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Organometallic methodologies for nucleic acid synthesis

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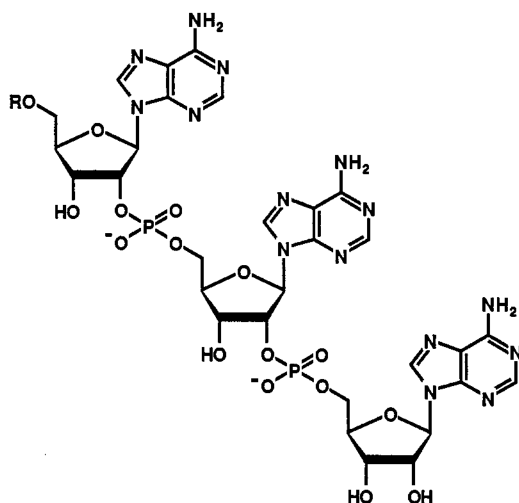
Abstract—A new, general preparation of 2'-5'-linked oligoadenylates has been developed, which relies on: (1) chemoselective *O*-phosphorylation of *N*-unblocked nucleosides via hydroxyl activation by Grignard reagents, (2) one-pot construction of internucleotide linkage using bifunctional phosphorylating agents, and (3) selective production of a 3',5'-di-*O*-protected adenosine. This solution-phase synthesis allows large-scale preparation of a wide range of related oligomers. Palladium chemistry coupled with allylic protection of functional groups leads to the development of an efficient solid-phase synthesis of DNA oligomers via a phosphoramidite approach. Allyl groups on internucleotide linkages and allyloxycarbonyl groups on amino moieties of nucleobases are removable all at once on the solid supports by exposure to a palladium(0) complex and nucleophiles. This procedure has been utilized for synthesis of a DNA 60mer of unprecedented high purity. In addition, this marks the realization of the first efficient preparation of solid-anchored DNA oligomers.

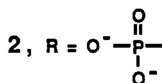
Oligonucleotides are one of the most significant classes of compounds from both the academic and industrial point of view. The chemical synthesis, however, poses an array of problems in the transformation and isolation of products, and high overall efficiency is secured only by using a combination of truly appropriate methods. Organometallic chemistry, though exerting general intense influence on contemporary organic synthesis, has seldom been used in nucleic acids synthesis, perhaps because of the multifunctional, polar nature of the substrates. We demonstrate here the potent utility of metallo-organic methodologies in this area by describing a solution-phase synthesis of some short-chain oligomers having unusual structures and a solid-phase preparation of a long-sequence DNA.

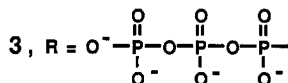
GENERAL SYNTHESIS OF 2'–5'-LINKED OLIGOADENYLATES

In solution-phase chemistry, we selected as targets oligoadenylates, **1**—**3**, possessing 2'-5'-internucleotide bonds (2-5As). This unusual class of compounds, which are produced from ATP by 2-5A synthetase induced by dsRNA in interferon-treated cells, play a major role in antiviral and antiproliferative actions of interferons (ref. 1 and 2). The 2-5A family exhibits a wide variety of biological activity, depending on the structures, and certain structural modifications are known to enhance their potency. Development of an efficient chemical synthesis has been strongly required, since the existing methods have three common drawbacks to practical synthesis: namely, (1) lack of chemoselectivity in phosphorylation of adenosine requiring undesirable protection/deprotection of the 6-amino group, (2) tedious isolation of the adenosine 2'-phosphodiester intermediate before the internucleotide-linkage formation, and (3) nonselective protection of 2',3'-unblocked adenosine generally yielding a mixture of comparable amounts of the 2'- and 3'-*O*-protected products, which needs chromatographic separation unsuitable for large-scale synthesis (ref. 3). We have cleared these impediments and opened a new, general way to 2-5A derivatives. The success relies mainly on the highly selective preparation of a 3',5'-di-*O*-protected adenosine and the chemoselective, one-pot formation of the internucleotide bonds by condensation of a bifunctional phosphorylating agent and *N*-unprotected nucleosides via hydroxyl activation.



