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A Novel Phosphoramidite for the Site-Selective Introduction of Functional Groups into Oligonucleotides via Versatile Tethers

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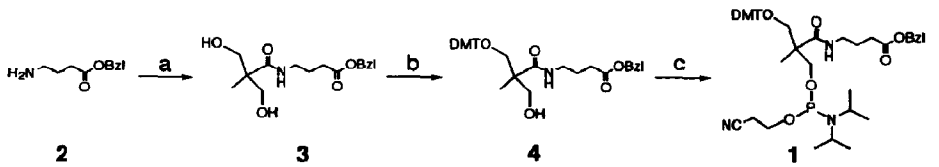
Abstract: A phosphoramidite monomer, which has a benzyl ester moiety in the side chain and is useful for the site-selective introduction of functional groups into oligonucleotides via various tethers, has been synthesized.

Chemical modification of oligodeoxyribonucleotides has been widely studied, because of potential applications to non-radioactive labeling, functionalization of oligonucleotides, and others.[1-5] Phosphoramidite monomers attached with functional groups were used for the site-selective introduction of the moieties to oligonucleotides.[6-9] In these cases, however, replacement of the functional groups and the tethers with others requires *de novo* synthesis of the corresponding phosphoramidite monomers. Oligonucleotides having latent reactive groups, which are substituted by varieties of functional residues when necessary, should be of great value for the purpose.

We report here the synthesis of a novel phosphoramidite monomer **1** which has a benzyl ester residue in the side chain (Scheme 1). The benzyl ester is stable during the DNA synthesis, but readily reacts with amino compounds in the subsequent treatment. Various functional residues are site-selectively introduced via versatile tethers to the oligonucleotides.

The phosphoramidite monomer **1** was synthesized as depicted in Scheme 1. Benzyl 4-aminobutylate (**2**), prepared from 4-aminobutylic acid in a usual fashion, was coupled with 2,2-bis(hydroxymethyl)propionic acid by use of 1-ethyl-3-[3'-dimethyl(aminopropyl)]-carbodiimide hydrochloride (WSCI), 1-hydroxybenzotriazole (HOBt) and triethylamine in DMF at r.t. for 24 h (62 % yield). The resultant diol **3** was reacted with 4,4'-dimethoxytrityl chloride (DMT-Cl), 4-dimethylaminopyridine (DMAP), and pyridine in dry dichloromethane

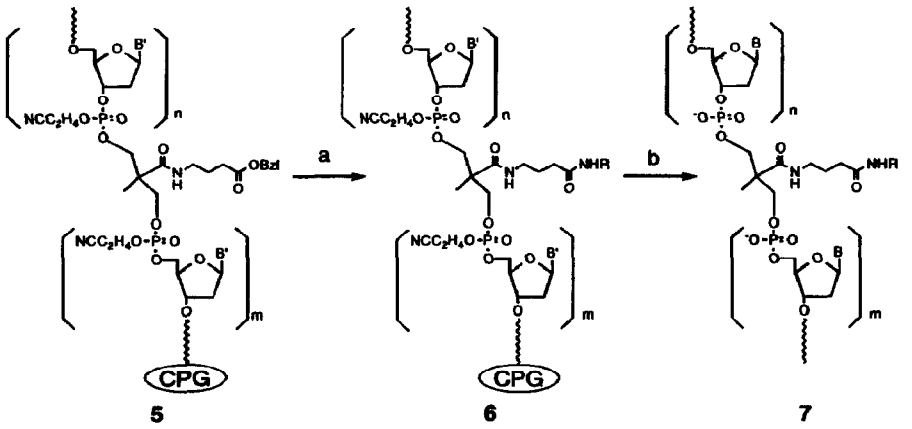


Scheme 1. a) 2,2-bis(hydroxymethyl)propionic acid, WSCI, HOBt, Et_3N, DMF. b) DMT-Cl, DMAP, pyridine, CH_2Cl_2. c) 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphoramidite, tetrazole, CH_3CN.

at r.t. for 12 h to afford **4** in 60 % yield. Phosphitylation of **4** was carried out by 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphoramidite with tetrazole in dry acetonitrile at r.t. for 2.5 h. The phosphoramidite **1** was desalted, coevaporated with dry acetonitrile, and was used for the DNA synthesis without further purification. The characterization of **1** was made by ^{31}P-NMR (152.37 and 152.41 ppm with respect to 80 % H_3PO_4 in D_2O) as well as by ^{1}H-NMR.

An oligonucleotide 5'-ATAACGGCCAXTCTTCGCCTG-3' (**5** in Scheme 2) was synthesized on an automated DNA synthesizer. Here X denotes the unit derived from the phosphoramidite **1**. The concentration of **1** in dry acetonitrile was 1.5 times as large as that employed for the normal Expedite agents.[10] The coupling efficiency at the step involving **1** was greater than 98 %, judging from the amount of DMT cation released.

