Journal of Chromatography, 253 (1982) 65–79
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,274

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN COMBINATION WITH FIELD DESORPTION MASS SPECTROMETRY: SEPARATION AND IDENTIFICATION OF BUILDING BLOCKS FOR POLYNUCLEOTIDE SYNTHESIS*

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(Received August 9th, 1982)

SUMMARY

Optimum conditions for analytical and preparative separation of suitably protected mono- and dinucleotides by high-performance liquid chromatography (HPLC) are described. These nucleotide units serve as standard building blocks for the synthesis of polynucleotides according to the triester method, and being of low volatility and low thermal stability can conveniently be identified and characterized by field desorption mass spectrometry in off-line mode. The use of HPLC techniques for analytical and preparative separations of larger oligonucleotide fragments is also demonstrated.

INTRODUCTION

Synthetic oligo- and polynucleotides have wide application, especially in molecular biology and genetic engineering. The increasing importance of this class of substances stimulates the search for new and simple synthetic procedures as well as fast, efficient methods for their separation and identification. Currently, the synthetic method most often employed is the "modified triester method", developed by several groups over the last years[4]. The key intermediates most commonly used in this approach are the blocked monomers (1), which after conversion into the functionalized monomers (2 and 3) can be combined to give a standard set of dinucleotide blocks (4)

* Synthesis with Nucleic Acid Constituents, Part XI; Part X: see ref. 16; Part IX: see refs. 1 and 2. Mass Spectrometry of Nucleotides, Part VI; Part V: see ref. 3.

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0021-9673/82/0000–0000/$02.75

(Fig. 1). These are terminally deblocked in the same way as 1 to yield, by further combination, longer oligonucleotide chains of defined sequence[5-9]. Due to the development of new and improved condensing agents, the time required for internucleotide bond formation has been drastically reduced. This calls for methods for the purification and identification of the intermediates which are both rapid and efficient.

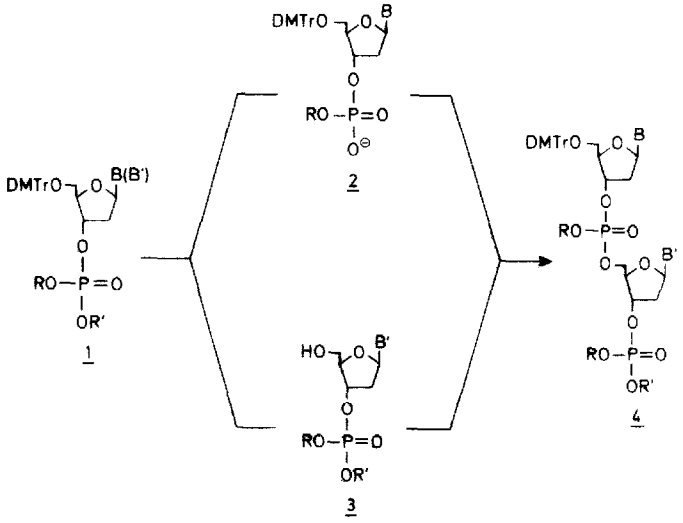

Fig. 1. Synthesis of a dinucleotide building block. B = B' = N-benzoyladenine (a); N-benzoylcytosine (b); N-isobutyrylguanine (c); thymine (d); R = *p*-chlorophenyl; R' = β-cyanoethyl; DMTr = *p,p'*-dimethoxytrityl.

A characteristic feature of the modified triester method is the fact that the monomer (1) as well as all products of internucleotide bond formation, such as 4, are fully blocked and, therefore, neutral and lipophilic substances. Various types of silicagel chromatography have been suggested for separation of the building blocks 4 from 2 and 3, such as thin-layer chromatography (TLC)[7-9], column chromatography on regular size[5,10] or "short" columns[11,12], possibly with the use of a medium pressure system[7-9]. High-performance liquid chromatography (HPLC) on an analytical or preparative scale is a fast and efficient alternative to these methods. The potential of this technique has been pointed out by Crea *et al.*[13], although details of the chromatographic systems used were not given. In pursuing synthetic studies using the modified triester method[14-16] we have been interested in optimizing the conditions for a standard purification of dinucleotide building blocks by liquid chromatography[17] and now report on these investigations.

Any separation has to be accompanied by the identification of the separated compounds and a check of their purity. The latter is particularly important, since the purity of the intermediates greatly influences the success of further condensation steps. Rechromatography of the separated products with reference substances may not suffice to exclude the presence of impurities or to give more than a rough estimate of their content. NMR spectroscopy has been used to assess the purity of the building blocks[18,19]. However, even though spectrometers of high magnetic field strengths are available, their use is limited by the amount of sample necessary for rapid analysis,

especially for the less complex and more instructive ^{13}C nuclear magnetic resonance spectra.

Mass spectrometry (MS), in contrast, owing to its high sensitivity requires only minute quantities of material and is expected to yield spectra which can readily be interpreted in respect of the nature and content of substances present in a mixture. However, the nucleotides used in the triester method are unsuitable for conventional ionization techniques in MS such as electron impact (EI) or chemical ionization (CI)[20–22]. Due to their sensitivity to thermal or impact-induced excitation, these compounds give no molecular ions but only fragments and hence solely indications as to partial structures. We have therefore employed field desorption mass spectrometry (FD-MS)[23,24], one of the soft ionization methods. As has recently been demonstrated[3,25–30], these newly developed ionization techniques are well suited to the analysis of protected mono- and oligonucleotides.

