Simplifications in the synthesis of short oligonucleotide blocks

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ABSTRACT

A rapid and convenient procedure has been developed for the synthesis of fully protected mono, di and triideoxyribo-nucleotides utilizing an aryl phosphoroditriazolidine. It affords advantages over coupling strategies employing condensing reagents, such as 2,4,6-triisopropylbenzenesulfonyl tetrazolidine in preparing small oligonucleotides and is relatively free of the drawbacks inherent in other approaches using bifunctional phosphorylating reagents. In particular, the synthesis of tri-nucleotide blocks without purification at the dimer stage is described.

INTRODUCTION

The chemical synthesis of larger oligodeoxyribonucleotides by the phosphotriester approach requires the easy availability of protected mono, di and trinucleotides for chain extension. One way of preparing these starting materials is first to synthesize fully protected mononucleotides by phosphorylation of the 3'-hydroxyl group of 5'-O-protected nucleosides. In the following step, the 3'-phosphodiester component prepared from the fully protected monomer is coupled with the 5'-hydroxyl group of a second nucleotide with a coupling reagent, such as 2,4,6-triisopropylbenzenesulfonyl derivatives.^{1,2} It is also possible to combine these two steps into one by phosphorylating the 3'-hydroxyl group with a bifunctional phosphorylating reagent, such as an aryl phosphorodichloridate, and then reacting the resulting monofunctional intermediate with the 5'-hydroxyl function of a second nucleotide in the presence of a nucleophilic catalyst.^{3,4} Although the former method has certain disadvantages, such as sulfonylation of the 5'-OH group⁵ and modification of guanine

base⁶, it is the most general method for the synthesis of not only starting oligonucleotide blocks, but also longer oligonucleotides^{1,2}. While the latter method employing a bifunctional phosphorylating reagent is rapid and simple, it has a serious drawback, the extensive formation of 3'-3' and 5'-5' symmetrical dimers which arise by reaction of two molecules of 3'- or 5'-hydroxyl components with the bifunctional phosphorylating reagent. Aryl phosphoroditriazolides were introduced as an alternative to dichloridates and other less selective reagents⁷. The first triazole moiety of the ditriazolide is a far better leaving group than the second and phosphorylation of the 3'-hydroxyl function of a 5'-O-protected nucleoside by this reagent proceeds readily with little formation of the 3'-3' symmetrical product. The next step, however, formation of the phosphotriester bond, requires either activation by a nucleophylic catalyst, such as 1-methylimidazole, or replacement of triazole by a superior leaving group⁸. By judicious choice of reaction conditions, it is possible to virtually eliminate the formation of symmetrical and other by-products and this has led to the development of the efficient and simple method for the synthesis of fully protected mono, di and trinucleotides.

RESULTS AND DISCUSSION

The reaction of O-chlorophenylphosphoroditriazolide (II) with a 5'-O-protected nucleoside (III) yielded the monotriazolide (IV) accompanied by other products which depend upon stoichiometry and solvent (Figure 1). Generally, 1.4 equivalent reagent (II) was sufficient to rapidly complete phosphorylation of the 3'-OH function (III) in dioxane or tetrahydrofuran and under these conditions very little or no 3'-3' dimer (IX) formation was observed. Pyridine was also used as a solvent for the phosphorylation and appears to function as a weak nucleophilic catalyst analogous to 1-methylimidazole. Nucleophilic reaction on phosphorous proceeds more quickly in this solvent than in dioxane. However, side-reactions in the phosphorylation of deoxyguanosine (III, B = N-isobutyrylguanine) made this solvent unsuitable for the preparation of IV. When treated with the reagent II in pyridine for 1 hour, followed by hydrolysis, deoxyguanosine de-

Figure 1

rivative (III) yielded two products which were separable by thin layer chromatography (T.L.C.) on C_{18} reversed-phase silica gel. The major product (about 80%) was the expected 3'-phosphodiester product (V). The other product was not identified thoroughly but appeared, from its UV spectrum, to contain a modified purine residue (typical UV absorption of the guanine base disappeared). Additionally, phosphorylations carried out in pyridine frequently resulted in the formation of darkly colored reaction mixtures and products. All monotriazolides are therefore prepared in dioxane.

