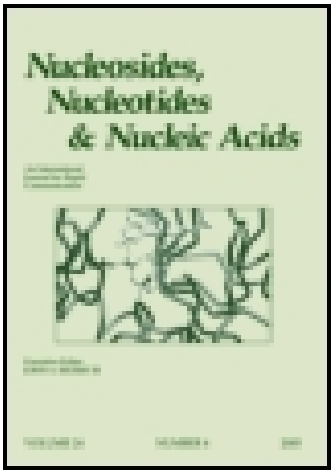

Nucleosides, Nucleotides and Nucleic Acids

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Improved Synthesis of Trinucleotide Phosphoramidites and Generation of Randomized Oligonucleotide Libraries

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IMPROVED SYNTHESIS OF TRINUCLEOTIDE PHOSPHORAMIDITES AND GENERATION OF RANDOMIZED OLIGONUCLEOTIDE LIBRARIES

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□ *A new method to produce a set of 20 high quality trinucleotide phosphoramidites on a 5–10 g scale each was developed. The procedure starts with condensation reactions of P-components with* N*-acyl nucleosides, bearing the 3′-hydroxyl function protected with 2-azidomethylbenzoyl, to give fully protected dinucleoside phosphates* ***13****. Upon cleavage of dimethoxytrityl group from* ***13****, dinucleoside phosphates* ***16*** *are initially transformed into trinucleoside diphosphates* ***19*** *and then the 2-azidomethylbenzoyl is selectively removed under neutral conditions to generate trinucleoside diphosphates* ***5*** *in excellent yield. Subsequent 3′-phosphitylation affords target trinucleotide phosphoramidites* ***7****. When mutagenic oligonucleotides are synthesized employing mixtures of building blocks* ***7*** *as well as following the new synthetic protocol, representative oligonucleotide libraries are generated in good yields.*

Keywords Trinucleotide phosphoramidites; 2-azidomethylbenzoyl protection group; randomized oligonucleotide libraries

INTRODUCTION

Protein mutagenesis can be used to fine tune a variety of properties, such as improved stability to high temperatures, denaturants, or nonaqueous solvents; higher affinity binding to a target molecule; increased rates

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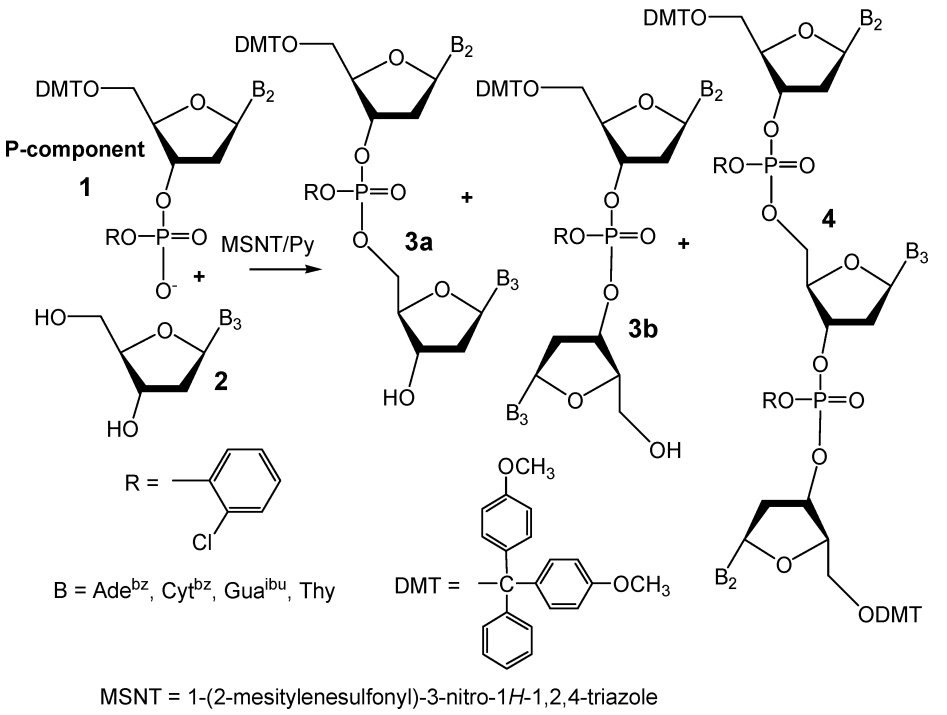
of enzymatic reactions; or changes of specificities. However, generating and finding these improved proteins can be a difficult task. One of the most popular methods is to make pools of degenerate oligonucleotides, which can be incorporated into the genes as cassettes or by PCR by using the degenerate oligo as a primer.[1] Degenerate oligonucleotides are synthesized as a mixture of A/C/G/T phosphoramidites (N) at the site of the codons to be mutated. Problems arise, though, from using an equimolar solution of each base. First there is a coding bias. Of the 64 possible codon combinations of A, C, G and T, eighteen code for leucine, arginine, or serine, but only two for tryptophan or methionine. As a result, only 3% of the mutagenic oligonucleotides will contain methionine or tryptophan, and over 28% will contain either leucine, arginine, or serine. In addition, the three nonsense codons will lead to chain termination in 4.7% of the sequences. There are ways to improve this situation. For instance, using two degenerate mixes of bases, N and G/C, on the DNA synthesizer to insert NNG/C into the sequence will halve the number of the most degenerate codons, but still code for all 20 amino acids. However, still 59% of the clones will code for just eight amino acids and 3% will have a stop codon inserted. The generation of redundant sequences and stop codons makes searching a clonal library inefficient. However, it is possible to improve the efficiency of this process by using a mixture of trinucleotide (trimer) phosphoramidites.[2–5] By synthesizing a set of trimers that cover all 20 amino acids, the mutation of a gene can be carried out at the codon level rather than at individual bases. Therefore, unlike other methods of mutagenesis, trimer phosphoramidites lead to no codon bias, no frame-shift mutations, and no production of stop codons, making them one of the most efficient tools to explore sequence space in protein regions that are important for function[6]—even in nonsaturating conditions.[7,8]

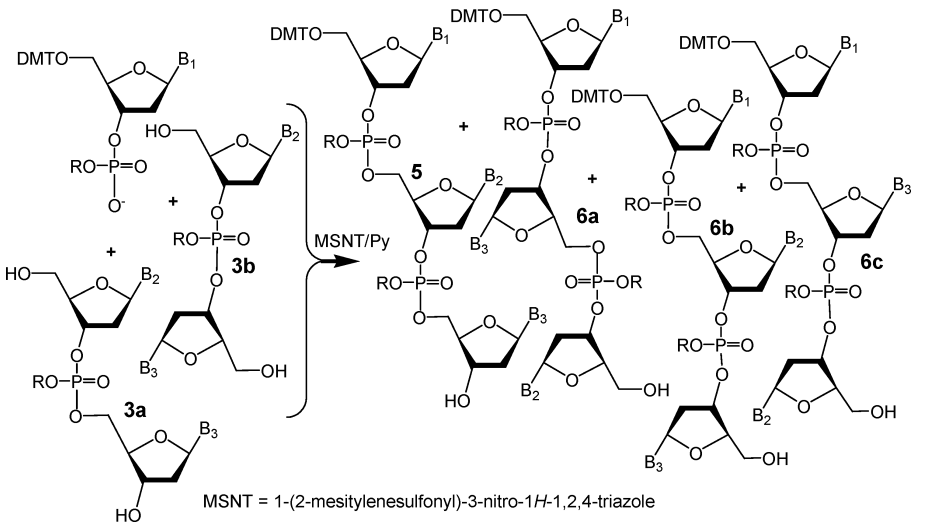
RESULTS AND DISCUSSION

Several reports[9–11] describing the synthesis of trimer phosphoramidites have been published. To our mind, the basic approach described by Kayushin et al.[2–4] still seems straightforward and timely. The general procedure, reported in detail in the initial publication[4] utilizes an approach employing condensation of the P-component (triethylammonium salt of 5′-*O*-dimethoxytrityl-*N*-acyl-2′-deoxynucleoside-3′-*O*-(2-chlorophenyl)phosphate or 5′-*O*-dimethoxytritylthymidine-3′-*O*-(2-chlorophenyl)phosphate) with the *N*-acyl nucleoside containing an unprotected 3′-hydroxyl [12,13] in the presence of 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT) in pyridine in the first step (Scheme 1). The mildest way for internucleotide link formation was selected in this case[7] in order to exploit the difference in reactivity of primary and secondary hydroxyl functions of *N*-acyl nucleosides.



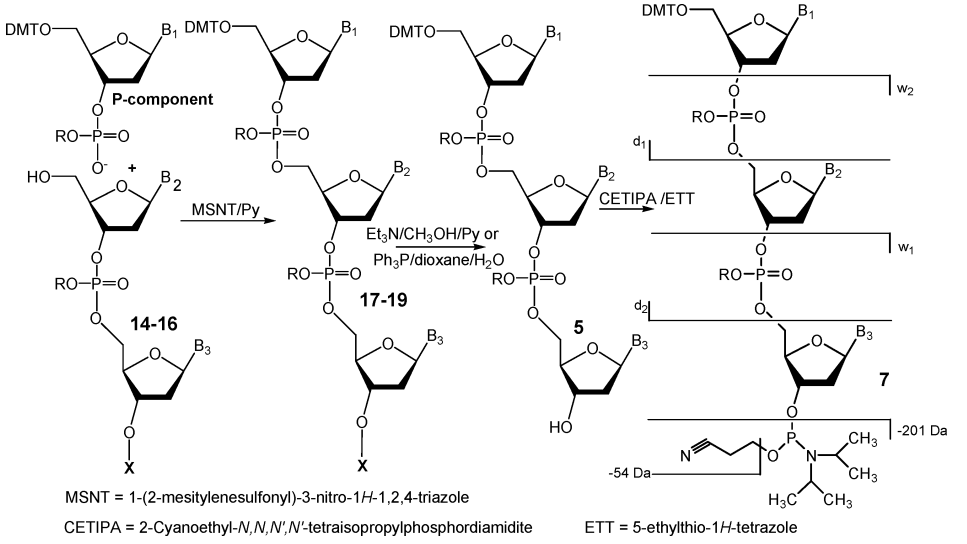
SCHEME 1 Synthesis of dinucleotide according to[7].

