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The Use of Liquid Phase Carriers for Large Scale Oligonucleotide Synthesis in Solution via Phosphoamidite Chemistry

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Abstract: Nucleoside derivatives coupled to a multifunctional highly symmetric primary amine **3** built the fundamental of a convenient method for large scale oligonucleotide synthesis in solution. The basic purification for the fast isolation of intermediates is obtained by gel permeation chromatography. Monomer and dimer phosphoamidites (**1a-d**; **2a,b**) are used for the preparation of short oligodeoxynucleotides. Total cycle yields between 81 and 95 % and average cycle yields of 87 % were obtained. MALDI-TOF-mass spectrometry was used for the analysis of the fully protected intermediates during synthesis.

INTRODUCTION

According to the antisense principle[1,2], oligodeoxynucleotide analogues are specific inhibitors of gene expression and can function as a new class of pharmaceuticals in the therapy of molecular diseases. One problem to overcome in the development of efficient antisense oligonucleotides is the lack of effective large scale methods for their synthesis. In this field oligonucleotide synthesis in solution offers some advantages over the commonly used solid phase strategy, such as easy upscaling, direct reaction control and products of high purity, especially recommended for therapeutical use. Only few efforts have been made to adapt β-cyanoethyl phosphoamidite chemistry[3-5] to solution based techniques, despite of the fact that this approach is regarded as the most effective for synthesis of oligodeoxynucleotides and their analogues[6-8]. Herein reported is the use of a multifunctional highly symmetric liquid phase carrier (LPC), bearing three primary nucleosides for simultaneous chain elongation in 3'→5' direction with monomer and dimer phosphoamidites. The procedure represents a way to a purification orientated and effective technique for the synthesis of short oligonucleotides and their analogues in preparative

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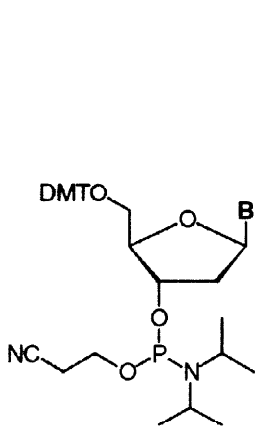
scales. The purification, mainly achieved by gel permeation chromatography (GPC) on Sephadex LH20, is faciliated through the coupling of the intermediates to the LPC. The considerable difference in size between reaction products and all other reagents including the excess phosphoamidites allows separation by the principle of gel filtration. The condensation product as the largest component is eluted first near the void volume. Moreover the LPC mediates the solubility in organic solvents during the different reaction steps and the chromatography process. Preferably after dedimethoxytritylation the reaction products can be analysed by MALDI-TOF mass spectrometry. A direct reaction control on the step of the fully base and phosphate protected intermediates is possible. The scale of synthesis is mainly determined by the dimension of the Sephadex LH20 column and can be varied in a wide range.

In this paper the synthesis of the decanucleotide d(GACGGCCAGT) **21** using monomeric phosphoamidites **1a-d** and the LPC **3**, based on a 1,3,5-benzene tricarboxylic acid derivative is described. Supplementary to this procedure also dimer phosphoamidites **2a,b** were tested in the synthesis of the pentanucleotide d(GCCCT) **25**. Such building blocks are ingenious tools especially for oligonucleotide synthesis in solution because the number of time consuming coupling cycles and purification steps is reduced. The synthesis of the used LPC **3** will be published in an other paper [9] (containing also the synthesis of a pentaerythrite derivative tested for the same purpose and different dedimethoxytritylation procedures).

RESULTS AND DISCUSSION

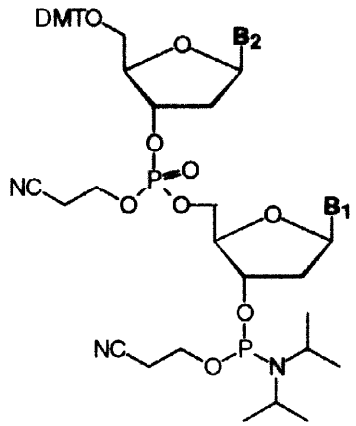
The synthesis of decanucleotide d(GACGGCCAGT) **21** was performed using 1,3,5-tris-[9-(2'-deoxythymidine-3'-O-yl)-2,5-diaza-1,6,9-trioxononyl]-benzene **3** as starting material (Scheme 1). The LPC consists of the symmetrical substituted 1,3,5-benzenetricarboxylic acid core with three alkylamide linkers to the 3'-oxygen of the deoxythymidine residue. As an abbreviation for compound **3** $(dT)_3$-aryl-LPC is proposed and used in the following. The analogous short forms are used for the corresponding compounds **4-19** and **22-24**, representing all intermediates in the synthesis of the decamer d(GACGGCCAGT) **21** and the pentamer d(GCCCT) **25**. $(dT)_3$-aryl-LPC **3** is easily obtained using reagents as 1,3,5-benzenetricarboxylic acid trimethylester, ethylenediamine and pyridinium 5'-O-dimethoxytrityl-deoxythymidine-3'-O-succinate[9]. The dedimethoxytritylated LPC **3** is then treated in similar cyclic procedures to that used in modern solid phase strategies on controlled pore glass (CPG), with the one inherent difference of homogenous reaction conditions in solution. Three oligonucleotide chains can grow simultaneously on $(dT)_3$-aryl-LPC **3** when treated with the activated coupling monomers, preferably deoxynucleoside H-phosphonates or the herein used β-cyanoethyl phosphoamidites (**1 a-d**).

$(dT)_3$-aryl-LPC **3** is the first 5'-hydroxyl component in the synthesis of decanucleotide d(GACGGCCAGT) **21**. Because of the weak solubility of $(dT)_3$-aryl-LPC **3** in acetonitrile, abso-



1 a-d

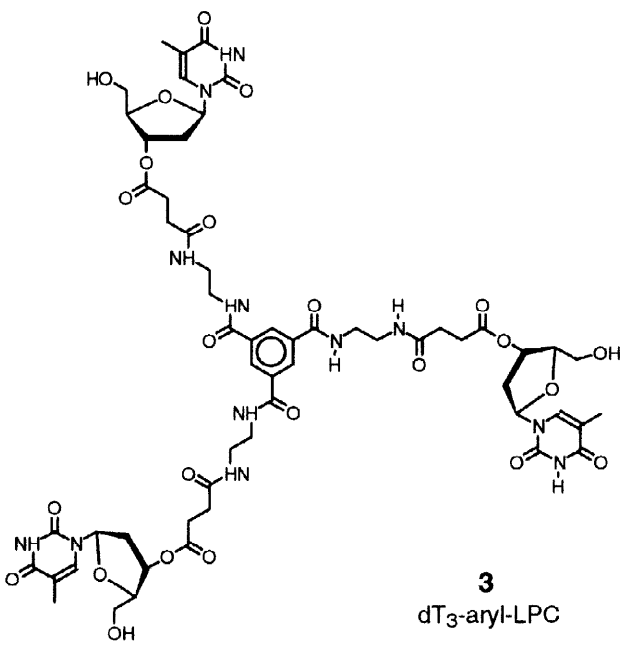
a: B = thymine, T
b: B = N-isobutyryl-guanine, G^{ib}
c: B = N-benzoyl-adenosine, A^{bz}
d: B = N-benzoyl-cytosine, C^{bz}



2 a,b

a: B_1,B_2 = N-toluoyl-cytosine, C^{tl}
b: B_1 = N-toluoyl-cytosine, C^{tl};
B_2 = N-isobutyryl-guanosine, G^{ib}

DMT = dimethoxytrityl-, dimethoxytriphenylmethyl



3
dT_3-aryl-LPC

Scheme 1

luted pyridine was used in the condensation reactions without any noticeable disadvantages. 166 μmol of LPC **3** (corresponds to 500 μmol 5'-hydroxyl groups) were dissolved in dry pyridine and together with a solution of 1H-tetrazole in acetonitrile dropped on 2.5 equivalents of the solide 5'-O-(DMT)-dG^{ib}-phosphoamidite **1b** under exclusion of moisture in argon atmosphere.

