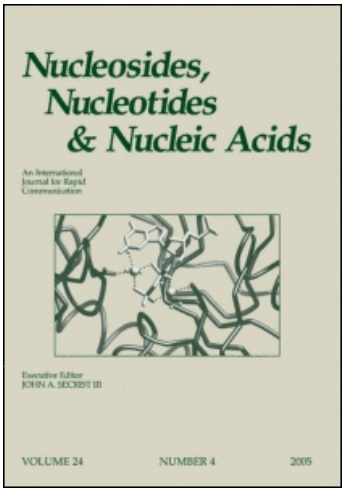

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Large-Scale Solid-Phase Preparation of 3'-Unprotected Trinucleotide Phosphotriesters - Precursors for Synthesis of Trinucleotide Phosphoramidites

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LARGE-SCALE SOLID-PHASE PREPARATION OF 3'-UNPROTECTED TRINUCLEOTIDE PHOSPHOTRIESTERS - PRECURSORS FOR SYNTHESIS OF TRINUCLEOTIDE PHOSPHORAMIDITES

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ABSTRACT: The approach to large-scale solid-phase synthesis of 3'-unprotected trinucleotide phosphotriesters has been developed. The trinucleotides have been synthesized in 5 g scale by phosphotriester approach using CPG with pore size 70A. Total yield of target products was 75-90%. The molar extinctions of trinucleotides at various wavelengths were calculated; the experimental UV-spectra of trinucleotides show a good agreement with theoretical ones. The trinucleotides synthesized were used for synthesis of trinucleotide phosphoramidites - synthons for generation of DNA/peptide libraries.

INTRODUCTION

The DNA and peptide libraries are widely used now as powerful tool in various investigations (see, for instance, [1]). The favored method for preparing of those libraries is the oligonucleotide-directed mutagenesis. The only method, that allows introduction of desired subset of codons into a defined position, is the synthesis of oligonucleotide mixture using trinucleotide synthons instead of traditional monomers. We have recently used such an approach for synthesis of several libraries of high complexity and controlled diversity [2]

One of the problems arising in the course of preparation of those synthons is high-yield synthesis of their precursors - 3'-unprotected trinucleotide phosphotriesters **4** (FIG. 1). The molecule of those trinucleotides contains both acid and base labile protective groups, so to find out the suitable 3'-protective group, that can be removed selectively, is rather difficult. If 3'-hydroxyl group remains unprotected, some quantity of 3'-3'-isomer is always generated, and total yield of the desirable product is decreased.

We report here another approach to the synthesis of 3'-unprotected trinucleotide phosphotriesters - the large-scale solid-phase procedure. This technique is being intensively developed now (see, for instance, [3-5]), but the main goal of those investigations was the large-scale synthesis of fully deprotected oligonucleotides. The solid-phase synthesis of partially protected trinucleotide triesters like **4** was not described yet (at least, we did not find the corresponding references.)

The applicability of the solid-phase approach to the synthesis of partially protected trimers was illustrated by synthesis of 6 trinucleotide phosphotriesters in 5 g scale with high yield. Those triesters were converted into corresponding phosphoramidites and successfully used in conventional oligonucleotide synthesis. We also developed procedures for recovering of nucleoside/nucleotide components that are used in excess during synthesis and for regeneration of a support.

RESULTS AND DISCUSSION

One of crucial points in any solid-phase synthesis is a choice of the support. We decided to take a support that allows the highest loading. In addition, we had to bear in mind the possibility of regeneration of support; otherwise, the large-scale synthesis would be too expensive.

The most popular support for oligonucleotide synthesis - CPG with 500-1000A pore size - was not acceptable for us because of relatively low loading (30-40 μmol per gram).

First, we have tried to use a polysterene-divinylbenzene copolymer (2% of DVB). We used as a starting material a chloromethylated resin (1 mmol Cl per gram). We succeeded to load up to 400 μmol/g of NH_2-groups using 6-aminocaproyl linker (it was loaded by treatment of the support by BOC-6-aminocaproic acid in presence of triethylamine followed by removing of BOC-group by 50% trifluoroacetic acid in dichloromethane), but the loading of nucleoside (through oxalyl anchor [6]) was low (70-80 μmol/g). Nevertheless, we have performed a synthesis of trinucleotide on this support and found that it is not suitable for large-scale synthesis. The main disadvantage of this support is a problem with washing of support by different solvents and solutions. Because of differences in swelling in different solvents it is impossible to remove traces of the previous solution by reasonable volume of a solvent.

