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Onium salts as coupling reagents in the preparation of silica polymer supports for oligonucleotide synthesis

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Abstract. Two types of silica polymer supports for solid phase oligonucleotide synthesis were prepared using onium salts, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), in the presence of 1-hydroxybenzotriazole (HOBT) as activating reagents in the reactions of carboxylic functions with amino or hydroxy groups. The HBTU-HOBT system was found to be a more efficient coupling reagent for polyamide linker chain elongation and nucleoside attachment to the carboxylated silica support than the BOP-HOBT one.

Keywords: oligonucleotides, polymer supports, coupling reagents, phosphonium salts, uronium salts.

Introduction. Natural and modified oligonucleotides are widely used for DNA sequencing, as nucleic acids probes, PCR primers, gene expression inhibitors, etc [1, 2]. The development of efficient new procedures for oligonucleotide synthesis is still in demand. Here we describe the convenient method of preparation of silica-based polymer supports for the solid phase oligonucleotide synthesis. Elongation of the polyamide linkers and attachment of nucleosides to the polymers containing COOH groups was performed using uronium and phosphonium coupling reagents originally developed for peptide synthesis.

Materials and methods. Onium salts, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and anhydrous 1-hydroxybenzotriazole (HOBT) were purchased from Aldrich, Fmoc-glycine, 3-aminopropyltriethoxsilane, piperidine and N,N-diisopropylethylamine (DIPEA) were from Fluka, 1-methylimidazole (MeIm) and 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) were from Merck. Other reagents and solvents were obtained from domestic suppliers. Diisopropylethylamine and piperidine were distilled from NaOH, dimethylformamide and MeIm were distilled under vacuum. Absolute acetonitrile was prepared by distillation over phosphorus pentoxide and then from calcium hydride. Pyridine was distilled over NaOH, ninhydrin and CaH_2. 5'-O-dimethoxytritylthymidine and its 3'-H-phosphonate were synthesized according to standard procedures [3, 4]. The concentration of amino and carboxylic groups on the polymer was determined by spectroscopic methods [5, 6]. Aminopropyl group was attached to Silochrom-2 silica as previously described [6]. The amine content in the polymer was 125 µmol/g.

Polymer I. Succinate group was introduced in the polymer by the treatment of aminopropyl silica Silochrom-2 with succinic anhydride in water maintaining pH below 5, until no free NH_2 groups were detected [5, 6]. Reaction of the obtained carboxylated polymer with ethylenediamine was also performed as previously described [6], pro-

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viding amino polymer that was then treated with succinic anhydride again. The silica containing 52 μmol/g of COOH groups was obtained and used for the attachment of nucleoside. 200 mg of the polymer, 54 mg of 5'-O-dimethoxytritylthymidine (0.1 mmol), HBTU (38 mg, 0,1 mmol), HOBT (14 mg, 0.1 mmol) and DIPEA (52 μl, 0.3 mmol) were slowly agitated in 2 ml of DMF for 4 hours. The silica was filtered off and washed with DMF (3x3 ml) and acetonitrile (3x3 ml). Polymer was suspended in 2 ml of dry pyridine and treated with TPSCl (150 mg, 0.5 mmol), 1-methylimidazole (70 μl, 1 mmol) and methanol (20 μl, 0.5 mmol) for an hour with occasional swirling to block remaining carboxylic groups. Silica support I was filtered off, washed with pyridine (3x3 ml), acetonitrile (3x3 ml), chloroform (3x3 ml) and finally with diethyl ether (3x3 ml) and dried. The nucleoside loading was 36 μmol/g as determined by dimethoxytrityl group acidic cleavage, according to [5].

Polymer I prepared using BOP at the last step under the same reaction conditions had nucleoside loading 30 μmol/g.

