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Synthesis of New Liquid Phase Carriers for Use in Large Scale Oligonucleotide Synthesis in Solution

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Abstract: The synthesis of multifunctional symmetric primary amines **4** and **8** and the covalent binding of 5'-O-dimethoxytrityl-deoxynucleoside derivatives to their amino groups (compound **5** and **9**) is described. Different strategies for dedimethoxytritylation including the use of strong acidic ion exchangers or protic acids and modified silica gels and/or gel permeation chromatography are developed. The resulting liquid phase carriers **6** and **10** are suitable for large scale oligonucleotide synthesis in solution using phosphoamidites and gel permeation chromatography for fast isolation of intermediates.

INTRODUCTION

With the development of automated solid phase methods using controlled pore glass (CPG)[1,2] and β-cyanoethyl phosphoamidites[1-3] DNA-synthesis has been perfectly adapted to the demands of modern research in molecular biology and biochemistry. Today oligonucleotides are easily available with a wide spectrum of functionalizations on their 3'- or 5'-termini, on the internucleotidic phosphodiester linkages or on the nucleobases[4-6]. Generally, oligonucleotides are needed in only small amounts (nano- to milligramms), for use in polymerase chain reaction[7,8] and as diagnostic tools[9-13], for example. But some therapeutic applications, according to the promising antisense principle[14,15], could lead to an immense demand for modified oligonucleotides (gramms to kilogramms), if this approach proved to be successful in the therapy of even just one important human disease. It is questionable, if such amounts of modified oligonucleotides can be economically synthesized by solid phase strategies in high purity, which is recommended for the therapeutical use. Synthesis in solution offers some advantages in large scale production, such as easy upscaling, direct control over reaction and products of high purity because of intensive purification in each reaction cycle. One major drawback of solution synthesis lies in the lower

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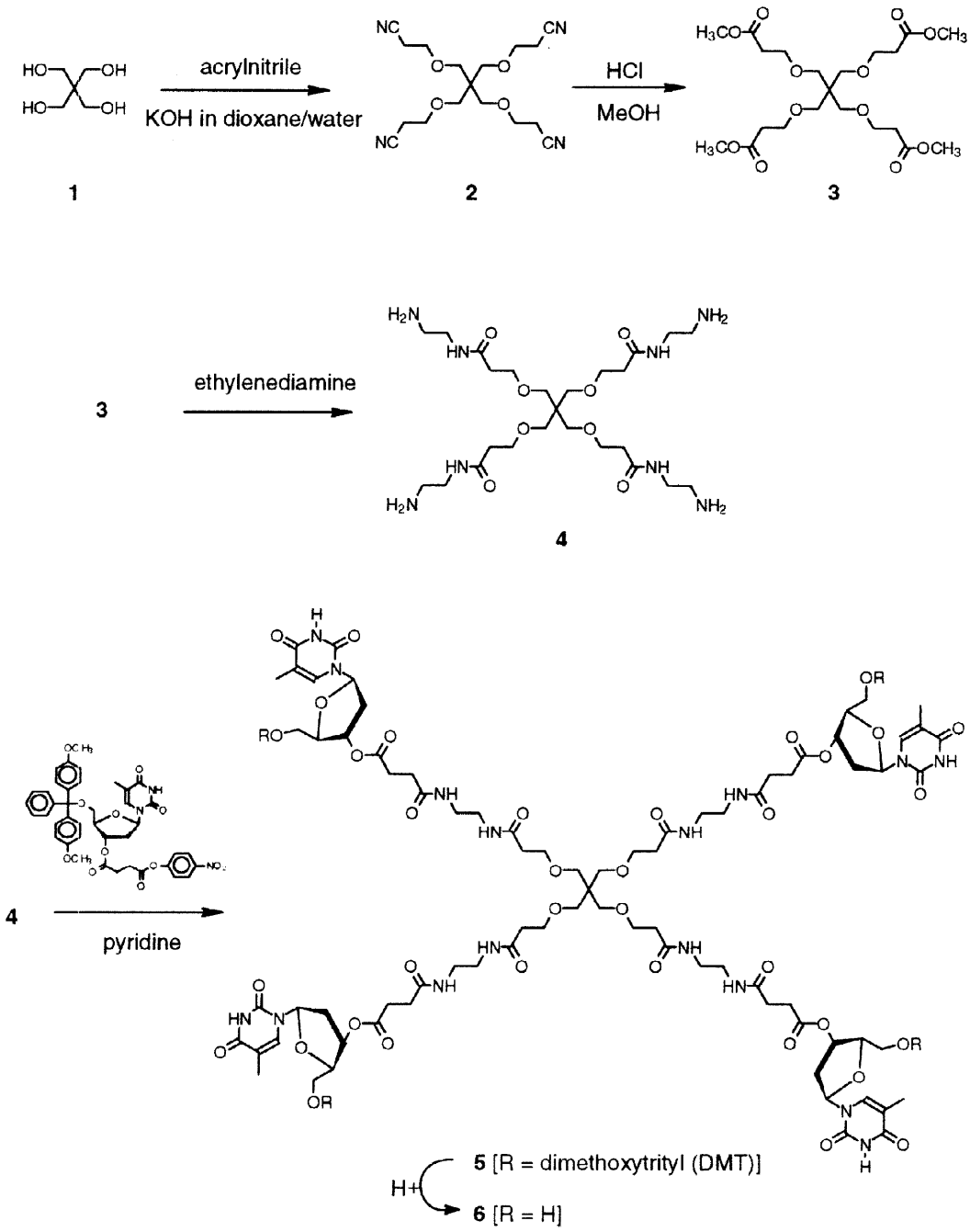
yields, caused by loss of product during purification and incomplete reactions, because reagents can not be used in larger excesses. To overcome these problems a concept for oligonucleotide synthesis in solution should be strictly orientated on the purification procedures.

