207. Nucleosides and Nucleotides. Part 17. A Simple Preparation of Protected Deoxynucleoside-3′-phosphates[1])

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Summary

A simple method for the preparation of fully protected 2′-deoxynucleoside-3′-phosphates is described. The main step, phosphorylation of partially protected deoxynucleosides, is performed with the monofunctional reagent *p*-chlorophenyl (2-cyanoethyl) phosphochloridate[3]) (**2**) in dioxane. This reagent is quickly prepared from readily accessible *p*-chlorophenyl phosphodichloridate by reaction with 3-hydroxypropionitrile and triethylamine in dioxane or ether. The crude reagent is used directly for phosphorylation. After chromatography, protected deoxynucleoside-3′-phosphates were isolated in yields of 80–87%. The method described here, which has been optimized in detail, represents a simple alternative to published methods.

Introduction. – The advances in our knowledge of genetics and genetic engineering have led to a growing demand for synthetic polynucleotides [2]. Since the middle seventies, the triester method has increasingly become established as the method of choice for their synthesis [2] [3]. There are already a large number of publications in this field, including the synthesis of several polynucleotides. However, numerous problems have to be solved before polynucleotide synthesis can be considered easy and economical. Today, technical simplification, improvement of reproducibility and shortening of the time required for synthesis are of particular significance (see *e.g.* [4–9]).

One important class of intermediates in the triester method consists of fully protected deoxynucleoside-3′-phosphate, which have permanent protecting groups on both the base and the phosphate, and temporary ones on the 5′-OH group and the phosphate. A typical example of this class is **1** ((DMTr)dN$^{p}\varphi$ (CE))[3]), which was introduced by *Itakura et al.* [3], and has been used by us in the synthesis of a polynucleotide. This compound is prepared by phosphorylation of the 3′-OH group of a 5′- and base-protected nucleoside ((DMTr)dN^{p}) with a suitable phosphorylating agent [3] [4]. In some cases the preparation of the latter, or the phosphorylation itself, consists of several steps [4] [10] [11].

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[3]) Abbreviations: dN = 2′-deoxynucleoside, p = base protecting group, (DMTr) = 5′-*O*-dimethoxytrityl, φ = 3′-*O*-*p*-chlorophenyl phosphate, (CE) = 2-cyanoethyl.

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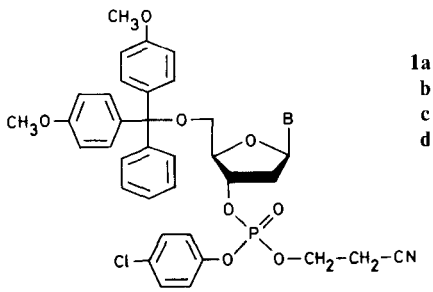
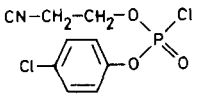


1a B = thymine
b N^4-anisoyl-cytosine
c N^6-benzoyl-adenine
d N^2-isobutyryl-guanine

The method of preparation of *Itakura et al.* [3], *via* *p*-chlorophenyl phosphobistriazolide, seemed to be the simplest. However, for unknown reasons we could not achieve the high yields reported, and we searched for another fast, cheap and technically simple method.

Phosphorylating agent. – One possibility, attractive in principle, is the one-step phosphorylation with a monofunctional phosphoric acid chloride, which already carries both necessary protecting groups, and reacts with a protected nucleoside to give **1** directly. Several compounds of this type with various protecting groups have been published (*e.g.* [5] [7] [8] [12] [13]). In our case, for the preparation of **1**, we wanted compound **2**, which has been employed [14] [15], but not described in detail. In the meantime, the authors seem to have preferred a different method [6]. However, we attempted to find a way of preparing this compound as simply as possible.



2

Firstly, we applied the method of *Grzeskowiak* [7]. This author had prepared a compound analogous to **2**, starting from $POCl_3$ in a one-pot procedure including consecutive reaction with two alcohols (one equivalent of each). However, we obtained no product sufficiently pure (cf. [7]), but a mixture containing a large amount of unreacted 3-hydroxypropionitrile. Distillation at 0.3 Torr caused decomposition. We decided to isolate the intermediate *p*-chlorophenyl phosphodichloridate, which then reacts in the second step with 3-hydroxypropionitrile. The pure dichloridate is prepared quickly and easily from cheap $POCl_3$ [16].

