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COMMUNICATION

A new and convenient approach for the preparation of β-cyanoethyl protected trinucleotide phosphoramidites†

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Herein we report a convenient approach for the preparation of fully protected trinucleotide synthons to be used for the synthesis of gene libraries. The trinucleotide synthons bear β-cyanoethyl groups at the phosphate residues, and thus can be used in standard oligonucleotide synthesis without additional steps for deprotection and work-up.

Over the past two decades, protein studies at the molecular and submolecular level have greatly gained momentum, with the focus more recently having shifted from analysis of structure–function relationship to synthesis, *i.e.* the generation of proteins, especially enzymes, with pre-deliberated, novel properties.[1] The latter development was critically facilitated by newly emerging methods of combinatorial and evolutionary protein engineering which combine random mutagenesis or combinatorial gene synthesis with functional screening or genetic selection applied at the phenotype level to an ensemble ("library") of many structural variants generated in parallel.[2,3]

Combinatorial gene synthesis offers the highly attractive option of restricting structural variation to residues that are not merely part of the structural scaffold of the protein studied, but can, instead, be expected to be directly involved in functions such as catalysis or others. Among a host of related methods,[4–7] the use of mixtures of pre-formed trinucleotide blocks representing codons for the 20 canonical amino acids stands out as allowing fully controlled partial (or total) randomization individually at any number of arbitrarily chosen codon positions of a given gene.[8–17] Hence, the accessibility of this uniquely flexible experimental regime has created substantial demand of fully protected trinucleotide synthons of good reactivity in standard oligonucleotide synthesis. Such synthons, however, are not easy to come by. Procedures reported in the literature so far,[8–17] suffer from one or more limitations. We have developed a new method that gives easy access to trinucleotide phosphoramidites capable of undergoing coupling reactions by the solid phase phosphoramidite approach, and allowing for oligonucleotide deprotection and cleavage from support in only one step.‡ In particular, our method is characterized by the use of a pair of orthogonal protecting groups for the 5′- and 3′-hydroxyl functions, and the standard β-cyanoethyl group for protection of the phosphate moiety. Thus, side products due to competition between the 5′- and 3′-OH group during trinucleotide synthesis is prevented, and deprotection of the synthesized oligomers is achieved under mild conditions, simultaneously with cleavage from the solid support, and without application of additional protocols as required for removal of the *ortho*-chlorophenyl group[11–12,15,17] or alkyl groups[9,16] from the phosphate.

The synthesis strategy is outlined in Scheme 1. An *N*-acyl-5′-dimethoxytrityl (DMT) protected nucleoside-3′-*O*-phosphoramidite is coupled to an *N*-acyl-3′-*O*-*tert*-butyldimethylsilyl (TBDMS) protected nucleoside under standard conditions of phosphoramidite chemistry in solution (for experimental details see the Supplementary Information†). The resulting dinucleotide can be extended in both the 3′- and 5′-direction. In the present study, we decided to remove the DMT group from the 5′-hydroxyl function followed by coupling of the 5′-deprotected dinucleotide to an *N*-acyl-5′-*O*-DMT protected nucleoside phosphoramidite, to give the fully protected trinucleotide (Supplementary Information†).

With 5′-DMT being the group of choice for solid phase DNA synthesis, the search for a suitable 3′-*O*-protecting group becomes a key feature of the procedure. Virnekäs *et al.* have used phenoxyacetyl (PAC) for protection of the 3′-OH group.[9] Removal of the PAC group requires a nucleophilic reagent, usually hydroxyl ions. Therefore, the β-cyanoethyl group used for protection of the phosphate moiety in standard DNA synthesis was replaced with the more stable methyl group. However, the methyl group needs to be cleaved off with thiophenol or another thiolating agent, requiring an additional step in oligonucleotide workup. Furthermore, in the mentioned study, the PAC group was removed with methanolic ammonia, conditions that are known to give rise to breakage of internucleotidic phosphotriester linkages.[18]