The use of a liquid phase carrier (LPC) for this purpose has been originally introduced by Köster for the separation of phosphotriester condensation products by Sephadex LH 20 gel filtration[16]. Herein two growing oligonucleotide chains were anchored on trityl groups and bridged by an octanedioic acid ester. This leads to a significant difference in size between the bridged reaction product and all other reagents including also the other nucleotidic components in the condensation reaction mixture. Gel permeation chromatography (GPC) on Sephadex LH20 can therefore be used for the rapid and primary isolation of the product, which elutes near the void volume of the column before all other components. Nevertheless the achieved yields in the condensation reaction were 85 % maximum and decreased to 35 % with increasing chain length. One fundamental reason therefore is the use of the phosphotriester method[17], another minor reason the use of an only bifunctional liquid phase carrier. For better results a method which guarantees higher condensation rates as by the use of H-phosphonate[18-20] or phosphoamidite chemistry[21,4] should be choosen. In spite of the fact that the β-cyanoethyl phosphoamidite approach[1-6] is regarded as most efficient for the synthesis of oligodeoxynucleotides and their analogues, only few efforts have been made to adapt it to solution based techniques[22-24]. Herein reported is the synthesis of liquid phase carriers for just that purpose. These compounds are designed for the covalent binding of four or respectively three units of 5'-O-DMT nucleoside derivatives (DMT = dimethoxytrityl- or dimethoxytriphenylmethyl-). The dedimethoxytritylation step, the first in each cycle in oligonucleotide synthesis, is varied in order to develope optimized conditions. This includes the use of strong acidic ion exchangers or protic acids together with modified silica gels or GPC. The synthesis of short oligonucleotides with the prepared liquid phase carriers will be described separately[25].

RESULTS AND DISCUSSION

For the synthesis of liquid phase carriers, which were successfully proved in preparation of dinucleotides and short oligonucleotides, two different types of multifunctional highly symmetric core molecules were used as starting materials, namely pentaerythrite **1** and tris-1,3,5-benzenetricarboxylic acid trimethylester **7**. The first step was the introduction of short linker units with terminal primary amino functions. The four or respectively three identical amino functions were used for the coupling of the p-nitrophenylesters of the 5'-O-dimethoxytrityl-deoxynucleoside-3'-O-succinates. This procedure is comparable to the modification and loading of CPG and other solid supports for use in automated solid phase DNA-synthesis[26].

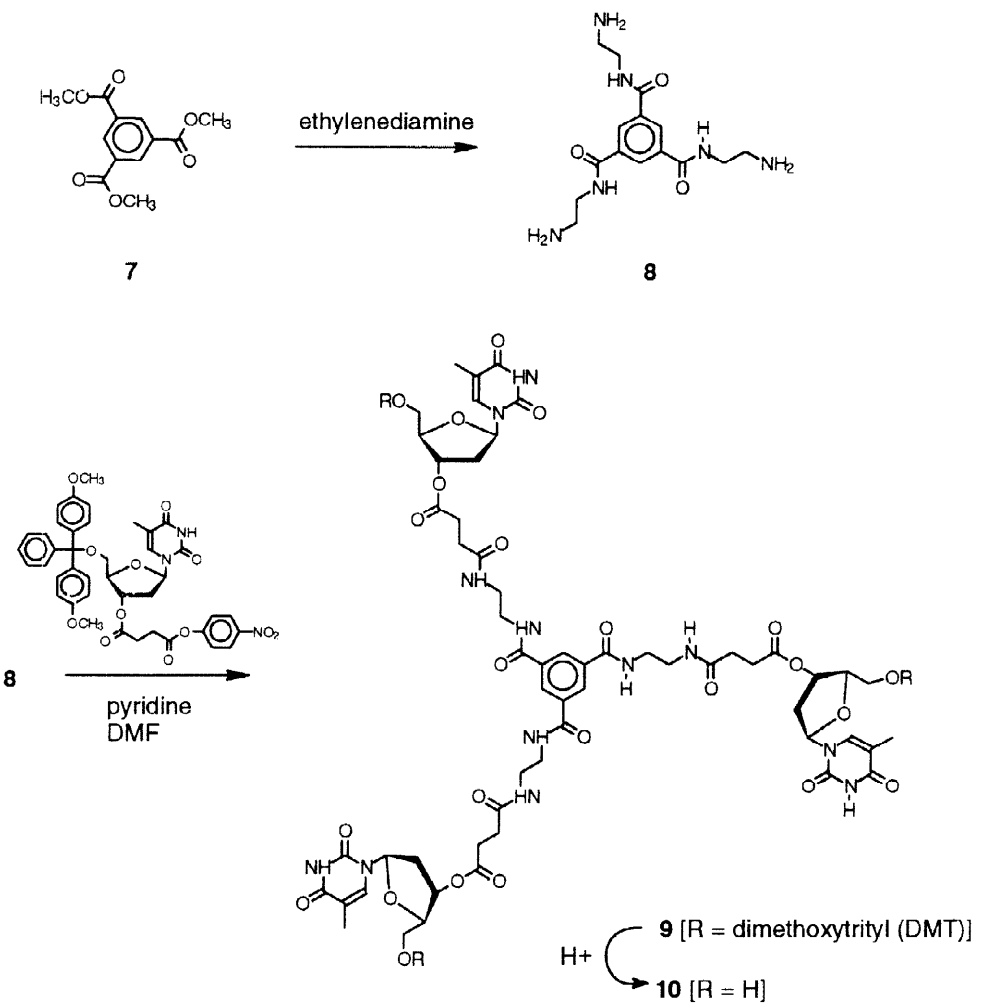
Treatment of pentaerythrite **1** with acrylnitrile in the presence of KOH gave tetrakis-[(cyanoethoxy)methyl]methane **2** (Scheme 1). When refluxed with HCl saturated methanol under exclusion of water nitrile **2** reacts to compound **3**, tetrakis-[((methoxycarbonyl)-ethoxy)methyl]-methane. Both reactions were performed following the work of Newkome[27-29].



Scheme 1

The methylester **3** is an intermediate in the synthesis of dendritic cascade polymers. The aminolysis with ethylenediamine leads to the tetravalente amine **4**, tetrakis-(8-amino-6-aza-2-oxa-5-oxooctyl)-methane, in a reaction also used in the synthesis of dendritic poly(amido-amines)[30]. The acylation of the four primary amino groups with the reactive p-nitrophenylester of 5'-O-dimethoxytrityl-deoxythymidine-3'-O-succinates in pyridine gave the highly symmetrical compound **5**. The isolation of the pentaerythrite type liquid phase carrier **5** (as an abbreviation $(DMT\text{-}dT)_4$-PE-LPC is proposed and used in the following) was somewhat difficult. After repeated low pressure liquid chromatography on silica gel and GPC on Sephadex LH20 (Pharmacia) pure material in a yield of only 45 % was isolated and characterized by ^1H- and ^{13}C NMR spectroscopy.

The synthesis of the second type of liquid phase carriers starts with the aminolysis of the commercially available 1,3,5-benzene tricarboxylic acid trimethylester **7** using ethylenediamine (Scheme 2).