The principle and environmental, medical and pharmaceutical applications of FD-MS in combination with HPLC have recently been outlined[31]. This technique is efficient and versatile in analysing polar substances in complex mixtures. Using the example of the terminal dinucleoside monophosphate (5′) and the dinucleoside diphosphate (5) (Fig. 2), we will demonstrate the use of the off-line combination of HPLC and FD-MS for separation, identification and purity control of such nucleotide building blocks.

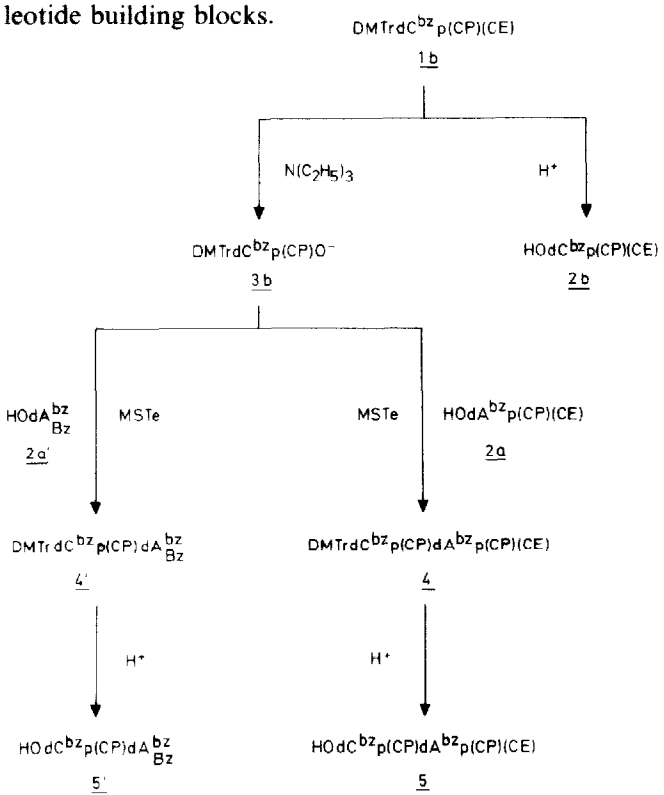

Fig. 2. Preparation of an intermediate (5) and a terminal (5′) block of protected dCpA(p). Nomenclature: dC = 2′-deoxycytidine; dA = 2′-deoxyadenosine; bz = N-benzoyl; Bz = 3′-O-benzoyl. Phosphate protecting groups: CP = *p*-chlorophenyl; CE = β-cyanoethyl. MSTe = Mesitylenesulphonyl tetrazolide.

MATERIALS AND METHODS

Preparation of protected mono- and oligonucleotides

The nucleosides were commercial products of Pharma Waldhof (Düsseldorf, G.F.R.). Chemicals used for the introduction of the protecting groups and for the preparation of activating agents were obtained from Ega-Chemie (Steinheim/Albuch, G.F.R.), E. Merck (Darmstadt, G.F.R.) and Fluka (Buchs, Switzerland). All solvents were distilled and, if necessary, dried according to standard procedures. *p*-Chlorophenyl phosphodichloridate was prepared as described in the literature[7–9], except that a three-fold excess of $POCl_3$ and a small amount of KCl as catalyst were used. The product was converted into *p*-chlorophenyl β-cyanoethyl phosphorochloridate by a modification of a procedure of Van Boom *et al.*[32]. The condensing agent, mesitylenesulphonyl tetrazolide, was prepared according to refs. 7–9.

Procedures for the preparation of protected nucleosides were as described[7–9].

Phosphorylation of protected nucleosides (preparation of 1). Three millimoles of base-protected nucleosides (for base protecting groups see Fig. 2), blocked at the 5′-position with a *p,p′*-dimethoxytrityl group, were dissolved in *ca.* 20 ml dry acetonitrile. Traces of water were removed by repeatedly evaporating and replenishing *ca.* 3–4 ml dry acetonitrile. To the dry solution 1 ml N-methylimidazole was added. Then 10 mmol of *p*-chlorophenyl β-cyanoethyl phosphorochloridate, dissolved in 5 ml dry acetonitrile, were added dropwise with cooling. After reaction for 1–2 h at 20°C, TLC indicated quantitative conversion into the fully protected nucleoside 3′-phosphate. The reaction was then stopped by slowly adding 5 ml of 1 *M* ammonium bicarbonate in an ice-bath. After hydrolysis (1 h at room temperature) the mixture was evaporated, the residue dissolved in 20 ml chloroform, extracted three times with the same volume of 0.1 *M* ammonium bicarbonate and once with water. The chloroform layer was dried and evaporated to dryness. The residue contained nearly pure product. Further purification was done by preparative liquid chromatography on a silica gel column with 2% methanol in methylene chloride as eluent. The purity of the compounds was checked by analytical HPLC (see example in Fig. 4). The yields of pure 1 were about 80–85%. Structural proof was obtained by FD-MS; the fragmentation pattern of an example is given in Fig. 3.