Phosphoamidite and tetrazole solution were added until no further reaction was observed, totally 2 g (2.4 mmol, 5 eq.) 5'-O-(DMT)-dG^{ib}-phosphoamidite **1b** and 12 ml (5.2 mmol, 10 eq.) tetrazole solution. The condensation was monitored by thin layer chromatography (TLC). The resulting mixture of product **4** and excess reagents including several equivalents of phosphoamidite was carefully reduced to a volume up to 10 ml and separated on a Sephadex LH20 column with THF/methanol 80:20 (v/v) as eluent. The characteristic elution profile (Figure 1) was obtained by measuring the UV absorption at a wavelength of 260 nm after 200-fold dilution with eluent. As expected, the condensation product (**A**) eluted near the void volume, followed by the excess phosphoamidite components (**B**) and finally the used solvent, pyridine (**C**).

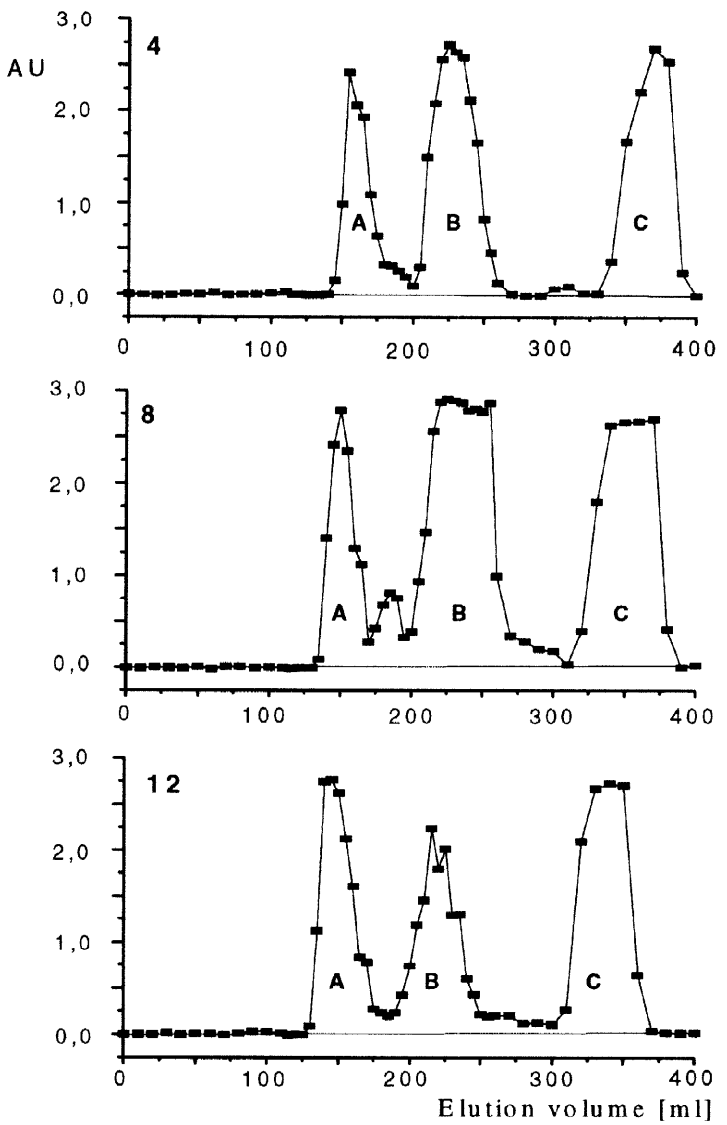


Figure 1. Elution profiles of condensation reaction mixtures, obtained by GPC on Sephadex LH20 (column: 530 mm x 30 mm, flow: 1ml/min, eluent: THF/MeOH 80:20, v/v), from solution synthesis of d(5'-O-DMT-G^{ib}T)$_3$-aryl-LPC) **4**, d(5'-O-DMT-C^{bz}A^{bz}G^{ib}T)$_3$-aryl-LPC **8** and d(5'-O-DMT-G^{ib}C^{bz}C^{bz}A^{bz}G^{ib}T)$_3$-aryl-LPC **12**, using phosphoamidite chemistry.

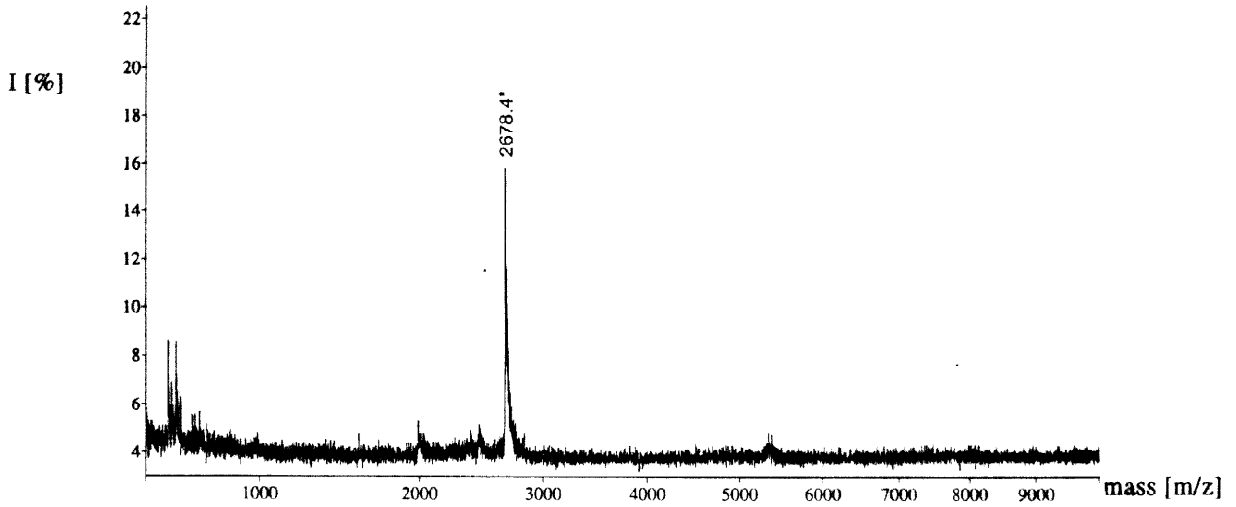
After GPC the fractions containing the condensation product **4** were combined and oxidized with t-butylhydroperoxide (t-BuOOH) at 0°C. In former studies the generally used oxidizing reagent in DNA-synthesis, iodine/water in THF/pyridine, showed to be incompatible with Sephadex LH20. During chromatography a complex between the dextrane matrix of the gel and iodine is formed, visible as a brownish zone with low mobility. Therefore, the recycling of gel filtration media for further separations is only reached by intensive washing with several column volumes. The alternative t-butylhydroperoxide (boiling point 81°C) can easily be removed by co-evaporation with the used solvents (THF and methanol).

