A general method for the synthesis of 5'-monophosphates of DNA fragments via phosphotriester intermediates

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ABSTRACT
A new and attractive phosphorylation procedure which allows the introduction, via phosphotriester intermediates, of 5'-phosphate functions of DNA fragments is described. The method is based on the activation of bifunctional phosphorylating agents with 1-hydroxybenzotriazole. The approach will be exemplified by the synthesis of pACGC using four different 5'-phosphotriester intermediates.

INTRODUCTION
The enzyme DNA ligase catalyses in the presence of ATP the formation of an internucleotide phosphodiester bond between a 5'-phosphorylated oligodeoxynucleotide (II, donor, Fig. 1) and an oligodeoxynucleotide with a free 3'-hydroxyl group (III, acceptor, Fig. 1) on a complementary oligodeoxynucleotide strand (I, template, Fig. 1). Several physico-chemical methods (X-ray analysis, NMR and CD spectroscopy) may give information on the specific interaction between the ligase and the DNA hybrid (IV, Fig. 1) containing a single stranded nick.

In order to perform these studies, pure deoxyribonucleotide fragments (i.e. I, II, III, Fig. 1) should be available in sufficient quantities.

At the moment the phosphotriester approach is the method of choice for the synthesis of DNA fragments with a defined sequence and length (e.g., I and III). However a reliable and general procedure for the introduction, via

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\begin{align*}
I & : 5' \text{GpCpGpTpGpCpG}^3' \\
II & : 5' \text{pApCpGpC}^3' \\
III & : 5' \text{CpGpCpA}^3' \\
\rightarrow & 5' \text{GpCpGpTpGpCpG}^3' \\
& \text{NICK}
\end{align*}
\]

Figure 1
phosphotriester intermediates, of a phosphate function at the 5'-end of a partially protected DNA fragment is not available. In a strategy based on the phosphotriester approach to the synthesis of DNA fragments we may discern two distinct stages. The first one involves the synthesis of fully protected DNA intermediates by condensation of properly protected building blocks. The last step consists of the complete removal of all the protective groups from the fully-protected intermediate to afford a deoxyribonucleotide fragment, containing naturally occurring 3'-5'-phosphodiester linkages as well as free hydroxyl and exocyclic amino functions.

Although different routes to the synthesis of fully-protected DNA intermediates have been developed, they all have in common the feasibility to remove a protective group at the 5'-end selectively in the presence of the other groups. This property of the protective group at the 5'-end is an essential part in a phosphotriester approach to the synthesis of DNA fragments. Thus selective removal of the protective group at the 5'-end allows elongation of a nucleic acid chain in the 5'-direction. On the other hand, a free hydroxyl group at the 5'-end of a protected DNA intermediate may also be phosphorylated with monofunctional phosphorylating agents (i.e. 1-3, in Fig. 2) to give a 5'-phosphotriester function, which after deblocking of all protective groups affords a 5'-phosphorylated oligodeoxynucleotide (e.g. II, in Fig. 1). Up to now, most monofunctional phosphorylating agents (i.e. 1a (R=4-NO2C6H4); 1b (R=BrC6H5); 2 and 3, Fig. 2) have been used for the introduction of a phosphotriester function at the 5'- or 3'-position of nucleosides and in a few cases for the introduction of a phosphotriester function at the 5'-end of a partially protected RNA dimer and trimer.

We now wish to report that 5'-phosphorylated tetramer II (Fig. 1) is easily accessible by the introduction of a phosphotriester function at the 5'-OH of partially protected tetramer (i.e. 10 in Scheme 2) using phosphorylating agents which have been activated with 1-hydroxybenzotriazole (i.e. reagents 2 and 12a-c in Scheme 1 and 3, respectively).