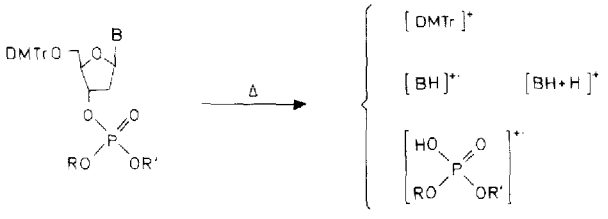

Fig. 3. Formation of structurally significant ions of protected mononucleotide triesters by thermally induced degradation on the emitter surface during field desorption.

Detritylation of compounds 1. The detritylation of compounds 1 was performed as described[5]. For further purification the compounds 2 were subjected to preparative liquid chromatography on silica gel as described in the caption to Fig. 7. The purity

check by analytical HPLC is also shown in Fig. 7; for identification by FD–MS see Table I.

Decyanoethylation of 1. Alternatively, the compounds 1 were decyanoethylated to compounds 3 by treatment with triethylamine in pyridine according to Narang and co-workers[7–9]. Complete decyanoethylation is indicated by a decrease of the TLC R_F value.

Preparation of 3′-benzoylated nucleosides (2′). One millimole of 5′-dimethoxytritylated base-protected nucleoside was dissolved in 15 ml dry pyridine and 1.2 mmol of benzoyl chloride were added dropwise with stirring at 0°C. The reaction was terminated after 4 h, as monitored by TLC. To the reaction mixture were added 10 ml water or 0.1 *M* ammonium bicarbonate solution. The mixture was extracted three times with chloroform, the chloroform phase back-extracted with water and dried over Na_2SO_4. The chloroform solution was filtered, evaporated to a small volume and pyridine was removed by evaporation to dryness with toluene. Then the dimethoxytrityl group was removed as described above. The purity of the compounds 2′ was checked by HPLC (see Fig. 5) and their identities were established by FD-MS.

Synthesis of fully blocked dinucleotides (4, 4′). 0.7 mmol of compound 3 and 0.5 mmol of 2 (2′) were dissolved in 10 ml dry pyridine and freed from water by repeated addition and evaporation of 5 ml dry pyridine. After adding *ca.* 2.1 mmol mesitylenesulphonyl tetrazolide, the mixture was again evaporated to a final volume of *ca.* 8 ml and then stirred at room temperature. After 45 min, TLC in chloroform–methanol (9:1) indicated that the reaction was essentially complete. It was then terminated by addition of 25 ml 5% ammonium bicarbonate solution with cooling, followed by 20 ml chloroform and stirring for 1 h. Then the chloroform phase was separated and the reaction mixture reextracted twice with 25-ml portions of chloroform. The combined chloroform extracts were washed with 20 ml 5% ammonium bicarbonate and 20 ml 10% NaCl solution, dried over anhydrous Na_2SO_4, filtered and coevaporated with excess of toluene, then with methanol under high vacuum. Each coevaporation should result in the formation of a gummy residue. This procedure removes all pyridine remaining in the mixture. The residue was dissolved in chloroform and separated by preparative HPLC on a silica gel column (see Fig. 8).

Preparation of functionalized dinucleotide building blocks.

Dinucleotides with a free 5′-OH group (5) were obtained by treatment of the compounds 4 as described for compounds 1. An example of the analytical HPLC of compounds 5 is shown in Fig. 6. The MS identification is demonstrated in Fig. 11 and the corresponding data are included in Table I.

Alternatively, the β-cyanoethyl group could be removed from compounds 4 by the treatment described previously for 1. The preparation of 3′-benzoylated terminal blocks (4′) and their detritylated derivatives (5′) was done analogously to the synthesis of 4 and 5. An example of the identification of a terminal block is included in Fig. 6, and the results of FD-MS are given in Table I.

Preparation of longer oligonucleotide chains. Functionalized di- or trinucleotide units, the latter obtained by reaction of di- and mononucleotides, were combined in the same fashion as described for dinucleotides, and subsequently functionalized by detritylation or decyanoethylation. The purification was done by silica gel chromatography, followed by column chromatography or preparative HPLC on reversed stationary phase.

As an example, the preparation of the octanucleotide DMTrdGpGpApCpCpTpApGp(CE) (A, C, G = N-benzoyladenosine, N-benzoylcytidine and N-isobutyrylguanosine; p = *p*-chlorophenylphosphoryl) from two tetranucleotide precursor blocks was as follows. DMTrdGpGpApCp(CE) was obtained from DMTrdGpGp$^-$ (0.3 mmol) and dApCp(CE) (0.26 mmol) using 1.2 mmol mesitylenesulphonyl tetrazolide (45 min, room temperature) in 56% yield. Similarly, DMTrdCpTpApGp(CE) was prepared from DMTrdCpTp$^-$ (0.36 mmol) and dApGp(CE) (0.3 mmol), using the same condensation conditions, in 66% yield. After functionalization of the two tetramer blocks, DMTrdGpGpApCp$^-$ (0.1 mmol) was condensed with dCpTpApGp(CE) (0.09 mmol) using 0.4 mmol mesitylenesulphonyl tetrazolide for 60 min at room temperature. After work-up and removal of pyridine, the residue was first subjected to preparative HPLC on a silica gel column (eluent: 4.5% methanol in chloroform), which removed all residual phosphodiester blocks as well as some other impurities. The crude product thus obtained (4210 absorbance units at 280 nm) was mainly a mixture of the octanucleotide and the tetranucleotide precursor with a free 5′-hydroxyl group. Its further separation by preparative HPLC on a silica gel C_{18} column is shown in Fig. 9, and the conditions are described in the legend. The success of the separation was controlled by parallel analytical HPLC before and after the separation (see Fig. 10 and conditions given in the legend). The yield of pure octanucleotide was 0.022 mmol [25%, based on dCpTpApGp(CE)].