Initially our goal was to synthesize all 20 Trimer phosphoramidites following the above referenced method (structures being based on codon usage in *E. coli*). We started with preparation of 12 intermediate dimers and in the process of this work we have noticed that indeed[4] in all 12 condensation reactions the main product was always the desired dinucleotide **3a**. The isomeric dimer **3b** (both **3a** and **3b** are regioisomeric pairs) along with the trimer **4** were always present in the reaction mixtures as well (Scheme 1). The amount of unwanted **3b** and **4** varied, depending on the target dimer in question, being up to 7–8% in certain cases (data not shown). The silica gel chromatographic separation of trimer **4** appeared to be an easy task. However, the separation of a few percent of unwanted dimer **3b** was never a straightforward procedure. In some cases, the complete separation of **3b** (if possible at all) required a long silica gel column and large volumes of chromatographic solvents. In any case, an analytical confirmation (RP HPLC) of the purity of **3a** as well as the complete absence of **3b** in the target dimer, was absolutely required before the next condensation step. In cases when the dimer **3b** was not removed completely from **3a** (fast flash chromatography), subsequent condensation generated trimer **5** contaminated with three isomeric trimers, **6a**, **6b**, and **6c** (Scheme 2). It appeared very difficult, if possible at all to remove these unwanted trimers



SCHEME 2 Synthesis of trinucleotides according to[7].

from the target product **5**. One has to keep in mind that all of these trimers were in fact mixtures of six diastereomers. Obviously, this fact seriously hampered the isolation of pure trimers **5**. Subsequent conversion of **5** into trimer phosphoramidite **7** was a straightforward and simple procedure (Scheme 3). However, preparative reverse phase (RP) chromatography does not allow separation of target trimer phosphoramidite **7** from undesired 3′-phosphoramidites of **6a**, **6b** and **6c,** in the case initial trimer 5, used



SCHEME 3 Synthesis of trinucleotides 7, employing intermediate protection of 3′-terminal hydroxy-groups. Typical fragment ions of **7**, labeled as reported earlier,[5] are also shown.

for conversion into trimer phosphoramidite **7,** was not pure enough. It is noteworthy that any trimer 3′-phosphoramidite comprises a mixture of 8 isomers, irresolvable by preparative low pressure RP chromatography.

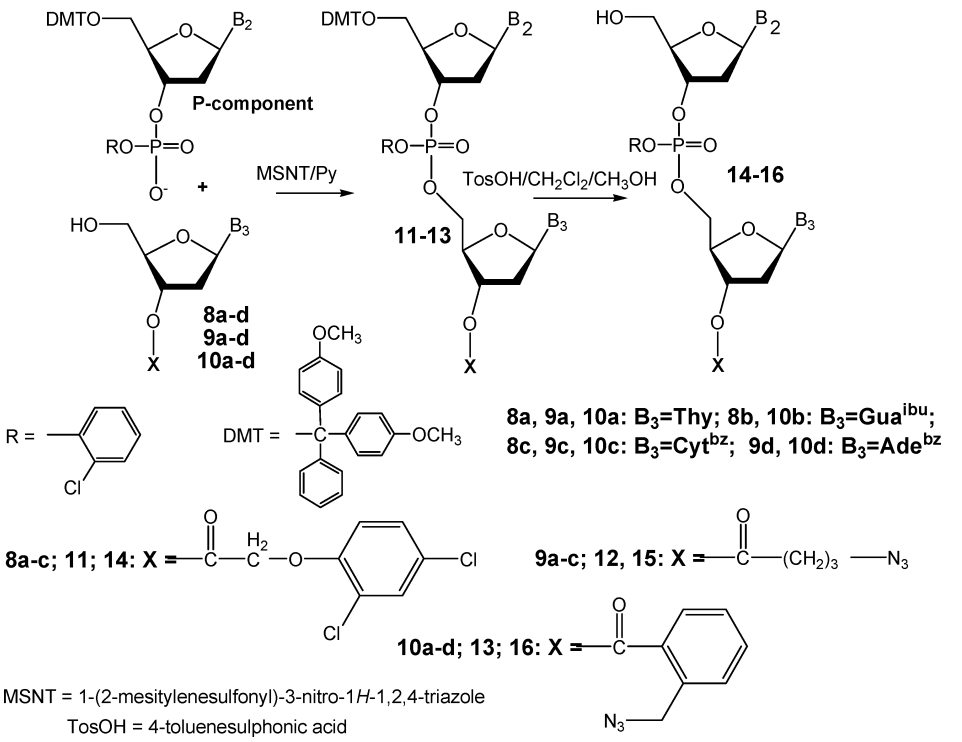
When a mutagenic oligonucleotide was synthesized using trimers 7 contaminated with 3′-phosphoramidites of **6a**, **6b**, and **6c**, and subsequently employed for construction of libraries by introducing it into a plasmid used for *E. coli* transformation, a number of resulting clones often incorporated DNA sequences with frame shifts and unexpected mutations.[14]

Evidently, the repetitive production of a set of 20 trimer phosphoramidites has to rely on a procedure which guarantees the absence of unwanted dimer **3b** and subsequently trimers **6a**, **6b**, and **6c** on the way to the final target trimer phosphoramidites **7.** In addition, the new approach has to be a straightforward synthesis, employing simple and fast flash chromatography purification of intermediate compounds. Hence, the method of production of trimer phosphoramidites has to be considerably revised and improved to meet these requirements.

SYNTHESIS AND CHARACTERIZATION OF TRIMER PHOSPHORAMIDITES

To our mind, a new strategy to produce a set of 20 high quality trimer phosphoramidites has to start from the condensation reactions of P-components with *N*-acyl nucleosides with properly protected 3′-hydroxyl group. This will ensure the absence of unwanted isomeric dimers and trimers present in the intermediate and final products and, more importantly, will considerably simplify the necessary purification steps. Due to the nature of blocking functions in P-components (DMTr, 2-chlorophenyl group on phosphorous and *N*-acyl group), this protection group must be neither acid-labile nor strongly base-labile. In another words, the protection of 3′-terminal nucleoside 3′-hydroxy group has to be easily removable either under very weak basic conditions or under neutral specific conditions.

In the present investigation, we tested the suitability of three such candidates—2,4-dichlorophenoxyacetyl-, 4-azidobutanoyl- and 2-azidomethylbenzoyl protection groups (Scheme 4). To our knowledge, the weakly base-labile 2,4-dichlorophenoxyacetyl group has not been used in nucleoside chemistry earlier. This function may be easily introduced into 5′-*O*-dimethoxytrityl-*N*-acyl-nucleoside by reaction with 2,4-dichlorophenoxyacetylimidazole prepared in situ to give the corresponding 5′-*O*-dimethoxytrityl-3′-*O*-(2,4-dichlorophenoxyacetyl)-*N*-acyl-nucleosides in a good yield. Subsequent removal of 5′-*O*-dimethoxytrityl functions under conditions specific for each *N*-acylnucleoside or thymidine (see Experimental section) gave 3′-*O*-(2,4-dichlorophenoxyacetyl)-*N*-acyl-nucleosides **8b–c** and 3′-*O*-(2,4-dichlorophenoxyacetyl)-thymidine **8a** in 80–90% overall



SCHEME 4 Synthesis of Dinucleotides **14–16**, bearing protection group at 3′- terminal hydroxyl.

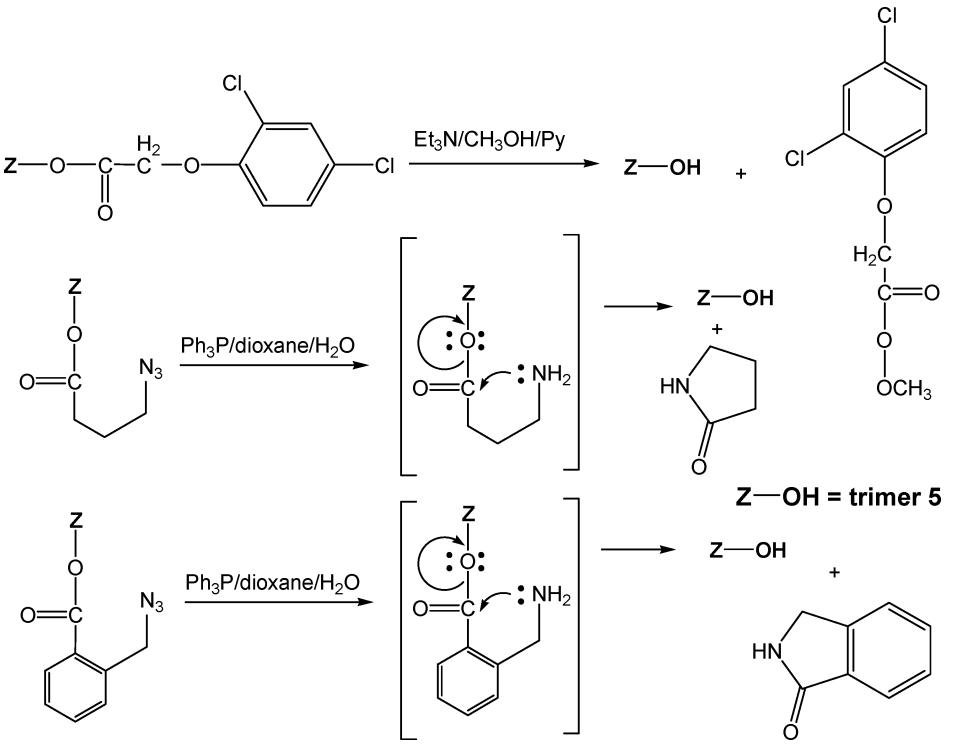
yield. Subsequent synthesis of completely protected trinucleotides was based on classical phosphotriester method in solution (Scheme 3) with minor technical modifications (see Experimental section). The overall yields of 3′-*O*-dichlorophenoxyacetyl-trinucleotides **17**, when calculated based on initial 3′-*O*-(2,4-dichlorophenoxyacetyl)-*N*-acylnucleosides **8a–c** were reasonably good. No unwanted isomeric trimers were found in reaction mixtures and therefore all chromatographic separations of intermediate 3′-*O*-dichlorophenoxyacetyl-dinucleotides and 3′-*O*-dichlorophenoxyacetyl-trinucleotides appeared to be straightforward.