![Figure 2](image-url)
In previous papers we demonstrated that 1-hydroxybenzotriazole (HOBT) can be used to convert several phosphorodichloridates (i.e. reagents 4 in Fig. 2 with $X=4-\text{NO}_2\text{C}_6\text{H}_4$, $X=\text{Br}_3\text{CCH}_2$ or $X=2,4-\text{diCl}_2\text{C}_6\text{H}_3$) into effective and efficient phosphorylating agents (i.e. 12a-c in Scheme 3). An interesting observation in these studies was the behaviour of phosphorylating agent 5 (Scheme 1), which is easy accessible by the reaction of morpholinophosphorodichloridate (4; $X=\text{morpholino}$, See Fig. 2) with two equivalents HOBT in the presence of two equivalents of pyridine. Reaction of reagent 5 with 3'-O-benzoyldeoxythymidine 6 (Scheme 1) proceeded smoothly, in the presence of 1-methylimidazole, to afford the relatively stable triester 7: the latter intermediate could be purified by short column chromatography and isolated in high yield. In contrast, triesters of type 7, which contain an alkyl or aryl protective group instead of the morpholino function (e.g. intermediates 13a,b,c in Scheme 3), could not be isolated but were hydrolysed immediately during the work-up of the reaction mixture. The identity of fully-protected product 7 was confirmed by $^{31}\text{P-NMR}$ spectroscopy. Further, product 7 was rapidly converted by $N^1,N^1,N^3,N^3$-tetramethylguanidinium syn 4-nitrobenzaldoximate (oximate) into the 5'-phosphoromorpholidate derivative 8, which, after acid and base treatment, afforded solely deoxythymidine 5'-phosphate. Another noteworthy property of triester 7 was its conversion into triester 9. Thus, derivative 7 reacted slowly in the presence of 1-methylimidazole with an excess of 3-hydroxypropionitrile to afford product 9 in satisfactory yield.
of morpholino and cyanoethyl protective groups may present a new and useful intermediate for the synthesis of phosphorylated nucleoside derivatives containing 5'-mono-, di- or triphosphate functions.

The favourable properties of phosphorylating agent 5 urged us to find out whether this reagent could be used to phosphorylate the 5'-hydroxyl function of protected tetramer 10 (Scheme 2). We therefore treated tetramer 10 with an excess of reagent 5 in the presence of 1-methylimidazole. The course of the reaction could not be followed by TLC analysis: no difference in R_f values was observed between starting compound 10 and product 11. The complete conversion of 10 into 11 was corroborated as follows. A small quantity of crude 11 was treated with aqueous pyridine at 20°C. TLC analysis of the reaction mixture, after 15 h, showed complete conversion of 11 into base-line material. The latter result indicates that 10 reacted quantitatively with 5 to afford 11. Having established complete reaction of 10 with reagent 5, we now removed all the protective groups from 11 by a three-step procedure. Thus, the internucleotide aryl protective groups were removed by treatment of 11 with a solu-
tion of 1,1,3,3-tetramethylguanidinium syn-4-nitrobenzaldoximate (oximate)\(^{11}\) in dioxane-water, followed by the removal of the acyl protective groups by aqueous ammonia. Subsequent cleavage of the morpholino function with acid (0.01 N HCl; pH=2) afforded the 5'-phosphorylated tetramer II (Scheme 2). HPLC analysis of the crude reaction mixture showed the presence of mainly one product having a longer retention time than the fully-unprotected tetramer d-ACGC. Crude tetramer II was now purified on a column of Sephadex G25. Pure II, thus obtained, was completely digested with Venom phosphodiesterase into the expected d-nucleotides. Furthermore \(^{31}\)P-NMR spectroscopy showed the presence of three distinct phosphodiester linkages and one phosphomonoester. These data clearly indicate that HOBT converts morpholinophosphorodichloridate into a phosphorylating agent which is suitable for the introduction of a phosphate function at the 5'-end of oligodeoxynucleotides.