Scheme 2

The gained amine **8**, 1,3,5-benzene tricarboxylic acid tris-N-(2-aminoethyl)amide, was treated with the p-nitrophenylester of 5'-O-dimethoxytrityl-deoxythymidine-3'-O-succinate in pyridine/DMF to give the aryl type liquid phase carrier **9** in excellent yields of up to 90 % (abbreviated as (DMT-dT)$_3$-aryl-LPC). Silica gel column chromatography and GPC on Sephadex LH20 were used for purification, ^{1}H- and ^{13}C NMR spectroscopy for identification. The two step synthesis of (DMT-dT)$_3$-aryl-LPC **9** showed to be much more successful and easier in comparison to the four step synthesis of (DMT-dT)$_4$-PE-LPC **5**. Therefore mainly compound **9** was used in the development of a route to oligonucleotide synthesis in solution.

The next step, dedimethoxytritylation of the liquid phase carriers **5** and **9** is the first necessary reaction for starting the synthesis of oligonucleotides and also repeated at the begin of each following cycle. It is important to develop a procedure, which guarantees fast and complete deprotection of the terminal 5'-hydroxyl groups. A partially uncomplete reaction will lead to failure sequences. Long reaction times under acidic conditions will favour another known side reaction, the depurination of guanosine and adenosine units in the growing oligonucleotide chain. To be competitive to solid phase strategies the method for oligonucleotide synthesis in solution has to be not too time consuming and labour intensive. For that reason and because the nucleotidic products after dedimethoxytritylation are very polar compounds (**6** and **10**), generally used methods like extraction out of aqueous solutions or silica gel column chromatography are unsuitable. A number of methods for dedimethoxytritylation and purification were tested to overcome the difficulties, leading to high yields but more or less also to disadvantageous aspects as discussed in the following. All compounds were well characterized by their ^{1}H- and ^{13}C-NMR spectra, dT$_3$-aryl-LPC **10** was also identified by MALDI-TOF mass spectrometry (Figure 1).

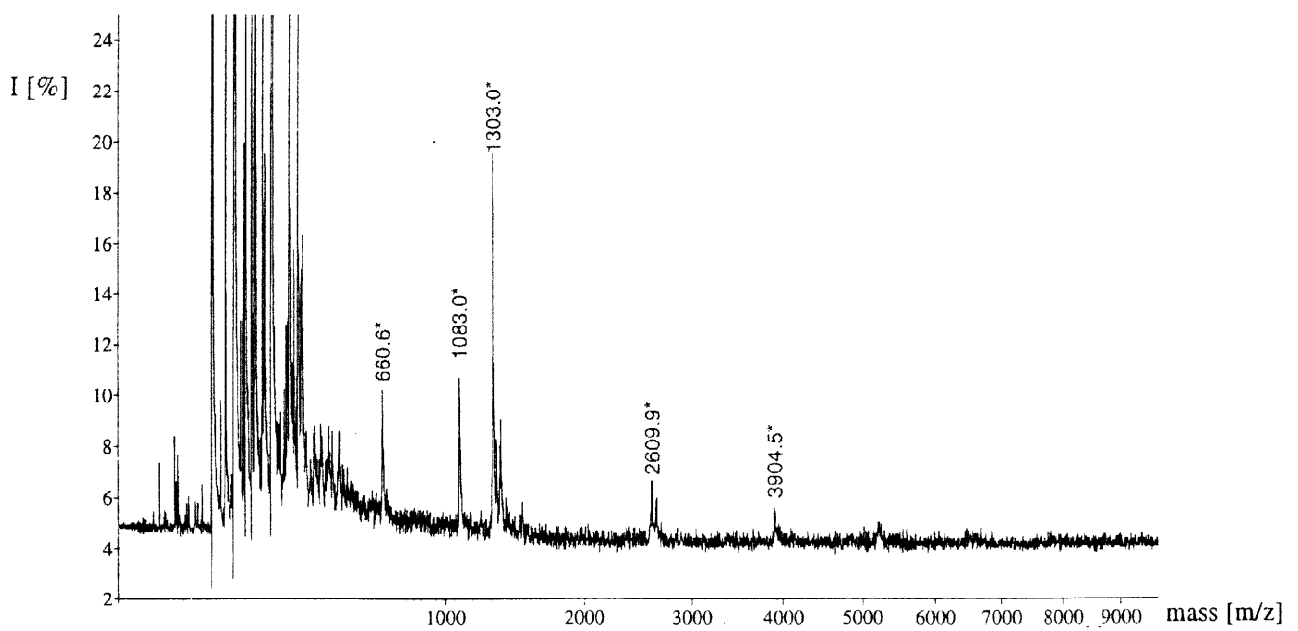


Figure 1: MALDI-TOF-mass spectrum of dT$_3$-aryl-LPC **10**; M+H$^+$: theoretical mass: 1309 Da, found: 1303 m/z

On the one side cationic ion exchangers in their protonated form (Fractogel® EMD-SO_3^- 650 (M) in H^+-form, Merck) were used for combined dedimethoxytritylation reaction and purification by stepwise variation of the polarity of the solvent system (solid phase extraction). Nonpolar solvent conditons (dichloromethane/ethanol mixtures) enable the fast separation of the dimethoxytrityl group with high flow rates, the 5'-O-deprotected LPC is eluted next with more polar solvent systems (ethanol or THF/ethanol mixtures). The yields were 93 % for dT_4-PE-LPC **6** and 82 % for dT_3-aryl-LPC **10** (with additional GPC using Sephadex LH20). A serious drawback is the partial decomposition of the ion exchanger in H^+-form after several uses, leading to acidic impurities in the isolated products. Also no primary isolation of product was attained. The relative long retention times under acidic conditions would favour depurination.

On the other side dedimethoxytritylation with TFA (trifluoroacetic acid, 2%) in 1,2-dichloroethane/nitromethane/methanol 80:19:1 (v/v) was used for rapid cleavage of the 5'-O-protecting group, completed in less than 5 minutes[23]. After neutralization (most times triethylamine) purification followed with different types of chromatography media:

a) by solid phase extraction using LiChroprep® DIOL (Merck) (yield: 97 % dT_3-aryl-LPC **10** ; product is probably contaminated with pyridine/TFA salt, no primary isolation of product is achieved),

b) by GPC using Sephadex LH20 and additional extraction of remaining dimethoxytritylcarbinol with diethylether (yield: 88 % dT_3-aryl-LPC **10**, extraction/filtration is labour extensive, but primary isolation of product is achieved),

c) neutralization with n-trioctylamine, purification by reversed phase chromatography using NUCLEOPREP® 300-30 C18 (Macherey/Nagel) (yield: 98 % dT_3-aryl-LPC **10**, expensive reagent for neutralization, water containing eluent mix necessary (acetonitrile/THF/water 56:14:30 v/v), but fast and primary isolation of product is achieved),

d) by reversed phase chromatography using NUCLEOPREP® 300-30 C18 in combination with gel permeation chromatography using Sephadex LH20 (yield: 97 % dT_3-aryl-LPC **10**, water containing eluent mix necessary (THF/water 70:30 v/v), but primary isolation of product is achieved).

