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A Novel Approach to Oligodeoxyribonucleotides Bearing Phosphoric Acid Esters at the 3'-Terminals via the Phosphoramidite Method with Allyl Protection: An Efficient Synthesis of Base-Labile Nucleotide–Amino Acid and –Peptide Conjugates

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Abstract: A new method for synthesis of 3'-end-phosphorylated DNA oligomers via the phosphoramidite method with allyl protection has been developed. This method is particularly useful for the preparation of derivatives with base-labile structures such as oligoDNA-OPO(OH)$OCH_2CH(R)Z$, in which Z is an electron-withdrawing function. For example, a oligonucleotide-amino acid conjugate, $^{5'}$TGTCGACACCCAATT$^{3'}$-OPO(OH)$OCH_2CH(NH_2)COOH$, and a oligonucleotide–peptide conjugate, $^{5'}$TGTCGACA-CCCAATT$^{3'}$-OPO(OH)$OCH_2CH(NH_2)CONHCH_2COOH$, have been obtained in high purity.

The synthesis of DNA oligomers with a phosphoric acid or its ester group at their 3'-terminal is a significant subject, since the introduction of the ester function at this position generally allows an increase of the nucleotide's resistance to 3'-exonuclease[1] which brings about the enhancement of some biological activities, *e.g.*, antisense activity. Further, enhancement of the nucleotide uptake through cell membranes in the absence of liposomal carriers,[1] which is a basic requirement for biological activity, is expected by the introduction, particularly, of dieters or triesters. Thus, not only oligodeoxyribonucleotide 3'-monophosphoric acid monoesters[2] but also a variety of the diesters with a functional group including an intercalator,[3] a lipophilic ligand,[4] a DNA/RNA-cleaving group,[5] a dye,[6] and a peptide[7] were prepared as candidates for antisense molecules, while some of the derivatives actually showed attractive antisense activities. The syntheses reported so far are useful for producing base-stable derivatives but do not always give satisfactory results for derivatives bearing the structure of oligonucleotide-OPO(OH)OC—CHZ, where Z is an electron-withdrawing group such as COOH, COOR, or CONHR. This is due to the structure not remaining stable under the basic conditions required for the removal of nucleobase protectors such as the acyls or [2-(*p*-nitrophenyl)ethoxy]carbonyl[4a] employed in the existing methods. By contrast, allyl protection[8] removable under mild, non-basic conditions is expected to be useful for the preparation of not only base-stable derivatives but also base-labile compounds. Thus, we planned the synthesis via the phosphoramidite approach using allyl protection and describe here a new, general approach to both base-labile and -stable DNA oligomers with a phosphate function at the 3'-terminal.

There are two typical methods for the synthesis of oligonucleotide 3'-monophosphoric acid esters: (1) a pathway starting from a nucleoside 3'-phosphate attached via a suitably modified linker to solid support and then

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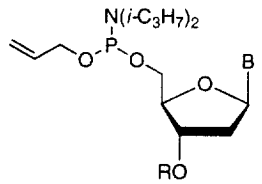
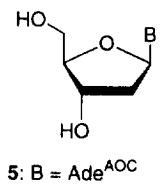
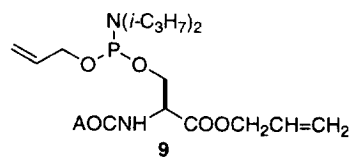
constructing the DNA chain by the (3' → 5')-directed elongation using nucleoside 3'-phosphoramidites as building blocks (method A),[2,3] and (2) a route preparing first the DNA chain through the (5' → 3')-elongation with nucleoside 5'-phosphoramidites as monomer units and, in the final stage, phosphorylating the 3'-end hydroxyl (method B).[4a] Compared with method A, method B is superior in regard to synthetic flexibility and generality. For example, in the preparation of nucleotide–peptide conjugates which are a class of targets in this work, the conjugate bond can be directly constructed on the solid phase in method B. While, multi steps are required in method A, such like detachment of an oligonucleotide with/without an alkyl chain and condensation of a peptide in solution phase.[7a] In addition, method B does not require any tedious modification of the long-alkyl linker. Therefore we carried out the synthesis via the (5' → 3')-elongation approach. The nucleoside 5'-phosphoramidites, **1**–**4**[9] (AOC = allyloxycarbonyl, All = allyl), requisite as monomer units for the assembly of oligonucleotides, were obtained from the corresponding nucleoside among **5**–**8**[10] via the room-temperature reactions of (1) 5'-*O*-silylation with *tert*-butyldimethylsilyl chloride and imidazole in DMF (24 h), (2) DBU-assisted 3'-*O*-tritylation with *p*-methoxytrityl chloride or *p*,*p*'-dimethoxytrityl chloride in pyridine (24 h), (3) desilylation using tetrabutylammonium fluoride in THF (4 h), and (4) phosphoramiditylation with CH_2=$CHCH_2OP[N(i\text{-}C_3H_7)_2]_2$ promoted by diisopropylammonium tetrazolide in acetonitrile (3 h). Other building blocks, **9**–**12**, for the synthesis reported in this article were prepared as follows: The serine phosphoramidite **9** was synthesized from the parent substance in 59% overall yield through the acid-catalyzed introduction of an allyl protecting group to the carboxylic acid moiety of *N*-(allyloxycarbonyl)serine[11] with allyl