Up to now, no monofunctional phosphorylating agent\(^{3-6}\) (e.g. \(\mathbf{1}, \mathbf{2}, \mathbf{3}\) in Fig. 2) having properties comparable with reagent \(\mathbf{5}\) (Scheme 1) has been reported. For instance, several monofunctional phosphorylating agents, having amide functions (e.g. \(\mathbf{2}; R=4-\text{NO}_2 \cdot \text{C}_6 \cdot \text{H}_4\) and \(\mathbf{6}\) in Fig. 2), have been designed to introduce amide phosphotriesters on a nucleoside level. However, these phosphorylating agents reacted very sluggishly with the 5'-hydroxyl of protected oligodeoxynucleotides. On the other hand, synthesis of phosphotriester containing an amide function (e.g. morpholino or anilino) can also be accomplished by a two-step procedure\(^{10}\). The first step involves phosphorylation of 10 with reagents \(\mathbf{12a,b,c}\) to afford intermediates \(\mathbf{13a,b,c}\) respectively (Scheme 3). In the second step, intermediates \(\mathbf{13a-c}\) are converted into the required triester by substitution of the benzotriazole function with a proper amine. For example, phosphorylation of tetramer \(\mathbf{\sim}\) with an excess of reagent \(\mathbf{12a}\) (R=4-\text{NO}_2 \cdot \text{C}_6 \cdot \text{H}_4) gave intermediate \(\mathbf{13a}\) (Scheme 3). The reaction of \(\mathbf{\sim}\) with \(\mathbf{12a}\) could, in contrast with the phosphorylation of \(\mathbf{\sim}\) with \(\mathbf{5}\) (Scheme 2), easily be monitored by TLC analysis: intermediate \(\mathbf{13a}\) (R=4-\text{NO}_2 \cdot \text{C}_6 \cdot \text{H}_4) hydrolysed instantaneously in the presence of aqueous pyridine to base-line material.

Addition of morpholine to the reaction mixture containing \(\mathbf{13a}\) afforded, after work-up and purification by short column chromatography, fully-protected tetramer \(\mathbf{14}\) in high yield. In an analogous way we prepared starting from \(\mathbf{\sim}\) and phosphorylating agent \(\mathbf{12b}\) (R=Br_3 \cdot \text{CCH}_2) intermediate \(\mathbf{13b}\) (Scheme 3), which was converted in the presence of 1-methylimidazole into fully-protected tetramer \(\mathbf{\sim}\) by reaction with aniline. After work-up and purification of the crude product by short column chromatography, fully-protected tetramers \(\mathbf{14}\) and \(\mathbf{15}\) were deblocked in the following way. The protective groups of \(\mathbf{14}\) were remove ac-
According to the same three-step procedure as described for the complete de-blocking of tetramer 11 (Scheme 2). Tetramer 15 was converted into end-product II by applying a different three-step procedure. Firstly, the 2,2,2-tribromoethyl group was selectively removed by treatment of 15 with activated zinc dust in pyridine in the presence of 2,4,6-triisopropylbenzenesulfonic acid\(^{11}\). The aniline\(^{12}\) group was removed by reaction with isopentyl nitrite in pyridine-acetic acid. Finally, the acyl and the internucleotide aryl groups were removed by treatment with aqueous ammonia.

HPLC analysis of the crude reaction mixtures of products II, which were
obtained by complete deblocking of 14 and 15, showed the presence of mainly one product having the same retention time as product II obtained earlier (See Scheme 2). Purification and identification of II, obtained by the procedures in Scheme 3, was performed by the same techniques described earlier for the preparation of tetramer II in Scheme 2. Having established the favourable properties of intermediate 13a and 13b for the introduction of amidate functions at the 5'-end of tetramer 10 (e.g. products 14 and 15 in Scheme 3), we were anxious to find out whether intermediate 13 was equally well suitable for the synthesis of a thioate phosphotriester at the 5'-end of tetramer 10.

We, therefore, treated tetramer 10 with phosphorylating agent 12c (R=2,4-Cl2C6H3, Scheme 3). TLC analysis, after 0.5 h, showed complete conversion of 10 into base-line material. The latter indicates the formation of intermediate 13c (R=2,4-Cl2C6H3, Scheme 3). Thiocresol and 1-methylimidazole were now added to the thus obtained intermediate 13c. TLC analysis after 4 h, showed that intermediate 13c was completely converted into product 16. Tetramer 16 was purified by short column chromatography and was isolated in high yield. The 5'-phosphorylated tetramer 16 was completely deblocked by a three-step procedure. Firstly, the aryl protective groups were removed by treatment of 16 with oximate under anhydrous conditions. Next, the thiocresol group13 was oxidized and hydrolyzed by treatment with iodine-water. Finally, the acyl groups were removed with aqueous ammonia. Purification and identification of the thus obtained product II was carried out similarly to the techniques described previously.