The methods using GPC on Sephadex LH20 and/or reversed phase silica gel NUCLEOPREP® 300-30 C_{18} revealed a central problem in fast and primary isolation of the 5'-O-deprotected LPC. GPC on Sephadex LH20 alone was not successful in complete separation of the dedimethoxytrityl group using the binary eluent system THF/water (70:30 v/v). The water containing polar solvent mix was necessary for dissolving all components in the reaction mixture (DMT group, LPC and TFA salt of triethylamine), but led to another dominating separation mechanism for the nonpolar DMT-component, separation by partition instead of gel filtration[31]. Sephadex LH20 gel preferably takes up the polar solvent (water), creating a marked difference between stationary and mobile phase. The nonpolar DMT group, weak soluble in water, is therefore mostly present in the mobile phase and elutes partially together with dT_3-aryl-LPC **10**, al-

though the third component, TFA salt of triethylamine, is completely removed. Figure 2 demonstrates, that separation by gel filtration in the same solvent system and under same conditions is dominating for components of similar polarity but significant differences in size (dT_3-aryl-LPC **10**, M=1309 g/mol and deoxythymidine , M=242 g/mol).

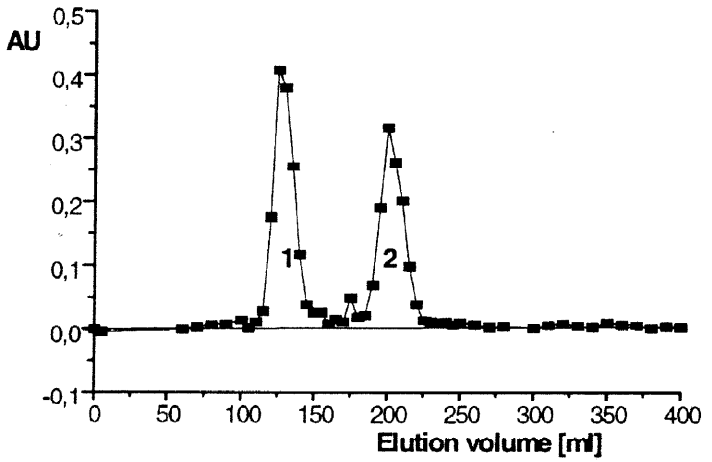
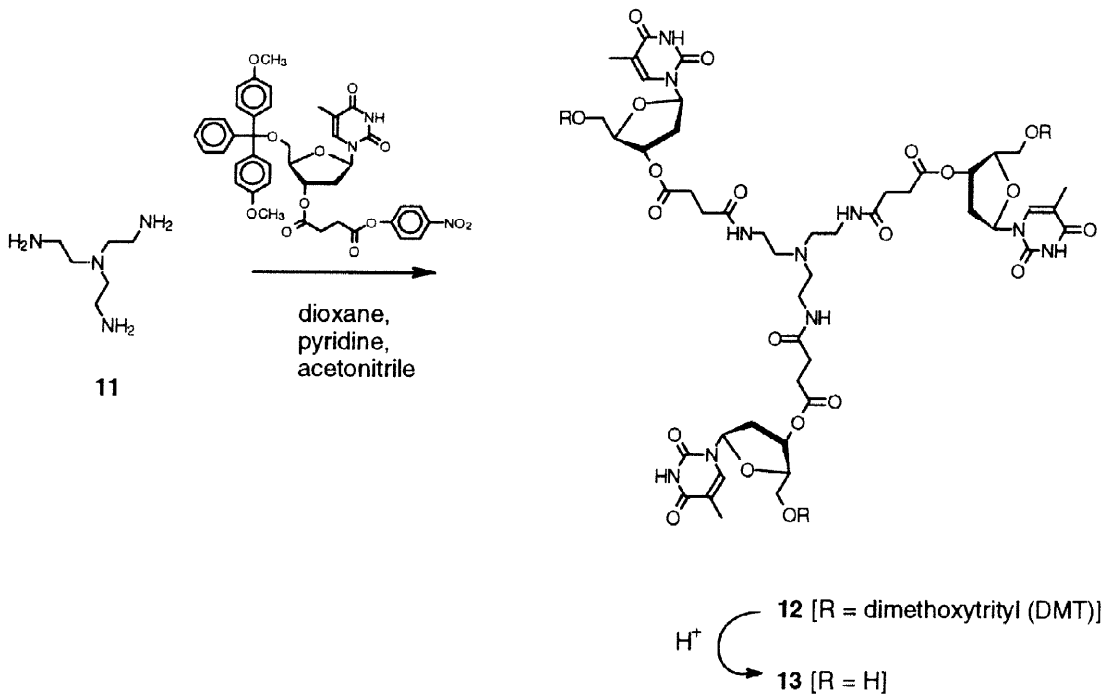


Figure 2: separation of 85mg dT_3-aryl-LPC {1} and 40 mg deoxythymidine {2} by gel filtration with Sephadex LH20 (column: 460x30mm, flow 1ml/min, eluent THF/water 70:30 v/v, 5 ml fractions, quantified by UV absorption at 260 nm, diluted 1:200 with eluent).

The RP-silica gel NUCLEOPREP® 300-30 C_{18} is suitable for fast and complete separation of the lipophilic DMT group at high flow rates, but not for removing of the TFA salt of triethylamine. More lipophilic long chain n-alkylamines in high purity as substitute for triethylamine in neutralization are expensive reagents, but their TFA salts are retained in chromatography giving pure deprotected dT_3-aryl-LPC **10** in up to 97 % yield. Water is an essential component in the eluent mix. The underlying separation mechanism uses hydrophobic interactions, which are far too weak in pure organic solvents, such as THF or acetonitrile. The later used method for synthesis of short oligonucleotides was a combination of both, using a NUCLEOPREP® 300-30 C_{18} filled precolumn for retention of DMT-components and Sephadex LH20 for removal of TFA salt of triethylamine (yield: 97 % for isolation of dT_3-aryl LPC **10**). Herein the use of water make intensive drying of nucleotidic products necessary before the following coupling reaction with phosphoamidites is started. In general water is no necessary eluent for chromatography using only Sephadex LH20. It can be avoided, when an eluent consisting of just one component is used. Separation by partition, as observed for very hydrophobic DMT-compounds in THF/water 70:30 (v/v), is impossible. Only few polar solvents are able to fulfill the demands for good solubility of all components and for other basic properties in chromatography (sufficient swelling of Sephadex LH20, low viscosity, low boiling points for easy remove after GPC). Pyridine is favoured for this purpose, also because this solvent proved not to disturb the condensation re-

action with phosphoamidites for chain elongation in contrast to water, alcohols, organic acids or strong bases. Experiments to improve the chromatography after dedimethoxytritylation avoiding the use of water are under way.