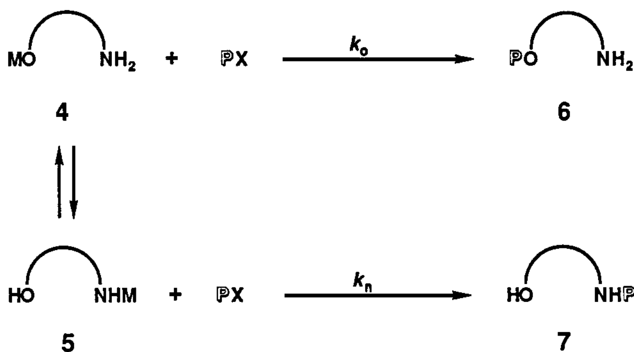
1 , R = H





O-phosphorylation of N-unprotected nucleosides

Although functional group protection/deprotection procedures have been believed essential for efficient oligonucleotide synthesis, the transformation without protective groups is obviously desirable, particularly in large-scale synthesis of short-sequence oligomers. Nucleosides that bear amino and hydroxyl groups such as adenosine, react with electrophilic phosphorylating agents, predominantly at the more basic nitrogen atom, to form phosphoramidates. This tendency necessitates the blocking of amino groups on the nucleobases. However, alternation of the selectivity profile can be expected when a nucleoside is treated with one equivalent of a strong base, leading to an equilibrium mixture of the alkoxide **4** and the amide **5** (M = metallic species). In principle, the *O*- and *N*-selectivity in phosphorylation of such equilibrating reactive species is determined by the equilibrium concentration of **4** and **5** and their relative reactivities. These changes in the chemical properties of OH, NH_2, or NH groups in nucleosides may result in a marked change in the chemoselectivity in such a way that the desired *O*-phosphorylation product **6** is formed.



ℙX = phosphorylating agent

Various organometallic reagents were then screened, along with other strong bases, as promotors of condensation of diethyl phosphorochloridate and *N*-unblocked nucleosides, **8**–**10**, and found that *tert*-butylmagnesium chloride in THF is one of the best general reagents for this purpose. Chemoselectivity of this reaction is controlled primarily by the acidity of OH and NH. The order of acidities of common nucleosides in THF is: NH of guanosine, thymidine, and uridine (pKa ca. 9) > OH of sugars (17 to 18) > NH_2 of adenosine and cytidine (ca. 20) (ref. 4). This approach, therefore, is particularly useful for the phosphorylation of adenosine and cytidine without *N*-protection; the *O*-selective condensation was simply accomplished at ambient temperature by using an equimolar amount of the Grignard reagent and 1.2–1.4 equiv of the phosphorylating agent. Whereas guanosine, thymidine, and uridine required 2 equiv of the Grignard reagent because of the highly acidic proton in the nucleobases. The hydroxyl groups are not activated solely by the use of an equimolar quantity of the magensium reagent; the clean *O*-phosphorylation is possible only by using dimagnesium salts. Some examples are listed in Table 1.