Up to now the methods, developed to introduce thioate functions by a phosphotriester approach at the 5'-terminus of properly protected oligonucleotides, are based on monofunctional phosphorylating agents. For instance, 0-2,4-dichlorophenyl-S-methyl phosphorochloridothioate (e.g. 3 in Fig. 2) suitable for the preparation of 5'-mono-, di- and triphosphates of ribonucleosides, was introduced by Reese et al.7,8 However, the latter reagent is not easily accessible. Moreover, the monitoring by TLC analysis of the reaction of reagent 3 (Fig. 2) with a properly protected oligonucleotide (i.e. 10 in Scheme 2) may be troublesome. The two-step procedure, outlined in Scheme 3, presents an alternative to the above described procedure for the preparation of thioate triesters at the 5'-end of properly-protected oligonucleotides.

CONCLUSION

The methodology described in this paper shows that phosphorylating agents
(e.g. Ɇ in Fig. 2) which are commonly used in nucleic acid chemistry can be converted with 1-hydroxybenzotriazole into effective phosphorylating agents (e.g. agents Ɇ and Ɋa,b,c in Scheme 1 and 3).

Thus phosphorylation of the 5'-OH function of protected tetramer Ɇ with agent Ɇ (Scheme 2) gave the relatively stable triester ɇ, which was deblocked to afford tetramer Ɉ.

On the other hand, phosphorylation of the 5'-OH function of protected tetramer Ɇ (Scheme 3) with reagents Ɋa,b,c gave intermediates ɉa,b,c (Scheme 3) which in turn can be converted into mixed triesters ɊɊ, ɊɊ, and ɊɈ, respectively, in Scheme 3. In this respect the HOBT-approach presents an attractive route for the introduction of valuable phosphotriesters at the 5'-end of oligonucleotides. It also presents an alternative route to the synthesis of mixed phosphotriester by using monofunctional phosphorylating agents. For instance, the fully-protected tetramers ɊɊ and ɊɊ (in Scheme 3) are in principle accessible by the phosphorylation of Ɇ with reagents Ɋa (R=N=N-C,H,) and Ɇ, respectively, in Fig. 2. However, a monofunctional reagent suitable for the preparation of fully-protected tetramer Ɉ (Scheme 3) is not available. The same holds for the introduction of the mixed phosphotriester function of compound Ɇ in Scheme 1.

Another favourable property of the phosphorylating agents mentioned in this paper (i.e. Ɇ and Ɋa-c) is the fact that no side-reactions occur on the heterocyclic bases thymine and guanine. We, therefore, believe that the phosphorylation procedure described in this paper may be of general value for the introduction of phosphate functions at the 5'-end of DNA fragments via phosphotriester intermediates.

**EXPERIMENTAL SECTION**

**General methods and materials**

Acetonitrile, pyridine and tetrahydrofuran were dried by refluxing in the presence of CaH_2 for 16 h and then distilled. Pyridine was redistilled from p-toluenesulfonyl chloride (60 g per liter). Tetrahydrofuran was redistilled from LiAlH_4 (5 g per liter). All solvents were stored over molecular sieves. 1-Methylimidazole and 3-hydroxypropionitrile were distilled under reduced pressure and stored over molecular sieves 4 Ǻ. 1-Hydroxybenzotriazole was purchased from Aldrich and dried in vacuo (P₂O₅) at 50°C.

Morpholine was distilled from sodium. Aniline was distilled from zinc dust. 4-Toluenethiol was purchased from Fluka.

Schleicher and Schüll DC Fertig folien F1500 LS 254 were used for TLC in sol-
vent system A (chloroform-methanol 92:8, v/v) and B (chloroform-methanol 85:15, v/v). Short column chromatography was performed on Kieselgel 60 (230–400 mesh ASTM) suspended in chloroform.