Therefore, we decided to use a CPG for our synthesis. We have tried CPGs with various porosity and found that CPG with pore size 70A is quite suitable for our goals. The loading of nucleoside was 120-130 μmol/g.

As an anchor group, we used the oxalyl residue, suggested by Letsinger [6]. We have found that phosphate protective groups (o-chlorophenyl) are stable to treatment with 5% solution of 25% aqueous ammonia in methanol for at least 40 min, while full removing of trinucleotide from support proceeds within nearly 20 min.

The synthesis itself was performed by traditional phosphotriester technique (FIG. 1). We used a mixture of 1-methylimidazole and 2,4,6-triisopropylbenzenesulphonyl-chloride as a condensing reagent [7, 8]. The reaction proceeds with high yield, and the P-components can be easily regenerated.

We used a solution of trifluoroacetic acid in acetonitrile for removing of 5'-dimethoxytrityl residue during synthesis. The solution of trifluoroacetic acid in dichloromethane is too active for large-scale solid-phase synthesis - it is impossible to keep the duration of acid treatment exactly.

The target trinucleotide **4** was removed from support by diluted solution of ammonia in methanol. It should be noted that the oxalyl anchor is sensitive to solution of pyridine in methanol - the trinucleotide can be released from the support by treatment with 20% pyridine in methanol during 12 hours.

The purity of trinucleotides synthesized was checked by HPLC, while the composition was confirmed by comparison of UV-spectra of samples with calculated ones. We have measured the molar extinctions of 5'-O-dimethoxytrityl-N-protected nucleosides and N-protected ones solutions in methanol at several wavelengths (shown in TABLE 1). The molar extinction of trinucleotide was then calculated by simple addition of component extinctions at given wavelength (the contribution of o-chlorophenyl was neglected) and compared with UV-spectrum of trinucleotide solution in methanol. A parameter R that presents a ratio of measured absorbance at some wavelength to the calculated extinction at this wavelength was used for the comparison. Theoretically, it must be the same for all wavelengths for given trinucleotide.

The results of the comparison for some trinucleotides are presented in TABLE 2. Additionally the identity of trinucleotides was confirmed by comparison of their HPLC retention times with those obtained for trinucleotides synthesized earlier [9]. Typical results

TABLE 1. Molar extinctions for 5'-O-dimethoxytrityl-N-protected and N-protected nucleosides

λ (nm)	ε ($mol^{-1}cm^{-1}$)							
	DTrbzA	bzA	DTrbzC	bzC	DTribG	ibG	DTrT	T
230	40800	10530	32080	14270	20910	7500	17660	6990
240	31500	8700	27970	13340	21050	8800	15930	5050
250	17370	6920	20620	16940	17160	13630	9730	6780
260	14350	5770	20190	19680	16150	14920	9300	9300
270	16730	6920	15140	13630	12980	11170	9730	9690
280	19100	9660	9300	7780	12620	11030	7140	6340
290	13550	9730	7210	7430	9730	9370	2090	2090
300	6340	6780	8440	8580	5410	5340	220	360
310	2310	2310	8070	8070	1300	1300	0	0
320	1660	430	5050	4760	140	140	0	0

TABLE 2. The results of comparison of calculated and experimental spectra of trinucleotides. λ - wavelength (nm), ε - the caculated extinctions, A - optical density (AU/ml), R - (A/ε)*10000. **A** - trinucleotide ATC, **B** - TAC, **C** - GAC, **D** - TCT, **E** - CAT, **F** - TGG.

A.

λ	230	240	250	260	270	280	290	300	310	320
ε	62060	49890	41090	43330	40050	33220	23070	15280	10380	6420
A	0.92	0.84	0.82	0.97	0.94	0.72	0.46	0.29	0.19	0.11
R	0.15	0.17	0.19	0.22	0.23	0.22	0.21	0.19	0.19	0.18

B.

λ	230	240	250	260	270	280	290	300	310	320
ε	42460	37970	33590	34750	30280	24580	19250	15580	10380	5190
A	1.24	1.15	1.07	1.23	1.18	0.96	0.68	0.47	0.32	0.19
R	0.29	0.30	0.32	0 35	0.39	0.37	0.36	0.30	0.31	0.36

C.

λ	230	240	250	260	270	280	290	300	310	320
ε	45710	43090	41020	41600	33530	30060	26890	20770	11680	5330
A	1.42	1.25	1.35	1.21	0.94	0.81	0.88	0.62	0.33	0 14
R	0.31	0.29	0.33	0.29	0.28	0.27	0.33	0.30	0.28	0.26

D.