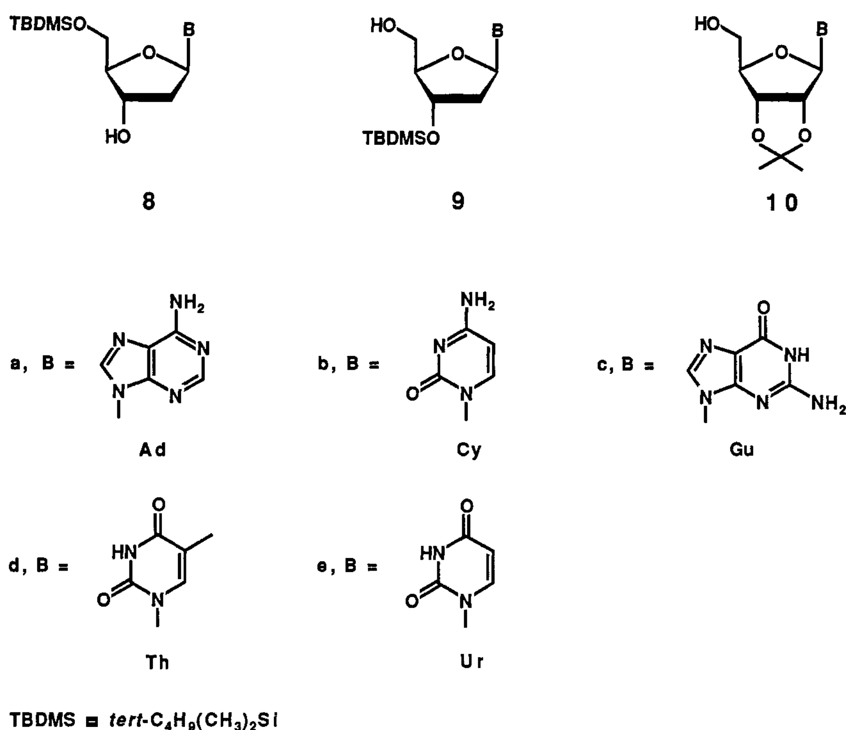


TABLE 1. Phosphorylation of *N*-unprotected nucleosides via magnesium alkoxides[a]

nucleoside	equiv of *tert*-C_4H_9MgCl	phosphorylating agent	time/h	yield of phosphate/%
8a	1	$(C_2H_5O)_2POCl$	1	96
8a	1	$(C_2H_5O)_2POOC_6H_4$-*p*-NO_2	1	93
8b	1	$(C_2H_5O)_2POCl$	0.5	95
8c	1	$(C_2H_5O)_2POCl$	6	88
8c	2	$(C_2H_5O)_2POCl$	0.5	94
8d	1	$(C_2H_5O)_2POCl$	6	92
8d	2	$(C_2H_5O)_2POCl$	0.5	98
10a	1	$(C_2H_5O)_2POCl$	1	91
10b	1	$(C_2H_5O)_2POCl$	2	76
10e	2	$(C_2H_5O)_2POCl$	1	91[b]

[a] Unless otherwise noted, the reaction was carried out in THF. [b] DMF as solvent.

One-pot formation of internucleotide linkage

Our aim is to create an internucleotide linkage giving dinucleoside phosphotriesters without isolation of the phosphodiester intermediates. Although no efficient methods for such an ideal process have been developed, the principle is very simple, *viz.*, consecutive condensation of two nucleosides and a phosphorylating agent of type (ArO)POXY in which X is a better leaving group than Y. Examination of the rates of the hydroxyl activated condensation of a 2'-deoxyadenosine and several kinds of phosphorylating agents revealed that chlorine for X and *p*-nitrophenoxy for Y satisfy this requirement, which led to the invention of (ArO)(p-$NO_2C_6H_4O$)POCl (**12**) as a bifunctional phosphorylating agent. Thus the first nucleoside **11** was treated with 1 equiv of *tert*-butylmagnesium chloride and 1.0–1.1 equiv of **12** (step A), and the resulting phosphotriester intermediate **13**, without isolation, was condensed with 0.9–1.1 equiv of the magnesium alkoxide of the second nucleoside **14** (step B), affording the phosphate **15** in a reasonable yield. Table 2 exemplifies the utility of this facile method.