The high performance liquid chromatography system used in this study has been described elsewhere. High-performance anion-exchange chromatography was performed with the anion-exchange resin Permaphase AAX (Dupont, USA) dry packed into a stainless-steel column (1 m x 2.1 mm; System I). Elution was effected using a linear gradient, starting with buffer A (0.005 M KH$_2$PO$_4$, pH=4.5) and applying 3% buffer B (0.05 M KH$_2$PO$_4$, 0.5 M KCl, pH=4.5) or buffer C (0.1 M KH$_2$PO$_4$, 1.0 M KCl, pH=4.5) per min.

In another system high-performance anion-exchange chromatography was performed on a column (25 cm x 4.6 mm) packed with Partisil PXS 10/25 SAX (Whatman) (System II). Isocratic elution was effected by buffer D (0.01 M KH$_2$PO$_4$, 0.1 M KCl, pH=4.1). $^1$H-NMR spectra were measured at 40.48 MHz. with a Jeol JNMFFt 100 spectrometer equipped with an EC-100 computer operating in the Fourier transform mode. Shifts are given in ppm ($\delta$) relative to 85% H$_3$PO$_4$ as an external standard. Product $\gamma$ was synthesized according to a previously described procedure.

Synthesis of morpholino-di-1-benzotriazole phosphate $\gamma$.

A solution of morpholinophosphorodichloridate (5.0 mmol) in anhydrous tetrahydrofuran (5.0 ml) was added dropwise to a stirred and cooled (ice-water bath) solution of 1-hydroxybenzotriazole (10 mmol) and pyridine (10 mmol) in anhydrous tetrahydrofuran (20.0 ml). The ice-water bath was removed and, after 1.5 h, the mixture was filtered off under anhydrous conditions. The thus obtained solution of phosphorylating agent $\alpha$ was used immediately.

Synthesis of 2,2,2-tribromoethyl-di-1-benzotriazole phosphate $\delta$b.

Reaction of 2,2,2-tribromoethylphosphorodichloridate with 1-hydroxybenzotriazole was carried out as described for the synthesis of $\gamma$.

Synthesis of 2,4-dichlorophenyl-di-1-benzotriazole phosphate $\delta$c.

Reaction of 2,4-dichlorophenylphosphorodichloridate with 1-hydroxybenzotriazole was carried out as described for the synthesis of $\delta$.

Synthesis of 4-nitrophenyl-di-1-benzotriazole phosphate $\delta$a.

Reaction of 4-nitrophenylphosphorodichloridate with 1-hydroxybenzotriazole was carried out as described for the synthesis of $\delta$ using acetonitrile instead of tetrahydrofuran.

Synthesis of 3'-0-benzoyldeoxythymidine 5'-0-(morpholino-1-benzotriazole) phosphate $\zeta$.

A solution of morpholino-di-1-benzotriazole phosphate $\delta$ (2.0 mmol) in anhy-
drous tetrahydrofuran (10 ml) was added to 3'-O-benzoyldeoxythymidine 6 (1.0 mmol). Pyridine (5 ml) and 1-methylimidazole (4.0 mmol) were then added. After 4 h, TLC analysis (System B) showed the reaction to be complete. The reaction mixture was diluted with chloroform (100 ml), washed with 1 M TEAB (pH=7.5; 50 ml) and water (50 ml). The organic layer was dried (MgSO₄) and concentrated to an oil. The oil was dissolved in chloroform (4.0 ml) and applied to a column of Kieselgel (14 g). The column was eluted with chloroform-methanol (96.5:3.5). The appropriate fractions were concentrated and precipitated from petroleum ether (40-60°C, 150 ml). The precipitate was filtered off and dried in vacuo (P₂O₅). Yield of 7 was 80%.

³¹P-NMR (CDCl₃) of a mixture of diastereoisomers: δ 9.48 and 8.88.

Synthesis of 3'-O-benzoyldeoxythymidine 5'0-(morpholino)phosphate 8.