The solution was reduced to dryness and the residue dissolved in dichloromethane/nitromethane/methanol 80:19:1 (v/v). For dedimethoxytritylation the resulting colourless solution was mixed with trifluoroacetic acid (TFA) in the same solvent system, so that a final acid concentration not higher than 2 % (by volume) was obtained. The removal of the 5'-O-protecting group was completed in less than 2 minutes and indicated by a deep red to orange colour. To avoid depurination the reaction was stopped by neutralization with triethylamine in an ice bath. For separation of the dedimethoxytritylated LPC compounds Sephadex LH20 chromatography with a RP silica gel filled precolumn (NUCLEOPREP® 300-30 C_{18}) was used. The precolumn was necessary to prevent contamination with dimethoxytrityl carbinol. In the first cycle the intermediate $d(G^{ib}T)_3$-aryl-LPC **5** was isolated as a colourless powder after drying by co-evaporation with dioxane. The identification by MALDI-TOF mass spectrometry[10,11] showed no significant side products at this stage of synthesis (Figure 2A).

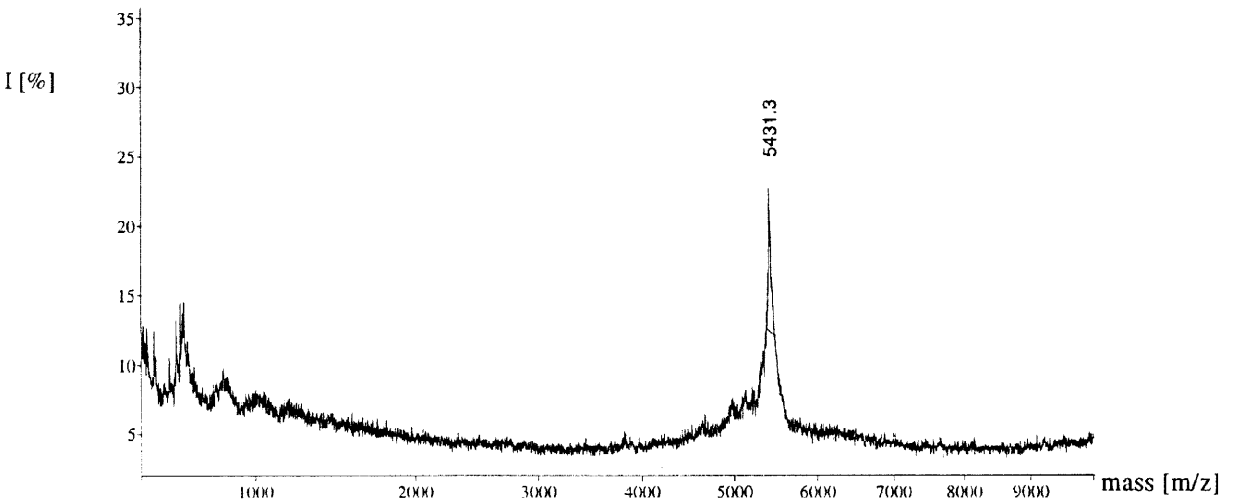
The further synthesis followed the procedure described for the first cycle without essential changes of the method. Table 1 shows the yields, achieved in nine cycles for condensation and oxidation (81-99 %), dedimethoxytritylation (81-99 %) and the whole cycle (81-99%). The total yield over all cycles amount to 33 % for d(5'-O-DMT-$G^{ib}A^{bz}C^{bz}G^{ib}G^{ib}C^{bz}C^{bz}A^{bz}G^{ib}T)_3$-aryl-LPC **20** , including 26 reaction steps and 17 chromatographic separations. The quantities of reagents used in the synthesis are listed in Table 2.

The used excess of β-cyanoethyl phosphoamidites **1a-d** varied between 5 and 12 equivalents, former experiences in the synthesis of di-, tri- and tetranucleotides showed, that 2 to 2.5 equivalents are sufficient for complete elongation. In these cases the 5'-hydroxyl components were dried in very intensive and therefore also very time consuming procedures. In Figure 1 the elution profiles of three condensation reactions are illustrated, demonstrating that separation is in general quantitative. The average yield of 96 % for the coupling reaction is somewhat, but not significant lower than that, obtained in solid phase synthesis and better than comparable synthesis in solution. The average yield of 87 % for dedimethoxytritylation is high, but also not too satisfactory. Improvements of the method can be reached by optimizing this step, for example through avoiding the use of water in chromatography. Other polar solvents in GPC on Sephadex LH20, such as pyridine[12] , N,N-dimethylformamide or dimethylsulfoxide are favoured for this purpose.

A: d(5'-HO-G^{ib}T)$_3$-aryl-LPC **5**: M+H$^+$: theoretical mass: **2666.4 Da, found: 2678.4 m/z**



B: d(5'-HO -C^{bz}A^{bz}G^{ib}T)$_3$-aryl-LPC **9**: M+H$^+$: theoretical mass: 5416.5 Da, found: 5431.3 m/z



C: d(5'-HO-G^{ib}C^{bz}C^{bz}A^{bz}G^{ib}T)$_3$-aryl-LPC **13**: M+H$^+$: theoretical mass 8112.8: Da, found: 8114.0 m/z

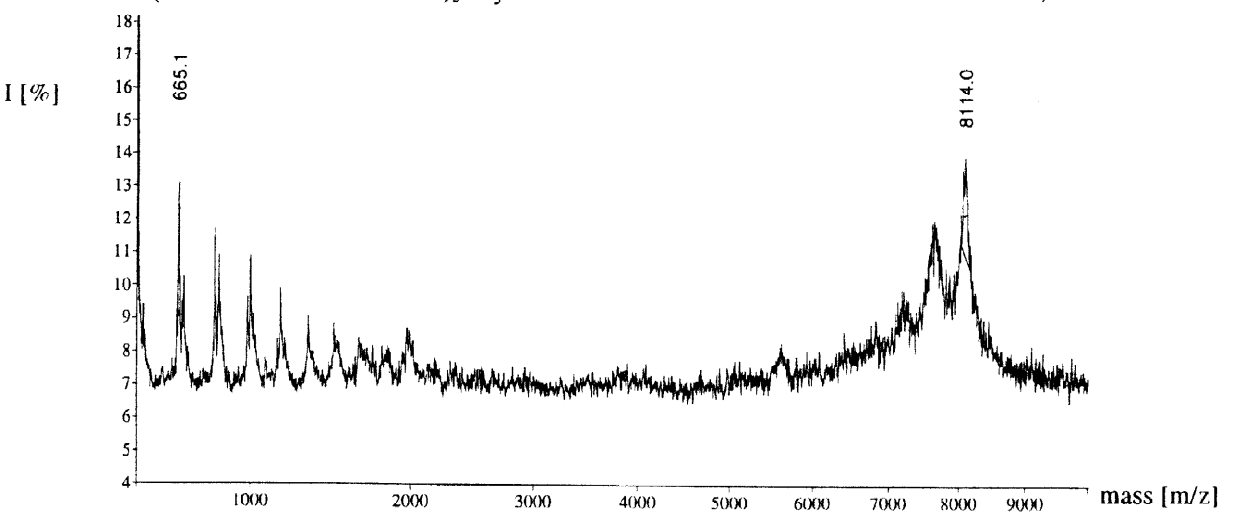


Figure 2. Analysis of intermediates from oligonucleotide synthesis via MALDI-TOF mass spectrometry.

Table 1. Yields, achieved during the synthesis of d(5'-O-DMT-$G^{ib}A^{bz}C^{bz}G^{ib}G^{ib}C^{bz}C^{bz}A^{bz}G^{ib}T)_3$-aryl-LPC **20** in solution, using 166 µmol dT_3-aryl-LPC **3** as starting material and phosphoamidite chemistry.