As we have found, the 2,4-dichlorophenoxyacetyl- protecting group may be removed under very mind conditions (10% triethylamine in pyridine-methanol, 2:1 for 3 hours at room temperature (rt)) from the 3′-hydroxyl of trinucleotides **17–19** (Scheme 3) to give trimers **5** in a reasonable yield of 65–85%. Under these conditions, most of the 2-clorophenyl groups as well as the *N*-acyl groups (Scheme 3) remained intact. However, minor amounts of initial 2,4-dichlorophenoxyacetylated products **17–19** (10–20%) were still present in reaction mixtures. It is noteworthy that even such a mild basic treatment of trinucleotides (especially incorporating 2′-deoxyguanosine nucleoside units) was always accompanied by considerable darkening of the reaction mixture. Although the longer (5 hours) treatment with

triethylamine-pyridine-methanol mixture reduced the amount of initial 2,4-dichlorophenoxyacetylated trimers **17–19** to about 2–3%, the loss of 2-clorophenyl groups was becoming already noticeable and, in general, reaction mixtures were turning very dark. The additional drawback of using the strategy described above, is the formation of stable emulsions upon cleavage of 3′-terminal 2,4-dichlorophenoxyacetyl protection group and subsequent workup (see Experimental section). This workup—successive washings of dichloromethane or chloroform solution of trinucleotide **5** containing a free 3′-terminal hydroxyl (Scheme 3) with sodium hydrogen carbonate and then triethylammonium hydrogen carbonate normally took several hours to accomplish due to very slow separation of the organic and aqueous layers.

Thus, although the use of 2,4-dichlorophenoxyacetyl protecting group basically helps to improve the quality of final trinucleotides by eliminating the problem of unwanted isomeric trimers in the target trimer phosphoramidites, it brings forward certain disadvantages as well. Darkening of reaction mixtures and hence colored target trimer phosphoramidites, the need to waste time on slow separation of layers during the workup and, more importantly, somewhat reduced yields of target compounds impelled us to probe other protecting groups for the 3′-terminal hydroxyl function.

In order to avoid the drawbacks of the method described above, we investigated the possibility to use 4-azidobutyryl group[15] for the protection of the 3′-terminal hydroxyls of trinucleotides (Scheme 4). 4-Azidobutyryl esters may be cleaved under neutral conditions via reduction (e.g., with triphenylphosphine in the presence of water) of the azido function to give esters of 4-aminobutyric acid. The formation of aminoacyl ester is then spontaneously followed by intramolecular cyclization, to generate the free hydroxyls (Scheme 5). The 4-azidobutyryl protection group may be easily introduced into 5′-*O*-dimethoxytrityl-*N*-acyl-nucleosides by reaction with 4-chlorobutyryl chloride to give corresponding 5′-*O*-dimethoxytrityl-3′-*O*-(4-chlorobutyryl)-*N*-acyl-nucleosides in good yield. Subsequent azidation reaction with sodium azide in DMF followed by removal of 5′-*O*-dimethoxytrityl functions (see Experimental section) gave 3′-*O*-(4-azidobutyryl)-*N*-acyl-nucleosides **9b–c** and 3′-*O*-(4-azidobutyryl)-thymidine **9a** in 70–80% overall yield. The subsequent chemistry leading to 3′-*O*-(4-azidobutyryl)-trinucleotides was basically the same as described above (see Experimental section). The overall yields of 3′-*O*-(4-azidobutyryl) trinucleotides **18** when calculated on initial 3′-*O*-(4-azidobutyryl)-*N*-acylnucleosides **9a–c** were in a range of 80–90%. As in the case with 2,4-dichlorophenoxyacetyl temporary protection, no unwanted isomeric trimers were found in these reaction mixtures. All chromatographic separations of intermediate 3′-*O*-(4-azidobutyryl)-dinucleotides **15** and 3′-*O*-(4-azidobutyryl)-trinucleotides **18** proved to be fast and simple. The 4-azidobutyryl protection group was removed from the 3′-hydroxyl



SCHEME 5 Cleavage of 3′-terminal protection groups, reported herein.

of trinucleotides (Scheme 3) upon reduction, followed by $O \rightarrow N$ acyl migration (Scheme 5 and Experimental section) in good yield. Under these conditions, no loss of 2-clorophenyl groups as well as N-acyl groups (Scheme 3) was detected and no initial 4-azidobutyrylated trimers were found in reaction mixtures. It is noteworthy that no darkening of reaction mixtures was observed. However, a small amount (5–10%) of a very polar DMT-containing material was present in all cases. The yields of trimers **5** when synthesized using 4-azidobutyryl temporary protection were 70–80%. Most of the resulting trimer phosphoramidites **7** generated by this method proved to be of high purity and were white to off-white in color.

Thus, the use of the 4-azidobutyryl protection group in the synthesis of trimer phosphoramidites appears to be a considerable improvement, when compared to the original procedure.[4] The potential to remove the blocking group from the 3′-hydroxyl function of trimers **5** under neutral conditions also has certain minor advantages, when compared to the method utilizing 2,4-dichlorophenoxyacetyl- protection (no coloration of reaction mixtures; no colored target trimer phosphoramidites). Nevertheless, the formation of 5–10% of side products in the process of 4-azidobutyryl cleavage makes these advantages, although significant, not absolutely satisfactory. In order to further improve the method to produce a set of

20 trimer phosphoramidites, we have investigated the possibility of using one more 3′-terminal protection group—2-azidomethylbenzoyl.[16] The 2-azidomethylbenzoylation of the 3′-hydroxy functions of 5′-*O*-dimethoxytrityl-*N*-acylnucleosides and 5′-*O*-dimethoxytritylthymidine was a simple procedure. The removal of 5′-*O*-dimethoxytrityl functions (see Experimental section) gave 3′-*O*-(2-azidomethylbenzoyl)-*N*-acyl nucleosides **10b–d** and 3′-*O*-(2-azidomethylbenzoyl)-thymidine **10a** in good yields (65–77%). It is noteworthy that adenine, cytidine, and thymidine 3′-*O*-(2-azidomethylbenzoyl)-nucleosides were purified by simple crystallization. Preparation of dinucleotides **16** and trinucleotides **19** was very similar to that of **15** and **18** and brings about high yields of the desired products. The 2-azidomethylbenzoyl group is basically similar to 4-azidobutyryl protection. It may be also removed from 3′-terminal hydroxyls of trinucleotides **19** (Scheme 3) under neutral conditions *via* reduction of the azido function (triphenylphosphine in the presence of water) to give esters of 2-aminomethylbenzoic acid, followed by spontaneous intermolecular $O \rightarrow N$ acyl migration to generate the free hydroxyls (Scheme 5). However, in this case, transformation of trinucleotides **19** into trimers **5** (Scheme 3) proceeded in nearly quantitative yield. The yields of trimers **5** when synthesized using 2-azidomethylbenzoyl temporary protection were 85–95%. Most of the target trimer phosphoramidites **7**, generated by this method in excellent yield, proved to be white in color and of high purity as shown below in Figures 1A and 1B.

Noteworthy that in all cases when reactions were performed with 2′-deoxyguanosine derivatives and MSNT, a somewhat higher content of side-products in reaction mixtures was observed. However, when the reaction conditions were strictly followed as described in the Experimental section, these unwanted modifications were minor and they did not hamper the isolation of desired products.

Quality control of trimer phosphoramidites appears to be very important and challenging. Trimer phosphoramidites have chiral centers at all three phosphorus positions. There are $2^3 = 8$ diastereomers in each phosphoramidite, which are at least partially separated on RP HPLC. The best separation of diastereomers is nearly achieved when the RP HPLC is performed under basic conditions (pH 8.4 or higher). Figure 1 A shows the RP HPLC traces of diastereomers of trimer phosphoramidite **7** (TAC), obtained at pH 8.7. When the trimer phosphoramidite **7** is converted into the corresponding trimer H-phosphonate by activation with tetrazole, followed by subsequent hydrolysis, all of the diastereomeric trimer phosphoramidites are converted into a faster eluting diastereomeric H-phosphonates (Figure 1B). This simple experiment normally proves the absence of contaminants not incorporating a phosphoramidite function. In addition to the HPLC data, the ^{31}P-NMR spectrum of trimer **7** always has to show the presence of