A number of reports describe the synthesis of trinucleotides by phosphotriester chemistry with *o*-chlorophenyl as the phosphate protecting group.[11–17] Here, the levulinyl group[13] or the azidomethylbenzoyl group[17] has been used for 3′-OH protection,

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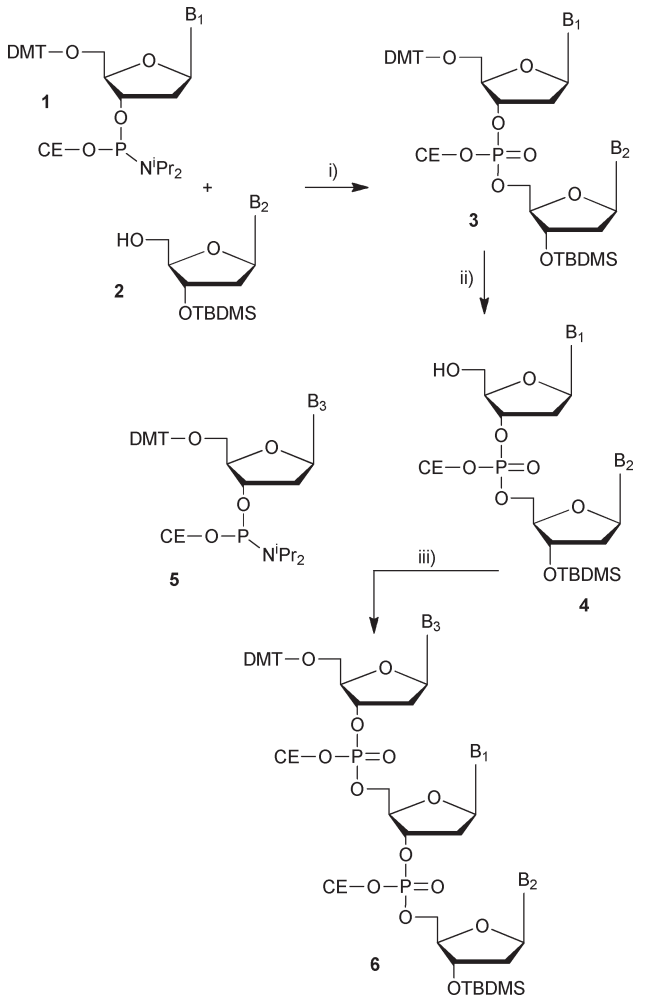


Scheme 1 Synthesis of fully protected trinucleotides: i) **1** (1 mmol), **2** (0.8 mmol), MeCN, tetrazole (10 mmol), 30 min, RT, iodine solution (0.1 M in lutidine/THF/water 1 : 2 : 1); ii) DCM with 3% trichloroacetic acid (1.2 mmol); iii) **4** (1 mmol), **5** (1.2 mmol), MeCN, tetrazol (10 mmol), 1 h, iodine solution (0.1 M in lutidine/THF/water 1 : 2 : 1).

or the 3′-hydroxyl function was left completely unprotected.[11,12,15] The latter strategy seems very convenient, and focuses on selective reaction of an activated nucleotide with the primary alcohol. However, not surprisingly, the authors mentioned the observation of by-products such as isomeric dimers and trimers.[17] This hampered all following purification steps and if incorporated into gene libraries, frame shifts and unexpected mutations occurred. Thus, protection of the 3′-OH group is strongly required to avoid unwanted by-products in intermediate and final products.