Another type of liquid phase carrier could easily be prepared starting with tris-(2-aminoethyl) amine **11** to give $(DMT\text{-}dT)_3$-amine-LPC **12** in good yields (74 %). Dedimethoxytritylation gave dT_3-amine LPC **13** (Scheme 3). Its use in oligonucleotide synthesis in solution was not studied intensively until now, because of the satisfactory results mainly achieved with dT_3-aryl-LPC **10**.



Scheme 3

EXPERIMENTAL

^1H (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[10-13]. 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[31]. The herein used columns from Kronwald (Sinsheim, Germany, flexible pressure glass columns, 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). Fractogel® EMD-SO_3^- (M) and LiChroprep®-DIOL were purchased from Merck (Darmstadt, Germany), NUCLEOPREP® 300-30 C_{18} from Macherey & Nagel (Düren, Germany)

Tetrakis-[(2-cyanoethoxy)methyl]-methane (2)[28]

Pentaerythrite **1** (6.84 g, 50 mmol) was dissolved in 20 ml dioxane and 2 ml water and mixed up with aqueous potassium hydroxid solution (40 % w/v, 1 ml). After cooling to 0°C in an ice bath, acrylnitrile (16.2 g, 300 mmol) was added and the mixture stirred for 48 h at room temperature. Solvents were evaporated, the remaining oil was diluted in 100 ml dichloromethane and washed with 10 % aqueous sodium chloride solution (w/v, 50 ml). After reextraction of the aqueous layer with dichloromethane (2x25 ml), the combined organic layers were dried over sodium sulfate and intensively evaporated. The nitrile **2** was obtained as a colourless syrup ; yield: 14.32 g (41 mmol, 82 %). - *^{1}H NMR* (400 MHz, $CDCl_3$): δ= 2.60 (t, 8H,-$C\underline{H}_2CN$), 3.50 (s, 8H, -$C_qC\underline{H}_2O$-), 3.65 (t, 8H, -$OC\underline{H}_2$-). - *^{13}C NMR* (100 MHz, $CDCl_3$): δ=18.86 (-$\underline{C}H_2CN$), 45.68 ($\underline{C}_q$), 65.88 (-$O\underline{C}H_2$-), 68.82 ($C_q\underline{C}H_2O$-), 118.22 ($\underline{C}N$) ppm.

Tetrakis-{[2-(methoxycarbonyl)ethoxy]methyl}-methane (3)[28]

Tetrakis-[(2-cyanoethoxy)methyl]-methane **2** (3.48 g, 10 mmol) was dissolved in dried methanol (30 ml) and saturated with dried hydrogen chloride at room temperature. The reaction mixture was refluxed for 1 h under continuous passing of hydrogen chloride gas. During cooling to room temperature argon was passed through the whole apparatus. After hydrolysis with ice cold water, the work up was performed (150ml) through extraction with diethylether (3x100 ml) and drying of the combined organic layers with sodium sulfate. Intensive evaporation leads to methylester **3** as a yellowish oil ; yield: 75 % (3.72 g, 7.5 mmol). - *^{1}H NMR* (400 MHz, $CDCl_3$): δ = 2.55 (t, 8H, -$C\underline{H}_2CO$-) , 3.32 (s, 8H, -C_q-$C\underline{H}_2O$-), 3.65 (t, 8H, -$OC\underline{H}_2$-), 3.70 (s , 12H, -

$OC\underline{H}_3$). - *^{13}C NMR* (100 MHz, $CDCl_3$): δ = 34.92 (-$\underline{C}H_2CO$-) , 45.35 ($\underline{C}_q$), 51.16 ($\underline{C}H_3$), 66.60 (-$O\underline{C}H_2$-), 69.23 ($C_q\underline{C}H_2O$-), 172.14 ($\underline{C}O$)

Tetrakis-(8-amino-6-aza-2-oxa-5-oxooctyl)-methane (4)[30]

Methylester **3** (3.91 g, 8 mmol) was dissolved in dried methanol (20 ml) and mixed up with a large excess of freshly distilled ethylenediamine (dried over CaH_2) at 0°C (200 ml, 3 mol). Under inert atmosphere (argon) the solution was stirred for 96 h at 4°C. Ethylenediamine was removed by evaporation and coevaporation after dissolving in methanol (10 ml) and the addition of toluene (100 ml, 3 times) ; yield: 88 % (4.01 g, 7 mmol). -*^1H NMR* (400 MHz, $CDCl_3$): δ = 1.60 (br, 8H, -$N\underline{H}_2$), 2.40 (t, 8H, -$C\underline{H}_2CO$-), 2.82 (m, 8H, -$C\underline{H}_2NH_2$), 3.30 (m, 8H, -$NHC\underline{H}_2$-), 3.32 (s, 8H, $C_qC\underline{H}_2O$-), 3.68 (t, 8H, -$OC\underline{H}_2$-), 7.05 (m, 4H, N$\underline{H}$).- *^{13}C NMR* (100 MHz, $CDCl_3$): δ = 36.94 (-$\underline{C}H_2CO$-), 41.57 (-$NH\underline{C}H_2$-), 42.34 ($\underline{C}H_2NH_2$), 45.37 ($\underline{C}_q$), 67.58 (-$O\underline{C}H_2$-), 69.73 ($C_q\underline{C}H_2O$-), 171.90 ($\underline{C}O$).