The preparation of **2** was first attempted under conditions similar to those of *Arentzen et al.* [5], but in dioxane with *N*-methylimidazole as catalyst. We again obtained an impure and undistillable product. An experiment according to *van Boom et al.* [12] [17] with equimolar amounts of each reactant, but in dioxane, yielded a product which showed too little cyanoethyl-H in the ^{1}H-NMR.; it could not be distilled, crystallized or precipitated, but it could be used directly for phosphorylation. However, this gave a by-product which was difficult to separate, and contained no

cyanoethyl group (supposedly symmetric dinucleoside monophosphate). We tried to optimize the conditions. The best results were obtained by reaction of 1.1 mol-equiv. each of 3-hydroxypropionitrile and triethylamine with 1 mol-equiv. of phosphodichloridate in dioxane at RT. or in ether at 0° during 2 h. Afterwards, the reaction mixture was filtered free of triethylammonium salt, evaporated and dried in high vacuum. The resulting liquid showed the expected signals in the ^{1}H-NMR. spectrum. It contained in addition residual traces of triethylammonium salt, bis(cyanoethyl)triester, and *ca.* 3% of dioxane (no ether), but practically no free 3-hydroxypropionitrile. This product turned out to be utilizable for the phosphorylation without purification, giving excellent results. Stored under Ar at RT., it decomposes within a few days (NMR.); it is stable for 1 month at −15° or for at least 3 months at −30°.

Acetonitrile appeared to be less suitable as solvent, because triethylammonium chloride is fairly soluble, and can only be partially removed by filtration. The reagent thus obtained was nevertheless usable.

Phosphorylation of nucleosides. – Several procedures have been published for the phosphorylation of protected nucleosides with monofunctional phosphorylating agents, *e.g.* [5][7][8][12–14]. The use of **2** has been described in one case [15]. Normally the nucleoside reacts in pyridine, dioxane, or acetonitrile with an excess of phosphorylating agent in the presence of a catalyst. *N*-Methylimidazole was a successful catalyst [6].

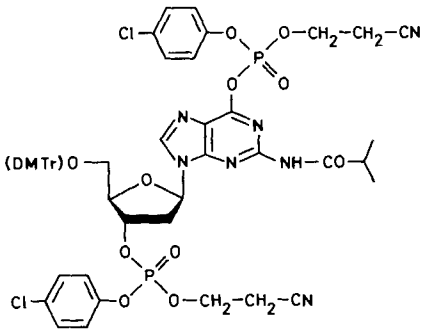
When reactions were carried out in pyridine, we obtained dark mixtures and a substantial amount of an anionic by-product. In acetonitrile or dioxane the reaction proceeded more cleanly. With two mol-equiv. of **2** in the presence of *N*-methylimidazole at RT., the reaction was complete in 30 min; for **1d** 60 min were necessary. TLC. showed quantitative conversion, a few percent of anionic by-product and a trace of less polar material. No difference was observable between the reaction mixtures of **1a**, **1b** and **1c**. After hydrolyses and extraction, the product was precipitated with ether/petrol ether. This allowed the separation of *N*-methylimidazole, which could neither be removed by extraction nor by column chromatography, and which can cleave the cyanoethyl group of **1** [6]. With ether/petrol ether 1:2 or 2:3 we obtained better precipitation and purification than with petrol ether or hexane alone. On the other hand, **1** is slightly soluble in ether.

Further, it was observed that the side product contained in the crude reagent, *p*-chlorophenyl bis(2-cyanoethyl) phosphate, was eliminated during work-up.

Finally the products were carefully chromatographed on short columns of silica gel (40–63 μm particle size), with methanol/dichloromethane. After a trace of by-product, **1a**, **1b** and **1c** were eluted with 1–2% methanol. Occasionally, it was difficult to remove a residual trace of by-product, especially in the case of **1b** and **1c**. However, HPLC. analysis showed only negligibly small amounts of by-product. After optimization, the yields of essentially pure material amounted to 87% (**1a**, **1b**) and 84% (**1c**).

Special problems arose in the preparation of **1d**. As the reaction proceeded more slowly than in the other cases, and seemed to need a greater excess of phosphorylating agent, we used 3.5 mol-equiv. of reagent and 6 mol-equiv. of *N*-methylimidazole. Independently of the excess, we observed, in addition to the desired product, a larger

amount of a less polar by-product (faster on TLC.). The ratio by-product/product was always about 4:1. After hydrolysis and extraction, chromatography on silica gel was attempted. Thus, the product mixture remained in contact with silica gel and 2% of methanol/dichloromethane for one to 3 h. Interestingly, no by-product was eluted, but about 60–65% of the desired **1d**. Spread over several fractions before and partly with the product, we found cleaved dimethoxytrityl group. This led us to the assumption, that the intermediate by-product is an unstable 6-*O*-phosphorylated derivative of **1d**. It is generally known that, in phosphorylations or under conditions of polynucleotide condensation, unwanted side reactions on guanine occur frequently, which can reduce yields considerably. In particular, 6-*O*-phosphorylated derivatives of guanine can be prepared [18]. We therefore propose structure **3** for the observed intermediate product.