Polymer II. The same aminopropyl silica Silochrom-2 containing 125 μmol/g of amino group was used for the preparation of Polymer II. 200 mg of the aminated polymer, Fmoc-glycine (89 mg, 0.3 mmol), HBTU (114 mg, 0.3 mmol), HOBT (42 mg, 0.3 mmol) and DIPEA (157 μl, 0.9 mmol) were slowly agitated in DMF (3 ml) for 2 hours at ambient temperature. Polymer was filtered off, washed with DMF (3x3 ml) and CH_3CN (3x3 ml) and treated with a solution of acetic anhydride (190 μl, 2 mmol) and MeIm (160 μl, 2 mmol) in 2 ml of dry acetonitrile with occasional swirling for 30 min to block unreacted amino groups. The silica was filtered off, washed with CH_3CN (3x3 ml), chloroform (3x3 ml), methanol (3x3 ml) and dried. Fmoc protecting group was removed by the treatment of the polymer with 20 % piperidine in DMF for 20 min [13], and the polymer was washed with DMF (5x3 ml). The second glycine residue was introduced by exactly the same procedure, including capping step. After piperidine deprotection of amino group, NH_2 content was 63 μmol/g. The silica polymer containing Gly-Gly aminolinker was succinylated with succinic anhydride in water, and then 5'-O-protected thymidine was attached to the support in the presence of HBTU/HOBT, as described above for the Polymer I. The nucleoside content in the resulting silica polymer support II was found to be 39 μmol/g.

The silica support synthesis performed using BOP instead of HBTU at each coupling step under exactly the same reaction conditions provided Polymer II with 22 μmol/g nucleoside loading.

Oligonucleotide synthesis. Solid phase synthesis of decathymidylate d$(Tp)_9$T was performed on polymer supports I and II by H-phosphonate method [4] with some modifications, in manual mode. Condensing reactions were carried out in acetonitrile-pyridine 4:1 mixture with pivaloyl chloride as coupling reagent, with 3 min coupling. Average coupling yields were in the range 97—97.5 %. After the chain elongation was over, hydrophosphoryl groups of the oligonucleotide sequence on the polymer were oxidized by 2 % iodine solution in pyridine-water (98:2) for 20 min [7], and oligonucleotide was cleaved from the support by concentrated ammonia (at room temperature overnight). Oligonucleotides were isolated by standard electrophoresis in 20 % denaturing polyacrylamide gel and desalted by gel filtration on PD-10 cartridge (Pharmacia, Sweden).

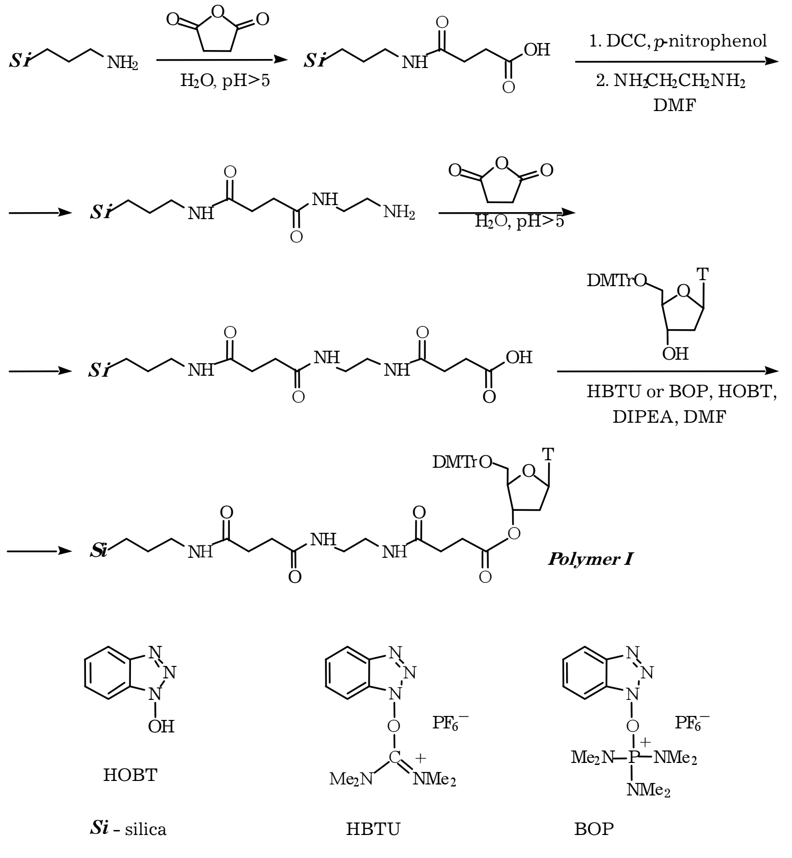
Results and discussion. An efficient polymer support for the synthesis of oligonucleotides should contain relatively long linker between polymer surface and attached nucleoside allowing the yields of the first coupling reactions to be sufficiently high. Also, both the yield and the purity of synthetic oligonucleotides depend on the linker structure [1, 3, 5, 8]. Highly efficient and simple linkers reported in the literature contain e.g. glycyl-glycyl-succinyl or succinyl-ethylenediamine-succinyl fragment [8]. These structures are sufficiently rigid due to intramolecular interactions to avoid bending, and therefore linked nucleoside residues are put apart from the polymer surface that allows free access of reagents to growing oligonucleotide chain. We have prepared supports containing both types of linkers. Polymer used for the preparation of modified support was previously described Silochrom-2 silica [6].