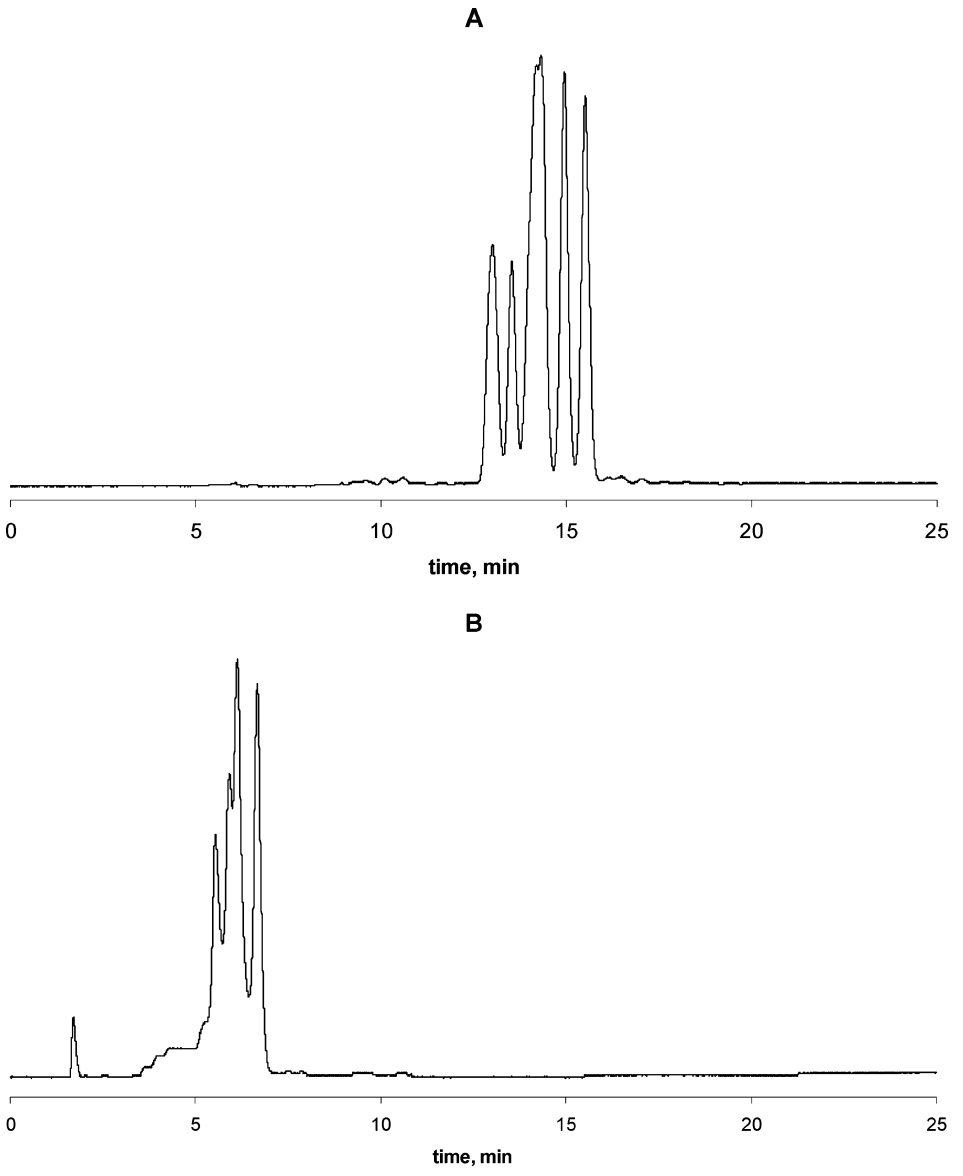


FIGURE 1 A) RP HPLC trace of trimer phosphoramidite **7** (TAC); B) RP HPLC trace of trimer phosphoramidite **7** (TAC) after treatment with ethylthiotetrazole and water (trimer H-phosphonate diester TAC).

phosphotriester and phosphoramidite functions in a ratio of 2:1 (Figure 2 is given as an illustrative example).

There is also the concern that the sequence of the trimers has to be verified (e. g., CTG, has to be differentiated from GTC). Electrospray mass spectrometric analysis[5] (ESI MS) of the trimers provides data confirming molecular mass (Figure 3A) and sequence (Figure 3B). In the latter

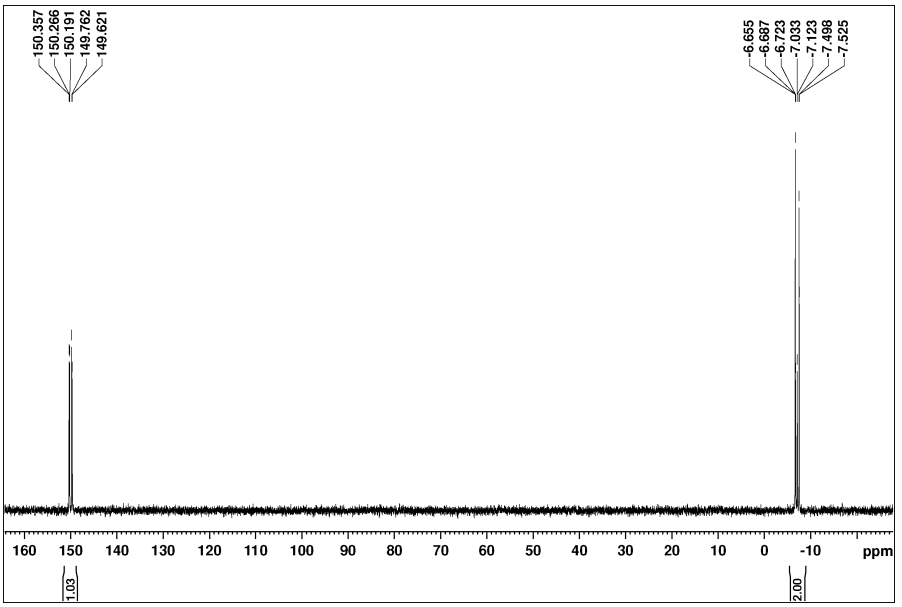


FIGURE 2 ^{31}P-NMR spectrum of trimer phosphoramidite **7** (TAC).

case, collision induced negative ion MS/MS analysis of the deprotonated molecule gives two types of complementary fragment ions, which clearly indicate the base sequence[5] (Figure 3b and Scheme 3).

In our opinion, all three methods (RP HPLC, ^{31}P-NMR, and ESI MS) have to be routinely employed for characterization of all 20 trimer phosphoramidites **7**, employed for generation of randomized oligonucleotides.

Thus, we have worked out an appropriate procedure to synthesize a set of 20 trimer phosphoramidites **7** on a production scale. The compounds generated following our method, utilizing protection of 3′-terminal hydroxyl function with 2-azidomethylbenzoyl group, are normally of a very high purity and are not contaminated with undesired isomeric 3′-phosphoramidites of **6a**, **6b**, and **6c**.

SYNTHESIS OF RANDOMIZED OLIGONUCLEOTIDE LIBRARIES

The set of 20 trimer phosphoramidites **7** was used in randomized oligonucleotide synthesis. As pointed out in the original communication on trimer preparation and oligosynthesis[4] reaction factor (RF) values have to be assigned to each trimer **7**. The reaction factor is critical since the trimers likely will be mixed and they have differing reactivity in the coupling reaction. For example, the RF for AAC is 1.0 and for TAC is 1.6. Therefore, 1.6 equivalents of TAC are needed for every 1.0 equivalent of AAC for equal coupling. Since the publication by Kayushin et al.[4] the values of RFs

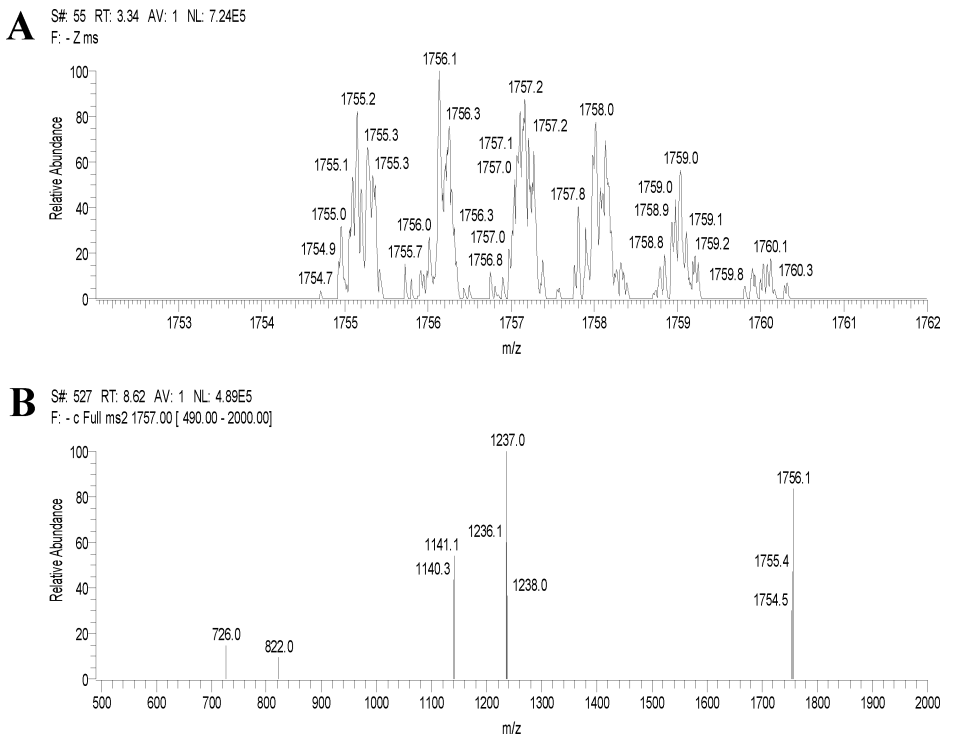


FIGURE 3 A) Negative ion zoom scan ESI spectrum of trimer phosphoramidite **7** (CTG) showing the isotopic pattern of the molecule; B) collision induced negative ion MS/MS spectrum of trimer phosphoramidite **7** (CTG), showing typical fragment ions.[15]

were re-evaluated and determined more precisely.[14] Several changes in RF values were introduced most probably because of much higher purity of trimers 7 reported in this study in comparison to trimer phosphoramidites tested in original communication.[4] The trimers, their coding amino acids and their revised reaction factors (RF) are listed in Table 1.

In the original communication[4] a "slightly modified standard cycles and procedures from the synthesizer producer" were used. These modifications were—double or triple couplings and 2-fold extended reaction time. In addition to these modifications, the trimers were dissolved in a mixture of acetonitrile—dichloromethane (1:3, v/v) to give 0.15 N concentration. It is noteworthy that since most of trimers **7** display a poor solubility in pure acetonitrile, the use of acetonitrile—dichloromethane mixture to dissolve trimers or trimer mixtures appears to be an obligatory and unavoidable measure. When running the oligosynthesis with the 19 trimer mixture it was observed that coupling yields ranged from 71% (the first trimer coupling) to almost 98%.[4]

In the present work we attempted to repeat the synthesis of randomized oligonucleotides, following the method described above with a one

TABLE 1 Trimer phosphoreamidites 7, their coding amino acids, and their revised reaction factors (RF)

Trimer*	MW	RF	Trimer*	MW	RF
AAA (Lys)	1911.5	1.1	GAA (Glu)	1893.5	1.9
AAC (Asn)	1887.5	1.0	GAC (Asp)	1869.5	1.3
ACT (Thr)	1774.5	1.3	GCT (Ala)	1756.5	1.5
ATC (Ile)	1774.5	1.2	GGT (Gly)	1762.5	1.1
ATG (Met)	1780.5	1.3	GTT (Val)	1667.5	1.9
CAG (Gln)	1869.5	2.0	TAC (Tyr)	1774.5	1.6
CAT (His)	1774.5	1.9	TCT (Ser)	1661.4	1.3
CCG (Pro)	1845.5	1.8	TGC (Cys)	1756.5	1.5
CGT (Arg)	1756.5	1.1	TGG (Thr)	1762.5	2.4
CTG (Leu)	1756.5	1.2	TTC (Phe)	1661.4	1.3

*Trimer phosphoramidites 7 are abbreviated with three-letter code for simplicity.