Cycle: [No.] ; phosphoamidite	condensation: [%] , [µmol], compound	dedimethoxytritylation: [%] , [µmol], compound	yield per cycle: [%]
1 ; **1b**	87 , 144 , **4**	94 , 135 , **5**	81
2 ; **1c**	93 , 126 , **6**	87 , 110 , **7**	81
3 ; **1d**	91 , 100 , **8**	99 , 100 , **9**	91
4 ; **1d**	98 , 98 , **10**	86 , 84 , **11**	84
5 ; **1b**	99 , 83 , **12**	96 , 80 , **13**	95
6 ; **1b**	99 , 80 , **14**	81 , 65 , **15**	81
7 ; **1d**	99 , 65 , **16**	83 , 54 , **17**	83
8 ; **1c**	99 , 54 , **18**	99 , 54 , **19**	99
9 ; **1b**	99 , 54 , **20**	-	-

An inherent advantage of large scale oligonucleotide synthesis in solution in comparison to solid phase strategies is shown by the use of MALDI-TOF mass spectrometry for analysis of the fully base and phosphate protected intermediates. In Figure 2 mass spectra of the 5'-hydroxyl components for the second, fourth and sixth cycle are illustrated [d(5'-HO-$G^{ib}T)_3$-aryl-LPC **5**, d(5'-HO -$C^{bz}A^{bz}G^{ib}T)_3$-aryl-LPC **9** and d(5'-HO-$G^{ib}C^{bz}C^{bz}A^{bz}G^{ib}T)_3$-aryl-LPC **13**, corresponding to condensation products **4**, **8** and **12** in Figure 1]. In contrast to solid phase synthesis, a direct control of the progressing elongation reaction is possible by analysis with this technique. For analysis of the condensation products on CPG the cleavage of the connecting linker is necessary. The hydrolysis is also leading to loss of phosphate and base protecting groups. Evidence about side reactions or incompleteness is therefore complicated. Control over the basic elongation step in oligonucleotide synthesis is however of great importance for economic large scale production.

The analysis of product d(GACGGCCAGT) **21**, synthesized on dT_3-aryl-LPC **3**, was performed after treatment with ammonium hydroxide, RP-HPLC and dedimethoxytritylation with acetic acid by SMART-HPLC (Mono Q 16/5) and MALDI-TOF mass spectrometry. Main and side products in comparison to the same sequence, obtained from a DNA synthesizer, are shown in Figure 3 and 4. The formation of a considerable amount of (n-1)-fragments during synthesis in solution occured, calculated to nearly 15 % by peak area (Figure 3B). MALDI-TOF mass spectrometry of the fractions, collected at the different peak maxima, gave the spectra 4**A-C** in Figure 4 (**A:** CPG synthesis, 13.64 min, $M+H^+$ = 3044.0 m/z; **B:** main product of solution synthesis of d(GACGGCCAGT) **21,** 13.45 min, $M+H^+$=3047.7 m/z, theoretical mass: 3.044.0 Da; **C:** (n-1)-fragments, 11.05 min , $M+H^+$ = 3047.5 and 2718.4 m/z, Δm = 329.1 m/z, corresponding to one missing dGp-unit, theoretical mass: 328.8 Da). The (n-1)-fragment was formed during synthesis in solution, because no capping reaction was carried out as part of each cycle. This is in contrary to standard protocols for solid support synthesis. It is evident, that the introduction of

capping is also absolutely necessary for improvement of the herein presented concept for oligonucleotide synthesis in solution.

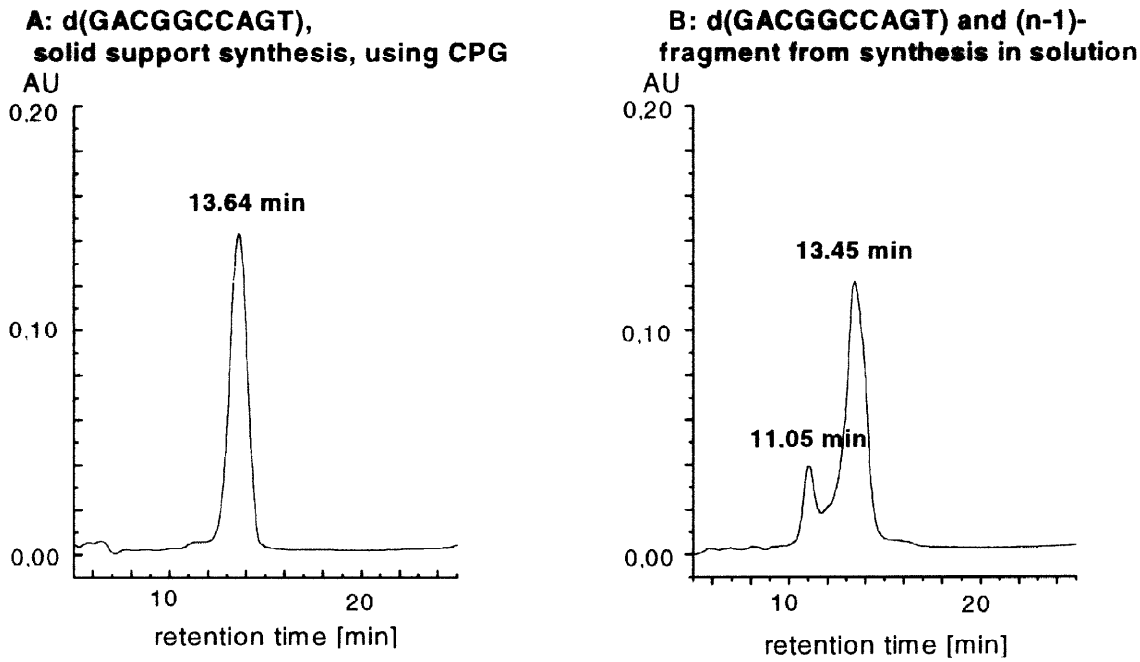


Figure 3. HPLC analysis of d(GACGGCCAGT) **21**, obtained by conventional solid support synthesis on CPG (**A**, with capping reaction) and by solution synthesis, using dT_3-Aryl-LPC **3** and phosphoamidite chemistry (**B**, without capping reaction); SMART-HPLC with Mono Q 1.6/5 (Pharmacia).

The competition of this method would also be increased, if blockwise synthesis could be performed using, for example, di- or trinucleotide phosphoamidites as synthons. This technique was used in the first chemical syntheses of gene fragments[13-15] and was an essential part in preparation of oligonucleotides with classical phosphodiester or -triester method in solution[16,17]. Only few examples are given in the literature for such a strategy using the phosphoamidite approach, mostly applied to solid phase synthesis[18-20].

The preparation of an oligonucleotide of the sequence d(GCCCT) **25** using the developed method for solution synthesis together with dimer phosphoamidites **2a,b**, demonstrated that these synthons are in general very useful (Figure 5 and 6). The quality of condensation product **22** proved to be unsufficient. Incorporated impurities, probably phosphomonochloridite derivatives, led to partial phosphorylation as one side reaction, making intensively purification of the dimer phosphoamidites (by silica column chromatography) inevitable. The phosphorylation is indicated by MALDI-TOF mass spectrometry (Figure 5B ; fragment ion at 3278.0 m/z, corresponding to d(5'-HO-$C^{tl}C^{tl}T)_2$(5'-pdT)-aryl-LPC **23b**, theoretical mass: 3272.9 Da). The steps in one elongation cycle are performed with no significant changes to that, described for monomeric phosphoamidites. Instead of 1H-tetrazole the more activating 5-(4-nitrophenyl)-tetrazole[21,22] is used.