Chromatography

Apparatus and conditions used for HPLC are described in the captions of Figs. 4–7. UV measurements were made with the spectrophotometer PMQ III and the spectrograph DMR 21, both from Carl Zeiss (Oberkochen, G.F.R.). HPLC was monitored with flow-through spectrophotometers LKB 8300 Uvicord II (LKB, Bromma, Sweden).

Mass spectrometry

The FD measurements were performed on a Type 731 Varian MAT double-focusing mass spectrometer equipped with a combined, commercial EI/FI/FD ion source. The FD emitters used were 10-μm tungsten wires activated at high temperature[23]. The length of the carbon microneedles was 40 μm on average. All spectra were produced using direct heating of the emitter by a emitter heating current (e.h.c.). The sample was transferred to the emitter by the modified syringe technique[23] and in general 3–5 μg of sample material were deposited on the centre of the front side of the emitter. The applied potentials were +8 kV for the field anode and −4 kV for the opposing cathode plate. The FD ion currents were recorded electrically in combination with the data system Varian SS 200. All spectra were acquired at a mass resolution of about 2000 (10% valley definition).

RESULTS AND DISCUSSION

Analytical and preparative separation by HPLC

To illustrate the analytical steps employed for controlling the synthesis of dinucleotide units, the path from a protected mononucleotide (1b) to the protected dinucleotides 4 and 4′ (as 3′-terminal unit of a corresponding sequence) is shown in

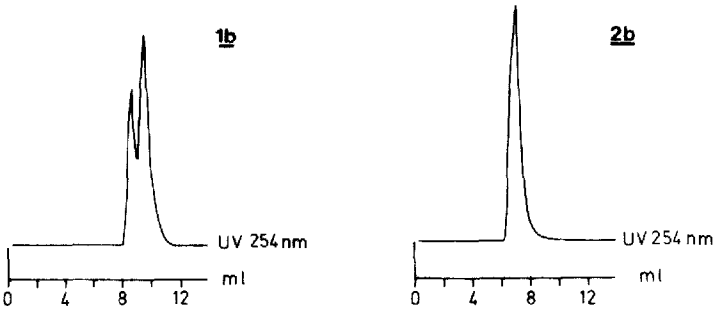

Fig. 4. HPLC purification of compounds 1b and 2b. Column: silica gel (MN/Nucleosil® 50-10). Eluent: methylene chloride–methanol (98:2 for 1b and 96.5:3.5 for 2b); flow-rate 2 ml/min. Pressure: 34 bar. Amplification range: 1.0.

Fig. 2. As can be seen from Fig. 1, functionalized building units must be gained on each further lengthening of the chain. Since the addition of such units occurs by quantitative reactions from the fully protected precursors (4), only the detritylated building units (5 and 5′) were controlled by HPLC. Thus, at the same time, the purity of the precursor 4 as well as that of the decyanoethylated block formed from it, itself difficult to separate by HPLC, is also monitored.

The elution diagrams of compounds 1b, 2b, 2a, 2a′, 5 and 5′ are shown in Figs. 4–6. The conditions for the separations are given in the captions. As can be seen, HPLC on silica gel phases is especially suitable as a simple and cheap method for the purification of neutral, protected mononucleotide derivatives. As eluents, methylene chloride–methanol mixtures are used, the compositions depending on the nature of the bases and protecting groups used.

A difficulty arises from the fact that with each fully protected phosphate group a chiral centre is introduced into the mono- and oligonucleotide molecules. The resulting mixture of stereoisomers can lead to broadening or splitting of the product

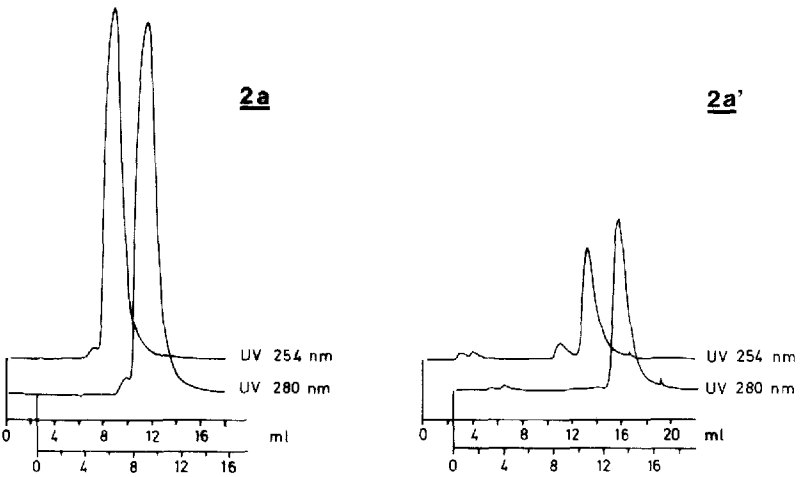

Fig. 5. HPLC purification of compounds 2a and 2a′. Column: silica gel (MN/Nucleosil® 50-10). Eluent: methylene chloride–methanol (96.5:3.5 for 2a and 98.5:1.5 for 2a′); flow-rate 2 ml/min. Pressure: 34 bar for 2a and 55 bar for 2a′. Amplification range: 1.28.

peak even with fully protected mononucleotides. With longer-chain oligonucleotides this considerably hinders purity control. However, as Fig. 6 shows, this splitting disappears when a reversed phase is employed. Thus, reversed-phase HPLC is preferred for purity control of protected dimers, such as 5 and 5′.