Usually nucleoside-containing polymer supports are prepared via the covalent attachment of nucleoside succinates (as activated esters or in the presence of coupling reagents like carbodiimides or arylsulfonylchlorides) to amino groups on polymers [1, 3, 8]. The use of activated esters requires an additional step of their preparation, whereas coupling reactions in the presence of activating reagents should be performed under strictly anhydrous reaction conditions. In the present work, linker elongation and nucleoside coupling to the polymer were accomplished using efficient coupling reagents, HBTU or BOP in the presence of HOBT. In peptide synthesis, during the past five years formerly predominant carbodiimide and active ester techniques have been increasingly replaced with onium salts approach. First reagents of this class, BOP (Castro's reagent) [9] and HBTU [10], were introduced about 25 years ago. Now a number of phosphonium and uronium reagents based on 1-hydroxybenzotriazole or 7-aza-1-hydroxybenzotriazole core structures are used in coupling reactions in peptide synthesis [11—13]. As for the other coupling reagents, the first step of coupling reaction is an activation of the carboxyl group with corresponding reagent, and the second one is a nucleophilic attack of amino or hydroxy component at activated carboxylic group. Recently onium reagents have became popular also in oligonucleotide chemistry since they are stable, nonhygroscopic and easy to use. The obvious field of their application is amide bond formation. There are reports on the use of onium activating reagents for polymer linker chain synthesis and nucleoside immobilization to solid supports [14—17], oligonucleotide functionalization or labeling [18—22], and on the preparation of peptide-oligonucleotide hybrids [23]. These reagents can hardly be used however for the activation of P-components for oligonucleotide sequence elongation since they are not sufficiently active to provide an alternative to modern methods of oligonucleotide synthesis. However, there are few reports in the literature on the use of onium salts for the activation of phosphate or H-phosphonate groups of nucleotides [24, 25].

The Silochrom-2 silica was first functionalized

Scheme 1

Preparation of silica polymer support containing succinyl-ethylenediamine-succinyl linker