Tetrakis-{6,9-diaza-13-[5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxythymidine-3'-O-yl]-2-oxa-5,10,13-trioxotridecyl}-methane ; $(DMT\text{-}dT)_4$-PE-LPC (5)

The synthesis of 5'-O-dimethoxytrityl-deoxythymidine-3'-p-nitrophenylsuccinate followed published procedures[26]. The obtained solution in dioxane/pyridine, containing the p-nitrophenylester (approx. 5.4 mmol) was concentrated under reduced pressure and dissolved in pyridine (10 ml). The amine **4**, tetrakis-(8-amino-6-aza-2-oxa-5-oxooctyl)-methane (600 mg, 1mmol) was added and stirred for 16 h at room temperature. Reaction mixture was concentrated and dissolved in eluent mix. The first chromatography on silica gel was performed using a stepgradient from dichloromethane/ethanol 95:5 (v/v) to dichloromethane/ethanol 60:40 (v/v). All solvents contained 0.5 % pyridine (v/v). Further purification with gel permeation chromatography on Sephadex LH20 gave only small portions of pure material (eluent: THF/isopropanol 80:20, v/v). Repeated column chromatography on silica gel (with dichloromethane/ethanol, 15 to 30 % ethanol, 0.5 % pyridine) and gel permeation chromatography gave the product in a colourless crystalline form, total yield: 45 % (1.4 g, 0.45 mmol). ^1H NMR (400 MHz, $CDCl_3$): δ = 1.37 (s, 12H, $C\underline{H}_3$-, Base), 2.40 (m, 8H, -$C\underline{H}_2CO$-), 2.40/2.50 (m, 8H, H2'), 2.49 (m, 8H, -$COC\underline{H}_2$-), 2.63 (m, 8H, -$C\underline{H}_2CO$-), 3.29 (s, 8H, $C_qC\underline{H}_2O$), 3.36 (m, 16H, 2x-$NHC\underline{H}_2$-) 3.43 (m, 8H, H5'), 3.68 (m, 8H, -$OC\underline{H}_2$-), 3.76 (s, 24H, $C\underline{H}_3O$-), 4.15 (m, 4H, H4'), 5.43 (m, 4H, H3'), 6.35 (dd, 4H, H1'), 6.8 - 7.4 (m, 52H, $H_{Ar.}$), 7.60 (s, 4H, C$\underline{H}$, Base) , 8.62 (dd, 2x4H, 2x -N$\underline{H}$-CH_2-), 9.90 (s, 4H, N$\underline{H}$, Base).- *^{13}C NMR* (100 MHz, $CDCl_3$): δ =11.63 (CH_3, Base), 29.74 (C12), 30.56 (C11), 36.79 (C4), 37.80 (C2'), 39.38 (C8), 40.01(C7), 55.28 (-OCH_3), 63.85 (C5'), 67.50 (C3),

69.24 (C1), 75.88 (C3'), 83.81/84.30 (C1'/C4'), 87.22 (C_Z, DMT), 111.93 (C5, Base), 113.34 (C3, DMT), 127.26/128.06/128.13 (C2'/C3'/C4', DMT), 130.09 (C2, DMT), 135.09 (C1, DMT), 135.40 (C6, Base), 144.16 (C1', DMT), 151.34 (C2, Base), 158,75 (C4, DMT), 164.24 (C4, Base), 171.96 (C5 und C10), 172.16 (C13).

Tetrakis-[13-(2'-deoxythymidin-3'-O-yl)-6,9-diaza-2-oxa-5,10,13-trioxotridecyl]-methane ; dT_4-PE-LPC (6)

Fractogel® EMD-SO_3^- 650 (M) (Merck) in the protonated form (counterion H^+) was used for dedimethoxytritylation and solid phase extraction. (DMT-dT)$_4$-PE-LPC **5** (466 mg, 150 µmol) was dissolved in THF/ethanol 95:5 (v/v) and transfered to a column (inner diameter: 20mm) containing 50 ml ion exchanger, equilibrated with n-hexane/THF 50:50 (v/v). Dimethoxytrityl compounds were washed of with n-hexane/THF/ethanol 50:50: 5 (v/v, 100 ml), THF/ethanol 95:5 (v/v, 100 ml) and THF/Ethanol 50:50 (v/v, 150 ml). dT_4-PE-LPC **6** was eluted with pure ethanol, solvent was removed and thoroughly coevaporation with toluene gave the product as a colorless solid, yield: 93 % (263 mg, 139 µmol). *^{1}H NMR* (400 MHz, d_6-DMSO): δ = 1.80 (s, 16H, C<u>H</u>$_3$-, Base), 2.20 (m, 8H, H2'), 2.25 (m, 8H, -C<u>H</u>$_2$CO-), 2.38 (m, 8H, -COC<u>H</u>$_2$-), 2.52 (m, 8H, -C<u>H</u>$_2$CO-), 3.08 (m, 16H, 2x-NHC<u>H</u>$_2$-), 3.21 (s, 8H, C_qC<u>H</u>$_2$O), 3.51 (m, 8H, -OC<u>H</u>$_2$-), 3.64 (m, 8H, H5'), 3.98 (m, 4H, H4'), 5.22 (m, 4H, H3'), 6.18 (dd, 4H, H1'), 7.75 (s, 4H, C<u>H</u>, Base), 7.80/7.85 (dd, 2x4H, 2x -N<u>H</u>-CH$_2$-).- *^{13}C NMR* (100 MHz, d_6-DMSO): δ =12.18 (CH_3, Base), 29.02 (C12), 29.94 (C11), 36.06(C4), 36.41 (C2'), 38.35 (C8), 38.84 (C7), 44.70 (C_q), 61.27 (C5'), 67.19 (C3), 69.00 (C1), 74.68 (C3'), 83.64/84.50 (C1'/C4'), 109.64 (C5, Base), 135.73 (C6, Base), 150.39 (C2, Base), 163.58 (C4, Base), 170.30 (C5), 170.75 (C10), 171.94 (C13).

1,3,5-Benzene tricarboxylic acid tris-N-(2-aminoethyl)amide (8)[30]

1,3,5-benzene tricarboxylic acid trimethylester **7** (1.04 g 4.0 mmol) was treated with ethylenediamine (66.7 ml, 1 mol), following the procedure described for synthesis of compound **4**. The reaction was complete after 24 h. After lyophilization with dioxane the product was obtained as a white solid ; yield: 99 % (1,35 g, 4.0 mmol). - *^{1}H NMR* (400 MHz, d_6-DMSO): δ = 1.55 (br, 6H, -N<u>H</u>$_2$), 2.72 (m, 6H, -C<u>H</u>$_2$NH$_2$), 3.32 (m, 6H, -NHC<u>H</u>$_2$-), 8.42 (s, 3H, C<u>H</u>$_{Ar.}$), 8.65 (t, 3H, N<u>H</u>). - *^{13}C NMR* (100 MHz, d_6-DMSO): δ = 42.16 (-<u>C</u>H$_2$NH$_2$), 44.16 (-NH<u>C</u>H$_2$-), 129.30 (<u>C</u>H$_{Ar.}$), 135.95 (<u>C</u>$_{Ar.}$), 166.56 (<u>C</u>O)

1,3,5-Tris-{2,5-diaza-9-[5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxythymidine-3'-O-yl]-1,6,9-trioxononyl}-benzene ; (DMT-dT)$_3$-aryl-LPC (9)