One of the first reports on the synthesis of trinucleotides describes application of standard phosphite triester chemistry in solution using DMT for 5′-OH protection, β-cyanoethyl for protection of the phosphite and *tert*-butyldimethylsilyl (TBDMS) for 3′-OH protection.[10] The TBDMS group can easily be removed by treatment with fluoride ions. This reaction however, was observed being accompanied by cleavage of phosphotriester bonds.[13] As an alternative, Lyttle *et al.*[10] used 6 N hydrochloric acid for removal of TBDMS, conditions that are rather harsh and known to cause depurination. Furthermore, the 5′-DMT group is

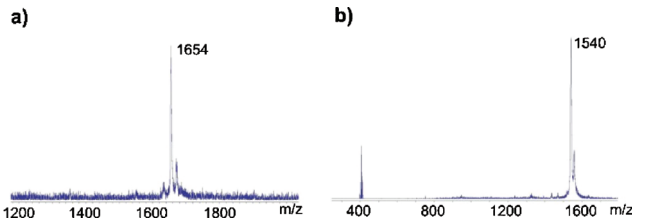
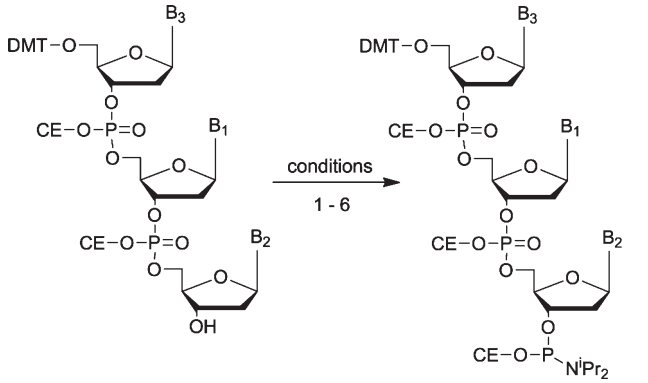


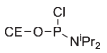
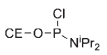
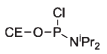
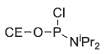
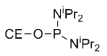
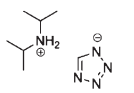
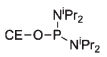
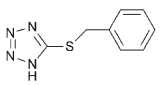
Fig. 1 MALDI-spectra of a) fully protected and b) 3′-OH-Trinucleotide CAC.

lost simultaneously, such that the free 5′-and 3′-OH functions compete in the coupling reaction. Accordingly, yields of the synthesized trinucleotides were rather low, and formation of by-products was a strong hurdle.[10] We have reinvestigated removal of the TBDMS group from di- and trinucleotides using fluoride ions. Traditionally, tetra-*n*-butylammonium fluoride (TBAF) has been used as fluoride ion source. Under these conditions, indeed, we observed a mixture of products, very likely resulting from cleavage of the phosphotriester bonds (not shown). Alternatively, we applied TEAx3HF, a reagent that is used in modern RNA chemistry for removal of the 2′-*O*-TBDMS group.[19] TEAx3HF is a milder reagent compared to TBAF, allowing for specific cleavage of the TBDMS group without attacking the phosphotriester bond. In liquid phase, HF forms long zigzag chains with linear F–H···F– units. Triethyl amine breaks off the structure and forms the ionic compound $HN(Et)_3^+$ $^-F\cdots(H–F)_x$, in which F^- based on its aggregated structure is a much softer nucleophile.[20] Thus, upon treatment of the protected trinucleotide with TEAx3HF, one main product was generated. Mass spectrometric analysis confirmed the nature of the product being the intact trinucleotide (Fig. 1, ESI Table S2†).

Next to the choice of an appropriate 3′-*O*-protecting group, a second important issue is the nature of the phosphate protecting group. It would be most desirable using the standard β-cyanoethyl group in the trinucleotide synthons in order to avoid additional steps during oligonucleotide work-up. As mentioned above, most protocols for the synthesis of trinucleotides use phosphotriester chemistry with *o*-chlorophenyl as the standard phosphate protecting group. Application of phosphite triester chemistry has been combined with methyl or ethyl groups for phosphite protection. In all cases, special deprotection protocols are required. We have studied the suitability of the β-cyanoethyl group for phosphate protection in trinucleotide synthesis, in particular looking at survival of the β-cyanoethyl group under the conditions used for the individual steps of trinucleotide preparation. The main problem is the strong sensitivity of the β-cyanoethyl group to basic conditions. Due to rather strong CH-acidity, β-cyanoethyl is easily removed by β-elimination. However, the acidity is only high if the neighbouring phosphorous is oxidized (P^V). As long as the phosphorus appears in the P^{III} state, the β-cyanoethyl group is stable, even in the presence of rather strong bases such as triethyl amine (TEA) or diisopropylethyl amine (DIPEA).[21] Thus, the first coupling reaction of the *N*-acyl-5′-dimethoxytrityl (DMT) protected nucleoside-3′-*O*-phosphoramidite to the 5′-OH group of the *N*-acyl-3′-*O*-*tert*-butymdimethylsilyl (TBDMS) protected nucleoside as outlined in Scheme 1 proceeds under standard conditions of phosphoramidite chemistry in solution without loss of the β-cyanoethyl