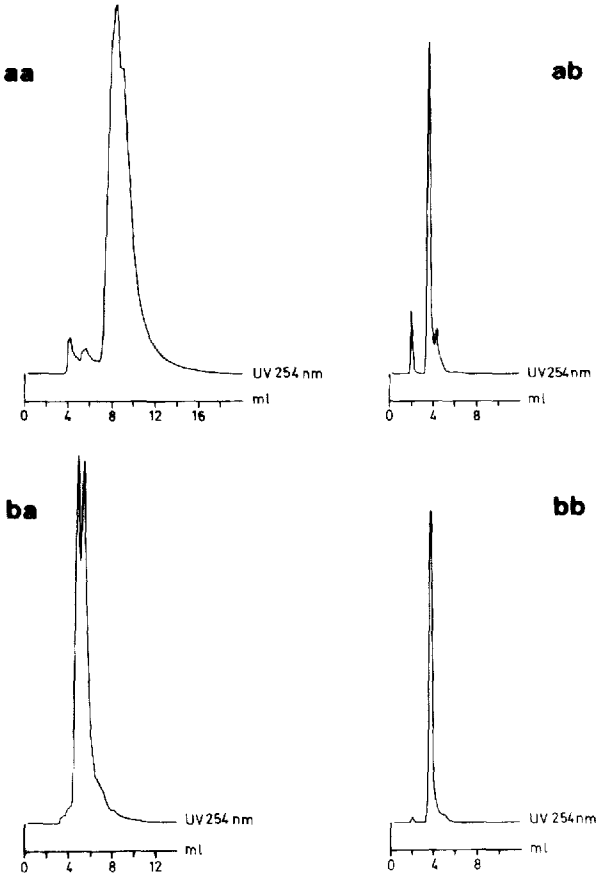

Fig. 6. Comparison of silica gel (aa,ba) and reversed-phase (ab,bb) columns for the HPLC purification of compounds 5 (a) and 5′ (b). aa and ba: column, silica gel (MN Nucleosil® 50-10); eluent, methylene chloride–methanol (94:6) for aa and chloroform–methanol (96.5:3.5) for ba, flow-rate 2 ml/min; pressure, 34 bar; amplification range, 0.1 for aa and 1.0 for ba, ab and bb; column, RP-18 (μBondapak); eluent, acetonitrile–water (80:20), flow-rate 2 ml/min; pressure, 69 bar; amplification range, 0.05 bar for ab and 0.5 bar for bb.

The reversed-phase systems described in Fig. 6 for separation and purity control can also be applied to longer-chain oligonucleotides, provided solubility problems do not interfere with the separation. This is demonstrated for the fully protected octanucleotide DMTrdGpGpApCpCpTpApGp(CE) in Fig. 10a. A phenomenon often encountered in reversed-phase separations of longer oligonucleotide chains with triester internucleotide linkages is also seen in Fig. 10b: due to the presence of a multitude of stereoisomers the product peak shows a splitting, although the compound is found to be homogeneous with respect to its chemical composition and base

sequence. A complete resolution of isomers, which could lead to their isolation and identification, has, so far, not been observed.

As a double check on the purity of the protected fragments a sample is stripped of all protective groups and subjected to HPLC in acetonitrile–water systems[18,19], followed by sequence analysis by the two-dimensional fingerprint method. The experimental conditions for these operations are described elsewhere[14–16].

For purification of larger quantities of substance (up to 15 mmol) HPLC is employed on a preparative scale. For mono- and dinucleotide building blocks, the use of the reasonably priced silica gel columns has proved to be adequate for good separations. Due to the large quantities involved, refractometry must be used for detection in addition to UV-spectroscopy. An example of purification of a protected mononucleotide unit is the separation of N-isobutyryldeoxyguanosine-3′-(*p*-chlorophenyl-*β*-cyanoethylphosphate (2c), shown in Fig. 7, after detritylation of the fully protected precursor 1c. In the procedure followed here the separation conditions were established first by analytical HPLC (Fig. 7ba). The preparative separation was then carried out with the same eluent (Fig. 7a). The purity of the product (retention time > 18 min) was controlled, parallel to the separation, by TLC (chloroform–methanol, 9:1; R_F = 0.21) and UV-spectroscopy (λ_{max} = 280.257 nm; λ_{min} = 272.248 nm) of single fractions, and then checked by analytical HPLC of the relevant com-