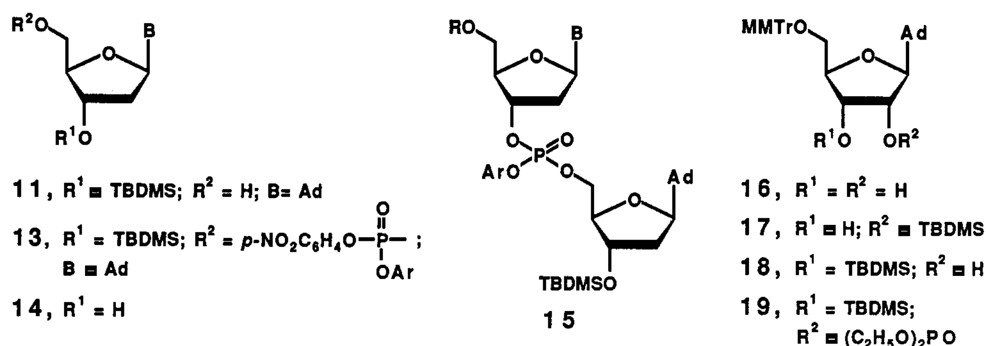


TABLE 2. One-pot synthesis of dinucleoside phosphate **15**

			conditions				
			step A[a]		step B		
B	R	Ar	time/h	solvent	temp/°C	time/h	yield/%
Ad	TBDMS	C_6H_5	3	THF	15	1.5	85
Ad	MMTr	o-ClC_6H_4	1	THF	15	2	86
Cy	TBDMS	C_6H_5	2.5	THF	60	2	81
Gu[b]	MMTr	o-ClC_6H_4	2	DMF–THF[c]	15	10	71
Th[b]	TBDMS	o-ClC_6H_4	2	DMF–THF[c]	15	12	80

[a] THF was used as solvent. [b] Two equivalents of *tert*-butylmagnesium chloride was employed. [c] A 1:2 mixture of DMF and THF.

Selective access to 3'-O-tert-butyldimethylsilylated adenosines

Differentiation between the 2'- and 3'-hydroxyls in adenosine is not easy because of their similar chemical properties. We have now found a very convenient route to the 3'-*O*-silyl-protected adenosine utilizing second-order regioselective protection. The standard silylation of 5'-*O*-*p*-methoxytrityladenosine (**16**) with *tert*-butyldimethylsilyl chloride in DMF containing imidazole gave, as expected, a ca. 1:1 mixture of **17** and **18**. These two isomers equilibrate rapidly in a 4:4:5:100 mixture of triethylamine, methanol, ethyl acetate, and ether (ref. 5) and, fortunately, desired **18** recrystallizes from this medium much more readily than **17** does. Thus we are able to obtain **18** selectively in large quantity and high yield (ref. 6). This isomer proved not to undergo silyl migration under the conditions of the magnesium alkoxide-mediated nucleotide formation. Treatment of **18** with 1 equiv of *tert*-butylmagnesium chloride followed by diethyl phosphorochloridate in THF gave the 2'-phosphate **19** as the single product.

Synthesis of 2-5As

With these new tools in hand, selective synthesis of 2-5As and related compounds is now possible. The 3',5'-di-*O*-protected adenosine **18** was activated with 1 equiv of *tert*-butylmagnesium chloride followed by condensation with *o*-chlorophenyl *p*-nitrophenyl phosphorochloridate (**12**, Ar = *o*-ClC_6H_4) to generate the reactive phosphorotriester intermediate **20**, which was directly treated with the magnesium alkoxide of **21** in a DMF—THF mixture. The product was detritylated by dichloroacetic acid in dichloromethane to give the diadenosine phosphate **22** in 70% yield. The extension of this dimer to the trimer **23** was performed in 63% yield (71% conversion) through a similar reaction sequence using 1.5 equiv of the phosphotriester **20**. Deprotection of **23** by successive treatments with (1) 1,1,3,3-tetramethylguanidium *syn*-4-nitrobenzaldoximate (NBO) in aqueous dioxane (ref. 7), (2) 2% ammonia, and (3) tetrabutylammonium fluoride (TBAF) furnished in 75% yield the 2-5A core (**1**). This was identical to the authentic sample in HPLC, electrophoresis, and enzymatic hydrolysis. The protected 2-5A core **23** was subjected to 2,6-lutidine-assisted condensation with chlorobis(2,2,2-trichloroethoxy)phosphine in THF followed by oxidation with aqueous iodine to give **24** in 80% yield (ref. 8). Deblocking of **24** was accomplished by treatments with (1) a Zn/Cu couple in the presence of acetylacetone in DMF, (2) Chelex 100 resin (NH_4^+ form), (3) NBO, (4) 2% ammonia, and (5) TBAF in THF. The product was purified by passage through a diethylaminoethyl (DEAE) cellulose (HCO_3^- form) column using 0.01—0.04 M triethylammonium hydrogencarbonate eluent. Thus p5'A2'p5'A2'p5'A (**2**) was obtained in 50% isolated yield (ca. 65% yield by HPLC assay), identical in all respects with the authentic sample. The trinucleotide **2** could be converted in 32% yield to ppp5'A2'p5'A2'p5'A (**3**) by treatment with 5 equiv of *N*,*N*-carbonyldiimidazole in the presence of triethylamine followed by 10 equiv of tributylammonium diphosphate in DMF (ref. 8 and 9).