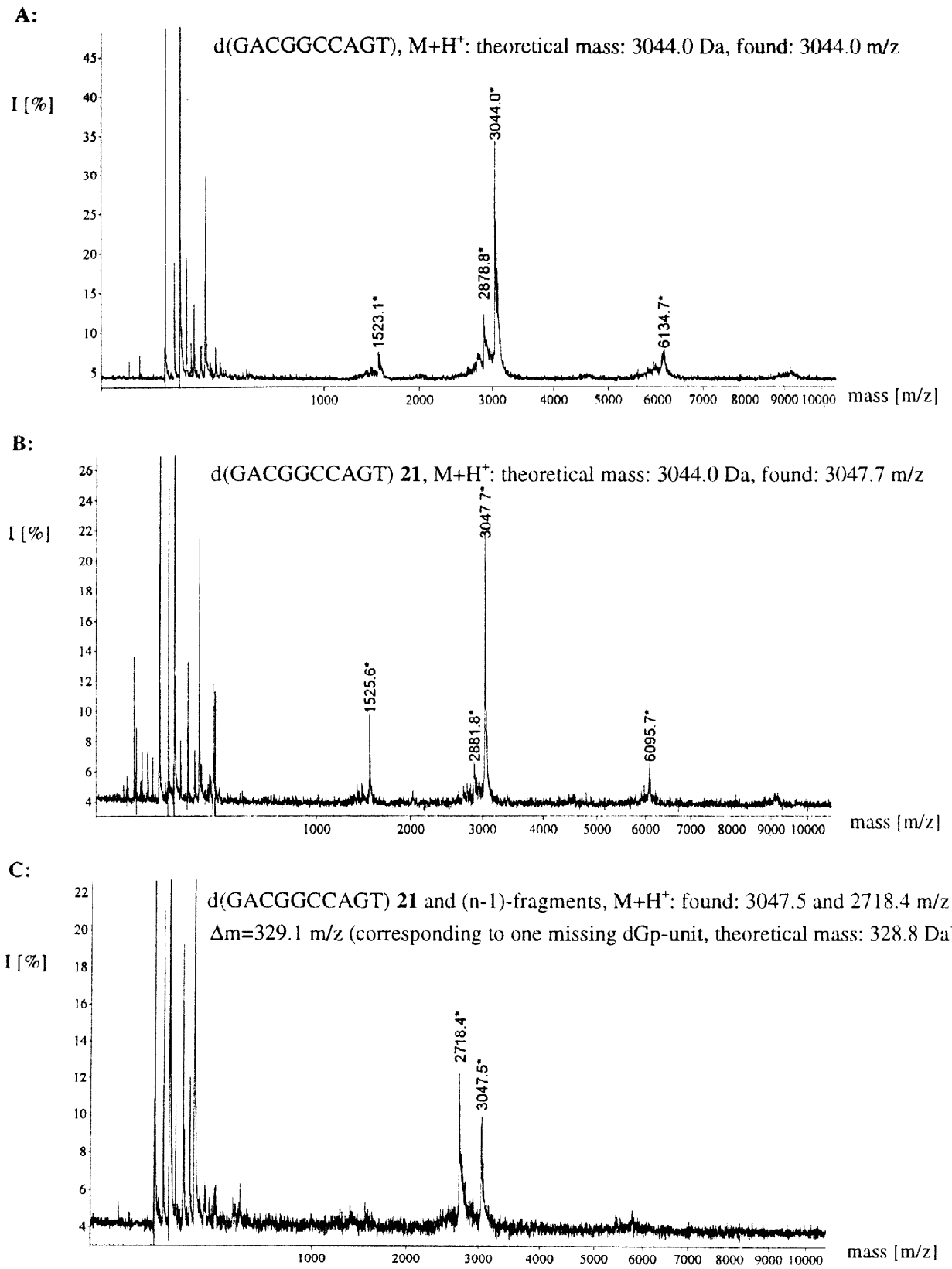
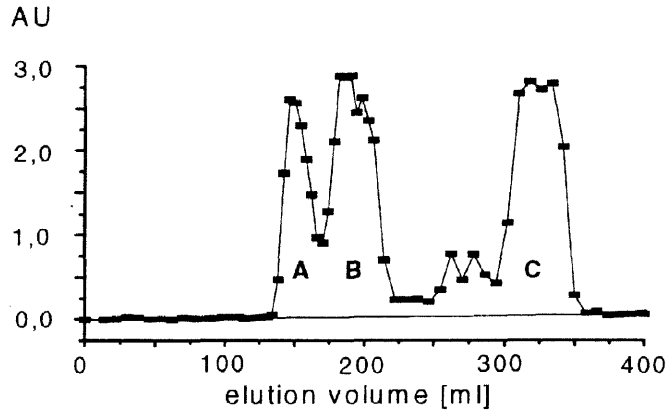


Figure 4. MALDI-TOF mass spectra of main (**B**) and side products (**C**), formed in the solution synthesis of d(GACGGCCAGT) **21** and comparison to the same sequence, synthesized on CPG as solid support (**A**).

A: Elution profile of condensation reaction mixture, obtained by GPC on **Sephadex LH20** (column: 530 mm x 30 mm, flow: 1ml/min, eluent: THF/MeOH 80:20, v/v), corresponding to solution synthesis of d(5'-O-DMT-$C^{tl}C^{tl}T)_3$-aryl-LPC **22**, using the dimer d(5'-O-DMT-$C^{tl}C^{tl}$) phosphoamidite **2a**:



B: MALDI-TOF mass spectra of d(5'-HO-$C^{tl}C^{tl}T)_3$-aryl-LPC **23a**: $M+H^+$: theoretical mass: 4077.6 Da, found: 4087.1 m/z and side product: d(5'-HO-$C^{tl}C^{tl}T)_2$(5'-pdT)-aryl-LPC **23b**: theoretical mass: 3272.9 Da, found: 3278.0 m/z, the probe corresponds to peak A in the above illustrated elution profile after dedimethoxytritylation.