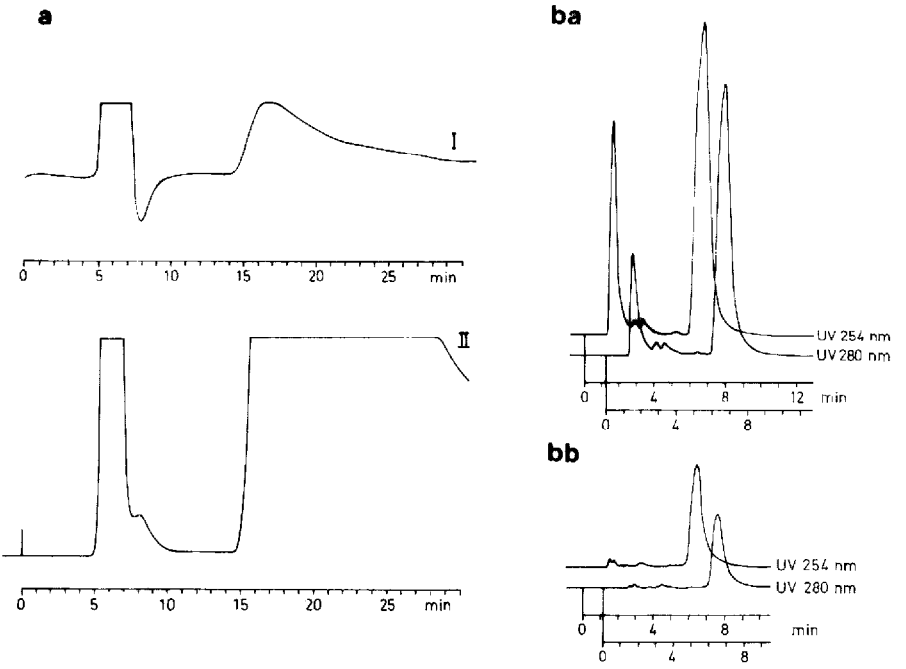

Fig. 7. a, Separation of the product of the detritylation of compound 1c (*ca.* 10 mmol) using the Prep-LC 500 system (Waters Assoc.), registered by differential refractometry (curve I) and UV-spectroscopy at 254 nm (curve II). Column: silica gel. Eluent: methylene chloride–methanol (95:5); flow-rate, 100 ml/min. b, Control of the preparative separation (a) by analytical HPLC: separation of the reaction product before preparative LC (ba) and check of the purity of compound 2c after preparative LC (bb). Column: silica gel (MN/Nucleosil® 50-10). Eluent: methylene chloride methanol (95:5); flow-rate, 2 ml/min. Pressure: 55 bar. Amplification range: 1.28. Detection: UV at 254 nm.

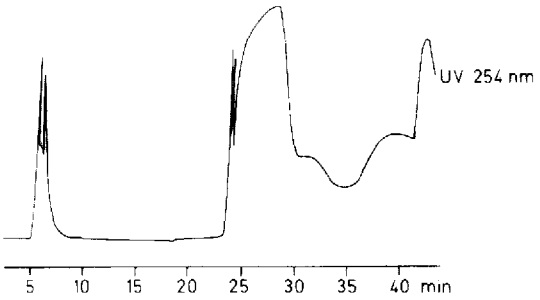

Fig. 8. Preparative separation of compound 4 using the Prep-LC 500 system. Column: silica gel. Eluent: chloroform–methanol (95:5); flow-rate, 100 ml/min. Loading: 7000 absorbance units at 280 nm, corresponding to 0.3 mmol. Detection: UV at 254 nm.

bined fractions (Fig. 7bb). As shown, almost complete removal of the side products can be achieved in a single preparative HPLC run.

The preparative HPLC separation of a fully protected dinucleotide is shown in Fig. 8 for 5′-dimethoxytrityl(N-benzoyl)deoxyadenosine-3′-(*p*-chlorophenylphosphoryl)-5′-(N-benzoyl)deoxycytidine-3′-(*p*-chlorophenyl-β-cyanoethyl)phosphate. In the course of this procedure, the methanol content of the eluent was raised stepwise from 1 to 5%. The product was eluted with a mixture of 2% methanol in chloroform and the purity checked in a manner similar to that described above.

The routine use of preparative HPLC for the purification of fully protected longer-chain oligonucleotides has not previously been reported. We found that in many cases a single preparative HPLC run on a silica gel column is sufficient to remove the product from both precursor blocks containing either a 3′-phosphodiester or a 5′-hydroxy terminus. However, the separation properties of the product and the 5′-hydroxyl-terminated block are often quite similar, and a good resolution on silica gel is not obtained. In these cases the separation was accomplished by passing the mixture first through a silica gel column (open column or HPLC), which removes the phosphodiester precursor. The residual material is then easily purified by preparative reversed-phase HPLC. This is demonstrated in Fig. 9 for the fully protected oc-

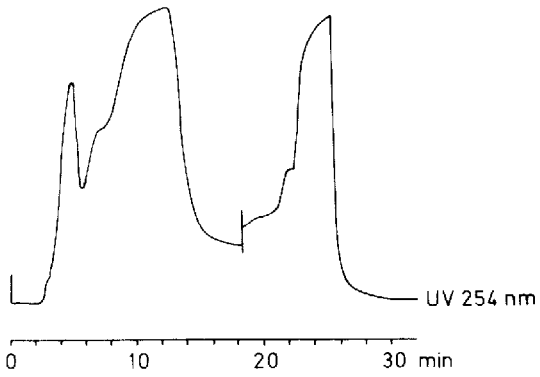

Fig. 9. Preparative separation of the fully protected octanucleotide GGACCTAG using the Prep-LC 500 system, registered by UV-spectroscopy at 254 nm. Column: RPC 18. Eluent: methanol–water–chloroform (64:16:20); flow-rate, 100 ml/min. Loading: 2400 absorbance units at 280 nm, corresponding to 0.05 mmol.

tanucleotide DMTrdGpGpApCpCpTpApGp(CE). Fig. 10a and 10b show the control analytical HPLC runs before and after the preparative separation. In this example *ca.* 20 μmol of octanucleotide were, in a single preparative separation, obtained sufficiently pure for further fragment combinations to yield longer polynucleotide chains. Thus, preparative HPLC can provide sizable quantities of oligonucleotides of defined sequence in a simple and rapid procedure. In view of the fact that large-scale syntheses of DNA fragments for physico-chemical or crystallographic studies are still formidable tasks, the methods developed in our laboratory should be a valuable addition to the preparative techniques of polynucleotide chemistry.