MMTrO — Ad; TBDMSO; o-ClC_6H_4O–P(=O)–OC_6H_4-p-NO_2

20

HO — Ad; TBDMSO, OTBDMS

21

HO — Ad; TBDMSO; o-ClC_6H_4O–P(=O)–O — Ad; TBDMSO, OTBDMS

22

RO — Ad; TBDMSO; o-ClC_6H_4O–P(=O)–O — Ad; TBDMSO; o-ClC_6H_4O–P(=O)–O — Ad; TBDMSO, OTBDMS

23, R = H

24, R = $(CCl_3CH_2O)_2PO$

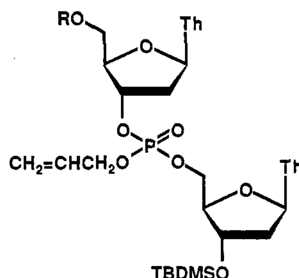
MMTr = p-$CH_3OC_6H_4(C_6H_5)_2C$

SOLID-PHASE SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES OF HIGH PURITY

Chemical synthesis of oligodeoxyribonucleotides in the solid phase is a key operation in the field of molecular biology. A variety of synthetic approaches have been developed and indeed commercial automated synthesizers are now routinely used in DNA-related research and technology. The efficiency of the current methods, however, does not always conform to today's high standard of synthetic organic chemistry which aims for quantitative yield and perfect selectivity. The purity of the synthesized DNAs is often variable depending on the sequence and length and is not generally satisfactory. The impurities are mainly short-chain oligomers formed by cleavage of the DNA strand during the removal of *N*-benzoyl and -isobutyryl groups on nucleoside bases under harsh conditions (ref. 10). We describe here a new, practical method for solid-phase synthesis of DNA oligomers of unprecedented high purity using allylic protection in conjunction with palladium chemistry.

Allyl protection of internucleotide linkage

The allyl group acts as a useful protecting group for an internucleotide bond, that can be removed by brief treatment with a palladium(0) complex and nucleophiles. For example, removal of the allyl group of the phosphotriester **25** was accomplished almost quantitatively by exposure to a mixture of 0.05 equiv of tetrakis(triphenylphoshine)-palladium(0), 0.2 equiv of triphenylphosphine, and an excess amount of a nucleophile in THF. Nucleophiles may be primary or secondary amines, their mixtures with formic acid, a butylamine—carbonic acid mixture, etc. Examples are given in Table 3. The deblocking conditions are milder than those for conventionally utilized protectors such as methyl and *o*- or *p*-chlorophenyl.



25, R = MMTr or TBDMS

TABLE 3. Deprotection of allyl dithymidine phosphate **25**

R	nucleophile	time/min	yield/%
MMTr	n-$C_4H_9NH_2$	75	>95
MMTr	$(C_2H_5)_2NH$	25	>95
MMTr	n-$C_4H_9NH_2$/HCOOH	50	>95
MMTr	$(C_2H_5)_2NH$/HCOOH	25	>95
TBDMS	n-$C_4H_9NH_2/H_2O/CO_2$	10	>95

Allyloxycarbonyl protection of nucleosides

In nucleic acids synthesis acyl groups are most widely used to protect the amino group, but removal of such groups is frequently accompanied by side reactions including cleavage of the internucleotide linkage, resulting in serious loss of products. In this regard, allyloxycarbonyl (AOC) is excellent for the protection of amino or imide moieties of nucleoside bases and sugar hydroxyls. Deblocking is easily performed with a palladium(0) catalyst and a variety of nucleophiles at room temperature. On the other hand, conditions for removal of the MMTr or DMTr and TBDMS protecting group do not affect the AOC protection. Thus the AOC group serves as both a specific and general

protector. Table 4 lists some examples illustrating the efficiency of this method. The *O*-AOC nucleoside **31** was also deprotected smoothly by mild palladium(0) catalysis. When the fully protected dinucleotide **32** was exposed to a mixture of terakis(triphenylphosphine)palladium(0) and triphenylphosphine (0.05 and 0.2 equiv/allyl), butylamine (10 equiv), and formic acid (10 equiv) in THF at room temperature for 30 min, the four allylic protecting groups were removed all at once from the nucleoside base, sugar hydroxyl, and phosphate moiety to give thymidylyl(3'-5')-2'-deoxyadenosine in 97% yield.