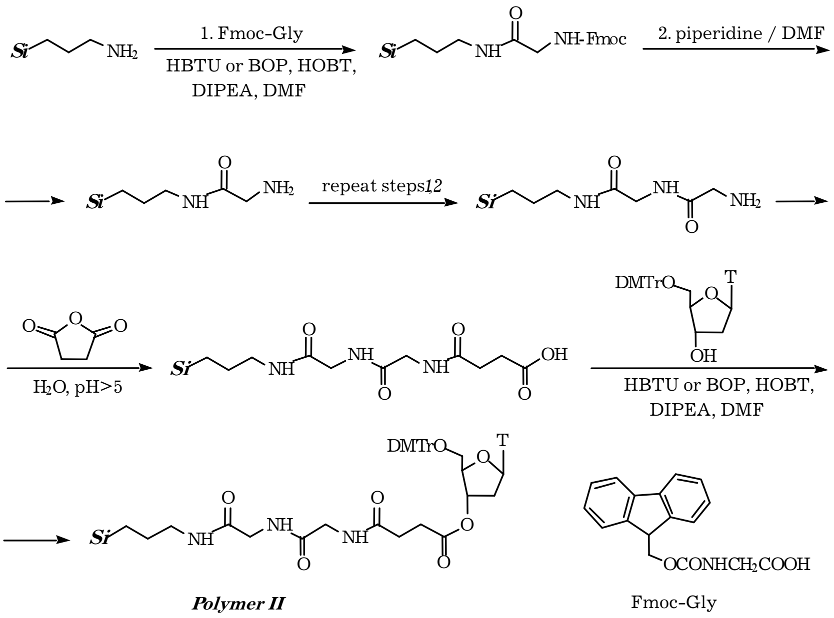
by the treatment with aminopropyltriethoxysilane in ethanol to provide aminopropyl support as previously described [3, 6]. The first type of silica polymer support was prepared according to the Scheme 1. Aminopropyl silica was treated with succinic anhydride in water to get COOH-containing polymer. Then, ethylenediamine was attached to the linker carboxy group activated via *p*-nitrophenyl ester formation, and an amino group was again acylated with succinic anhydride [5, 6]. 5'-Protected 2'-deoxythymidine was linked to the polymer succinate via free 3'-hydroxy group. This convenient approach eliminates the laborious preparation of nucleoside 3'-succinates to be coupled to amino linker in the classic method. The coupling of nucleoside to the silica support was performed in the presence of HBTU or BOP reagent, with addition of HOBT and diisopropylethylamine. The reaction of polymeric COOH functions with nucleoside secondary hydroxyl group resulted in a good nucleoside loading (30—36 μmol/g) that is well within the range most commonly used in solid phase oligonucleotide synthesis (20—50 μmol/g).

The preparation of the second type of polymer support started from the same aminopropyl silica (Scheme 2). Two consequent additions of N-protected glycine (Fmoc-Gly) were performed with HBTU or BOP activating reagent in the presence of HOBT and the base. The capping with acetic anhydride was used to block remaining amino groups after each coupling step, followed by amine deprotection by piperidine treatment. The end amino group of the Gly-Gly linker was acylated with succinic anhydride, and then a nucleoside was attached to the carboxylated polymer in the presence of onium salt via the ester bond. For both Polymers I and II, after the nucleoside attachment the remaining free carboxylic groups were blocked as methyl esters by coupling reaction with methanol in pyridine in the presence of arylsulfonyl chloride and MeIm as a nucleophilic catalyst [26].

Generally, the reaction of N-protected amino acid with amino polymer has become quite a standard technique of modern solid phase peptide synthesis [12, 13]. But the ester bond formation using onium reagents is not so common. The reduced nucleophilicity of hydroxyl groups relative to amino groups makes coupling through ester linkages more difficult, and coupling reactions are slower [27, 28]. Only limited reports are available in the literature on the use of onium activating reagents for ester bond formation in solid phase chemistry, e.g. on the attachment of amino acids to hydroxyl resins for peptide [29] or peptide nucleic acids (PNA) synthesis [30], or coupling of nucleoside 3'-succinates to hydroxyl

Scheme 2

Preparation of silica polymer support containing Gly-Gly linker



polymers [31]. However, «reverse mode» coupling of nucleosides to COOH-containing polymer supports with phosphonium/uronium activators we describe here was not reported before.