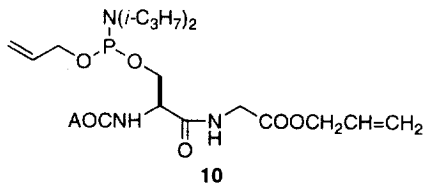
1: B = Ade^{AOC}, R = DMTr
2: B = Cyt^{AOC}, R = DMTr
3: B = $Gua^{All, AOC}$, R = MMTr
4: B = Thy, R = DMTr



5: B = Ade^{AOC}
6: B = Cyt^{AOC}
7: B = $Gua^{All, AOC}$
8: B = Thy



9



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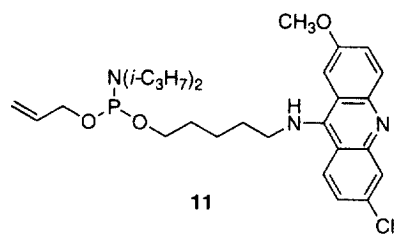
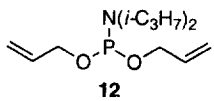


11



12

13: R = $HOCOCH(NH_2)CH_2$
14: R = $HOCOCH_2NHCOCH(NH_2)CH_2$
15: R = $AcrNH(CH_2)_4CH_2$
16: R = H

alcohol in toluene (reflux, 3 h). This was followed by the phosphoramiditylation of the hydroxy group with CH_2=$CHCH_2OP[N(i\text{-}C_3H_7)_2]_2$ promoted by diisopropylammonium tetrazolide in acetonitrile (2 h). The preparation of the peptide phosphoramidite **10** was performed in 64% overall yield via (1) condensation of *N*-(allyloxycarbonyl)serine and allyl glycinate[12] assisted by a mixture of DCC, and 1-hydroxybenzotriazole in THF (25 °C, 4 h), and (2) the phosphoramidite formation described above. The acridinylalkyl phosphoramidite **11** was obtained in 52% yield by the 1*H*-tetrazole-promoted condensation of 6-chloro-2-methoxy-9-(hydroxypentylamino)acridine [$AcrNH(CH_2)_5OH$][13] and CH_2=$CHCH_2OP[N(i\text{-}C_3H_7)_2]_2$. The phosphoramidite **12** was prepared according to the known procedure.[14] The ^{31}P NMR spectra of new phosphoramidite showed signals at δ 147 to 149 ppm, supporting their structures. The structures were also confirmed by elemental analysis.