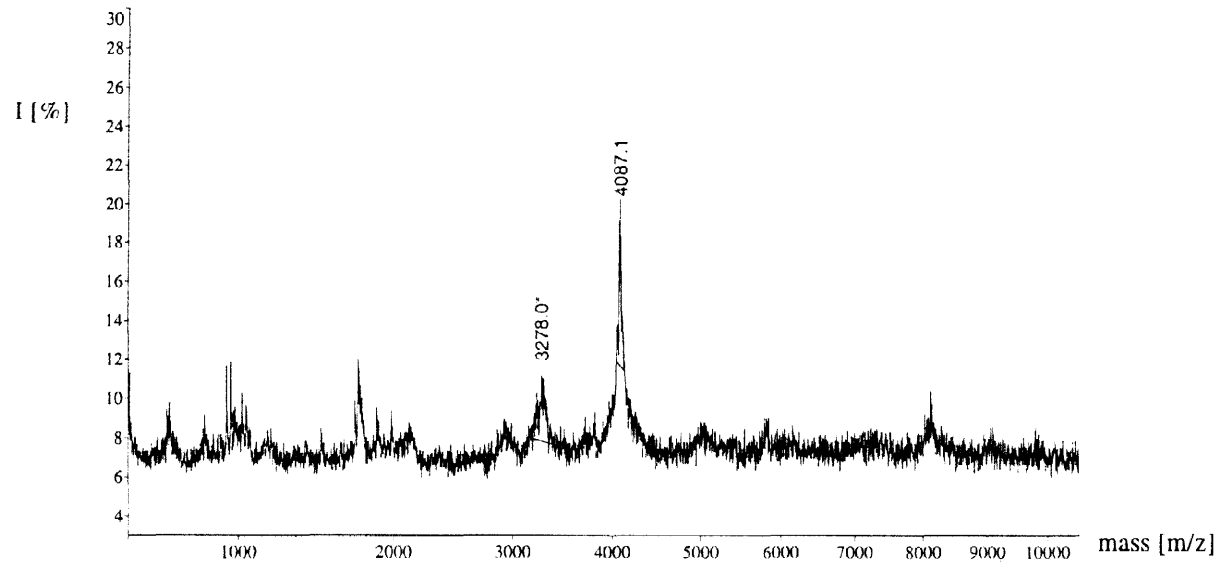


Figure 5. Purification and analysis of the first cycle using the dimer d(5'-O-DMT-$C^{tl}C^{tl}$) phosphoamidite **2a**.

The coupling yield was 83 % but in contrast to the use of monomeric phosphoamidtes 1a-d (elution profiles in Figure 1), no complete separation of condensation product (peak A) and excess dimer phosphoamidite (peak B) can be observed in the elution profile (Figure 5A). This makes the simple adaptation of Sephadex LH20 chromatography to longer columns necessary.

Without complete separation in this step a further side reaction occurs in the following cycle. This is shown in figure 6, the analysis of d(GCCCT) **25** with MALDI-TOF mass spectrometry after cleavage from LPC, deprotection, RP-HPLC and dedimethoxytritylation. Some fractions from RP-HPLC showed pure pentamer **25** (Figure 6A), but most product containing fractions included a significant amount of a side product, which could not be separated by ion

exchange SMART-HPLC (Mono Q, 1.6/5, Pharmacia). This impurity should therefore content the same number of phosphate groups as d(GCCCT) **25** has. With MALDI-TOF mass spectrometry the side product was identified as d(GCCC)-3'-phosphate **26** (Figure 6B). It was originated from reaction between excess dimer GC-amidite **2b** and the dimer impurities out of the first cycle, which could not be separated completely by GPC on Sepheadex LH20 (Figure 5A).

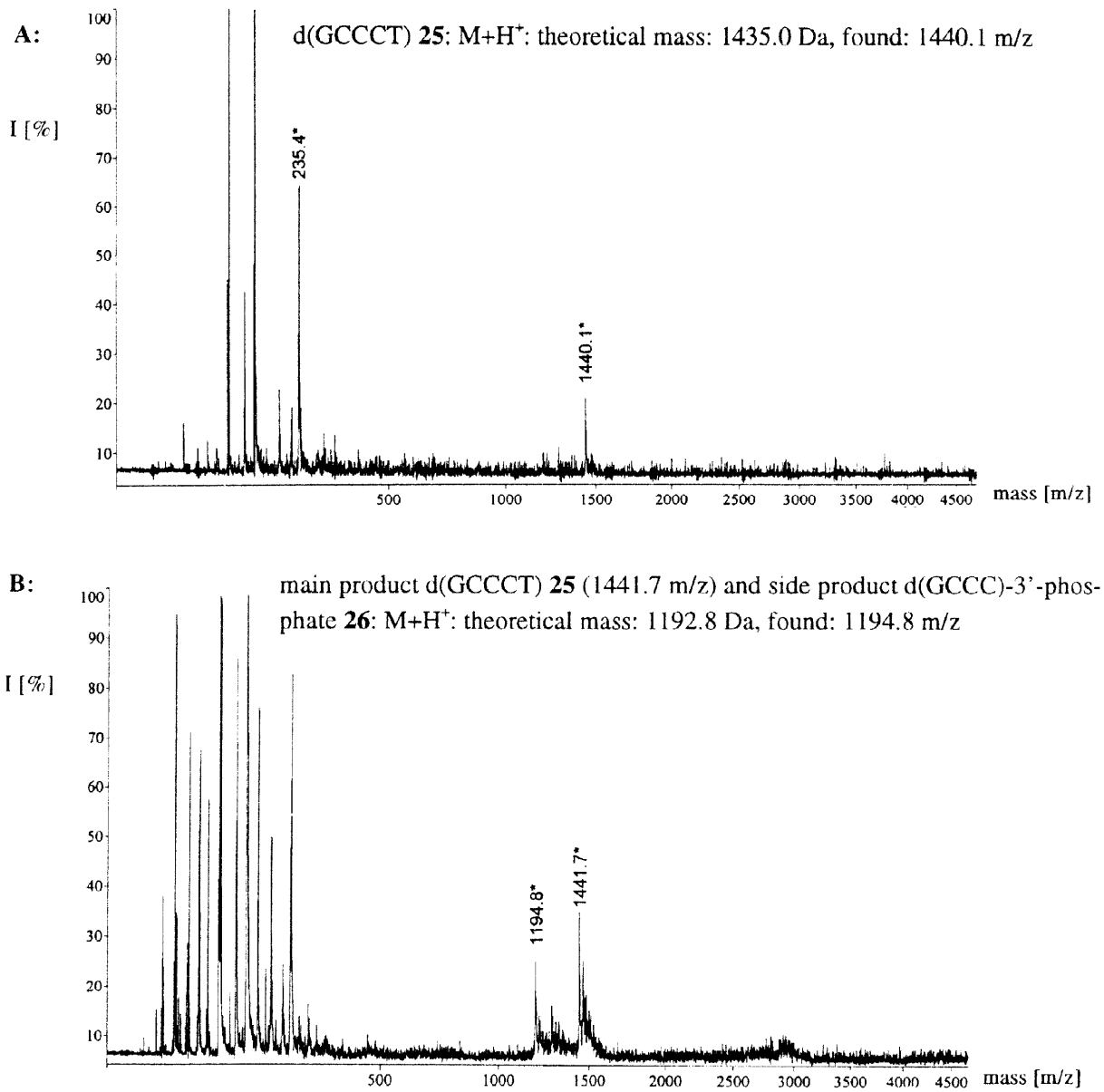


Figure 6. Synthesis of d(GCCCT) with dimer phosphoamidites **2a,b** and product analysis via MALDI-TOF MS.

The discussed inprovements together with the already achieved results, give evidence about the usefulness of the underlying concept of synthesis, straight orientated at purification. The described synthesis in solution represents a promising alternative to solid phase strategies for large scale production of oligonucleotides and analogues. The use of dimer or trimer conden-

sing blocks together with the use of the phosphoamidite chemistry and the developed purification procedures can be regarded as a real progress in oligonucleotide synthesis in solution. It is noticed that the use of liquid phase carrier based syntheses could also be adapted to peptide or carbohydrate chemistry or in general for simplifying complex syntheses of biopolymers and other organic compounds.