3'-O-Benzoyldeoxythymidine 5'-O-(morpholino-1-benzotriazole)phosphate 7 (0.5 mmol) was dissolved in a solution of N,N,N',N'-tetramethylguanidinium syn-4-nitrobenzaldoximate (0.3 M) in dry tetrahydrofuran (8.0 ml). The solution was stirred for 16 h at 20°C. TLC analysis (System B) showed complete conversion of 7 into base-line material. P-NMR spectroscopy of the reaction mixture showed the presence of one resonance; δ = 5.78. Crude product 8 was now completely deblocked to give the expected deoxythymidine 5'-phosphate. Thus, aqueous ammonia (25%, 10 ml) was added and the mixture was left for 24 h at 20°C. The reaction mixture was concentrated to a small volume. The residue was dissolved in 0.01 N HCl (20 ml) and the pH was adjusted to 2.0 by the addition of 0.1 N HCl. After 2 h at 20°C, the solution was washed with chloroform (2 x 10 ml) and ether (2 x 10 ml). The aqueous layer was neutralized with aqueous ammonia (25%, 1.0 ml), concentrated to a small volume and analyzed by HPLC (System II). Crude product 8 thus obtained had the same retention time as thymidine-5'-phosphate.


To a solution of product 7 (0.5 mmol) in tetrahydrofuran (2.5 ml) was added 3-hydroxypropionitrile (25.0 mmol) and 1-methylimidazole (2.0 mmol). The reaction mixture was stirred for 70 h at 20°C. TLC analysis (System B) showed the reaction to be complete. The reaction mixture was diluted with chloroform (100 ml) and washed with 1 M TEAB (2 x 50 ml). The organic layer was dried (MgSO₄) and concentrated to an oil. The oil was dissolved in chloroform (2 ml) and applied to a column of Kieselgel (5 g). The column was eluted with chloroform-methanol (96:4, v/v). The appropriate fractions were concentrated and precipitated from petroleum-ether (40-60°C, 10 ml). The precipitate was fil-
tered off and dried in vacuo (P₂O₅), to afford 2 as a fluffy solid in 70% yield.

³¹P-NMR (CDCl₃): mixture of diastereoisomers; δ 7.88 and 7.70.

Deblocking of 2 by treatment with triethylamine in acetonitrile, followed by aqueous ammonia and finally acid (0.01 N HCl, pH = 2) afforded, as corroborated by HPLC analysis (System II), thymidine-5'-phosphate.

Synthesis of fully-protected tetramer 11.

To a solution of tetranucleotide 10 (0.0225 mmol) in anhydrous acetonitrile (0.5 ml) was added a solution of phosphorylating agent 9 (0.1 mmol) in tetrahydrofuran (0.5 ml). 1-Methylimidazole (0.25 mmol) was added and the reaction mixture was stirred for 3.5 h at 20°C. The reaction mixture was diluted with chloroform (50 ml) and washed with 1 M TEAB (3 x 25 ml). The organic layer was dried (MgSO₄) and concentrated to a small volume. The thus obtained product 11 was precipitated from petroleum ether (40-60°C, 150 ml). The precipitate was dissolved in chloroform (1.0 ml) and applied to a column of Kieselgel (20 g). The column was eluted with chloroform-methanol (97:3, v/v). The appropriate fractions were concentrated and precipitated from petroleum-ether (40-60°C). The precipitate was filtered off and dried in vacuo (P₂O₅).

Yield of product 11 was 78%.

Deprotection of fully-protected tetramer 11.

A solution of syn-p-nitrobenzaldoxime (0.9 mmol) and N¹,N¹,N³,N³-tetramethylguanidine (0.9 mmol) in dioxan-water (1:1, v/v, 3 ml) was added to crude product 11 (0.0225 mmol). The reaction mixture was stirred for 24 h at 20°C after which aqueous ammonia (25%, 30 ml) was added. The reaction vessel was sealed and kept at 50°C for a period of 50 h. The reaction mixture was concentrated and the resulting solution was diluted with 0.01 N HCl (15 ml) and the pH was adjusted to 2.0 by the addition of 0.1 N HCl. After 2 h at 20°C, the solution was washed with chloroform (2 x 20 ml) and ether (2 x 20 ml). The aqueous layer was neutralized with 25% aqueous ammonia, concentrated to a small volume and analyzed by HPLC (System I).