The monotriazolide (IV) is a versatile synthetic intermediate which is hydrolyzed to the charged phosphate (V)⁹, cyano-ethylated with 3-hydroxypropionitrile to give the fully protected mononucleotide (VII) or coupled with the 5'-hydroxyl function of another nucleotide (VI) to yield a dinucleotide (VIII). While the monotriazolide (IV) was prepared in dioxane, the coupling reaction to prepare VIII was performed in dioxane or tetrahydrofuran in the presence of 1-methylimidazole. Under these conditions, the reaction went to completion in 2-3 hours with little formation of by-products. Without the addition of 1-methylimidazole, often some unreacted starting material remained.

A few analogues of 1-methylimidazole, including 1-n-butyl¹⁰, 1-benzyl¹⁰ and 1-ethyl-2-methyl-5-chloroimidazoles⁴, and 4-N,N-dimethylaminopyridine (DMAP)^{11,12} were tested as nucleophilic catalysts, but none was superior to 1-methylimidazole. Although DMAP was found to be a more powerful catalyst than 1-methylimidazole, it was sufficiently basic to cause the rapid β -elimination of the cyanoethyl protecting group and gave lower yields of nucleotides containing this protecting group. However, if the oligonucleotide chain is synthesized stepwise from the 3'-end to the 5'-end on a solid-phase, DMAP appears to work better than 1-methylimidazole (the oligonucleotide is attached at the 3'-position to the solid-phase support by an ester bond)¹². 1-Methylimidazole also slowly removed the cyanoethyl group and it was essential to remove the reagent thoroughly by extraction with 0.1 N sodium biphosphate solution before the purification of the product.

For the synthesis of the dinucleotides (VIII), the use of an excess of the phosphorylating reagent (II, 1.4 mmole equivalent) over the 3'-hydroxyl compound (III) naturally resulted in formation of the 5'-5' symmetrical dimer (X) upon reaction of the excess of II with 2 molecules of 5'-hydroxyl component (VI). This problem could be effectively overcome by the addition of aqueous pyridine sufficient to just hydrolyze the excess of II. Little monotriazolide (IV) was hydrolyzed in this process, since the water reacted far more quickly with the ditriazolide (II). In one experiment, 1 mmole of 5'-O-dimethoxytrityl nucleoside (III) was phosphorylated with 2.00 mmole of the ditriazolide

(II) for 1 hour, 1.00 mmole of water was added to the reaction mixture and, after 10 minutes, the resulting monotriazolide (IV) was coupled to 0.7 mmole of the 5'-hydroxyl nucleotide (VI) in the presence of 1-methylimidazole. The fact that all the 5'-hydroxyl components reacted after 3 hours and that no 5'-5' symmetrical dimer (X) formation was observed, demonstrated the selective hydrolysis of the ditriazolide (II). Without the addition of water, the 5'-5' dimer (X) was formed as expected. Thus, by using an excess of ditriazolide (II) over the 3'-hydroxyl nucleoside, driving the phosphorylation to completion, and in turn, hydrolysing this excess, it is possible to carry out the coupling in high yields. In addition, the reaction mixtures obtained were significantly less contaminated with by-products, particularly 5'-O-sulfonylated compounds and modifications of the guanine base, which are often produced in reactions with coupling reagents^{5,6}. The chromatographic purification of the product on silica gel is generally quite simple and the isolated yields of 16 kinds of dimers were 75 to 85%¹³ (Table 1). This method, therefore, provided a route to fully protected trinucleotide blocks which did not require time-consuming chromatographic purification of the intermediate dimer (see Figure 2). The reaction mixture from the dinucleotide synthesis was treated with 2% benzenesulfonic acid solution (chloroform-MeOH 7:3) at 0°C for 5 minutes to remove the dimethoxytrityl group. This mixture was extracted with 5% aqueous sodium bicarbonate to remove the excess of the charged mononucleotide (XI), leaving the 5'-hydroxyl dimer (XII) in the organic phase. The resulting dimer product was freed of dimethoxytrityl alcohol by precipitation with petroleum ether and was sufficiently pure to be coupled with a second monotriazolide as described above (IV), or with the phosphodiester component (V) under the influence of a coupling reagent, 2,4,6-triisopropylbenzenesulfonyl tetrazolide (TPST)¹⁴.

