process[1] (Fig. 1). We have modified and simplified this process by coupling together these up-to-now generally uncoupled reactions[2] into one continuous and abbreviated reaction sequence (Fig. 2). These modifications lead to an increase in the overall yield to near quantitative as well as to a decrease in the working time by at least a factor of 4.

FIGURE 1

A) 3' PHOSPHORYLATION STEP; 30-90% yield; 2-4 days.



B) 5' PHOSPHORYLATION STEP; 30-90% yield; 2-4 days.

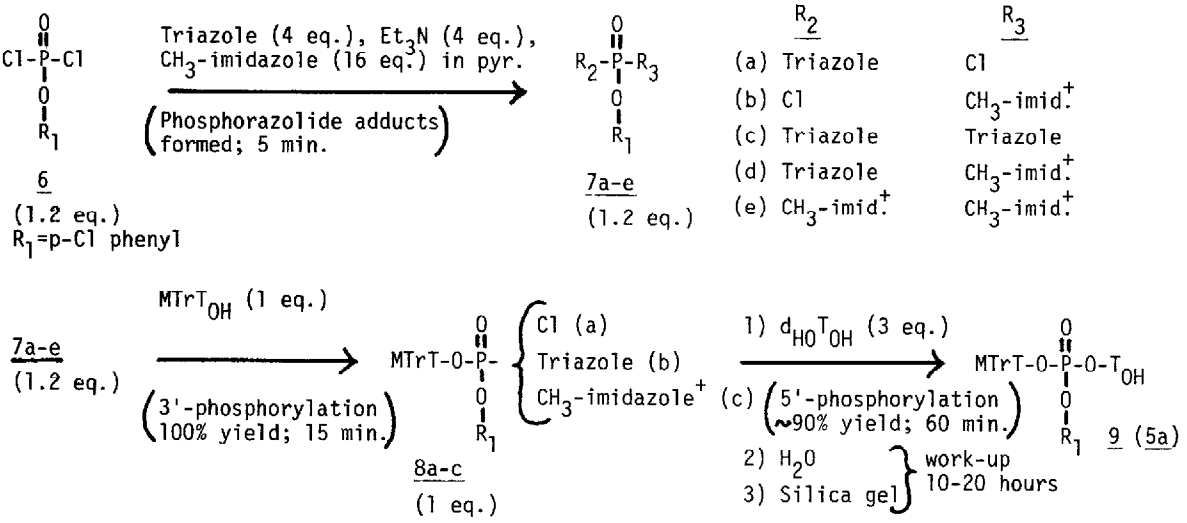


$MTrT_{OH}$=5'-O-Mono-p-methoxytrityl thymidine; R=substituted benzene, β,β,β-trichloroethyl or β-cyanoethyl; CE=β-cyanoethyl; TPSCl=2,4,6-triisopropylbenzene sulfonyl chloride; Et_3N=triethylamine.

In order to make this approach feasible experimentally, it was necessary to drive the initial 3'-phosphorylation step to completion, (Fig. 2) otherwise the unreacted MTrT would tend to co-isolate with the product MTrTpT 9 on preparative silica gel columns. This is particularly important for oligonucleotides of longer chain lengths since separation of oligonucleotides with only one or two nucleotides difference in length becomes less. To effect this, we sought to supplement the very efficient phosphorylating reagent 7c[2d, 3a] with corresponding N-methyl imidazolide adduct 7e or the mixed-azolide adduct 7d and less successfully with others reasoning that they would be more activated than the former[3b]. These intermediates effected not only the

FIGURE 2

3' and 5' PHOSPHORYLATIONS COUPLED; 90% yield; 10-20 hours.