λ	230	240	250	260	270	280	290	300	310	320
ε	38920	34320	33450	38280	33050	21260	11610	9160	8070	4760
A	0.66	0.58	0.60	0.69	0.56	0.36	0.17	0.14	0.12	0.07
R	0.17	0.17	0.18	0.18	0.17	0.17	0.15	0.15	0.15	0.14

E.

λ	230	240	250	260	270	280	290	300	310	320
ε	49600	41720	34320	35260	31750	25300	19030	15580	10380	5480
A	1.05	1.04	0.79	0.74	0.67	0.61	0.38	0.33	0.20	0.11
R	0.21	0.25	0.23	0.21	0.21	0.24	0.20	0.21	0.19	0.20

F.

λ	230	240	250	260	270	280	290	300	310	320
ε	32660	33530	36990	39140	32070	29200	20830	10900	2600	280
A	1.21	1.17	1.18	1.34	1.15	1.02	0.69	0.37	0.08	0.01
R	0.37	0.35	0.32	0.34	0.36	0.35	0.33	0.34	0.31	0.36

of this analysis (trinucleotide TCT) are presented on FIG. 1. The chromatogram **a** is the chromatogram of trinucleotide synthesized in solution [9], the chromatogram **b** is the chromatogram of trinucleotide synthesized by solid-phase method, and the chromatogram **c** was resulted after chromatography of a mixture of those substances. It can be seen that HPLC properties of all four diastereomers for both trinucleotides are absolutely identical. The same results were obtained for other trinucleotides.

The trinucleotides synthesized earlier were fully characterized by ^{1}H-NMR- and mass-spectra, and by analysis of their nucleoside/nucleotide composition. We believe that the full coincidence of spectral and chromatographic properties of newly synthesized trinucleotides with old ones is an exhaustive evidence of their identity.

The trinucleotide phosphoramidites were synthesized, isolated, and tested exactly as in [9].

All starting materials (nucleosides, nucleotides, and CPG) can be regenerated and successfully used in other syntheses. CPG was regenerated by treatment with 25% aqueous ammonia (20 hours), followed by washing by ethanol and drying. The aminogroup and nucleoside were loaded as usual; loading of nucleoside was 110-120 μmol/g. The regeneration procedure for nucleosides and nucleotides is described in the ***Experimental*** section.

Two trinucleotides - TAC and GAC - have been synthesized using the recovered materials. The recovered CPG and the native nucleoside and nucleotides were used for the synthesis of the first one with overall yield 84%. The trinucleotide GAC has been prepared using regenerated materials only - the yield of the final product was 79%.

We believe that our approach to the synthesis of 3'-unprotected trinucleotide phosphotriesters has some advantages to the synthesis in solution. First, the total yield of the target product is higher, second, the synthesis proceeds faster (a trinucleotide can be synthesized in one day), third, it is less expensive (all starting materials can be regenerated). In our opinion, this technique may be applied to the synthesis of other compounds that contain both acid- and base-labile groups.

EXPERIMENTAL

General. Base-protected dimethoxytrityl nucleosides were obtained from "Vostok Ltd." (Russia). Synthesis of 5'-O-dimethoxytrityl-N-acylnucleoside-3'-O-(2-chloro-

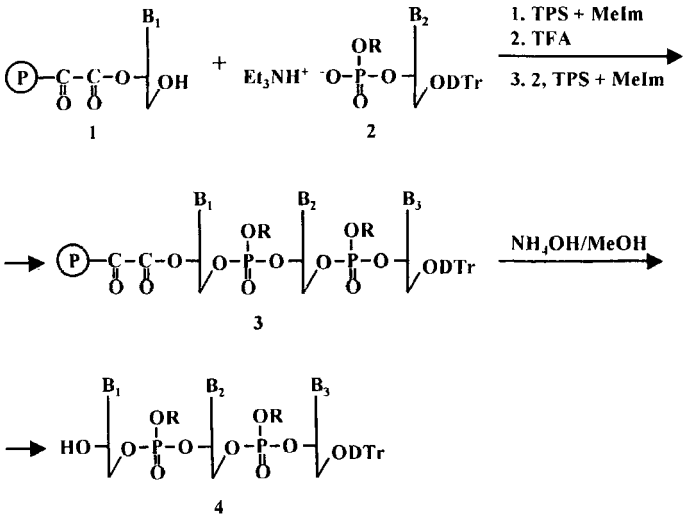

FIG. 1. The synthetic route of 3'-unprotected trinucleotide phosphotriesters. **P** – aminoderivatized CPG, B_1, B_2, B_3 – Thy or N-protected Ade, Cyt, or Gua, R – o-chlorophenyl.

