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POLYMER SUPPORTED DNA SYNTHESIS USING HYDROXYBENZOTRIAZOLE ACTIVATED PHOSPHOTRIESTER INTERMEDIATES

J.E. Marugg[#], L.W. McLaughlin[+*], N. Piel[+], M. Tromp[#], G.A. van der Marel[#] and J.H. van Boom[#*]

[#]Gorlaeus Laboratories, State University of Leiden, P.O. Box 9502, 2300 RA Leiden, The Netherlands

[+]Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Hermann-Rein-Str. 3, D-3400 Göttingen, GFR

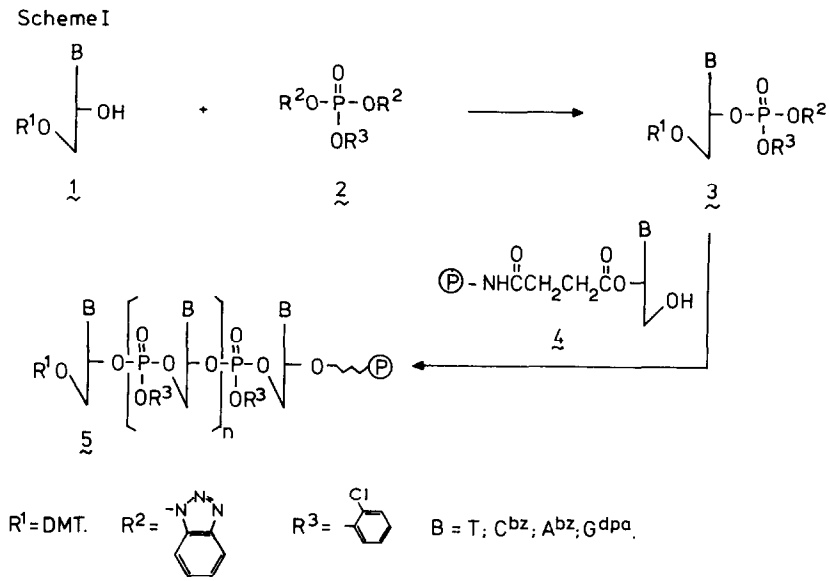
ABSTRACT

In the present method appropriate N-protected-5'-O-(4,4'-dimethoxytrityl)-2'-deoxynucleosides are phosphorylated with the reagent 2-chlorophenyl-O,O-bis(1-benzotriazolyl)-phosphate and the 3'-phosphorylated intermediates thus obtained are used directly to elongate the polymer support Kieselgur-polydimethylacrylamide. According to this procedure, we prepared two DNA fragments: one decamer and a heptadecamer.

One of the essential steps in the synthesis of DNA on a solid support is the introduction of an intermediate 3'-5'-internucleotide phosphotriester linkage. In one of the existing approaches, i.e., the phosphotriester approach[1], the formation of a triester linkage between an immobilized DNA fragment and an incoming nucleotide is achieved as follows: The immobilized DNA fragment with a free 5'-OH (e.g., 4 in Scheme 1) is coupled, in the presence of an activating agent, with a suitably protected 2'-deoxynucleoside carrying a 3'-phosphodiester function (e.g., 3; R^2 = H). An alternative route to achieve the same goal would be to circumvent not only the use of the 3'-phosphodiester intermediate 3 (R^2 = H), but also the activating agent.

We now wish to report that the bifunctional phosphorylating agent 2-chlorophenyl-O,O-bis(1-benzotriazolyl)phosphate[2] (2) can be used as an alternative for the introduction of triester linkages between an immobilized DNA fragment and an incoming suitably protected 2'-deoxynucleoside. The route we have employed is illustrated in Scheme 1. The intermediate phosphotriester 3, which will be coupled directly with the immobilized deoxynucleoside 4, was obtained by the following procedure. The 5'-protected 2'-deoxynucleoside 1 (R^1 = 4,4'-dimethoxytrityl; 1.0 mmol) was dissolved in anhydrous pyridine and evaporated to give an oil. An equimolar amount of phosphorylating agent 2 withdrawn from a stock solution of 2 (0.2 M) in dioxane[3] was added to the above oil. TLC-analysis ($CHCl_3$-MeOH; 92:8, v/v), after 15 min at 20°C,