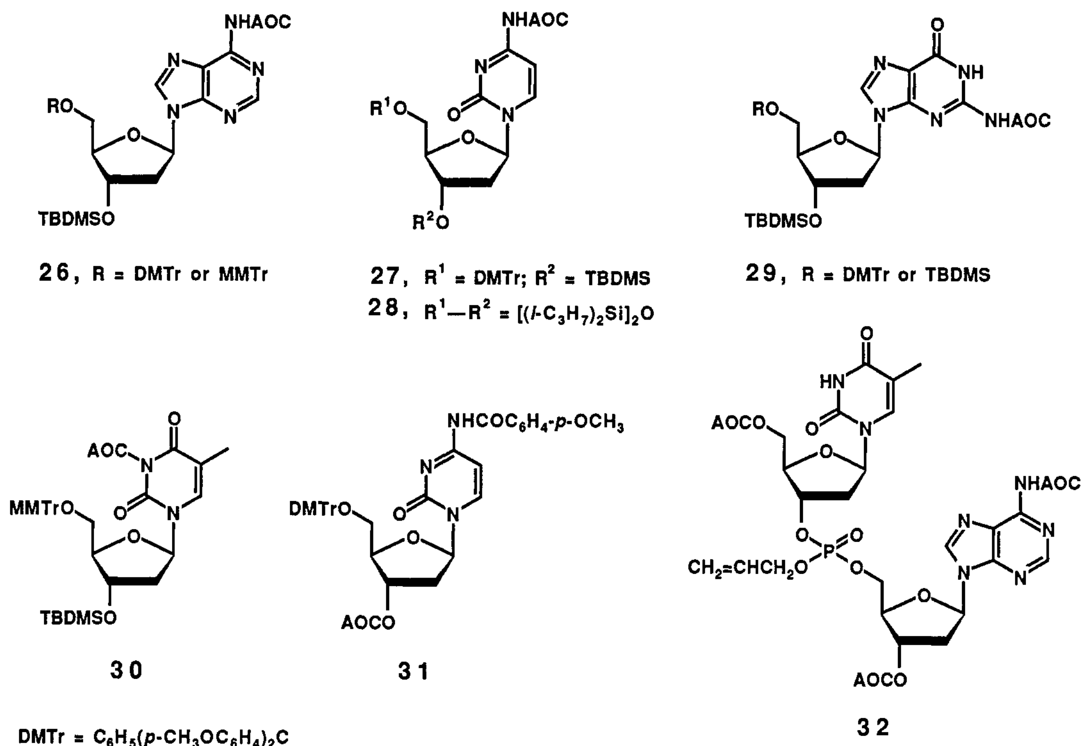


TABLE 4. Deprotection of allyloxycarbonylated nucleosides[a]