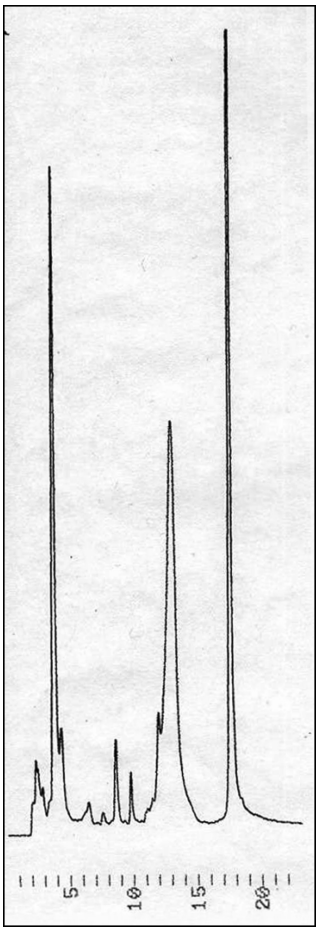


FIGURE 4 RP HPLC traces of randomized oligonucleotide **RO-3.** Target **RO-3** elutes at about 18 minutes.

exception—we employed 0.1 N concentration of the 20 trimer mixture rather than using 0.15 N concentration. Obviously our attempt to reduce trimer consumption and hence the cost of randomized oligonucleotides looked reasonable. However, the concentration reduction resulted in yields of target randomized oligonucleotides ranging in total from 2% to 5%. Under these circumstances, we decided to adjust the oligosynthesis procedure to achieve better overall yields but still employing 0.1 N concentration of trimers. When reviewing the synthetic procedure published earlier[4] it seems obvious that, whereas trimers were initially dissolved in a mixture of acetonitrile—dichloromethane, their solutions become diluted with acetonitrile when passing through the synthesizer tubings, mixing with activator and finally on the synthesis column, pre-washed with acetonitrile. Obviously, dilution with acetonitrile seemed to be undesirable since we observed the formation of emulsions with some trimers upon dilution of their acetonitrile—dichloromethane (1:3, v/v) solution with acetonitrile. To avoid this we decided to dissolve activator (5-ethylthio-1H-ethylthiotetrazole, 1 g) in acetonitrile-dichloromethane mixture (1:3 v/v, 31 mL), introduce a washing step with acetonitrile-dichloromethane mixture (1:3 v/v) before the trimer mixture addition step and employ a 10 min trimer mixture addition step (in three increments) into the randomized oligonucleotide synthesis protocol.

Hereafter, we report the comparison of results of synthesis of three randomized oligonucleotides, obtained by the original method[4] and the procedure described above. These randomized oligonucleotides **RO-1** and **RO-2** were libraries of 20^4 48mers and **RO-3** was a library of 20^{10} 60mers:

5′-TTGTGTCACGACGTTGTGTCACGA$(X)_4$AGCTCAGATCAC-3′ **RO-1**,

5′-TTGTGTCACGAC$(X)_2$GTTGTGTCACGA$(X)_2$AGCTCAGATCAC-3′ **RO-2**,

5′-TTGTGTCACGAC$(X)_5$GTGTCA$(X)_5$AGCTCAGATCAC-3′ **RO-3**,

where X = 20 trimer phosphoramidite mixture.

In all of our test syntheses (0.1 μmolar scale), we programmed the synthesizer to perform "trityl-on" for more convenient randomized oligonucleotide isolation from the reaction mixture. Since we performed all our syntheses on the Universal solid support,[17] our cleavage /deprotection procedure differed slightly from the original one.[4] We employed a 45 minutes treatment of oligomer-bound solid support with 3.5 N ammonia in methanol before 3 times diluting the methanolic solution with 32% aqueous ammonia and leaving the resulting mixture for 36 hours at rt.

In the case of the original procedure, yields of target **RO-1—RO-3** upon deprotection were as low as 3–4% for **RO-1** and **RO-2** and less than 1.5% for **RO-3**. However, our new modified method allowed preparation of the same randomized oligomers in a yield of about 30%. In both

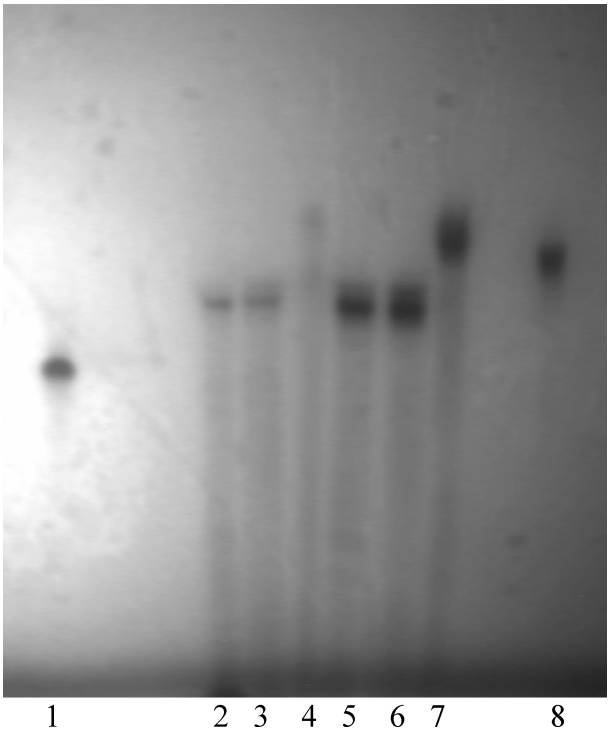


FIGURE 5 Photograph of 10% PAGE. Lane 1: standard 40 mer; Lanes 2, 3, 4: randomized oligonucleotides **RO-1**, **RO-2,** and **RO-3** correspondingly, synthesized according to original protocol[7] and purified as in Figure 6; Lanes 5, 6, 7: randomized oligonucleotides **RO-1**, **RO-2,** and **RO-3** correspondingly, synthesized according to modified protocol, reported herein and purified as in Figure 6; Lane 8: standard 57 mer.

cases, yields of compounds were assessed using RP HPLC by integrating total "trityl-on" peaks at 260 nm. Figure 4 shows the analytical RP HPLC trace of crude **RO-3** as an illustrative example. The chromatographic run was performed by using a linear gradient of acetonitrile concentration (0–20%) in 0.1 M triethylammonium acetate (pH 7.5) over 12 minutes, then sharply raising the acetonitrile concentration from 20 to 64% over 1 minutes, and finally performing an isocratic elution with 64% acetonitrile in 0.1 M triethylammonium acetate (pH 7.5) over additional 10 minutes. According to integration of peaks from this trace, the yield of randomized **RO-3** (the last peak, eluting at 18 minutes) was 37%. All three oligomers, synthesized employing original[4] and new procedures were purified on a preparative scale using conditions, described above, de-tritylated and finally analyzed on the 10% PAGE (Figure 5). One can clearly see that, whereas all three randomized oligonucleotides were synthesized very well employing our new synthetic procedure, only reasonably pure RO-1 and RO-2 were obtained following original protocol.[4] The PAGE also has supported the length of target oligomers and the absence of shorter failure

sequences, separated by RP HPLC. This evidence supports the claim that the new synthetic protocol is superior to original one.[4] The new procedure generates reasonable amounts of randomized oligonucleotides, more than sufficient for subsequent biological experiments.

CONCLUSION

We have developed a new procedure to produce valuable reagents—high quality trinucleotide phosphoramidites, on a 5–10 g scale each. The trimers are now commercially available (Metkinen Chemistry, Finland Kuopio) individually, as a mixture of all 20 trimers or any other specific mixture.

We conclude that when mutagenic oligonucleotides are synthesized using these trimers 7 following the modified synthetic protocol, oligonucleotide libraries resulting from cloning will be most likely free from oligomers containing DNA sequences with frame shifts and unexpected mutations.

EXPERIMENTAL

General

5′-*O*-Dimethoxytrityl-N^6-benzoyl-2′-deoxyadenosine, 5′-*O*-dimethoxytritylN^4-benzoyl-2′-deoxycytidine, 5′-*O*-dimethoxytrityl-N^2-isobutyryl-2′-deoxyguanosine, and 5′-*O*-dimethoxytrityl-thymidine were purchased from Rasayan Inc. (Encinitas, CA, USA). *N,N*′-Carbonyldiimidazole was obtained from Aldrich (St. Louis, MO, USA) and recrystallized from dry toluene. Dichlorophenoxyacetic acid was obtained from Lancaster (Eastgate, Lancashire, UK), 4-chlorobutyryl chloride, 1-(2-mesitylenesulfonyl)-3-nitro-1 *H*-1,2,4-triazole (MSNT) and 7 N ammonia in methanol were obtained from Aldrich and used without further purification. 2-Azidomethylbenzoyl chloride was synthesized as described earlier.[16] Benzenesulfonic acid and 4-toluenesulfonic acid monohydrate were purchased from Fluka. Acetonitrile, pyridine, and 1-methylimidazole (Fluka Buchs, Switzerland) were distilled over calcium hydride. 2-chlorophenyldichlorophosphate was from Sigma (St. Louis, MO, USA). Triethylammonium salts of 5′-*O*-dimethoxytrityl-*N*-acyl-2′-deoxynucleoside-3′-*O*-(2-chlorophenyl)phosphates and 5′-*O*-dimethoxytritylthymidine-3′-*O*-(2-chlorophenyl)phosphate (P-components) were prepared as reported earlier[4] or as described below. column flash chromatography was run using B-681 and B-688 piston pumps and borosilicate glass columns (Büchi). Silica gel 60 (40–63 μm) or Lichroprep RP-18 (40–63 μm), both from Merck (Darmstadt, Germany), were used for column flash chromatography. A gradient of methanol in dichloromethane (from 0 to 5–7%, flow rate 50 ml/minute) was used in a