rapid (15 min) and quantitative phosphorylation of the 3'-OH group in the first phase of the coupled reaction, but also the rapid (60 min) and near quantitative (ca. 90%) phosphorylation of the 5'OH group in the second phase resulting in the formation of internucleotide bonds. It should be noted that Reese has used N-alkyl imidazole derivatives to promote the synthesis of ribooligonucleotides[2c], cyclic nucleotides[4a], and the phosphorylation of nucleosides[4b]; recently Letsinger has used N-methyl imidazole in the synthesis of oligothymidylic acids on solid phase[4c]. Table I shows the compounds synthesized to date. Thus yields consistently close to 90% were achieved for the synthesis of a variety of dinucleoside phosphates with yields falling off only slightly for tri- and tetra- nucleotides. Lines 8 and 9 of the table indicate that omission of CH_3-imidazole or lowering of its concentration to 4 equivalents lowers the yield somewhat; line 10 shows that omitting all three amines lowers the yield to 30%; line 11 shows that CH_3-imidazole alone[5a] at 4 equivalents concentration while affording a rapid (15 min) and quantitative phosphorylation of $MTrT_{OH}$ was unable to increase the overall yield beyond the 30% mark (i.e. that achievable by the dichloridate 6 alone)[5b]. The fact that the highest yields are achieved only when triazole (4 eq.) is supplemented with a considerable excess (16 eq., but not 4) of methyl imidazole suggests that at these higher concentrations the more activated methyl imidazolium diester 8c must begin to be formed together with the (expected and less activated) triazolide diester 8b. Finally it may be seen from line 12 that dT may be lowered from 3 to 1.5 equivalents without substantially lowering the yield if at the same time the corresponding equivalents of the 3 catalytic amines are increased 2.5X from the values in Figure 2. It should be noted from Figure 2 that we are using a 20% molar excess of the phosphorylating reagent 6 (relative to MTrT) and hence after forming the 3'-phosphorylated intermediates 8a-c, there will still be 0.2 equivalents of 7a-d available to react with the 3 equivalents of incoming nucleoside (e.g. dT) to form diesters or untritylated triester side products (leaving only 2.8 equivalents of dT). In any case given

the nature of these side products, they do not interfere with the isolation of the tritylated uncharged product 9 on silica gel. It is our experience under the present set of conditions that blocking of the 3'-OH group of the incoming nucleoside is unnecessary, based upon the susceptability of the fully deprotected deoxyoligomers to total digestion by both venom and spleen phosphodiesterases. This is, in fact, in keeping with the observations of others[6, 1(a)] using the phosphotriester method involving intermediates with similar steric properties and reactivity as the presumed intermediates 8a-c; as well as in keeping with the properties of compounds shown to be specific 5'-phosphorylating reagents[7], namely that the 3'-OH group need not always be blocked.

It should be noted that there is a minimum of acid catalyzed detritylation (<4%) observed during these reactions even when prolonged for 10-20 hours. It may be calculated that 2 equivalents of H^+ are released (in the form of $Et_3NH^+Cl^-$) when the ditriazolide 7c is formed, but that none are generated with the corresponding methyl-imidazolide adduct 7e. In addition 2 equivalents of H^+ are formed per phosphorylation cycle (one each for the 3' and 5' phosphorylation steps respectively). It has been our experience[8] using mono- or dimethoxytrityl-terminated oligomers that rather severe detritylation (15-25%) can occur using TPSCl or its aryl sulfonyl triazolide derivatives, because in either case one is creating an aryl sulphonic acid with a very low pKa (ca. 2). This latter undesirable situation will be circumvented, however, using the present azole-promoted method, thus reducing undesirable side reactions and at the same time increasing the yield of the coupling products.

Table 1. Protected Oligonucleotides Synthesized.