protected nucleoside	R	Pd catalyst	nucleophile	time/min	yield/%[b]
26	MMTr	$Pd[P(C_6H_5)_3]_4$	dimedone	5	96[c]
26	MMTr	$Pd[P(C_6H_5)_3]_4$	HCOOH	30	100[c]
26	MMTr	$Pd[P(C_6H_5)_3]_4$	n-$C_4H_9NH_2$/HCOOH	5	94
26	MMTr	$Pd_2(dba)_3{\cdot}CHCl_3$[d]	n-$C_4H_9NH_2$/HCOOH	5	100[c]
26	MMTr	$PdCl_2$	n-$C_4H_9NH_2$/HCOOH	240	99
26	MMTr	$PdCl_2(C_6H_5CN)_2$	n-$C_4H_9NH_2$/HCOOH	240	100[c]
26	MMTr	$Pd(OCOCH_3)_2$	n-$C_4H_9NH_2$/HCOOH	45	96
26	DMTr	$Pd[P(C_6H_5)_3]_4$	n-$C_4H_9NH_2/H_2O/CO_2$	10	97
27		$Pd[P(C_6H_5)_3]_4$	n-$C_4H_9NH_2$/HCOOH	5	96
27		$Pd[P(C_6H_5)_3]_4$	n-$C_4H_9NH_2/H_2O/CO_2$	10	98
28		$Pd[P(C_6H_5)_3]_4$	n-$C_4H_9NH_2$/HCOOH	5	94
29	TBDMS	$Pd[P(C_6H_5)_3]_4$	n-$C_4H_9NH_2$/HCOOH	5	80[e]
29	DMTr	$Pd[P(C_6H_5)_3]_4$	n-$C_4H_9NH_2/H_2O/CO_2$	10	95
30		$Pd[P(C_6H_5)_3]_4$	n-$C_4H_9NH_2$	5	97

[a] The reaction was carried out in THF containing the protected nucleoside (1 equiv), the Pd catalyst (5 mol %), triphenylphosphine (20–30 mol %), and the nucleophile (2 equiv) at 20–25 °C. [b] Isolated yield, unless otherwise noted.
[c] The yield was estimated by HPLC (ODS Develosil, a 1:15:2 mixture of acetonitrile, methanol, and water, 50 °C). [d] dba = dibenzylideneacetone. [e] The reaction was achieved in a 1:5 mixture of hexamethylphosphoric triamide and THF.

Synthesis of long-chain DNA oligomers

Allylic protection provides a truly powerful tool in synthesis of long-sequence oligonucleotides on solid supports. We choose as a target a 60mer, d(5'TATGGG-CCTTTTGATAGGATGCTCACCGAGCAAAACCAAGAACAACCAGGAGATTTTATT3') (**33**), known as a part of a DNA sequence of yolk sac tumor proteoglycan cDNA pPGl (ref. 11). The requisite phosphoramidite monomer units, **34**–**37**, were obtained in high yields by condensation of the corresponding 3'-*O*-free nucleosides and allyloxybis(diisopropylamino)phosphine assisted by 1*H*-tetrazole and diisopropylamine. The chain elongation was achieved on an Applied Biosystems Model 381A DNA synthesizer starting from **38**, thymidine covalently attached at the 3'-hydroxyl to controlled pore glass (CPG) supports (500 Å pore size) via a long-chain alkylamine spacer arm (ref. 12).