3

Because of his guanine phosphate structure, **3** would be expected to be fairly reactive towards nucleophiles, which could conceivably attack at 6-*O*-phosphorus or C(6)[18][19]. However, the fact that methanolysis on the silica gel column produces **1d** almost exclusively shows that attack of methanol at phosphorus is preferred, with guanine as leaving group. Furthermore, in the presence of traces of water, a phosphoric acid is expected to be produced as a by-product, which catalyzes detritylation. We consider this a probable explanation for the observed detritylation during chromatography.

From these considerations, **3** was expected to be converted back to **1d** completely by a sufficiently long hydrolysis in the presence of pyridine. Effectively hydrolysis was complete after 60 min. No detritylation was observed during the subsequent column chromatography (as for **1a**–**1c**, methanol gradient 1–3%). Thus, the yield of **1d** was increased from *ca.* 60 to 80%.

Products were checked by TLC., HPLC., UV. and ^{1}H-NMR. spectroscopy. The cyanoethyl group was cleaved by triethylamine in pyridine according to [20]. Occasionally, a trace of unpolar material remained.

Remarks. – The preparation of the reagent **2** is simple and carried out within a few hours; **1** can be prepared easily within one day. The method described here starts from cheap phosphorusoxychloride and represents an easy alternative to the published

methods [3] [4] [10]. We consider this a simplification of preparation of the central synthon **1**. Moreover, we are convinced that compounds analogous to **2** may be prepared by the same procedure with various protecting groups, especially with groups other than *p*-chlorophenyl.

As a general result, practical details in this topic are of great importance for reproducibility, yield and purity of products.

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Experimental Part

General. The following materials were purchased from *Fluka* AG., Buchs: pyridine *(puriss. p.a.)*, dioxane (dried, *puriss. p.a.*), triethylamine *(puriss. p.a.)*, *N*-methylimidazole *(purum)*, petrol ether (40–60°, *puriss. p.a.*), 3-hydroxypropionitrile *(puriss.)*, unprotected 2′-deoxynucleosides (*puriss.* CHR), 4-chlorophenol *(puriss.)*. Pyridine was distilled and stored over CaH_2, *N*-methylimidazole was distilled and, like 3-hydroxypropionitrile, stored over 4 Å molecular sieves *(Union Carbide)*. Triethylamine was distilled and kept over KOH. 4,4′-Dimethoxytriphenylmethylchloride was purchased from *EGA-Chemie*, Steinheim. *p*-Chlorophenyl phosphodichloridate was prepared from distilled phosphoryl chloride, 4-chlorophenol and $AlCl_3$ [16] and distilled (b.p. 95°/0.25 Torr; d = 1.508). The protected nucleosides (DMTr)dT, (DMTr)dA^{bz}, (DMTr)dC^{an} and (DMTr)dG^{ib} were prepared esentially according to published procedures. Solvents were evaporated on the rotatory evaporator at 40° and 14 or 0.5 Torr. After evaporation of moisture-sensitive solutions, the rotatory evaporator was filled with Ar. Reactions were kept under Ar. Manipulations were carried out in an Ar-filled beaker. Products were isolated as foams by evaporation on the rotatory evaporator from CH_2Cl_2 or $CHCl_3$ at 50°, dried at 0.5 Torr and powdered. For TLC. plates precoated with Kieselgel 60 F 254, 0.25 mm, from *Merck*, Darmstadt were used. Solvent systems: CH_2Cl_2/methanol 90:10 (A) and 75:25 (B). Spots were visualized by spraying with 10% aq. $HClO_4$-solution and subsequent heating. For column chromatography we used Kieselgel 60, particle size 40–63 μm, from *Merck*. UV. spectroscopy was performed on a *Varian Cary* 219 spectrometer, 90-MHz-^{1}H-NMR. spectroscopy on a *Bruker* WH 90 FT-spectrometer. HPLC. was carried out on a *Dupont 830* liquid chromatograph; column: 2.27 × 50 cm, *Lichrosorb 60–10;* solvent: 2.5% of methanol and 0.25% of water in CH_2Cl_2; pressure: 27 bar; flow rate: 18 ml/min.