When the oligonucleotide **5**, as obtained on the CPG column, was treated with primary amines in dry dioxane at 45°C for 48 h, aminolysis of the benzyl ester took place almost quantitatively and the amines were connected to the oligonucleotide by amide bonds (the step a in Scheme 2). Detachment of **5** from the CPG column hardly occured here, as shown by the absorption spectroscopy. By use of bifunctional amino compounds such as diamines, triamines, and cystamine, various tethers were also introduced to **5**. The resultant oligonucleotides **6** were deprotected by concentrated ammonium hydroxide at r.t. for 1 h, and were purified on a reversed-phase HPLC (Fig. 1 (left)).



Scheme 2. a) NH_2R, dioxane, 45 °C, 48 h. b) deprotection, HPLC purification.

The products **7a–c** were digested by snake venom phosphodiesterase and alkaline phosphatase, and the resulting products were analyzed by HPLC (Fig. 1 (right)). The molar ratios dC:dG:T:dA:dApX were exactly identical with the theoretical values (7:4:5:3:1). Assignment of the signals for dApX's was made by coinjection of the authentic samples, which were independently prepared from **1**.

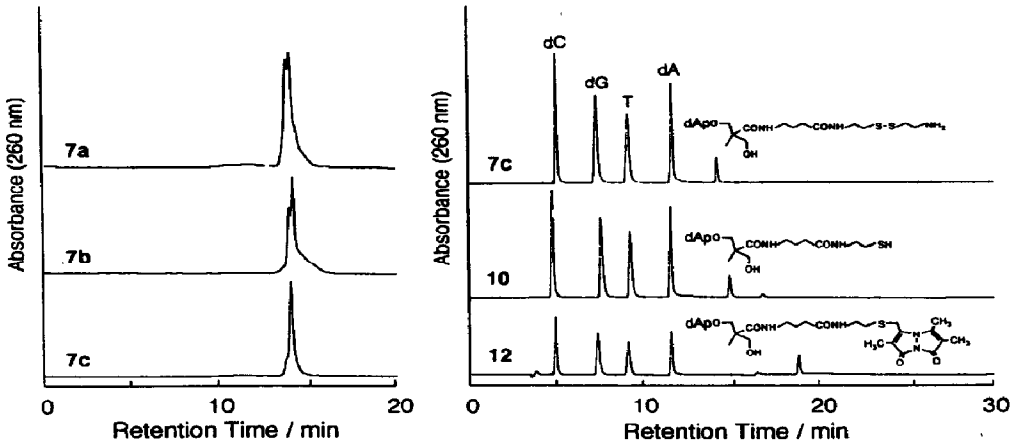
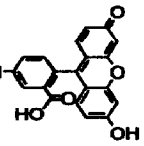
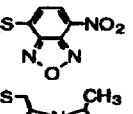
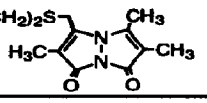


Figure 1. HPLC profiles of the oligonucleotides **7a–c** (left) and the enzymatically digested products of the oligonucleotides **7c**, **10**, and **12** (right). Conditions: Shiseido SG120 ODS column, 1.0 ml/min, 260 nm; (left) a linear gradient 5–20 % (20 min) acetonitrile/water containing 50 *mM* ammonium formate; (right) a linear gradient 5–50 % (40 min).

Table 1. Modified oligonucleotides prepared by use of the phosphoramidite **1**[1].

product	R	retention time / min	retention time of dApX / min
7a	$-(CH_2)_2NH_2$	13.4 13.7	11.5
7b	$-(CH_2)_2NH(CH_2)_2NH_2$	13.6 13.7	12.1
7c	$-(CH_2)_2SS(CH_2)_2NH_2$	13.7	13.7
8 [2]	$-(CH_2)_2NHCSNH-$ 	15.5	19.4
9 [2]	$-(CH_2)_2NHCOCH_2N(CH_2CO_2Et)_2$	15.4	20.4
10 [3]	$-(CH_2)_2SH$	13.8	14.7
11 [4]	$-(CH_2)_2S-$ 	15.3	23.4
12 [4]	$-(CH_2)_2S-$ 	14.5	18.1

1) The retention times of the oligonucleotides and of dApX generated from the enzymatic digestion are also presented. HPLC conditions are the same as described in Fig. 1. 2) prepared from **7a**. 3) from **7c**. 4) from **10**.

Furthermore, several functional groups were introduced to the tethered-oligonucleotides **7a-c** by the well-known procedures [4,11,12] (see Table 1). The modified oligonucleotides **8** and **9** were synthesized by reacting **7a** directly with fluorescein isothiocyanate [11] and *N,N*-bis(ethoxycarbonylmethyl)glycine *p*-nitrophenyl ester, respectively. For the preparation of **11** and **12**, the oligonucleotide **7c** was first converted to **10** by dithiothreitol, and was then reacted with 4-chloro-7-nitrobenz-2-oxa-1,3-diazole [4] or with monobromobimane.[12]

The modified oligonucleotides **8, 9, 11**, and **12** were enzymatically digested, and the resulting products were analyzed by HPLC, in a similar way as described for the tethered-oligonucleotides. The dApX's attached with the corresponding residues were formed in the expected amounts, as confirmed by coinjection with the authentic samples (see Fig. 1 and Table 1).

In conclusion, the phosphoramidite monomer **1**, which has a benzyl ester moiety, is quite useful for the site-selective introduction of functional residues to oligonucleotides. Study on the applications of these functionalized oligonucleotides is under way.

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