Table 1 Different conditions for 3′-phosphorylation of trinucleotides



Entry	Phosphorylation reagent	Base	Activator	Yield[a]
1		DIPEA	—	n. d.
2		Pyridine	—	n. d.
3		Lutidine	—	n. d.
4		Collidine	—	n. d.
5		—		n. d.
6		—		quantitative

[a] n. d.: not detectable

group. After oxidation of the phosphorous, however, it is crucial to avoid contamination with strong bases. Cleavage of the TBDMS group with TEAx3HF as described above is not problematic, since the reagent is weakly acidic.[22] According to the protocol used for 2′-*O*-deprotection of RNA, the reaction is quenched by addition of water. This is not possible here, since hydrolysis of the reagent results in a strongly acidic solution, which would cleave off the DMT group. Therefore, we quenched the reaction by addition of saturated bicarbonate solution with stringent control of the pH, in order to keep neutral conditions that leave the DMT group as well as the phosphate β-cyanoethyl group intact. Following this procedure, 20 trinucleotides of high purity were prepared (see ESI, Table S2†). The crucial step is the following preparation of the trinucleotide phosphoramidite (Table 1). We have evaluated two different ways using either 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite or 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (Table 1). Using 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite requires the presence of DIPEA functioning as scavenger for HCl, that is generated upon reaction. As expected, preparation of phosphoramidites following this protocol led to removal of the β-cyanoethyl group, as analyzed by TLC. Our attempts to replace DIPEA with less strong bases such as pyridine, lutidine or collidine (Table 1) failed. In these cases, no formation of the phosphoramidite could be observed. The alternative reagent 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite requires activation by tetrazole derivatives, and the reaction is not dependent on the presence of strong bases. According to the original protocol,[23] diisopropylammonium tetrazolide is used for activation of 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite. Preparation of trinucleotide phosphoramidites following this protocol, however, was again accompanied by significant loss of the β-cyanoethyl group. Reaction of one equivalent of the tetrazolide with one equivalent of the nucleotide produces two equivalents diisopropylamine, which obviously is strong enough to initiate β-elimination of the β-cyanoethyl group. In an attempt to circumvent this problem, we replaced the tetrazolide with benzylmercapto tetrazole (Table 1). This reaction produces only one equivalent of diisopropyl amine, which is neutralized by the benzylmercaptotetrazole released back after reaction. Since benzylmercaptotatrazole is more strongly acidic than normal tetrazole or ethylmercaptotetrazole,[24] it acts as a perfect scavenger for diisopropyl amine converting it into the ammonium salt. Under these conditions, preparation of phosphoramidites was most successful; only traces of by-products could be detected. Following this protocol, trinucleotide phosphoramidite synthons representing codons for all 20 amino acids were prepared. All trinucleotides were successfully incorporated in short test oligonucleotides (see ESI, Table S3†).

The trinucleotide phosphoramidites presented here, are advantageous over previously described trinucleotide synthons in terms of quality and application. Due to the choice of a superior 3′-*O*-protecting group and their mild and efficient removal, no by-products or isomeric di- and trimers were observed. Furthermore, using β-cyanoethyl as phosphate protecting group allows for easy application of the trinucleotide phosphoramidites in standard DNA synthesis without the need for specific protocols during work-up and deprotection.

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