Purification of tetramer 11.

The fully deprotected tetranucleotide 11 obtained above was purified on a column (2 m x 3 cm³) of Sephadex G 25 suspended in 0.05 M TEAB. Elution was effected with the same buffer at a flow rate of 14 ml per h. Fractions of 3 ml were taken and analyzed by HPLC (System I). The fractions containing the pure product were collected and lyophilized. The pure tetranucleotide 11 was brought into the ammonium form by running it through a column (5 cm x 2 cm³) of Dowex 50W cation-exchange resin (100-200 mesh, ammonium-form). The resulting aqueous
solution was relyophilized to afford II (20 mg, NH₄⁺-salt).

$^{31}$P-NMR ($D_2$O): δ 3.61 (5'-phosphomonoester) and -1.03, -1.21 and -1.27 (3'-5'-phosphodiester).

Enzymatic hydrolysis of tetramer II.

Venom phosphodiesterase (VPD): Tetranucleotide II (0.2 mg) was incubated with snake venom phosphodiesterase (Crotalis ter. ter. Boehringer) in a buffer (100 µl) containing 25 mM Tris-HCl (pH=9) and 5 mM MgCl₂ at 37°C for 2 h. HPLC analysis (System I and II) showed complete digestion of II into the expected products and in the correct ratio; pC:pA:pG=2:1:1.

Synthesis of fully-protected tetramer 14.

A solution of phosphorylating agent 12a (0.2 mmol) in anhydrous acetonitrile (1.0 ml) was added to a cooled (ice-water bath) solution of tetranucleotide 10 (0.05 mmol) in anhydrous acetonitrile (1.0 ml). After 5 min, the ice-water bath was removed and the reaction mixture was stirred for 20 min at 20°C. TLC analysis (System B) of the reaction mixture showed that 10 was completely converted into base-line material. To the thus obtained solution of intermediate 13a was added morpholine (1.0 mmol). After stirring at 20°C for 1.5 h, TLC analysis (System B) showed complete conversion of 13a into a product with higher Rf-value. The reaction mixture was diluted with chloroform (40 ml) and washed with 1 M TEAB (2 x 20 ml) and water (20 ml). The organic layer was dried (MgSO₄) and concentrated to a small volume. Crude product 14 was precipitated from petroleum ether (40-60°C, 150 ml). The precipitate was dissolved in chloroform (2.0 ml) and applied to a column of Kieselgel (3.0 g). The column was eluted with chloroform-methanol (97.5:2.5, v/v). The appropriate fractions were concentrated and precipitated from petroleum ether (40-60°C). The precipitate was filtered off and dried in vacuo (P₂O₅). Yield of product 14 was 80%.

Deprotection of fully-protected tetramer 14.

Deprotection of product 14 to afford tetramer II was carried out according to the same procedure as described for the deprotection of product 11. The thus obtained tetramer II was purified by the same procedure as described before.

Synthesis of fully-protected tetramer 15.

A solution of phosphorylating agent 12b (0.25 mmol) in tetrahydrofuran (1.0 ml) was added to a cooled (ice-water bath) solution of tetramer 10 (0.05 mmol) in anhydrous acetonitrile (10 ml). After 5 min, the ice-water bath was removed and the reaction mixture was stirred for 25 min at 20°C. TLC analysis (System B) of the reaction mixture showed that 10 was completely converted into base-line material. To the thus obtained solution of intermediate 13b was added
aniline (1.0 mmol) and, after 15 min, 1-methylimidazole (1.0 mmol). After stirring for 3 h at 20°C, TLC analysis (System B) showed the reaction to be complete. The reaction mixture was diluted with chloroform (60 ml) and washed with 0.1 N HCl (20 ml), 1 M TEAB (2 x 20 ml) and water (20 ml). The organic layer was dried (MgSO₄) concentrated to a small volume and precipitated from petroleum ether (40-60°C, 150 ml). The precipitate was dissolved in chloroform (2.0 ml) and applied to a column of Kieselgel (2.5 g). The column was eluted with chloroform-methanol (97:3, v/v). The appropriate fractions were concentrated and precipitated from petroleum-ether (40-60°C). The precipitate was filtered off and dried in vacuo (P₂O₅). Yield of product 15 was 55%.