	MTr-cmpd (equiv.)	5'-OH cmpd (equiv.)	$pClP_2Cl_2$[(a)] (equiv.)	Triazole (equiv.)	Et_3N (equiv.)	CH_3-imid. (equiv.)	Product[(b, c)]	Yield[(d, e)]
1.	MTrT (1)	dT (3)	1.2	4	4	16	MTrTpT	89-92%
2.	MTrT (1)	dA^{Bz} (3)	1.2	4	4	16	$MTrTpH^{Bz}$	80-90%
3.	MTrT (1)	dC^{An} (3)	1.2	4	4	16	$MTrTpC^{An}$	80-90%
4.	MTrTpT (1)	dA^{Bz} (3)	1.2	4	4	16	$MTrTpTpA^{Bz}$	65-75%
5.	MTrTpT (1)	dC^{An} (3)	1.2	4	4	16	$MTrTpTpC^{An}$	65-75%
6.	MTrTpT (1)	dT (3)	1.2	40	4	16	MTrTpTpT	69%
7.	MTrTpT (1)	dTpT (3)	1.2	4	4	16	MTrTpTpTpT	64%
8.	MTrT (1)	dT (3)	1.2	4	4	0	MTrTpT	75%
9.	MTrT (1)	dT (3)	1.2	4	4	4	MTrTpT	75%
10.	MTrT (1)	dT (3)	1.2	0	0	0	MTrTpT	30%
11.	MTrT (1)	dT (3)	1.2	0	0	4	MTrTpT	30%
12.	MTrT (1)	dT (1.5)	1.2	10	10	40	MTrTpT	80-90%
13.	$DMTrA^{Bz}$(1)	dG^{Ac} (3)	1.2	4	4	16	$DMTrA^{Bz}pG^{Ac}$	80-90%

(a) Abbreviation for 6. (b) Ø=p-Cl phenyl. (c) All compounds were characterized further by deprotecting with NH_3 and acid and treating the purified (Sephadex G-10) oligomer with venom diesterase and in most cases with spleen diesterase. Spectra, chromatographic mobility (TLC,

paper) and base ratios were close to anticipated values. (d) Yields were calculated by determining at 470 nm the relative absorbances of the product 9 and 3' phosphorylated intermediate 8 bands scraped from thin layer plates in 35% $HClO_4$. (e) Most reactions on 0.2 mM scale; but reactions on 2-10 mM scale also done.

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2. Direct coupling of these two steps has however been reported on occasion: (a) F. Eckstein et al, Chem. Ber. 102, 2362 (1969); (b) Ref. 1 (b) above; (c) J. H. van Boom et al, Chem. Comm, 869 (1971); (d) K. Itakura et al, J. Biol. Chem. 250, 4595 (1975). References a-c involve coupling with nucleosides, whereas (d) involves 2-cyanoethanol.
3. (a) K. Itakura et al, Can. J. Chem. 52, 3689 (1974) footnote #2; (b) Although we have no direct experimental evidence for the existance of these intermediates 7a-e, their occurance can be inferred from that of homologous properly characterized phosphorazolides (of imidazole[1c] or of triazole[2d, 3a]) made under similar conditions.
4. (a) J.H. van Boom et al, Chem. Comm., 618 (1974); (b) J.H. van Boom et al, Tetrahedron 31, 2953 (1975); (c) R.C. Pless et al, Nucleic Acids Res. 2, 773 (1975).
5. (a) We are aware of two groups (2c and 4c) who report that using 1-alkyl imidazolides of aryl phosphodichloridates causes the formation of side products; in one case (4c) this side product has been identified as a fluorescent imidazolium pyrimidone. At no time, using the conditions detailed in this paper, have we detected this fluorescent compound; MTrTpT synthesized under a variety of conditions has the expected spectral and chromatographic properties; likewise for its deprotected counterpart-TpT. The basis for this difference must involve the fact that in the present experiments all reactions are done in pyridine (not acetonitrile or acetonitrile/dioxane as in the other two cases) with two other bases present (Et_3N and triazole). (b) This is ascribable probably to the inability of methyl imidazole to in fact form the bis-methyl imidazolide adduct 7e, but to form only the mono-chloro mono-methyl imidazolide derivative 7b. It is not unlikely that the methyl imidazolium group of 7b is such a good leaving group relative to -Cl that whenever a second molecule of methyl imidazole reacts with 7b, the methyl imidazolium group is displaced preferentially to merely reform 7b.
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