EXPERIMENTAL

^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Bruker AMX 400 instrument. Samples were dissolved in the presence of tetramethylsilane as internal standard. Chemical shifts are given in ppm. Mass spectra were obtained on a Finnigan MAT Vision 2000 under MALDI-TOF conditions (matrix: 3-HPA, solvent: THF/water 70:30 v/v). Thin layer chromatography (TLC) was carried out on 60 PF_{254} silica gel coated alumina sheets (Merck, Darmstadt, Germany, No. 1.05562). Dimethoxytrityl and sugar containing compounds were visualized with a sugar spray reagent (0.5 ml 4-methoxybenzaldehyde, 9 ml ethanol, 0.5 ml concentrated sulfuric acid and 0.1 ml glacial acetic acid) and heating with a fan. Column chromatography was performed using silica gel from Merck (No. 1.09385). Gel permeation chromatography using Sephadex LH20 (Pharmacia, Upsala, Sweden, No. 17-0090) was performed following the instructions of the manufacturer. The herein used columns from Kronwald (Sinsheim, Germany, HPP-columns of the FPGC series) with an inner diameter of 30 mm and a length of 530 mm for separations of compounds with dimethoxytrityl group or respectively of 460 mm for separations of dedimethoxytritylated compounds were stable to all solvents, especially to THF (it is noted that THF can damage many materials which are used as seals or column couplers).

1,3,5-Tris-[9-(2'-deoxythymidine-3'-O-yl)-2,5-diaza-1,6,9-trioxononyl]-benzene; dT_3-aryl-LPC [3]:

$(DMT\text{-}dT)_3$-aryl-LPC (850 mg, 384 μmol) was dissolved in 1,2-dichloroethane/nitromethane/methanol 80:19:1 (v/v, 33 ml) and mixed with a TFA solution in the same solvent mixture (11.5 mmol TFA in 10 ml). After 2 minutes a triethylamine solution was added for neutralization under cooling in an icebath (11.5 mmol, 5 ml in the same solvent system). The reaction mixture was concentrated under reduced pressure and dissolved with the eluent THF/water 60:40 (v/v, 5 ml). Chromatography was performed with a precolumn filled with NUCLEOPREP 300-30 C18 (310x20 mm) and a second column with Sephadex LH20 (460x30 mm, flow: 1 ml/min). Product containing fractions were combined, concentrated and coevaporated with dioxane (2x40 ml). Final lyophilization with dioxane (10 ml) gave dT_3-aryl-LPC **3** as a colorless

solid, yield: 97% (490 mg, 374 μmol).-*^{1}H NMR* (400 MHz, d_6-DMSO): δ = 1.78 (s, 9H, C<u>H</u>$_3$-, Base), 2.25 (m, 6H, H2'), 2.40 (m, 6H, -COC<u>H</u>$_2$-), 2.55 (m, 6H, -C<u>H</u>$_2$CO-), 3.24/3.34 (m, 12H, 2x-NHC<u>H</u>$_2$-), 3.60 (m, 6H, H5'), 3.97 (m, 3H, H4'), 5.20 (m, 3H, H3'), 6.17 (dd, 3H, H1'), 7.70 (s, 3H, C<u>H</u>, Base) , 8.00 (dd, 3H, -N<u>H</u>CH$_2$-), 8.40 (s, 3H, C<u>H</u>$_{Ar.}$), 8.68 (dd, 3H, -N<u>H</u>CH$_2$).-*^{13}C NMR* (100 MHz, d_6-DMSO): δ =12.19 (CH_3, Base), 29.02 (C8), 29.76 (C7), 36.38 (C2'), 38.18 (C4), 39.13 (C3), 61.26 (C5'), 74.68 (C3'), 83.60/84.47 (C1'/C4'), 109.64 (C5, Base), 128.48 ($CH_{Ar.}$), 134.78 ($C_{Ar.}$), 135.74 (C6, Base), 150.39 (C2, Base), 163.57 (C4, Base), 165.51 (C1), 170.79 (C6), 171.95 (C9).

Synthesis of d(5'-O-DMT-G^{ib}pA^{bz}pC^{bz}pG^{ib}pG^{ib}pC^{bz}pC^{bz}pA^{bz}pG^{ib}pT)$_3$-aryl-LPC [20]:

The 5'-hydroxyl component dT$_3$-aryl-LPC **3** (228 mg 166 μmol) was coevaporated with dried pyridine (3x10 ml) and dissolved in the same solvent (10 ml). (DMT)-dG^{ib}-phosphoamidite **1b** (1.0 g 1.2 mmol) was submitted in a 100 ml-two-necked reaction flask (argon atmosphere). The solution of the 5'-hydroxyl component **3** and tetrazole (6 ml, 30.8 mg/ml in acetonitrile, 2.6 mmol) were dropped in 500 μl portions via syringe through septum to phosphoamidite **1b**. In case of uncomplete reaction phosphoamidite as solid and tetrazole solution were added (controlled by TLC). After complete reaction the mixture was concentrated under reduced pressure. The condensation product was isolated by GPC using Sephadex LH20 (eluent: THF/methanol 80:20 v/v, column: 530x30 mm, flow: 1 ml/min) without oxidation. Product containing fractions were combined, reduced to a volume of 10 ml and oxidized at 0°C with t-butylhydroperoxide (80 % solution in di-t-butylperoxide, 200 μl, 1.5 mmol) for 5 min. After concentrating and intensive drying in vacuo (5'-O-DMT-G^{ib}pT)$_3$-aryl-LPC **4** was obtained as a colourless solid, yield 87 % (520 mg, 144 μmol).

The compound **4** was dedimethoxytritylated with TFA reagent, using dichloromethane instead of 1,2-dichloroethane, following the procedure described for dT$_3$-aryl.LPC (see above). The amounts of the herein used reagents are given in Table 2, the chromatographic conditions were the same as described above. The intermediate d(G^{ib}pT)$_3$-aryl-LPC **5** was analysed via MALDI-TOF mass spectrometry (Figure 2A, M+H$^+$: theoretical mass 2666.39 Da, found: 2678.4 m/z).

The further elongation steps (including condensation, oxidation and dedimethoxytritylation) for the synthesis of d(5'-O-DMT-G^{ib}pA^{bz}pC^{bz}pG^{ib}pG^{ib}pC^{bz}pC^{bz}pA^{bz}pG^{ib}pT)$_3$-aryl-LPC **20** were performed, according to the above described cycle and using the amounts of reagents listed in Table 2. The crude compound d(5'-O-DMT-G^{ib}pA^{bz}pC^{bz}pG^{ib}pG^{ib}pC^{bz}pC^{bz}pA^{bz}pG^{ib}pT)$_3$-aryl-LPC **20** was over all nine cycles obtained in a yield of 33% (54 μmol , 750mg).

In Table 3 the MALDI-TOF mass spectrometry data, achieved from monitoring the complete synthesis are listed. The dedimethoxytritylated intermediates were dissolved in THF/water

70:30 (v/v) and mixed with matrix solution in the same solvent (3-hydroxy picolinic acid, 0.7mol/l). The 5'-hydroxyl components could be satisfactory identified up to cycle 6. Significant deviations from the calculated mass and mass differences are observed in the last three cycles, probably through the increasing occurance of side products in synthesis and fragmentation during desorption and ionization, leading to signals, which are strongly broadened.