former case and gradient of acetonitrile in water (from 50% to 100%, 25 ml/minute) was used in a latter case for purification of compounds. RP HPLC of Trimer phosphoramidites **7** and trimer phosphoramidites **7** after treatment with ethylthiotetrazole and water was performed employing a Gilson gradient chromatograph on a xBridge C18 column (4.6 × 150 mm, 5 μm, Waters (Milford, MA, USA)), using a gradient elution from 60 to 80% acetonitrile in 0.1 M triethylammonium acetate buffer, pH 8.7, over 20 minutes, followed by isocratic elution with 80% acetonitrile in 0.1 M triethylammonium acetate buffer, pH 8.7 over 10 minutes at a flow rate 1 ml/minute. RP HPLC of randomized oligonucleotides was performed employing a Merck-Hitachi (Darmstadt, Germany) gradient chromatograph on a xTerra C18 column (4.6 × 150 mm, 5 μm, Waters), using a gradient elution from 0 to 20% acetonitrile in 0.1 M triethylammonium acetate buffer, pH 7.5 over 12 minutes, followed by gradient elution from 20 to 80% acetonitrile in the same buffer over 1 min., and finally isocratic elution with 80% acetonitrile in the same buffer over 10 minutes; flow rate 1 ml/minute.

ESI MS data were obtained with Finnigan (San Jose, CA, USA) MAT LCQ ion trap mass spectrometer, using negative ion mode. The structures of trimer phosphoramidites were verified as described earlier.[5] NMR spectra were taken with Avance 500 spectrometer (Bruker, Silberstetten, Germany) in DMSO-d_6 (^{1}H-NMR) or $CDCl_3$ (^{31}P-NMR). Oligonucleotide syntheses were performed with an 8-column ASM-800 synthesizer (Biosseti Novosibirsk, Russia) on 0.1 μmolar scale, employing a Universal solid support USII-AMPS-M-40 (Metkinen Chemistry, Kuopio, Finland). Cleavage/deprotection of oligonucleotides was performed as recommended by the manufacturer.

Synthesis of 3′-*O*-2,4-dichlorophenoxyacetyl)-*N*-acyl-2′-deoxynucleosides and 3′-*O*-(2,4-dichlorophenoxyacetyl)thymidine

The synthesis of 3′-*O*-2,4-dichlorophenoxyacetyl)-*N*-acyl-2′-deoxynucleosides and 3′-*O*-(2,4-dichlorophenoxyacetyl)thymidine may be divided in two steps. Step A, the preparation of a 5′-*O*-dimethoxytrityl-3′-*O*-dichlorophenoxyacetyl-*N*-acyl-2′-deoxynucleosides, is common for all four 2′-deoxynucleosides. Step B, the removal of 5′-*O*- dimethoxytrityl residue and further workup is base-specific and will be described for each nucleoside separately hereafter.

3′-O-(2,4-Dichlorophenoxyacetyl)thymidine (8a)

Step A. The solution of carbonyldiimidazole (8.910 g, 55 mmol) in 100 ml of anhydrous acetonitrile was added to the solution of 2,4-dichlorophenoxyacetic acid (17.155 g, 55 mmol) in 200 ml of anhydrous acetonitrile under intensive stirring within 10 minutes. In nearly 5 minutes, the precipitate started to form. In 45 minutes the precipitate was

filtered off and the resulted solution was added to a suspension of 5′-*O*-dimethoxytritylthymidine (15 g, 27.5 mmol) in 200 ml of anhydrous acetonitrile. The suspension was concentrated to a gum by evaporation; during evaporation the precipitate started to dissolve and finally the clear solution has been obtained. In 2 hours 2 ml of water was added, in 20 minutes the solution was evaporated to dryness, the residue was dissolved in nearly 200 ml of chloroform and washed with 200 ml of 0.1 M triethylammoniumbicarbonate (TEAB). The aqueous layer was extracted with chloroform (3 × 200 ml), the combined organic layers were washed with 0.1 M TEAB (3 × 200 ml), dried over sodium sulfate and evaporated to dryness.

Step B. The residue obtained after completing the Step A was dissolved in 100 ml of 1,2-dichloroethane. The solution of (10.1 ml, 137.5 mmol) anhydrous trifluoroacetic acid in 100 ml of 1,2-dichloroethane was added under intensive stirring. In 1 minute 140 ml of saturated aqueous solution of sodium hydrogen carbonate was added, the aqueous layer was washed with chloroform (3 × 150 ml), the combined organic layers were washed with 0.1 M TEAB (3 × 150 ml), dried over sodium sulfate and evaporated to a gum. The residue was evaporated with chloroform (3 × 100 ml) and the desired product was isolated by column chromatography on silica gel (column 4 × 34 cm, linear gradient of methanol in chloroform, 0–6%, 2 l, flow rate 1 l/hour). Yield 10.89 g (89%); ^{1}H NMR (DMSO-d_6), δ_{TMS}, ppm; *J*, Hz: 11.33 (s, 1H, NH of Thy), 7.73 (d, 1H, J = 1.1, H-6 of Thy), 7.59 (d, 0.5 H, J = 2.6, H-3 of 2,4-dichlorophenoxyacetyl), 7.34 (dd, 1H, J = 2.6, J = 8.9, H-5 of 2,4-dichlorophenoxyacetyl), 7.18 (d, 1H, J = 9.0, H-6 of 2,4-dichlorophenoxyacetyl), 6.19 (dd, 1H, J = 6.0, J = 8.6, H-1′), 5.34 (m, 1H, H-3′), 5.21 (t, 1H, J = 5.2, 5′-OH), 5.01 (s, 2H, $\underline{CH_2}$-OAryl), 4.02 (m, 1H, H-4′), 3.63 (m, 2H, H-5′$_{a,b}$), 2.29 (m, 2H, H-2′$_{a,b}$), 1.78 (d, 5-CH_3 of Thy); MS (ESI-MS, CH_3CN): Calcd. for $C_{18}H_{18}Cl_2N_2O_7$ [M-H]$^-$: 445.25. Found: 443.1.

3′-O-(2,4-Dichlorophenoxyacetyl-N^2-isobutyryl-2′- deoxyguanosine (8b)

Step A was performed with (20 g, 31.25 mmol) of 5′-*O*-dimethoxytrityl-N^2-isobutyryl-2′-deoxyguanosine. Step B was performed as described for 3′-*O*-(2,4-Dichlorophenoxyacetyl)thymidine, except for duration of acidic treatment (30 seconds instead of 1 minute). Yield 15.38 g (91%); ^{1}H NMR (DMSO-d_6), δ_{TMS}, ppm; *J*, Hz: 12.08 (s, 1H, NH of Gua), 11.66 (s, 1H, 2-NH of Gua), 8.27 (s, 1H, H-2 of Gua), 7.61 (d, 0.5 H, J = 2.6, H-3 of 2,4-dichlorophenoxyacetyl), 7.36 (dd, 2H, J = 2.6, J = 8.9, H-5 of Cyt and H-5 of 2,4-dichlorophenoxyacetyl), 7.18 (d, 1H, J = 9.0, H-6 of 2,4-dichlorophenoxyacetyl), 6.21 (dd, 1H, J = 5.6, J = 9.0, H-1′), 5.45 (m, 1H, H-3′), 5.13 (t, 1H, J = 5.5, 5′-OH), 5.02 (d, 2H, J = 3.9, $\underline{CH_2}$-OAryl), 4.10 (m, 1H, H-4′), 3.60 (m, 2H, H-5′$_{a,b}$), 2.88 (m, 1H, H-2′$_a$), 2.77 (m, 1H, J = 6.9, CH of *i*bu), 2.54 (m, 1H, H-2′$_b$), 1.13 (d, 6 H, J = 6.8, 2CH_3 of *i*bu); MS (ESI-MS, CH_3CN): Calcd. for $C_{22}H_{23}Cl_2N_5O_7$ [M-H]$^-$: 538.10. Found: 538.2.

3′*-O-*(2,4-Dichlorophenoxyacetyl*-N^4*-benzoyl-2′-deoxycytidine (8c)

Step A was performed with (15.85 g, 25 mmol) of 5′-*O*-dimethoxytrityl-N^4-benzoyl-2′-deoxycytidine. Step B was performed as described for 3′-*O*-(2,4-Dichlorophenoxyacetyl)thymidine. After addition of sodium hydrogen carbonate the precipitate was formed. The precipitate was filtered off, the aqueous layer of the filtrate was washed with chloroform (5 × 100 ml), combined organic layers were washed with 0.1 M TEAB (3 × 150 ml), dried over sodium sulfate and evaporated to dryness. The residue was combined with the precipitate obtained above and re-crystallized from isopropanol. Yield 10.55 g (79%); m.p 198–201°C; ^{1}H NMR (DMSO-d_6), δ_{TMS}, ppm; *J*, Hz: 11.33 (s, 1H, 4-NH of Cyt), 8.37 (d, 1H, J = 7.6, H-6 of Cyt), 8.01 (m, 2H, Bz), 7.62 (m, 1H, Bz), 7.59 (d, 0.6 H, J = 2.6, H-3 of 2,4-dichlorophenoxyacetyl), 7.51 (m, 2H, Bz), 7.36 (m, 2H, H-5 of Cyt and H-5 of 2,4-dichlorophenoxyacetyl), 7.19 (d, 1H, J = 9.0, H-6 of 2,4-dichlorophenoxyacetyl), 6.19 (dd, 1H, J = 5.9, J = 8.0, H-1′), 5.37 (m, 1H, H-3′), 5.28 (br. s, 1H, 5′-OH), 5.02 (s, 2H, $\underline{CH_2}$-OAryl), 4.18 (m, 1H, H-4′), 3.67 (m, 2H, H-5′$_{a,b}$), 2.54 (m, 1H, H-2′$_b$), 2.30 (m, 1H, H-2′$_a$); MS (ESI-MS, CH_3CN): Calcd. for $C_{24}H_{21}Cl_2N_3O_7$ $[M-H]^-$: 532.08. Found: 532.1.