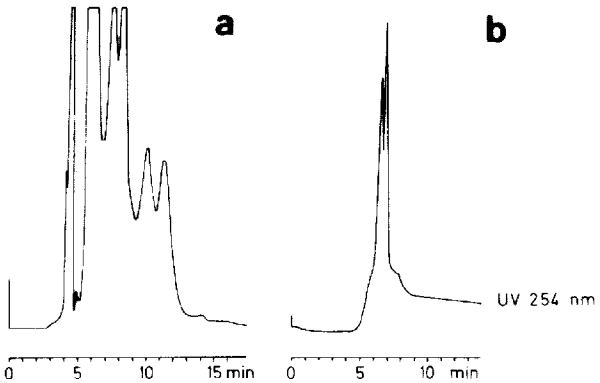

Fig. 10. Analytical separation of the fully protected octanucleotide GGACCTAG before (a) and after (b) preparative separation (see Fig. 9). Column: RP-18 (μBondapak). Eluent: methanol–chloroform–water (8.2:0.9:0.9); flow-rate, 2 ml/min. Pressure 75 bar. Amplification range: 0.1. Detection: UV at 254 nm.

Identification by FD-MS

Molecular-weight determination. The molecular weights of the triesters can be derived from the abundant ions $[M + H]^+$ and/or the $[M + Na]^+$ obtained by protonation or cationization of the original molecules as listed in Table I. The ratio of the abundances of $[M + H]^+$ and $[M + Na]^+$ changes from one sample to another and is dependent on the amount of sodium salts present as trace impurities in the authentic specimen. By adding *ca.* 1% of NaCl, the formation of $[M + Na]^+$ can be greatly enhanced. Thus, for compound 5′, the following relative abundances were obtained: 20% for $[M + H]^+$ at *m/z* 963; 100% for $[M + Na]^+$ at *m/z* 985 and 5% for $[M + {}^{39}K]^+$ at *m/z* 1001.

If a mass spectrum shows minimal or no fragmentation, the correct assignment of the molecular weight can essentially be confirmed, first by determining the elemental composition and secondly by measuring the isotopic abundances. Since the FD ion currents of nucleotides are of short duration and fluctuate due to salt effects[33,34], the high mass resolution necessary for the first solution is difficult to attain using electric detection. In contrast, the alternative technique employing a multichannel analyser in the low resolution mode and averaging a large number of repetitive magnetic scans has proved to be highly successful[35].

In general, therefore, confirmation of the molecular ions of the nucleotide triesters was performed by measurements of their isotopic abundances. This was of course facilitated by elements with distinctive isotopic distributions such as the ^{35}Cl

TABLE I
MOLECULAR WEIGHT OF NUCLEOTIDE TRIESTERS DETERMINED BY FD-MS

Compound	*Molecular weight*		
	*Theoretical**	*Found*	
		$[M + H]^+$	$M + Na^+$
1b	876.233	–	899
1c	882.254	–	905
2a	598.133	599	621
2b	574.102	575	597
2c	580.124	581	603
5	1101.178	1102	1124
5′	962.219	963	985

* Precise masses obtained by combination of the elements ^{12}C, ^{1}H, ^{14}N, ^{16}O, ^{31}P and ^{35}Cl respectively.