We selected an oligonucleotide–amino acid conjugate **13** and an oligonucleotide–peptide conjugate **14** as main targets, which both are not absolutely stable to bases. The synthesis was carried out on an ABI 392 DNA synthesizer, starting from the thymidine covalently attached at the 5'-hydroxyl to controlled pore glass (CPG) supports with 500 Å pore size via a long-chain alkylamine space arm. The chain elongation for construction of the 3'-O-free oligonucleotide was performed according to a standard procedure[15] using benzimidazolium triflate[16] as the activator of the phosphoramidite. In this method, the phosphitylation required 2 min for the completion. The overall coupling yield estimated by the trityl assay was >99% (average yield for one-base elongation: 99.9%). The 3'-hydroxyl of the solid-anchored oligonucleotide was phosphitylated with the serine phosphoramidite **9** and benzimidazolium triflate and the resulting product was oxidized by *tert*-butyl hydroperoxide (TBHP). Subsequently all allylic protectors were removed by treatment with a mixture of $Pd_2[(C_6H_5CH{=}CH)_2CO]_3{\cdot}CHCl_3$, $P(C_6H_5)_3$, and diethylammonium formate in THF at 50 °C. Finally, the target nucleotide–amino acid conjugate **13** was released from the solid supports by brief exposure to conc. ammonia at ambient temperature. The isolation yield of **13** was 75%. Similarly, the nucleotide–peptide conjugate **14** was prepared using **10** in place of **9**. The HPLC indicated that products **13** and **14** have excellent purity in crude forms as shown in Figure 1. These compounds could not be obtained in such a purity by the method employing acyl protecting groups for the nucleoside bases since removal of the acyl protectors with

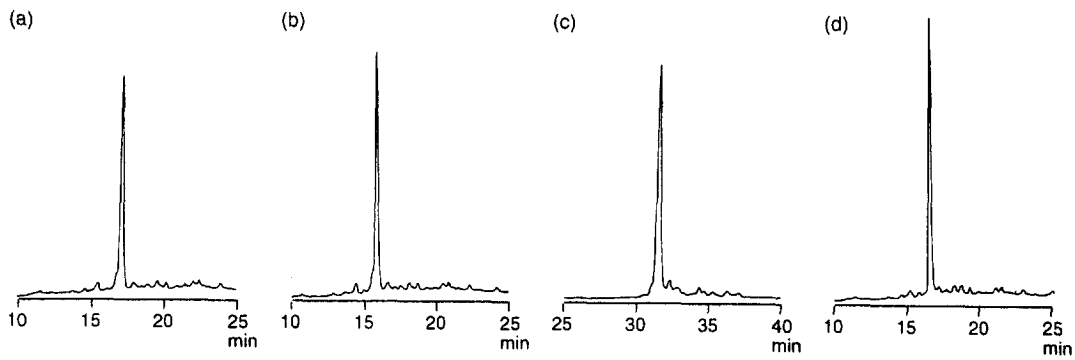


Figure 1. HPLC profiles of crude products of **13–16**: (a) **13**, (b) **14**, (c) **15** , and (d) **16**. Conditions: COSMOSIL 5C18-MS column; buffer A: 5% CH_3CN–0.1 M ammonium acetate (pH 7.0); buffer B: 25% CH_3CN–0.1 M ammonium acetate (pH 7.0); gradient: linear 0 to 40% B in 30 min; detection: 260 nm; flow rate: 1.0 mL/min; temperature: 40 °C.

conc. ammonia at 55 °C caused considerable decomposition of the serine–nucleotide linkage.[17]

This new approach is also effective for the synthesis of other compounds fairly stable to bases. For example, the acridinylalkyl ester **15**, attractive as an oligoDNA with an intercalator, was prepared via a similar process described above, in which **11** was used as a phosphoramidite in the phosphitylation. As well, the oligonucleotide 3'-monophosphate **16** was prepared using the amidite **12**. The purity of **15** and **16** was also very high as illustrated in Figure 1.

In conclusion, we developed an efficient, general method for the synthesis of oligonucleotides with phosphoric acid esters at the 3'-end. This synthesis is particularly useful for base-labile derivatives such as **13** and **14**. Since the introduction of the phosphoric acid moiety to the 3'-end position of the oligonucleotide can not be performed by enzymes, this approach also is useful as a tool for the production of the nucleotide 3'-monophosphoric acid such as **16**.

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17. The reaction gave oligonucleotide 3'-monophosphates in reasonable yields. However, dehydroalanine and dehydroalanylglycine, which are other conceivable fragments arising from the amino acid and peptide moieties of **13** and **14**, respectively, were not detected, because these compounds were decomposed under the reaction conditions. Structures of the degradation products were not determined.