Scheme I



showed complete conversion of starting material 1 into a product with zero mobility[4]. A stock solution of 3 (0.2 M) prepared in dioxane can be stored for several weeks at -20°C without any appreciable loss of activity. We have examined a number of the presently available polymer supports for DNA synthesis by the present approach[5]. It would appear that the polymer Kieselgur-polydimethylacrylamide[1a,b,6], in which the first deoxynucleoside is immobilized by a succinate linkage to the solid support (capacity: 83 μmol/g), is most effective.

In a typical elongation process, intermediate 3 (0.1 mmol) was mixed with N-methylimidazole/N,N-diisopropylethylamine/dioxane[7] (2:1:1, v/v, 100 μl) and injected using a glass syringe, onto the solid support 4 (120 mg) packed into a column which is part of a continuous flow-bench synthesizer[1b], and left in contact with the support for 15 min at 20°C. The effective-

Table 1. Deprotection, coupling and wash cycle.

Steps	Solvents and reagents	Time (min)
1.	10 % Trichloroacetic acid/1,2-dichloroethane	3
2.	DMF	2
3.	Pyridine	2
4.	Dioxane	5
5.	Coupling (stop flow)	15
6.	Dioxane	2
7.	Capping (Ac_2O/Et_3N/N-methylimidazole/dioxane: 4.5:4.5:1:30, v/v)	5
8.	Dioxane	3
9.	1,2-Dichloroethane	3

ness of the coupling process was monitored by measuring the release of the DMT-cation by UV spectroscopy at 498 nm. Based on the spectroscopic analysis the coupling process proceeds with an efficiency of greater than 95 % (based on 4). A complete elongation step, which includes the removal of the DMT group, the introduction of a 3'-5'-phosphotriester bond and subsequent capping of unreacted 5'hydroxyl functions is illustrated in Table 1. From the steps described in Table 1 one complete cycle (i.e., conversion of 5: n = 0; R^1 = DMT) into 5: n = 1; R^1 = DMT) can be performed in 40 min.

In applying the present elongation procedure, we prepared two immobilized DNA fragments: a decamer (i.e., 5; n = 8; sequence d(ApTpTpApTpTpApTpTpG)) and an heptadecamer (i.e., 5; n = 15; sequence d(ApApGpGpTpGpCpCpCpTpGpTpTpCpApTpC)). The fully protected and immobilized DNA fragments were deblocked and released from the solid support by a three step procedure. In the first step, the aryl protective groups (R^3) of the phosphotriesters and the succinate linkages, were removed by treating the immobilized fragments with (E)-2-nitrobenzaldehyde oxime[8] and N^1,N^1,N^3,N^3-tetramethylguanidine in dioxane/water. The N-acyl protective groups [i.e., benzoyl (bz) and diphenylacetyl (dpa)] were deblocked by a short treatment with aqueous ammonia for 24 h at 50°C. Finally, the dimethoxytrityl groups were deblocked by a short treatment with aqueous acetic acid. The HPLC analyses[1b,9] of the crude DNA fragments obtained for the decamer and heptadecamer are illustrated in Figures 1A and 1B respectively. It can be seen that the hydroxybenzotriazole (HOBT) approach affords DNA fragments of rather high quality. The two purified[10] (Sephadex G50) DNA fragments were completely digested, as judged from HPLC analysis, by venom and spleen phosphodiesterase to give the expected 2'-deoxynucleotides in the correct ratios.