phenyl)phosphates **2** was described earlier [9]. Controlled pore glass (CPG) was purchased from Serva (CPG-10, 120-200 mesh, pore size 70A). 3-Aminopropyltriethoxysilane and trimethylchlorosilane (Fluka) were distilled immediately before using. Triazole (Fluka) was recrystallized from toluene. 2,4,6-Triisopropylbenzenesulphonylchloride (TPS) (Fluka) was recrystallized from pentane. 1-Methylimidazole (MeIm) (Fluka) and pyridine were distilled over calcium hydride and stored over molecular sieves (4A, Serva). The HPLC was performed on a Gilson system (models 305 and 303) using the Kratos UV-detector Spectroflow 757. Zorbax-ODS column 4.6x250mm (DuPont Instruments) was used for HPLC-analysis of preparations (gradient 75%-100% acetonitrile in water, 60 min, flow rate 1 ml/min, detection at 280 nm).

1. Derivatization of CPG. 3-Aminopropyltriethoxysilane (33 ml) was added to suspension of 100 g CPG in approx. 100 ml of anhydrous ethanol. After 5 days the support was filtered, washed by methanol (3x100 ml), dried under vacuum at room temperature, and heated for 4 hours at 120°C. Nearly 100 ml of pyridine and 17 ml of trimethylchlorosilane were added and the suspension was left overnight. The support was filtered, washed by pyridine (3x100 ml), and treated by 200 ml of 50% aqueous pyridine. After 30 min the support was filtered, treated by 200 ml of 15% triethylamine in pyridine

(30 min), filtered, washed by 50% aqueous pyridine (3x100 ml), methanol (3x100 ml), and dried under vacuum. The loading was estimated with help of picrate test; the loading was 440-460 μmol of aminogroups per gram of CPG.

2. Synthesis of trinucleotides 4 (general procedure).

a. Attachment of nucleoside to CPG. 1,2,4-Triazole (26.7 g, 387 mmol) was dissolved in mixture of 400 ml acetonitrile and 80 ml pyridine. Oxalyl chloride (7.3 ml, 85 mmol) was added dropwise under cooling in ice. The solution of 5'-O-dimethoxytrityl-N-acylnucleoside (90 mmol) in approx. 50 ml pyridine (nucleoside was preliminarily twice co-evaporated with pyridine) was added. After 20 min aminoderivatized CPG (45 mmol NH_2-groups, nearly 100 g) was added, and the solvent was evaporated to nearly 100 ml. After 45 min the support was filtered, washed by pyridine (2x200 ml), and suspended in approx. 100 ml pyridine. Acetic anhydride and 1-methylimidazole (10 ml each) were added to the suspension, and the mixture was incubated for 45 min. The support was filtered, washed by pyridine (2x100 ml), methanol (4x100 ml) and dried *in vacuo*. The loading of nucleoside was 120-13 μmol/g (determined by measuring of dimethoxytrityl amount, released after treatment of aliquot by mixture of 70% $HClO_4$-MeOH, 1:1).

b. Phosphotriester synthesis of trinucleotides. 5'-O-dimethoxytrityl-N-acylnucleoside-CPG **1** (9 mmol of nucleoside, 70-80 g of support, depending on loading) was suspended in acetonitrile and placed into column. The column was washed by acetonitrile (nearly 100 ml) followed by 5% solution of trifluoroacetic acid in acetonitrile. The duration of acid treatment depends on the terminal nucleoside - 15 min for A, 20 min for C, 5 min for G, and 30 min for T. The column was then washed by acetonitrile, the washings were pooled, and the total amount of dimethoxytrityl cation was measured (ε_{500} 66240, ε_{410} 27360).