We have compared an efficiency of the phosphonium compound BOP and uronium derivative HBTU as coupling reagents for the reaction of COOH functions with amino and hydroxy groups, i.e. Fmoc-Gly coupling to aminated polymer and nucleoside attachment to succinylated silica. In both cases, the reaction yields for BOP reagent were somewhat lower than those obtained for HBTU under the same reaction conditions. For example, nucleoside content in the Polymer I prepared with HBTU and BOP reagent was 36 and 30 μmol/g, respectively. During the synthesis of the Polymer II, onium reagent-assisted coupling was performed at three elongation steps, and final nucleoside content in the polymer prepared using BOP was only 22 μmol/g, as compared to 39 mol/g for the same support II obtained with HBTU. It has been also shown that phosphonium derivatives are less stable than uronium analogues [12]. It should be noted that coupling efficiency of uronium or phosphonium salts alone, in the absence of HOBT additive, is quite poor.

The efficiency of onium reagents was also compared to that of more common condensing reagent, triisopropylbenzenesulfonyl chloride in the presence of MeIm [26]. In the reaction of Fmoc-Gly with aminoalkyl linker and the reaction of nucleoside with carboxylated polymer (both reactions in pyridine) the latter activating system was found to be more reactive than uronium reagents, and corresponding coupling reactions normally ended within an hour. However, in both cases the reaction yields were 10—30 % lower for TPSCl reagent, perhaps due to the competitive sulfonation of amino or hydroxy group of nucleophilic component. It seems that analogous yield-decreasing side reactions do not proceed to a significant extent with onium salts. However, their possible side reactions were not studied in this paper.

To demonstrate the utility of supports prepared in solid phase oligonucleotide synthesis, a model decathymidylate sequence (T_{10}) was successfully synthesized on Polymers I and II by a manual variant of H-phosphonate method [4, 7]. Average coupling yields were very close for the two supports (97—97.5 %), however the purity of oligonucleotide synthesized at Polymer I containing succinate-ethylenediamine linker was noticeably higer, as observed by the gel electrophoresis patterns of the crude oligonucleotides, perhaps because it is 2 atoms longer than Gly-Gly linker of the Polymer II.

In summary, Silochrom-2 based silica supports containing two types of polyamide linkers have been prepared using onium condensing reagents, HBTU or BOP in the presence of the HOBT. The efficiency of this activating system makes it a good choice for the preparation of polymers for oligonucleotide synthesis. The uronium coupling reagent HBTU was found to be more satisfactory than the phosphonium derivative BOP.

Abbreviations. BOP, benzotriazol-1-yloxytris (dimethylamino)phosphonium hexafluorophosphate; DCC, N,N'-dicyclohexylcarbodiimide; DIPEA, diisopropylethylamine; DMF, dimethylformamide; DMTr, 4,4'-dimethoxytrityl; Fmoc, 9-fluorenylmethoxycarbonyl; Gly, glycine; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetraethyluronium hexafluorophosphate; HOBT, 1-hydroxybenzotriazole; MeIm, 1-methylimidazole; Py, pyridine; TPSCl, 2,4,6-triisopropylbenzenesulfonyl chloride.

Онієві солі як конденсуючі реагенти для одержання полімерних носіїв для олігонуклеотидного синтезу на основі силікагелю

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Резюме. Два типи полімерних носіїв на основі силікагелю для олігонуклеотидного синтезу було отримано з використанням онієвих солей, гексафторфосфатів — 2-(1Н-бензотріазол-1-іл)-1,1,3,3-тетраметилуронію (HBTU) та бензотріазол-1-ілокситріс(диметиламіно)фосфонію (BOP) — у присутності 1-гідроксибензотріазолу (HOBT) як реагентів-активаторів у реакціях карбоксильних функцій з аміно- та гідроксигрупами. Система HBTU-HOBT виявилась більш ефективним конденсуючим реагентом для елонгації поліамідного лінкерного ланцюга та приєднання нуклеозидів до карбоксильованого полімерного носія.

Ключові слова: олігонуклеотиди, полімерні носії, конденсуючі реагенти, фосфонієві солі, уронієві солі.

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