Table 2: The amounts of reagents used in the different reaction steps and cycles in solution synthesis of d(5'-O-DMT-$G^{ib}pA^{bz}pC^{bz}pG^{ib}pG^{ib}pC^{bz}pC^{bz}pA^{bz}pG^{ib}pT)_3$-aryl-LPC **20**.

cycle [No.]	5'-hydroxyl-component [μmol][a)]	phosphoamidite [mmol] ; [eq.]	tetrazole[b)] [mmol] ; [eq.]	t-BuOOH[c)] [mmol] ; [eq.]	TFA and Et_3N[d)] [mmol] ; [eq.]	yield of product [%]
1	**3**, 166	**1b**, 2.4 , 5	5.2 , 10	1.5 ; 3	15 ; 30	**5**, 81
2	**5**, 135	**1c**, 3.5 , 8.75	7.8 ; 19	1.5 ; 3.7	15 ; 30	**7**, 81
3	**7**, 110	**1d**,1.8 , 5.5	5.2 ; 16	1.5 ; 4.5	7.5 ; 23	**9**, 91
4	**9**, 100	**1d**, 2.4 , 8	5.2 ; 17	1.5 ; 5	7.5 ; 25	**11**, 84
5	**11**, 84	**1b**, 1.8 , 7	3.9 ; 15	1.5 ; 6	7.5 ; 30	**13**, 95
6	**13**, 80	**1b**, 1.8 , 7.5	3.9 ; 16	1.5 ; 6	7.5 ; 31	**15**, 81
7	**15**, 65	**1d**, 2.4 , 12	5.2 ; 26	1.5 ; 7	7.5 ; 38	**17**, 83
8	**17**, 54	**1c**, 1.6 , 10	3.9 ; 24	1.5 ; 10	7.5 ; 46	**19**, 99
9	**19**, 54	**1b**, 2.4 , 15	5.2 ; 30	1.5 ; 10		

a) dissolved in 10 ml pyridine;

b) as solution in acetonitrile (30.8 mg/ml);

c) 80 % solution in di-t-butylperoxide,

d) trifluoroacetic acid and triethylamine were dissolved in dichloromethane/nitromethane/methanol 80:19:1 (v/v)

Table 3: Data, achieved from 5'-hydroxyl components via MALDI-TOF mass spectrometry.

intermediates analysed after dedimethoxytritylation	theoretical mass [Da]	mass found: $M+H^+$ [m/z]	mass difference introduced/found [m/z]
$d(T)_3$-aryl-LPC **3**	1309.26	1325,9	-
$d(G^{ib}pT)_3$-aryl-LPC **5**	2666.39	2678.4	1357/1352
$d(A^{bz}pG^{ib}pT)_3$-aryl-LPC **7**	4077.49	4093.7	1411/1415
$d(C^{bz}pA^{bz}pG^{ib}pT)_3$-aryl-LPC **9**	5416.54	5431.3	1339/1332
$d(C^{bz}pC^{bz}pA^{bz}pG^{ib}pT)_3$-aryl-LPC **11**	6755.59	6764.6	1339/1338
$d(G^{ib}pC^{bz}pC^{bz}pA^{bz}pG^{ib}pT)_3$-aryl-LPC **13**	8112.83	8114.0	1357/1366
$d(G^{ib}pG^{ib}pC^{bz}pC^{bz}pA^{bz}pG^{ib}pT)_3$-aryl-LPC **15**	9469.11	9346.8	1357/1234
$dC^{bz}pG^{ib}pG^{ib}pC^{bz}pC^{bz}pA^{bz}pG^{ib}pT)_3$-aryl-LPC **17**	10808.96	10774.8	1339/1428
$d(A^{bz}pC^{bz}pG^{ib}pG^{ib}pC^{bz}pC^{bz}pA^{bz}pG^{ib}pT)_3$-aryl-LPC **19**	12219.97	12143.2	1411/1368

Deprotection and Cleavage from Liquid Phase Carrier, d(GACCGGCAGT) [21]:

For the cleavage of decamer d(GACCGGCAGT) **21** from the liquid phase carrier and for the simultaneous base and phosphate deprotection of synthesized compound **19** d(5'-O-DMT-$G^{ib}pA^{bz}pC^{bz}pG^{ib}pG^{ib}pC^{bz}pC^{bz}pA^{bz}pG^{ib}pT)_3$-aryl-LPC (13 mg,1 μmol) was treated with ammonium hydroxide (32%, 3 ml) for 20 h at 55°C. The characterization of main and side products starts with reversed phase HPLC using the DMT group for separation. 200 μmol were purificated using a Waters chromatography system Delta Prep 4000 (column: Whatman, Partisil 10 ODS M9, 500x9.4 mm, 10 μm particles, eluents were: 0.1 M triethylammonium acetate at pH 7.0 (A) and acetonitrile (B), flow: 4 ml/min, 5 to 40 % B in 40 min, monitored at 260 nm). Fractions were collected around the peak maximum at 32 min, followed by dedimethoxytritylation with acetic acid (80 %, 500 μl). The product was analysed by ion exchange HPLC using the Pharmacia SMART system with Mono Q 1.6/5 column (inner diameter: 1.6 mm, length: 5 cm, eluents were 25 mM tris-HCl, 1mM EDTA, pH 8.0 (A) and 25 mM Tris-HCl, 1mM EDTA, 1M NaCl, pH 8.0 (B), flow: 100 μl/min: start: 20 % B, 4 min isocratic, 20 to 60 % B in 26 min). Results were compared to d(GACCGGCAGT), achieved from solid phase synthesis on CPG with included capping step (Figure 3 and 4).

Synthesis of d(GCCCT) [25] with dimer phosphoamidites [2a,b]:

The 5'-hydroxyl component dT_3-aryl-LPC **3** (131 mg, 100 μmol, in 5 ml pyridine) was treated with d(5'-O-DMT-$C^{tl}pC^{tl}$) phosphoamidite **2a** (total: 1.79 g, 1.36 mmol, 4.5 equivalents) according to the above described procedures. Instead of 1H-tetrazole 5-(4-nitrophenyl)-tetrazole was used (1.4 mmol, as suspension in acetonitrile). Oxidation with t-butylhydroperoxide (80 % in di-t-butylperoxide, 3.75 mmol, 500 μl, at 0°C) was performed before GPC with Sephadex LH20 (Eluent: THF/methanol 80:20, same conditions as described above, see also Figure 5A). Yield: 99 % crude (5'-O-DMT-$C^{tl}pC^{tl}pT)_3$-aryl-LPC **22** (516 mg, 99 μmol). In dedimethoxytritylation step TFA (760 μl) in dichloromethan/nitromethane/methanol 80:19:1 (32 ml, v/v). and triethylamine (1.4 ml at 0°C) were used. Purification was obtained by the described chromatography using the eluent THF/water 60:40 (v/v) and a precolumn filled with NUCLEOPREP 300-30 C18 (310x20 mm) combined with a second column with Sephadex LH20 (460x30 mm, flow: 1 ml/min). Yield per cycle: 83% (340 mg, 83 μmol d($C^{tl}pC^{tl}pT)_3$-aryl-LPC) **23a**. MALDI-TOF-MS: (MH^+=4087.1 m/z, $M_{calc.}$=4077.56, see also Figure 5B). In the second condensation 4.4 equivalents of d(5'-O-DMT-$G^{DPC/ib}pC^{tl}$-phosphoamidite **2b** (1.1 mmol, 1.65 g) and 5-(4-nitrophenyl)-tetrazole (1.2 mmol) were used leading to compound d(5'-O-DMT-$G^{DPC/ib}pC^{tl}pC^{tl}pC^{tl}pT)_3$-aryl-LPC **24** (570 mg, 69 μmol, 83 %). Cleavage, deprotection and analysis were performed as described above, but varying the eluent conditons in SMART-HPLC

(Start: 0 % B, 4 min isocratic, 0 to 60 % B in 26 min). MALDI-TOF-MS for d(GpCpCpCpT) **25**: M+H$^+$: 1440.1 m/z ($M_{calc.}$=1435.0 Da), d(GpCpCpCp) **26**: M+H$^+$: 1194.8 m/z ($M_{calc.}$= 1192.8 Da) (see also Figure 6 A and B).

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