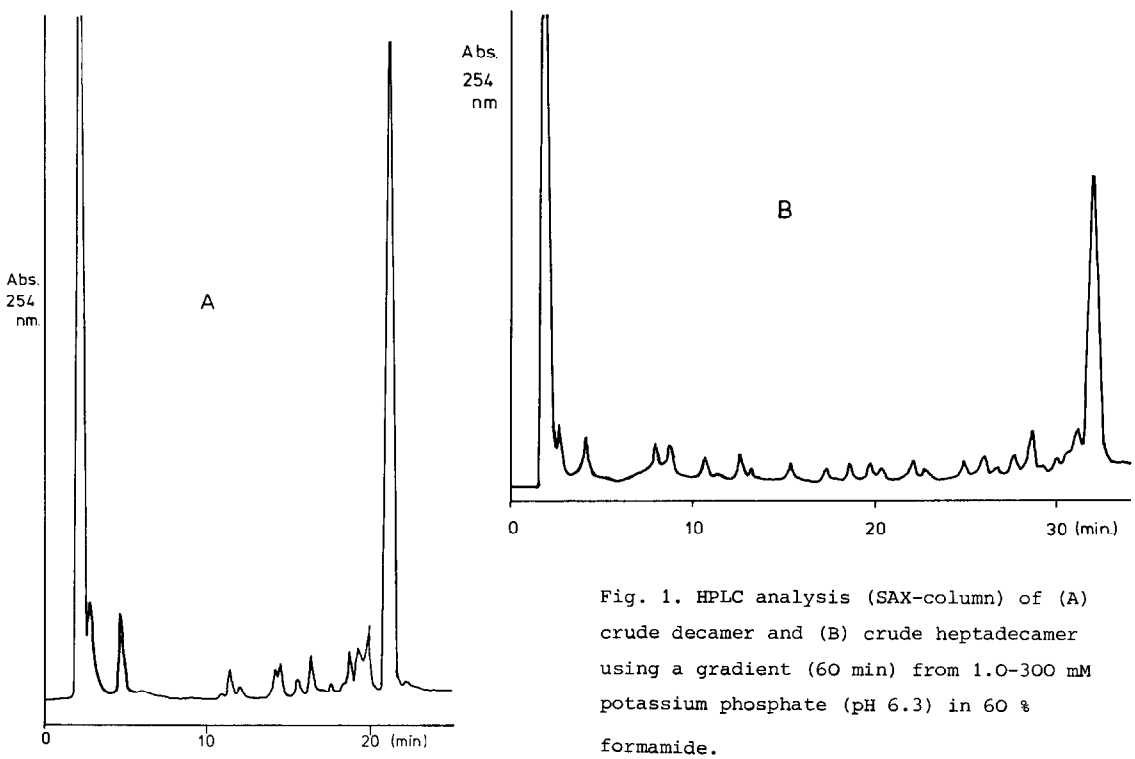


Fig. 1. HPLC analysis (SAX-column) of (A) crude decamer and (B) crude heptadecamer using a gradient (60 min) from 1.0-300 mM potassium phosphate (pH 6.3) in 60 % formamide.

The results obtained above indicate that the HOBT approach is in several aspects superior over the generally accepted phosph otriester procedure. For instance, the preparation of the key intermediate 3 is straightforward and does not involve a time consuming purification step. Furthermore, no activating agents [e.g., mesitylenesulfonyl-3-nitro-1,2,4-triazole[11] (MSNT)], which may give rise to side reactions with thymidine[12] or guanosine[13], are required. Another interesting feature of the HOBT approach is that the coupling step (i.e., conversion of 5; n = 0 to 5; n = 1) is nearly three times faster than the same step in a phosphotriester approach in which 3 (R^2 = triethylammonium salt) is coupled in the presence of the well established activating agent MSNT[1a]. The HOBT coupling time is comparable with that required for the introduction of a phosphotriester linkage by the phosphite-triester approach[14].

In conclusion, we believe[15] that the HOBT approach will be an economical and simple procedure for the synthesis of DNA fragments on a solid support.

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3. The phosphorylating agent 2 can be kept, under the exclusion of moisture, for several months at 0°C without any appreciable loss of activity.
4. This observation was also supported by ^{31}P-NMR studies: J.E. Marugg et al., to be published.
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15. Preliminary experiments showed that the HOBT approach could also be applied on a Synthesis Automation Machine.

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