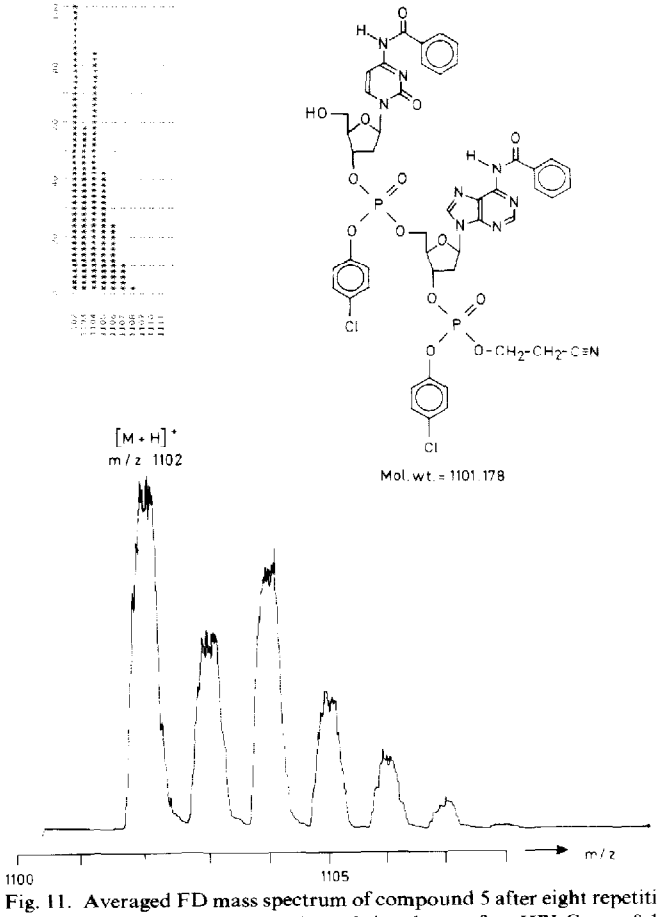

Fig. 11. Averaged FD mass spectrum of compound 5 after eight repetitive magnetic scans between e.h.c. of 10 and 30 mA. The concentration of the eluate after HPLC was 0.16 μg/μl; for sample application a concentration of 0.24 μg/μl was used. As an exception, the solvent in this case was acetonitrile–water (9:1).

and ^{37}Cl of the *p*-chlorophenyl protecting group. In Fig. 11 the mass region of the $[M + H]^+$ of the dinucleoside diphosphate 5 is compared with the theoretical isotopic pattern.

Structural information and purity control. The predominant formation of molecular ions is a characteristic feature of FD-MS. This first step in FD analyses assists detection of a compound and the recognition of impurities or side products of an individual synthesis step, but does not allow further identification via the formation of characteristic fragments, as is typical of EI-MS. A solution to this problem is in principle offered by the combination of field desorption and collisional activation. However, this technique is relatively complex, and has not yet been applied to substances in the high mass range. An alternative and experimentally simpler way of obtaining extra structural information is the controlled thermolysis of the compound on the emitter during desorption. As has recently been shown[36], this "chemistry on the emitter"[33] gives a series of significant degradation products, which can also be used in the identification of the nucleotide triesters.

For purity control of synthetic or natural products two main facts should be borne in mind. First, as has recently been demonstrated[37], FD-MS can be more sensitive than conventional HPLC detectors by a few orders of magnitude. This of course depends on characteristics such as the UV absorbance of the individual compounds when UV detectors are employed.

Secondly, MS yields not only the mass number and intensity. If the FD-emitter with the adsorbed sample is heated linearly or programmed with time, fractionated desorption of the adsorbed chemical species occurs. This means that the more volatile substances in the sample mixture such as solvents, reagents, etc. desorb at lower temperatures and, for instance, inorganic or organic salts desorb at higher temperatures than the protected nucleotide one is looking for. Thus, a search for the compound in question within a certain time (or temperature) window in ion desorption reduces the influence of contaminants in a manner comparable to a chromatographic separation. Examples of this effect have been described for ppt concentrations of biocides from a very complex matrix such as Rhine river water[38,39]. On the other hand, fractionated desorption can also be utilized to monitor certain classes of substances which differ in polarity (and thus desorption temperature) as they desorb one after another. This has been demonstrated convincingly by Lehmann[40] for lipids in human plasma.

The characteristic fragment of the fully protected mononucleotide 1b is the dimethoxytrityl ion of mass number 303, already intense at low emitter temperatures. Higher temperatures then lead to liberation of the protected nucleobase and its registration at *m/z* 215 and *m/z* 216 as $[BH]^+$ and $[BH + H]^+$ respectively, by cleavage of the N-glycosidic bond. At the same time, $[M + H]^+$ at *m/z* 261, corresponding to the thermal formation of the phosphoric acid diester with its typical isotope pattern, also appears in the spectrum (Fig. 3).

In the spectrum of compound 2a the ion of mass number 303 is missing, as expected. Structural information enables the assignments of $[BH]^+$ at *m/z* 239 for the nucleobase and the ion at *m/z* 261 to the phosphate remainder.

Due to the ease with which the dimethoxytrityl protective group can be eliminated, compounds 4 and 4′ were identified mass spectrometrically only after functionalization to 5 and 5′. The ions $[BH + H]^+$ of the two protected nucleobases at

m/z 216 and *m/z* 240 also give, in this case, important signals for identification. The base peak of the spectrum of the dinucleoside diphosphate 5 at higher emitter temperature is an ion at *m/z* 314, obtained by cleavage of the 3′-ester bond of the 5′-terminal nucleoside, forming a protonated 2′,3′-dideoxy-2′,3′-didehydroriboside. Since an analogous ion for the 3′-terminal nucleoside is missing, the spectrum clearly gives the sequence of the dinucleoside diphosphate.

A considerable amount of time is required for MS analysis of the molecular weight and structural features of a synthesized (or isolated natural) substance and its purity control. This time becomes even longer when accurate mass measurements or determination of the isotopic abundances of the compound are required, especially when compounds of mass $\geqslant$ 1000 have to be examined. In view of these facts, and also our experience with the HPLC–FD-MS combination, the off-line mode is preferred.

ACKNOWLEDGEMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Se 168/15 - 16; Schi 168/4; Schu 416/1-5). H.-H. Görtz is grateful for a doctoral fellowship of the Studienstiftung des Deutschen Volkes. We are, furthermore, indebted to Dr. Tittel, University of Munich, and Waters GmbH, Munich, for sample separations by liquid chromatography as well as to Dr. P. B. Monkhouse, University of Bonn, for valuable assistance in preparing this manuscript.

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