3′-*O*-(4-Azidobutyryl)thymidine (9a) and 3′-*O*-(4-Azidobutyryl)-*N*-acyl-2′-deoxynucleosides (9b–9d). General Procedure

A solution of 4-chlorobutyryl chloride (2.1 g, 14.9 mmol) in dichloromethane (10 ml) was added dropwise with stirring to a cold (0°C) solution of 1-methylimidazole (1.5 g, 18.3 mmol) in dichloromethane (10 ml). A solution of dried 5′-*O*-(4,4′-dimethoxytrityl)-*N*-acyl-2′-deoxynucleoside or 5′-*O*-(4,4′-dimethoxytrityl)thymidine (10 mmol) in dichloromethane (50 ml) was then added dropwise with stirring and the resulting solution was left at (0°C) overnight. The solution was washed with saturated sodium hydrogen carbonate (2 × 50 ml), 5% oxalic acid until pH < 5, water. organic layer was dried over sodium sulfate, evaporated to dryness and the residue was dissolved in N,N′-dimethylformamide (100 ml). Sodium azide 1.3 g (20 mmol) was added to the solution and the reaction mixture was stirred at 60°C for 12 hours. The reaction mixture was then evaporated to dryness, the residue was dissolved in toluene (150 ml). The toluene solution was washed with water (3 × 100 ml). The organic layer was separated, dried over sodium sulfate and evaporated to dryness. A 1% solution of 4-toluenesulphonic acid monohydrate in a mixture of dichloromethane–methanol (7:3, 150 ml) was added to the oily residue with vigorous stirring. After 10 min at rt, the acidic reaction mixture was neutralized by the addition of saturated sodium hydrogen carbonate

(50 ml). The organic layer was separated, additionally washed with saturated sodium hydrogen carbonate, dried over sodium sulfate and evaporated to dryness. The residue was either chromatographed on the silica gel in a gradient of methanol in dichloromethane (from 0 to 5%) to give 2.9 g of **9a** and 3.4 g of **9d** as foams in 79 and 72% overall yield correspondingly, or crystallized from acetone to give 2.9 g of **9c** in 66% yield.; m.p 170–172°C.

9a: ^{1}H NMR (DMSO-d$_6$), δ_{TMS}, ppm; J, Hz: 11.31 (s, 1H, NH of Thy), 7.73 (d, 1H, $J = 1.2$, H-6 of Thy), 6.18 (dd, 1H, $J = 5.9$, $J = 8.7$, H-1′), 5.24 (m, 1H, H-3′), 5.20 (t, 1H, $J = 5.2$, 5′-OH), 3.98 (m, 1H, H-4′), 3.63 (m, 2H, H-5′$_{a,b}$), 3.38 (t, 2H, $J = 6.8$, -CH$_2$-N$_3$), 2.44 (t, 2H, $J = 7.3$, -CH$_2$-CO-), 2.26 (m, 2H, H-2′$_{a,b}$), 1.80 (m, 2H, -CH$_2$-CH$_2$-CH$_2$-), 1.78 (d, 5-CH$_3$ of Thy); MS (ESI-MS, CH$_3$CN): Calcd. for $C_{14}H_{19}N_5O_6$ [M-H]$^-$: 352.13. Found: 352.1.

9c: ^{1}H NMR (DMSO-d$_6$), δ_{TMS}, ppm; J, Hz: 11.26 (s, 1H, 4-NH of Cyt), 8.39 (d, 1H, $J = 7.4$, H-6 of Cyt), 8.01 (m, 2H, Bz), 7.63 (m, 1H, Bz), 7.51 (m, 2H, Bz), 7.38 (d, 1H, H-5 of Cyt), 6.17 (dd, 1H, $J = 6.0$, $J = 7.8$, H-1′), 5.26 (m, 1H, H-3′), 5.22 (t, 1H, $J = 5.2$, 5′-OH), 4.14 (m, 1H, H-4′), 3.67 (m, 2H, H-5′$_{a,b}$), 3.39 (t, 2H, $J = 6.8$, -CH$_2$-N$_3$), 2.50 (m, 1H, H-2′$_b$), 2.45 (t, 2H, $J = 7.3$, -CH$_2$-CO-), 2.26 (m, 1H, H-2′$_a$), 1.81 (m, 2H, -CH$_2$-CH$_2$-CH$_2$-); MS (ESI-MS, CH$_3$CN): Calcd. for $C_{20}H_{22}N_6O_6$ [M+H]$^+$: 442.16. Found: 441.2, 442.1. **9d**: ^{1}H NMR (DMSO-d$_6$), δ_{TMS}, ppm; J, Hz: 11.21 (s, 1H, 6-NH of Ade), 8.76 (d, 0.6 H, H-8 of Ade), 8.71 (s, 1H, H-2 of Ade), 8.05 (m, 2H, Bz), 7.65 (m, 1H, Bz), 7.55 (m, 2H, Bz), 6.51 (dd, 1H, $J = 6.1$, $J = 8.4$, H-1′), 5.44 (m, 1H, H-3′), 5.20 (t, 1H, $J = 5.7$, 5′-OH), 4.14 (m, 1H, H-4′), 3.65 (m, 2H, H-5′$_{a,b}$), 3.41 (t, 2H, $J = 6.8$, -CH$_2$-N$_3$), 3.06 (m, 1H, H-2′$_a$), 2.58 (m, 1H, H-2′$_b$), 2.48 (t, 2H, J= 7.3, -CH$_2$-CO-), 1.84 (m, 2H, -CH$_2$-CH$_2$-CH$_2$-); MS (ESI-MS, CH$_3$CN): Calcd. for $C_{21}H_{22}N_8O_5$ [M-H]$^-$: 465.17. Found: 465.3.

3′-*O*-(2-Azidomethylbenzoyl)-*N*-acyl-nucleosides (10b–d) and 3′-*O*-(2-Azidomethylbenzoyl)thymidine (10a). General Procedure

A solution of 2-azidomethylbenzoyl chloride (2.9 g, 15 mmol) in dichloromethane (10 ml) was added dropwise with stirring to a cold (0°C) solution of 1-methylimidazole (1.5 g, 18.3 mmol) in dichloromethane (10 ml). A solution of dried 5′-*O*-(4,4′-dimethoxytrityl)-*N*-acylnucleoside or 5′-*O*-(4,4′-dimethoxytrityl)thymidine (10 mmol) in dichloromethane (50 ml) was then added dropwise with stirring and the resulting solution was left at (0°C) overnight. The solution was washed with saturated sodium hydrogen carbonate (2 × 50 ml), 5% oxalic acid until pH < 3, water. organic layer was dried over sodium sulfate and evaporated to dryness. A 1% solution of 4-toluenesulphonic acid in a mixture of dichloromethane–methanol (7:3, 150 ml) was added to the oily residue with vigorous stirring. After 10 minute

at rt, the acidic reaction mixture was neutralized by the addition of saturated sodium hydrogen carbonate (50 ml). The organic layer was separated, additionally washed with saturated sodium hydrogen carbonate, dried over sodium sulfate and evaporated to dryness. Crystallization of the residue from dichloromethane gave 3.1 g (77% yield) of pure **10a**, m.p 204–207°C; ^{1}H NMR (DMSO-d_6), δ_{TMS}, ppm; J, Hz: 11.33 (s, 1H, NH of Thy), 8.00 (dd, 1H, $J = 1.2$, $J = 7.8$, H-6 of 2-azidomethylbenzoyl), 7.79 (d, 1H, $J = 1.2$, H-6 of Thy), 7.68 (dt, 1H, $J = 1.4$, $J = 7.5$, H-5 of 2-azidomethylbenzoyl), 7.58(dd, 1H, $J < 1.0$, $J = 7.0$, H-3 of 2-azidomethylbenzoyl), 7.54 (dt, 1H, $J = 1.1$, $J = 7.5$, H-5 of 2-azidomethylbenzoyl), 6.29 (dd, 1H, $J = 6.5$, $J = 8.3$, H-1′), 5.49 (m, 1H, H-3′), 5.26 (t, 1H, $J = 5.2$, 5′-OH), 4.80 (s, 2H, -$\underline{CH_2}$-N_3), 4.20 (m, 1H, H-4′), 3.72 (m, 2H, H-5′$_{a,b}$), 2.43 (m, 2H, H-2′$_{a,b}$), 1.80 (d, 5-CH_3 of Thy); MS (ESI-MS, CH_3CN): Calcd. for $C_{18}H_{19}N_5O_6$ [M-H]$^-$: 400.13. Found: 400.1.