p-*Chlorophenyl(2-cyanoethyl) phosphochloridate* (**2**). A solution of 0.1 mol (16.3 ml) of *p*-chlorophenyl phosphodichloridate in 150 ml of dry dioxane, 0.11 mol (7.48 ml) of 3-hydroxypropionitrile were mixed. Then, over a period of 30 min with continuous stirring, 0.11 mol (15.3 ml) of triethylamine was added dropwise, which immediately led to a white precipitate. The mixture was kept under Ar and immersed in a water bath at RT. It was stirred for 4 h longer. The precipitate was then removed by fast filtration through a glass fiber filter (*Whatman* GF/F) under Ar with suction. The clear filtrate was evaporated at 40°/1 Torr. The resulting yellow liquid was dried at RT./0.5 Torr for 30 min. This crude product contained *ca.* 3% of dioxane, *ca.* 8% of *p*-chlorophenyl bis(2-cyanoethyl) phosphate and a trace of triethylammonium chloride. The content of **2** was estimated to be *ca.* 88% (NMR.); d = 1.39 (crude product). – ^{1}H-NMR. (90 MHz, (D_6)acetone): 7.27–7.56 (*m*, 4 H–C(arom.); 4.66 (*d* × *t*, $J_{P,H}$ = 8.5, $J_{H,H}$ = 5.9, 2 H–C_α); 3.10 (*d* × *t*, $J_{P,H}$ = 1.5, $J_{H,H}$ = 5.9, 2 H–C_β); additional small signals from *p*-chlorophenyl bis(2-cyanoethyl)phosphate: 4.48 (*d* × *t*, $J_{P,H}$ = 7.6, $J_{H,H}$ = 5.9, 2 × 2 H–C_α); 2.99 (*d* × *t*, $J_{P,H}$ = 1.2, $J_{H,H}$ = 5.9, 2 × 2 H–C_β).

Preparation of fully protected 3′-O-phosphorylated deoxynucleosides **1a–1d**. *General procedure.* Base-protected 5′-dimethoxytrityl deoxynucleoside ((DMTr)dN^p) was twice evaporated with 5 ml of dry pyridine at 20°/1 Torr. Afterwards, the rotatory evaporator was filled with Ar. After addition of 6 ml of dry dioxane, 2.5 mol-equiv. (0.57 ml) of crude **2** were added (in the case of (DMTr)dG^{ib}, 3.5 mol-equiv. = 0.80 ml), followed by 3 mol-equiv. (0.24 ml; for (DMNTr)dG^{ib} 6 mol-equiv. = 0.48 ml) of *N*-methylimidazole, which immediately led to a precipitate. The mixture was kept under Ar and stirred for 30 min. TLC. (A) showed the absence of the starting material, the main spot being the desired product. In the case of **1d** it was possible to recognize both diastereoisomers of the product in a double spot, and, in addition, the main product which moved 0.12 Rf-

units faster. The mixture was hydrolyzed with 4 ml of H_2O/pyridine 1:1 for 15 min (**1d**: 60 min). After evaporation of dioxane at 1 Torr, 100 ml of 4% of aq. $NaHCO_3$-solution was added, and the product was extracted with CH_2Cl_2 (4× 100 ml). The organic phases were combined, concentrated to a volume of 15 ml, filtered through cotton wool and concentrated to 4 ml. This solution was added dropwise to a mixture of dry ether/petrol ether 2:3 (500 ml), through a capillary at a rate of about 0.3 ml per min. The flask and the capillary were washed with CH_2Cl_2 (2× 2 ml). The product formed a white or slightly coloured precipitate and the liquid phase became clear after 15 to 30 min. After filtration through a glass fiber filter (*Whatman* GF/F) with suction, the precipitate was dissolved in CH_2Cl_2, the solution was filtered as before and concentrated *in vacuo* to a small volume. The TLC. (A) looked identical to the former one, except in the case of **1d**, where the previous main product had disappeared and the wanted product appeared as the main product. This material was carefully chromatographed on a short Kieselgel column (70 g, 4× 11 cm, prepared with 2% of methanol in CH_2Cl_2). Flow rate: 4 ml/min; fraction volume: 50 ml. After washing with 1% of methanol in CH_2Cl_2, the product was eluted with 2% of methanol (in the case of **1d**: 2–3%). Total volume 600–1000 ml. Fractions containing pure product were evaporated and the product isolated as a dry foam. Yields: 87% (**1a**, **1b**), 84% (**1c**) and 80% (**1d**).

Rf-Values. System A: **1a** 0.58; **1b** 0.67; **1c** 0.74; **1d** 0.60. System B: triethylammonium salts of: (DMTr)dT$\varphi^{\ominus}$ 0.38; (DMTr)dC$^{an}\varphi^{\ominus}$ 0.42; (DMTr)dA$^{bz}\varphi^{\ominus}$ 0.39; (DMTr)dG$^{ib}\varphi^{\ominus}$ 0.37.

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