The choice of the method is dependent on the scale of synthesis, because the exclusion of ambient water becomes increasingly difficult in the former method when working on scales of less than 0.5 mmole. The fully protected trinucleotides were isolated in 50~60% yields by short column chromatography on silica gel to

TABLE 1. Yields and Rf of Dinucleotide Blocks (VIII)

Nucleotide Sequence ^a	Yield ^b (%)	Rf on Silica Gel TLC ^c
TT	82	0.58
TC	76	0.64
TA	77	0.56
TG	75	0.53
CT	88	0.63
CC	84	0.66
CA	79	0.61
CG	82	0.45
AT	76	0.58
AC	75	0.63
AA	80	0.58
AG	76	0.41
GT	83	0.51
GC	81	0.57
GA	79	0.48
GG	81	0.35

- a TT stands for the fully protected dinucleotide (VIII, in Figure 2). The 5'-end base is thymine and the 3'-end base is also thymine.
- b Yield is based on the 5'-hydroxyl component (VI).
- c Silica gel plates were developed in CHCl_3 -MeOH (9:1 v/v).

remove charged compounds (V), followed by column chromatography on μ Bondapak C_{18} reversed-phase silica gel¹⁵ (Table 2). The overall yields of the trimer blocks by this method were similar to those prepared by our published method¹⁶, but the reaction mixtures obtained were cleaner and easier to purify. This rapid and large-scale synthesis of dimer and trimer blocks has proven to be very convenient for not only the solution synthesis, but also for the solid-phase synthesis of oligonucleotides.¹⁷

EXPERIMENTAL SECTION

The following chemicals were purchased from commercial

Figure 2

TABLE 2. YIELDS AND CHROMATOGRAPHIC BEHAVIOR OF TRINUCLEOTIDE BLOCKS (XIII)

Nucleotide Sequence	Method ^{a,b}	Yield (%) ^c	Rf on Silica Gel TLC ^d	Rf on Reversed-Phase TLC ^e
A G T	b	65	0.49	0.34
C G T	b	61	0.48	0.35
G G A	a	45	0.41	0.39
C C A	a	59	0.59	0.31
A G C	a	57	0.51	0.39
A C A	a	65	0.59	0.34
T A C	a	68	0.52	0.33

a Using the coupling reagent TPST.

b Using the phosphomonotriazolide to make XIII.

c Yield is based on the 5'-hydroxyl mononucleotides (VI).

d Solvent system is the same as the dinucleotide blocks.

e C₁₈ reversed-phase TLC was developed in acetone-water (7:3 v/v).

sources: deoxyguanosine and deoxyadenosine (Calbiochem), thymidine and deoxycytidine hydrochloride (Sigma, 4,4'-dimethoxytrityl chloride, (1H)-1,2,4-triazole, 1-methylimidazole, 3'-hydroxypropionitrile and 4-N,N-dimethylamino pyridine (Aldrich), silica gel F₂₅₄ TLC plates and silica gel H for column chromatography (E. Merck) and reversed-phase silica gel TLC plates FL-18 (Whatman). The 5'-dimethoxytritylated-amino protected nucleosides¹⁸, *o*-chlorophenyl phosphorodichloridate¹⁹ and TPST¹⁴ were prepared by the published methods. Silica gel plates were developed in chloroform-methanol (9:1 v/v) and reversed-phase plates in acetone-water (7:3 v/v). For the reversed-phase column chromatography, Prep LC System 500 and μ Bondapak C₁₈ cartridges (Waters Associates) were used.