A solution of 5'-O-dimethoxytrityl-deoxythymidine-3'-p-nitrophenylsuccinate (approx. 10 mmol) in dioxane/pyridine was concentrated under reduced pressure and dissolved in DMF (25 ml) and pyridine (10 ml). The amine **8**, 1,3,5-benzene tricarboxylic acid tris-N-(2-amino-ethyl)amide (673 mg, 2 mmol) was added, the reaction mixture stirred for 16 h at room temperature, concentrated and dissolved in eluent mix. Silica gel column chromatography (stepgradient with dichloromethane and 2 to 20 % ethanol, 0.5 % pyridine, v/v) led to mostly pure product fractions. Gel permeation chromatography on Sephadex LH20 (eluent: THF/methanol 80:20 v/v) of the impure fractions gave additional product, total yield: 90 % (4.0 g, 1.8 mmol). - *^{1}H NMR* (400 MHz, $CDCl_3$): δ = 1.32 (s, 9H, C<u>H</u>$_3$-, Base), 2.40/2.50 (m, 6H, H2'), 2.44 (m, 6H, -COC<u>H</u>$_2$-), 2.50 (m, 6H, -C<u>H</u>$_2$CO-), 3.40 (m, 6H, H5'), 3.54 (m, 12H, 2x-NHC<u>H</u>$_2$-), 3.77 (s, 18H, C<u>H</u>$_3$O-), 4.15 (m, 3H, H4'), 5.40 (m, 3H, H3'), 6.32 (dd, 3H, H1'), 6.8 - 7.4 (m, 39H, H$_{Ar.}$), 7.60 (s, 3H, C<u>H</u>, Base) , 8.19 (dd, 2x3H, 2x -N<u>H</u>-CH$_2$-), 8.40 (s, 3H, C<u>H</u>$_{Ar.}$). - *^{13}C NMR* (100 MHz, $CDCl_3$): δ =11.66 (CH_3, Base), 29.97 (C8), 30.66 (C7), 37.57 (C2'), 39.38 (C4), 40.57(C3), 55.26 (-OCH_3), 63.79 (C5'), 75.80 (C3'), 83.58/84.27 (C1'/C4'), 87.21 (C_Z, DMT), 112.06 (C5, Base), 113.34 (C3, DMT), 127.24/128.13/128.23 (C2'/C3'/C4', DMT), 129.04 ($CH_{Ar.}$), 130.10 (C2, DMT), 134.93 (C6, Base), 135.16 (C1, DMT), 135.27 ($C_{Ar.}$), 144.21 (C1', DMT), 151.34 (C2, Base), 158,79 (C4, DMT), 164.08 (C4, Base), 166.57 (C1), 171.95 (C6), 172.16 (C9).

1,3,5-Tris-[9-(2'-deoxythymidin-3'-O-yl)-2,5-diaza-1,6,9-trioxononyl]-benzene ; dT$_3$-aryl-LPC (10)

1.) Dedimethoxytritylation and solid phase extraction with Fractogel® EMD-SO_3^- 650 (M) (counterion H^+), additional purification with gel permeation chromatography using Sephadex LH20:

(DMT-dT)$_3$-aryl-LPC **9** (1.1g, 500 µmol) was dissolved in THF/ethanol 99:1 (v/v) and transfered to a column (inner diameter: 30 mm), containing 150 ml ion exchanger, equilibrated with n-hexane/THF/ethanol 60:35:5 (v/v). Dimethoxytrityl compounds were washed of with n-hexane/THF/ethanol 60:35:5 (v/v, 200 ml), THF/n-hexane/ethanol 60:35:5 (v/v, 200 ml) and THF/ethanol 95:5 (v/v, 200 ml). dT$_3$-aryl-LPC **10** was eluted with THF/ethanol 65:35 und THF/ethanol 50:50 (v/v, 300 ml each of). The crude product was purificated with gel permeation chromatography on Sephadex LH20 (eluent: THF/isopropanol 80:20 v/v), giving a colorless solid after removal of solvents, yield 82 % (530 mg, 410 µmol).

2) Dedimethoxytritylation with TFA reagent, purification with solid phase extraction using LiChroprep®-DIOL (Merck):

(DMT-T)$_3$-aryl-LPC **9** (580 mg, 260 μmol) was dissolved in (10 ml) 1,2-dichloroethane/nitromethane/methanol 80:19:1 (v/v) and TFA solution in the same solvent system was added (3%, 7.85 mmol TFA, 20 ml). After 3 minutes the reaction mixture was transfered to a column (inner diameter: 25 mm) filled with LiChroprep®-DIOL (90 ml), equiliberated with dichloromethane/ethanol 95:5 (v/v). The same solvent mixture was used for elution of dimethoxytrityl compounds (300 ml). Washing with dichloromethane/pyridine/ethanol 50:40:10 (v/v) gave the product dT$_3$-aryl-LPC **10** after removal of solvents and coevaporation with toluene (2 x 20 ml) as a colorless crystalline solid, yield: 97 % (330 mg, 252 μmol).

3) Dedimethoxytritylation with TFA reagent, purification by gel permeation chromatography using Sephadex LH20 and additional precipitation in diethylether:

A TFA solution in 1,2-dichloroethane/nitromethane/methanol 80:19:1 (v/v, 2 % TFA, 10 mmol) was dropwise mixed with a solution of (DMT-dT)$_3$-aryl-LPC **9** (739 mg, 330 μmol) in the same solvent system (5ml) under stirring at room temperature. After 2 minutes the reaction mixture was cooled in an icebath and triethylamine (10 mmol in 4 ml of the solvent system) was added. Solvents were removed under reduced pressure and the residue dissolved in N,N-dimethylformamide (5 ml). After chromatography on Sephadex LH20 (eluent: THF/water 70:30, v/v) the most product containing fractions were contaminated with traces of dimethoxytrityl compounds. After removal of the solvents and coevaporation with dioxane (40 ml), the residue was dissolved in THF/methanol (10 ml) and precipitated into heavily stirred diethylether (300 ml). Decantation of the ether layer, filtration of the precipitate under washing with diethylether and coevaporation with pyridine (5 ml) and toluene (2x10ml) gave dT$_3$-aryl-LPC **10** as a colorless solid, yield: 88 % (380 mg, 290 μmol).