Deprotection of fully-protected tetramer 15.

Tetranucleotide 15 (0.025 mmol) and 2,4,6-triisopropylbenzensulfonic acid (1 mg) were dissolved in pyridine (0.1 ml) and activated zinc was added. The suspension was stirred magnetically and the course of the reaction was followed by monitoring the evolvement of heat with a thermocouple. After 60 sec., the temperature of the reaction mixture rose and, after 4 min, the mixture was filtered off to remove excess zinc. TLC (System B) of the filtrate showed a complete conversion of the starting compound 12 into base-line material. The filtrate was diluted with chloroform (50 ml) and washed with 1 M TEAB (1.5 ml) and 0.1 M TEAB (1.5 ml). The organic layer was concentrated to an oil and precipitated from petroleum-ether (40-60°C). The precipitate was filtered off and dissolved in pyridine-acetic acid (1:1, v/v, 2.0 ml). Freshly distilled isoamyl nitrite (0.4 ml) was added and the reaction mixture was kept in the dark for a period of 24 h. The mixture was poured into petroleum ether (40-60°C) - ether (1:1, v/v, 100 ml). The precipitate was filtered off and dissolved in dioxan (4 ml). Aqueous ammonia (25%, 20 ml) was now added. The reaction vessel was sealed and kept at 50°C for a period of 48 h. The reaction mixture was concentrated to a small volume and analysed by HPLC (System I).

The thus obtained tetranucleotide II was purified by the same procedure as described before.

Synthesis of fully-protected tetramer 16.

A solution of phosphorylating agent 12c (0.36 mmol) in tetrahydrofuran (1.5 ml) was added to a cooled (ice-water bath) solution of tetramer 10 (0.074 mmol) in anhydrous acetonitrile (10 ml). After 5 min, the ice-water bath was removed and the reaction mixture was stirred for 25 min at 20°C. TLC analysis (System A) of the reaction mixture showed complete conversion of 10 into base-line material. To the thus obtained solution of intermediate 13c was
added thiocresol (0.82 mmol) and, after 15 min, 1-methylimidazole (1.0 mmol). After stirring for 4.5 h at 20°C, TLC analysis (System B) showed the reaction to be complete. The reaction mixture was diluted with chloroform (60 ml), washed with 1 M TEAB (30 ml) and water (30 ml). The organic layer was dried (MgSO₄), concentrated to a small volume and precipitated from petroleum ether (40-60°C, 150 ml). The precipitate was dissolved in chloroform (2.0 ml) and applied to a column of Kieselgel (3.0 g). The column was eluted with chloroform-methanol (97:3, v/v). The appropriate fractions were concentrated and precipitated from petroleum-ether (40-60°C, 150 ml). The precipitate was filtered off and dried in vacuo (P₂O₅). The yield of product 16 thus obtained was 74%. Deprotection of fully-protected tetramer 16.

A solution of p-nitrobenzaldoxime (1.0 mmol) and H₁,N₁,N₃,N₃-tetramethylguanidine (0.86 mmol) in dioxan/acetonitrile (1:1, v/v, 4 ml) was added to tetranucleotide 13 (0.01 mmol). The reaction mixture was stirred for 30 h at 20°C. Dowex 50W cation-exchange resin (100-200 mesh ammonium-form, 5 g) was added. After 5 min, the resin was filtered off. The filtrate was concentrated, co-evaporated with pyridine (3 x 5 ml) and concentrated. The residue was dissolved in pyridine-water (2:1, v/v, 50 ml) and sodium iodide (0.6 mmol) was added to the mixture. After 45 min, the reaction mixture was poured into water/ether (5:1, v/v, 50 ml). The aqueous layer was concentrated and aqueous ammonia (25%, 30 ml) was added. The reaction vessel was sealed and kept at 50°C for a period of 48 h. The reaction mixture was concentrated to neutrality and analyzed by HPLC (System I). The thus obtained unprotected tetranucleotide II was purified by the same procedure as described before.

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REFERENCES