Preparation of *o*-Chlorophenyl Phosphoroditriazolide (II)

A solution of 1,2,4-triazole (3.17 g, 46 mmole) in pyridine was evaporated to complete dryness, and the triazole and *o*-chlorophenyl phosphorodichloridate (3.43 g, 14 mmole) were dissolved in dry dioxane (70 ml). To the solution was added anhydrous triethylamine (3.03 g, 30 mmole) at 0°C under vigorous stirring, and the stirring was continued for 1 hour. The reaction mixture was filtered and this solution (0.2 M solution in

dioxane) could be stored for several days at -20°C .

General Method for the Synthesis of Fully Protected Mononucleotides (VIII)

The 5'-O-dimethoxytrityl nucleoside (III, 10 mmole) was dried by co-evaporation with pyridine twice and taken up in dioxane (50 ml). To the dioxane solution of the ditriazolide (II, 14 mmole) prepared above was added the dioxane solution of III at 0°C , and the reaction mixture was concentrated to 40 ml *in vacuo* and stirred for 1 hour at room temperature. After the nucleoside (III) had completely reacted with the phosphorylating reagent (II) to give the monotriazolide (IV), 3'-hydroxypropionitrile (1.8 g, 25 mmole) and 1-methylimidazole (2.4 g, 30 mmole) were added to the reaction mixture. After the reaction was complete (1 hour), the mixture was evaporated to a gum and dissolved in CHCl_3 (150 ml). The CHCl_3 solution was washed with 0.1 M NaH_2PO_4 solution²⁰ three times and water twice, dried over Na_2SO_4 and evaporated. The product was chromatographed on a silica gel column (150 g) and eluted using a stepwise gradient of methanol (0-5%) in CHCl_3 . The fractions containing the pure product were pooled and evaporated. The yields of the fully protected nucleotides were 80-90%.

General Method for the Preparation of 3'-(o-chlorophenyl) Phosphates (V)

The monotriazolide (IV) prepared above was hydrolyzed with aqueous pyridine (90%, 10 ml) for 10 minutes at 0°C , and the mixture was concentrated to a small volume (ca. 20 ml) and taken up in CHCl_3 (150 ml). The CHCl_3 solution was washed with 0.1 M triethylammonium (TEAB) buffer (50 ml) once and then with water twice, dried (Na_2SO_4) and concentrated. A solution of the residual gum in CHCl_3 (50 ml) was added to stirred petroleum ether (1 l). The resulting colorless precipitate was collected by centrifugation and dried in the desiccator. The yields of the 3'-phosphates were 85-90%. These phosphates were completely identical with the authentic samples prepared from the fully protected mononucleotides (VII) with base.

General Method for the Synthesis of Fully Protected Dinucleotides (XII)

The solution of the monotriazolidine (IV) obtained above was treated with 1 M solution of water in pyridine (4 ml) for 10 minutes at 0°C. To this mixture was added 5'-hydroxyl component (VI, 6.5 mmole) in dioxane (60 ml), immediately followed by 1-methylimidazole (2.4 g, 30 mmole) and the reaction mixture was concentrated to 40 ml. After 2-3 hours (disappearance of the starting material; VI), the product was isolated in the same way as described above for VII. The yields of the dinucleotide were 75-85% (see Table 1).