4) Dedimethoxytritylation with TFA reagent and neutralization with trioctylamine, purification by chromatography using NUCLEOPREP 300-30 C$_{18}$:

(DMT-dT)$_3$-aryl-LPC **9** (113 mg, 50 μmol) was dissolved in 1,2-dichloroethane/nitromethane/methanol 80:19:1 (v/v, 4 ml) and a TFA solution in the same solvent system (1.5 mmol TFA in 1 ml) was added dropwise. After 3 minutes the reaction mixture was cooled in an icebath and neutralisized by trioctylamine (1.5 mmol, 1 ml solvent system). Solvents were removed under reduced pressure and the residue dissolved in acetonitrile/THF/water 56:14:30 (v/v, 4 ml), also the eluent in the following chromatography using NUCLEOPREP 300-30 C18 (column: 310 x 20 mm, flow 10 ml/min). Product is present in fractions with a retention time between 7 and 9 minutes and well separated from all other components of the probe. After removal of solvents under reduced pressure and lyophilisation with dioxane dT$_3$-aryl-LPC **10** is obtained as a colorless solid, yield: 98 % (64 mg, 49 μmol).

5) Dedimethoxytritylation with TFA reagent, purification by combined chromatography using NUCLEOPREP 300-30 C18 and Sephadex LH20:

(DMT-dT)$_3$-aryl-LPC **9** (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 **10** as a colorless solid, yield: 97% (490 mg, 374 µmol). - *^{1}H NMR* (400 MHz, d_6-DMSO): δ = 1.78 (s, 9H, C$\underline{H}_3$-, Base), 2.25 (m, 6H, H2'), 2.40 (m, 6H, -COC$\underline{H}_2$-), 2.55 (m, 6H, -C$\underline{H}_2$CO-), 3.24/3.34 (m, 12H, 2x-NHC$\underline{H}_2$-), 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$\underline{H}$, Base) , 8.00 (dd, 3H, -N$\underline{H}$CH$_2$-), 8.40 (s, 3H, C$\underline{H}_{Ar.}$), 8.68 (dd, 3H, -N$\underline{H}$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).

Tris-{3-aza-4,7-dioxo-7-[5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxythymidine-3'-O-yl]-heptyl}-amine; (DMT-dT)$_3$-amine-LPC (12)

To a solution of 5'-O-dimethoxytrityl-deoxythymidine-3'-p-nitrophenylsuccinate (approx. 9.4 mmol) in dioxane/pyridine a solution of tris-(2-aminoethyl)-amine **11** (292 mg, 2 mmol) in dried acetonitrile (10 ml) was added and stirred for 12 h at room temperature. The reaction mixture was concentrated and the residue dissolved in dichloromethane (containing 0.5 % pyridine). Silica gel column chromatography was started with dichloromethane/methanol (0.5 % pyridine), increasing the methanol content stepwise up to 15 %. Product containing fractions were combined, concentrated and precipitated into n-hexane/diethylether 3:1 (v/v). (DMT-dT)$_3$-amine-LPC **12** was isolated by filtration as a colorless solid, yield: 74 % (2.99 g, 1.48 mmol). -*^{1}H NMR* (400 MHz, $CDCl_3$): δ = 1.35 (s, 9H, C$\underline{H}_3$-, Base), 2.40/2.50 (m, 6H, H2'), 2.50/2.52/ 2.62 (m, 3x6H, -COC$\underline{H}_2$-,/-C$\underline{H}_2$N$_{central}$/-C$\underline{H}_2$CO-), 3.23 (m, 6H, -CONHC$\underline{H}_2$-), 3.45 (m, 6H, H5'), 3.76 (s, 18 H, C$\underline{H}_3$O-), 4.15 (m, 3H, H4'), 5.40 (m, 3H, H3'), 6.38 (dd, 3H, H1'), 6.8 - 7.4 (m, 39H, $H_{Ar.}$), 7.58 (CH, Base) , 9.55 (NH, Base). - *^{13}C NMR* (100 MHz , $CDCl_3$): δ = 11.60 ($\underline{C}H_3$, Base), 29.77 (C6), 30.46 (C5), 37.75/37.75 (C2'/C2), 54.63 (C1), 55.26 (-O$\underline{C}H_3$), 63.88 (C5'),

75.89 (C3'), 83.84/84.39 (C1'/C4'), 87.19 (C_Z, DMT), 111.89 (C5, Base), 113.35 (C3, DMT), 127.21/128.04/128.23 (C2'/C3'/C4', DMT), 130.08 (C2, DMT), 135,18 (C1, DMT), 135.39 (C6, Base), 144.24 (C1', DMT), 150.91 (C2, Base), 158.78 (C4, DMT) 163.87 (C4, Base), 171.77 (C4), 172.25 (C7).

Tris-[3-aza-7-(2'-deoxythymidine-3'-O-yl)-4,7-dioxoheptyl]-amine, dT_3-amine-LPC (13)

Dedimethoxytritylation of (DMT-dT)$_3$-amine-LPC **12** (660 mg, 325 µmol) was performed with TFA (2 %) in nitromethane/methanol (96:2, v/v, 40 ml). Purification was achieved by solid phase extraction using LiChroprep®-NH_2 (Merck, 50 ml, column: 150x20 mm). Dimethoxytrityl compounds were eluted with n-hexane/ethylacetate 2:1 (v/v, 50 ml), the nucleotidic compounds with ethylacetate/ethanol 2:1 (v/v, 150 ml). Product containing fractions were concentrated and gel permeation chromatography was used for further purification (Sephadex LH60, eluent: THF/ethanol 90:10, v/v ; column: 310x25 mm, flow 1ml/min). dT_3-amine-LPC **13** was isolated as a colorless solid, yield: 61 % (220 mg, 197 µmol). -*^1H NMR* (400 MHz, d_6-DMSO): δ = 1.77 (s, 9H, $C\underline{H}_3$-, Base), 2.25 (m, 6H, H2'), 2.40 (m, 6H, -$COC\underline{H}_2$-), 2.48 (m, 6H, -$C\underline{H}_2N_{central}$), 2.55 (m, 6H,-$C\underline{H}_2CO$), 3.08 (m, 6H, -$CONHC\underline{H}_2$-), 3.62 (m, 6H, H5'), 3.98 (m, 3H, H4'), 5.18 (m, 3H, H3'), 6.18 (dd, 3H, H1'), 7.72 (C<u>H</u>, Base), 7.75 (dd, 3H,-N<u>H</u>CH_2-).- *^{13}C NMR* (100MHz, d_6-DMSO): δ = 12.18 ($\underline{C}H_3$, Base), 29.06 (C6), 29.70 (C5), 36.43 (C2'), 36.98 (C2), 53.35 (C1), 61.27 (C5'), 74.67 (C3'), 83.63/84.51 (C1'/C4'), 109.63 (C5, Base), 135.72 (C6, Base), 150.38 (C2, Base), 163.57 (C4, Base), 170.59 (C4), 171.94 (C7).

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