Crystallization of the residue from methanol gave 3.5 g (72% yield) of pure **10c**, m.p 144–147°C; ^{1}H NMR (DMSO-d_6), δ_{TMS}, ppm; J, Hz: 11.28 (s, 1H, 4-NH of Cyt), 8.39 (d, 1H, $J = 7.4$, H-6 of Cyt), 8.00 (m, 3 H, H-6 of 2-azidomethylbenzoyl and Bz), 7.69 (t, 1H, $J = 7.5$, H-5 of 2-azidomethylbenzoyl), 7.63 (t, 1H, $J = 7.4$, Bz), 7.59 (d, 1H, $J = 7.6$, H-3 of 2-azidomethylbenzoyl), 7.55 (t, 1H, $J = 7.6$, H-4 of 2-azidomethylbenzoyl), 7.52 (t, 2H, $J = 7.7$, Bz), 7.41 (d, 1H, H-5 of Cyt), 6.28 (dd, 1H, $J = 6.2$, $J = 7.5$, H-1′), 5.51 (m, 1H, H-3′), 5.29 (t, 1H, $J = 5.2$, 5′-OH), 4.81 (s, 2H, -$\underline{CH_2}$-N_3), 4.35 (m, 1H, H-4′), 3.75 (m, 2H, H-5′$_{a,b}$), 2.70 (m, 1H, H-2′$_b$), 2.39 (m, 1H, H-2′$_a$); MS (ESI-MS, CH_3CN): Calcd. for $C_{24}H_{22}N_6O_6$ [M-H]$^-$: 489.16. Found: 489.2. crystallization of the residue from ethanol gave 3.4 g (67% yield) of pure **10d**, m.p 110–113°C; ^{1}H NMR (DMSO-d_6), δ_{TMS}, ppm; J, Hz: 11.22 (s, 1H, 6-NH of Ade), 8.78 (d, 0.6 H, H-8 of Ade), 8.76 (s, 1H, H-2 of Ade), 8.05 (m, 3H, H-6 of 2-azidomethylbenzoyl and Bz), 7.70 (dt, 1H, $J = 1.4$, $J = 7.6$, H-5 of 2-azidomethylbenzoyl), 7.65 (m, 1H, Bz), 7.61 (dd, 1H, $J<1.0$, $J = 7.6$, H-3 of 2-azidomethylbenzoyl), 7.57 (m, 3H, H-4 of 2-azidomethylbenzoyl and Bz), 6.63 (dd, 1H, $J = 6.0$, $J = 8.4$, H-1′), 5.69 (m, 1H, H-3′), 5.28 (t, 1H, $J = 5.7$, 5′-OH), 4.85 (s, 2H, -$\underline{CH_2}$-N_3), 4.34 (m, 1H, H-4′), 3.74 (m, 2H, H-5′$_{a,b}$), 3.20 (m, 1H, H-2′$_a$), 2.78 (m, 1H, H-2′$_b$); MS (ESI-MS, CH_3CN): Calcd. for $C_{25}H_{22}N_8O_5$ [M-H]$^-$: 513.17. Found: 513.2.

Purification of the residue on a silica gel, using a gradient of methanol in methylene chloride from 0 to 5% gave 3.22 g (65% yield) of pure **10b** as foam; ^{1}H NMR (DMSO-d_6), δ_{TMS}, ppm; J, Hz: 12.08 (s, 1H, 1-NH of Gua), 11.62 (s, 1H, 2-NH of Gua), 8.32 (s, 1H, H-2 of Gua), 8.05 (dd, 1H, $J = 1.0$, $J = 7.7$, H-6 of 2-azidomethylbenzoyl), 7.70 (dt, 1H, $J = 1.2$, $J = 7.5$, H-5 of 2-azidomethylbenzoyl), 7.60 (d, 1H, $J = 7.6$, H-3 of 2-azidomethylbenzoyl), 7.56 (dt, 1H, $J<1.0$, $J = 7.6$, H-4 of 2-azidomethylbenzoyl), 6.60 (dd, 1H, $J = 5.7$, $J = 8.9$, H-1′), 5.59 (m, 1H, H-3′), 5.19 (t, 1H, $J = 5.5$, 5′-OH), 4.82 (s, 2H, -$\underline{CH_2}$-N_3), 4.27 (m, 1H, H-4′), 3.74 (t, 2H, $J = 4.8$, H-5′$_{a,b}$), 2.99 (m,

1H, H-2′$_a$), 2.75 (m, 1H, $J = 6.9$, CH of *i*bu), 2.69 (m, 1H, H-2′$_b$), 1.11 (d, 6 H, $J = 6.8$, 2CH_3 of *i*bu); MS (ESI-MS, CH_3CN): Calcd. for $C_{22}H_{24}N_8O_6$ [M-H]$^-$: 495.18. Found: 495.2.

Triethylammonium Salts of 5′-*O*-dimethoxytrityl-*N*-acyl-2′-deoxynucleoside-3′-*O*-(2-chlorophenyl)phosphates and 5′-*O*-dimethoxytritylthymidine-3′-*O*-(2-chlorophenyl)phosphate (P-Components). General Procedure

A solution of 2-chlorophenyldichlorophospate (8 ml, 49 mmol) in dichloromethane (40 ml) was added to a cold (0°C) mixture of triazole (8 g, 116 mmol), pyridine (20 ml, 250 mmol) and dichloromethane (80 ml). A solution of 5′-*O*-dimethoxytrityl-*N*-acyl-2′-deoxynucleoside or 5′-*O*-dimethoxytritylthymidine (30 mmol) in dichloromethane (100 ml) was added dropwise after 30 minutes stirring and the resulting mixture was stirred for additional 2 hours at 0°C. A 1 M triethylammonium hydrogen carbonate (80 ml) was added to the reaction mixture. The organic layer was separated, washed additionally with 1M triethylammonium hydrogen carbonate (3 × 70 ml) and dried over sodium sulfate. The solution was evaporated, co-evaporated with toluene (2 × 80 ml) to give pure P-components as white foams in nearly quantitative yield. These foams were used for preparation of di- and trinucleotides without further purification.

5′-*O*-Dimethoxytrityl-3′-*O*-(acyl) trinucleotides (17–19). General Procedure

A mixture of 3′-acyl-2′-deoxynucleoside **8a–d**, **9a–d,** or **10a–d** (15 mmol) and P-component (20 mmol) was dissolved in dry pyridine (150 ml). MSNT (12.4 g, 42 mmol) was added in portions with stirring. The reaction mixture was left for 1 hour at rt and quenched with water. The mixture was evaporated to dryness, co-evaporated with toluene (2 × 100 ml) and dissolved in dichloromethane (150 ml). The organic solution was washed with aqueous 5% oxalic acid until pH<3, dried over sodium sulfate and evaporated to dryness. A 1% solution of 4-toluenesulphonic acid in a mixture of dichloromethane—methanol (7:3, 150 ml) was added to the oily residue with vigorous stirring. After 10 minutes at rt, the acidic reaction mixture was neutralized by the addition of saturated sodium hydrogen carbonate (50 ml). The organic layer was separated, additionally washed with saturated sodium hydrogen carbonate (50 ml), dried over sodium sulfate and evaporated to dryness. purification of the residue on a silica gel, using a gradient of methanol in dichloromethane from 0 to 7 gave pure **14–16** as foams in 70–80% overall yield.

A mixture of 3′-*O*-acyl dinucleotides **14–16** (15 mmol) and P-component (20 mmol) was dissolved in dry pyridine (150 ml). MSNT (12.4 g, 42 mmol) was added in portions with stirring. The reaction mixture was left for 1

hour at rt and quenched with 1 M triethylammonium hydrogen carbonate buffer, pH 7.8 (TEAB). The reaction mixture was concentrated in vacuum, the residue was co-evaporated with toluene (2 × 100 ml), dissolved in dichloromethane (150 ml). The dichloromethane solution was washed with saturated hydrogen sodium carbonate (2 × 100 ml), dried over sodium sulfate and evaporated to dryness. Purification of the residue on a silica gel, using a gradient of methanol in dichloromethane from 0 to 7 gave pure **17–19** as foams in 80–90% yield. These foams were used for preparation of trinucleotides without further purification and characterization.

5′-*O*-Dimethoxytrityltrinucleotides (5)

Removal of 3′-O-(2,4-Dichlorophenoxy)acetyl Group. General Procedure

Fully protected trinucleotide **17** (5 mmol) was dissolved in a mixture of 100 ml pyridine, 50 ml MeOH, and 17 ml triethylamine. In 3 hours the reaction mixture was evaporated to a gum, the residue was co-evaporated with toluene (2 × 100 ml) and with chloroform (2 × 100 ml). The residue was dissolved in 200 ml chloroform, washed with saturated aqueous sodium hydrogen carbonate (3 × 100 ml), 0.1 M TEAB (2 × 100 ml), dried over sodium sulfate and concentrated. The traces of pyridine were removed by co-evaporation with toluene (3 × 50 ml), the residual gum was dissolved in chloroform and concentrated to a minimal volume. The desired products **5** were isolated by column chromatography on silica gel. Yields 65–85%. These trinucleotides were used for preparation of trinucleotides phosphoramidites **7** without further purification and characterization.

Removal of 4-Azidobutyryl Group. General Procedure

Fully protected trinucleotide **18** (10 g) was dissolved in a solution of triphenylphosphine (2.5 g, 9.5 mmol) in a mixture of dioxane (200 ml) and water (20 ml) and heated at 50–55°C for 7 hours. The solution was evaporated to dryness. Purification of the residue on a silica gel, using a gradient of methanol in dichloromethane from 0 to 7% gave pure trimers **5** as foams in 80–90% yield. These trinucleotides were used for preparation of trinucleotides phosphoramidites **7** without characterization.

Removal of 2-Azidomethylbenzoyl Group. General Procedure

Fully protected trinucleotide **19** (10 g) was dissolved in a solution of triphenylphosphine (2.5 g, 9.5 mmol) in a mixture of dioxane (200 ml) and water (20 ml) and left overnight at rt. The solution was evaporated to dryness. purification of the residue on a silica gel, using a gradient of methanol in dichloromethane (0–7%) gave pure trimers **5** as foams in 85–95% yield. These trinucleotides were used for preparation of trinucleotides phosphoramidites **7** without characterization.

Trinucleotide 3′-Phosphoroamidites 7. General Procedure

Trinucleotide **5** (5 mmol) was dissolved in a mixture of dry dichloromethane (22 ml), dry acetonitrile (7 ml) and 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphorodiamidite (4.75 ml, 4.5 g, 15 mmol). A 0.25M solution of 5-ethylthio-1H-tetrazole (4 ml, 1 mmol) was added and the mixture was stirred at rt overnight. The reaction was quenched by addition of saturated sodium hydrogen carbonate (20 ml). The organic layer was separated, dried over sodium sulfate, and evaporated to dryness. The residue was vigorously shaken with n-hexane (20 ml), the hexane extract was decanted and discarded. The residue was dried in vacuum, dissolved in acetonitrile (30 ml). Water (30 ml) was added to the acetonitrile solution and the resulting emulsion was applied onto the Lichroprep RP-18 column (4 × 19 cm), equilibrated with 50% aqueous acetonitrile. Gradient elution of acetonitrile in water (50–100%) afforded pure trimer phosphoramidites **7** as foams in 87–93% yield. All 20 Trimer phosphoramidites were characterized as described above. Their structure was confirmed as reported earlier.[5]

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