General Method for the Synthesis of Fully Protected Trinucleotides (XIII)

The product of the last reaction, without chromatographic purification, treated with 2% benzenesulfonic acid solution in CHCl_3 -MeOH (7:3 v/v, ca. 20 ml/mmole) at 0°C for 5 minutes to remove the dimethoxytrityl group from the 3'-phosphate (V) and the dinucleotide (VIII) components. After the reaction, the mixture was extracted with saturated NaHCO_3 solution (removal of 5'-hydroxyl-3'-phosphate, XI) twice and water, dried (Na_2SO_4) and evaporated. A solution of residual gum in CHCl_3 (5 ml/mmole) was added to stirred ether-petroleum ether solution (1:1 v/v, 100 ml/mmole). The resulting precipitate was collected by centrifugation and dried by co-evaporation with pyridine twice. The 5'-hydroxyl dimer (XII) was coupled with a second 1.5 equivalents of monotriazolidine (IV) in the same way as described for the synthesis of VIII to yield a fully protected trinucleotide (XIII). Alternatively, it was coupled with a 3'-phosphate (V, 1.5 equivalents) under the influence of TPST (3 equivalents) as described elsewhere. The product after working up was first passed through a short silica gel column (10 g/mmole) to remove the excess of charged phosphate (V) and then purified by a Prep LC System 500 on a reversed-phase silica gel C_{18} cartridge. Elution buffer: acetone- H_2O (7:3 v/v) solution with 0.1 M diethylenetriammonium acetate (pH = 7.0). After collection of the peak containing the pure product, the buffer was evaporated to a small volume and extracted with CHCl_3 . The CHCl_3 solution was dried and evaporated. The yields of the fully protected trimers

were 50-60% (Table 2).

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REFERENCES

1. Itakura, K.; Katagiri, N.; Bahl, C.P.; Wightman, R.H. and Narang, S.A. (1975) *J. Am. Chem. Soc.* **97**:7327.
2. Caltlin, J.C. and Cramer, F. (1973) *J. Org. Chem.* **38**:245.
3. Reese, C.B. and Saffhill, R. (1968) *Chem. Commun.* 767.
4. Van Boom, J.H.; Burgers, P.M.; Owen, G.R.; Reese, C.B. and Saffhill, R. (1971) *Chem. Commun.* 869.
5. Van Boom, J.H.; Burgers, P.M.J.; van der Marel, G.; Verdegall, C.M.M. and Wille, G. (1977) *Nucl. Acids Res.* **4**: 1047.
6. Gough, G.R.; Collier, K.J.; Weith, H.L. and Gilham, P.T. (1979) *Nucl. Acids Res.* **7**:1955.
7. Katagiri, N.; Itakura, K. and Narang, S.A. (1975) *J. Am. Chem. Soc.* **97**:7332.
8. Agarwal, K.L. and Riftina, F. (1978) *Nucl. Acids Res.* **5**: 2809.
9. Chattopadhyaya, J.B. and Reese, C.B. (1979) *Tetrahedron Letters* 5059.
10. Myles, A.; Hutzenlaub, W.; Reitz, G. and Pfeleiderer, W. (1975) *Chem. Ber.* **108**:2857.
11. Höfle, G.; Steglich, W. and Vorbrüggen, H. (1978) *Angew. Chem. Intl. Ed., Engl.* **17**:569.
12. Miyoshi, K. and Itakura, K. (1980) *Nucl. Acids Res.*, in press.
13. Generally, the yield of the coupling reaction between nucleotides with thymine base is best by employing a coupling reagent, and the worst is the reaction between nucleotides with guanine base.
14. Stawinski, J.; Hozumi, T.; Narang, S.A.; Bahl, C.P. and Wu, R. (1977) *Nucl. Acids Res.* **4**:353.
15. Hsiung, M.H.; Brousseau, R.; Michniewicz, J.J. and Narang, S.A. (1979) *Nucl. Acids Res.* **6**:1371.
16. Hirose, T.; Crea, R. and Itakura, K. (1978) *Tetrahedron Letters* 2449.
17. See the following three papers in this series.
18. Schaller, H.; Weimann, G.; Lerch, B. and Khorana, H.G. (1963) *J. Am. Chem. Soc.* **85**:3841.
19. Owen, G.R.; Reese, C.B.; Ransom, C.J.; van Boom, J.H. and Herscheid, J.D.H. (1974) *Synthesis* 704.
20. This condition to remove excess N-methylimidazole was suggested by Dr. M. Wiewiórowski of the Institute of Organic Chemistry in Poznań.