The corresponding 5'-O-dimethoxytrityl-N-acylnucleoside-3'-O-(2-chlorophenyl)phosphate **2** (3-fold excess relatively to free nucleoside hydroxyls) was twice co-evaporated with pyridine. 2,4,6-Triisopropylbenzenesulphonylchloride (2.5-fold excess relatively to phosphate) was added, and the solution was evaporated to 2-fold dead volume of the column (dead volume of 10 g of CPG - 11.5 ml). The solution was divided in two equal portions, 1.1 equivalent (relatively to TPS) of 1-methylimidazole was added to one of them, and the mixture was immediately applied to the column. After 20 min, the same quantity of 1-methylimidazole was added to the second portion, and the mixture

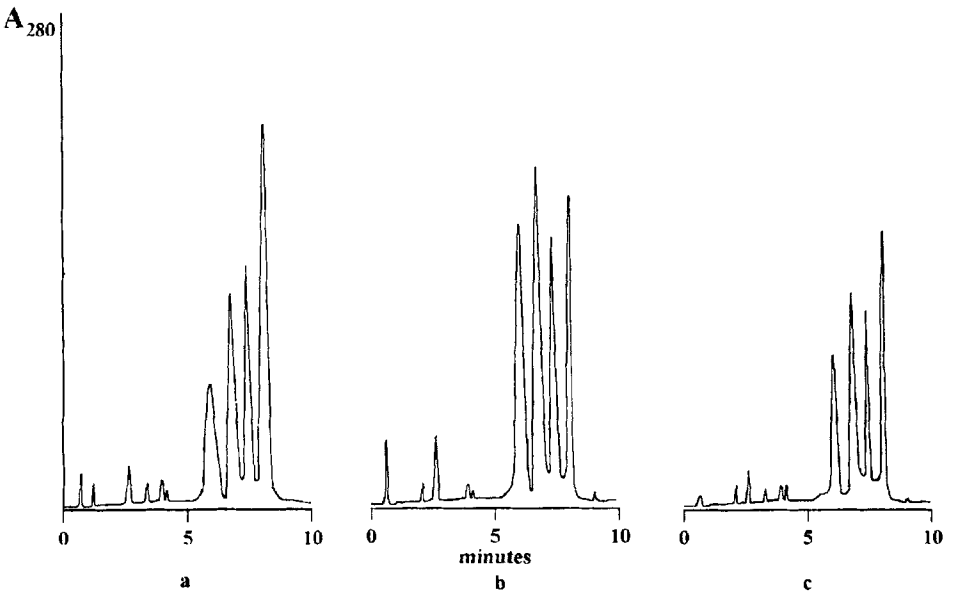

FIG. 2. HPLC-chromatograms of trinucleotide TCT. **a** - trinucleotide synthesized earlier [9], **b** - newly synthesized trinucleotide, **c** - a mixture of those substances See text for details.

was applied to the column too. After 20 min the column was washed by pyridine, the solid phase was treated by solution of acetic anhydride and 1-methylimidazole (2 ml each) in 50 ml of pyridine (30 min) and washed by pyridine and acetonitrile (nearly 200 ml each). The 5'-O-dimethoxytrityl residue was then removed as described earlier.

The addition of the next base was performed by the same manner (except of acetylation and detritylation steps). The column was washed by methanol (nearly 200 ml), and the trinucleotide was released from the support by 5% solution of 25% aqueous ammonia in methanol (approx. 20 min, 100-120 ml). The desirable product was isolated by column chromatography on silica gel (4x30 cm, gradient 0 - 15% MeOH in $CHCl_3$, 2l, flow rate 15 ml/min). The overall yields of the final products (calculated for the starting nucleoside) were: ATC - 87%, TAC - 84%, GAC - 79%, TCT - 90%, CAT - 76%, TGG - 75%.

The purity of trinucleotide synthesized was checked by HPLC under conditions, mentioned in the ***General*** section. The identity was confirmed by analysis of UV-spectra and by comparison of their chromatographic properties with trinucleotides synthesized earlier [9] (FIG 2).

3. Regeneration of 5'-O-DMTr-N-acylnucleosides. The washings obtained after **2a**, were evaporated to oil, treated with 5% solution of 25% aqueous ammonia in methanol (nearly 30 ml, 20 min), evaporated to oil, dissolved in 100 ml $CHCl_3$, washed by 0.1M triethylammoniumbicarbonate (TEAB) (3x100 ml), dried, and evaporated. The desirable product was isolated by chromatography on silica gel under the same conditions as trinucleotides (gradient 0 - 12% MeOH in $CHCl_3$). Recovery of nucleoside was 70%-80%.

4. Regeneration of 5'-O-DMTr-N-acylnucleoside-3'-O-chlorophenylphosphates 2. The eluates from column obtained after performing of condensation and washing by pyridine were pooled and evaporated. The resulted oil was dissolved in 100 ml $CHCl_3$, washed by TEAB (3x100 ml), dried, and evaporated to the oil. The desirable product was isolated by chromatography on silica gel under the conditions described above (gradient 0-15% MeOH in $CHCl_3$). Recovery - 60%-70%.

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