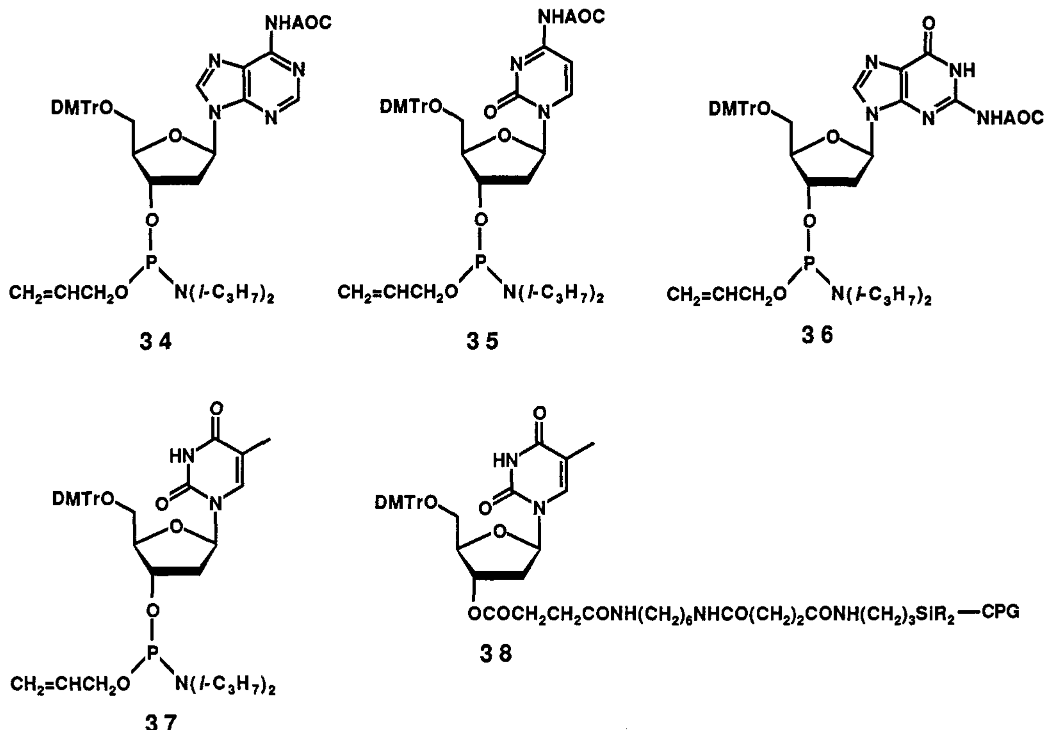


Table 5 outlines the synthetic cycle requiring 10.8 min, where the phosphite intermediates were oxidized by *tert*-butyl hydroperoxide (ref. 13) instead of the conventional aqueous iodine–pyridine mixture. After the final condensation, the 5'-DMTr protector was removed by trichloroacetic acid to afford a CPG-anchored DNA 60mer in which all NH_2 moieties of 2'-deoxyadenosine (dA), 2'-deoxycytidine (dC), and 2'-deoxyguanosine (dG) units were blocked by single AOC groups and the internucleotide bonds were protected by allyl groups. This product was then deblocked by treatment with a mixture of tris(dibenzylideneacetone)dipalladium(0) chloroform complex (2.5 equiv/allyl), triphenylphosphine (25 equiv/allyl), and a large excess of butylamine and formic acid in THF at 50 °C for 1 h, and treated with sodium *N,N*-diethyldithiocarbamate solution for 0.5 h. The target DNA was detached from the solid support by exposure to conc. ammonia at room temperature for 2 h. HPLC analysis of the products obtained by digestion of the 60mer by snake venom phosphodiesterase and bacterial alkaline phosphatase confirmed the full removal of the protective groups. The experimentally derived base composition, dA:dC:dG:T = 20.5:12.5:12.6:15.0 (T = thymidine), agreed well with the calculated ratio, 20:12:13:15. Furthermore, the digested product was inert to 5'-^{32}P-labeling with adenosine 5'-[γ-^{32}P]triphosphate using T4 polynucleotide kinase. This fact showed the absence of oligomers longer than a dimer, ensuring the complete elimination of the allyl protectors for phosphate linkages. A reference sample of **33** was prepared by using commercially supplied nucleoside phosphoramidites as monomer units with acyl (benzoyl for dA and dC and isobutyryl for dG) and 2-cyanoethyl for

protection of the amino and phosphate functionalties, respectively (ref. 12c). After detritylation, decyanoethylation and support-detachment were effected under the standard ammonium hydroxide treatment at ambient temperature for 2 h. Finally, heating with conc ammonia at 55 °C for 12 h accomplished deacylation completing the synthesis of free **33**.

TABLE 5. Reaction sequence of the solid-phase synthesis

step	operation	reagent	time/min
1	washing	CH_3CN	0.5
2	detritylation	3% CCl_3COOH/CH_2Cl_2	1.8
3	washing	CH_3CN	3.0
4	coupling	0.1 M phosphoramidite/CH_3CN + 0.5 M 4-nitrophenyltetrazole/ CH_3CN/THF	1.1
5	washing	CH_3CN	0.2
6	capping	Ac_2O/2,6-lutidine/THF (1:1:8) + 6.5% DMAP/THF	0.4
7	oxidation	1.1 M *tert*-C_4H_9OOH/CH_2Cl_2	0.8
8	washing	CH_3CN	0.6

The average coupling yield in the allyl-AOC method was 99.3% (assay of DMTr cation) resulting in an overall yield of 66% for protected 60mer **33**, which compares well with a 47% yield obtained by the current procedure. Fig. 1 illustrates the autoradiogram of the ^{32}P-labeled 5'-monophosphates of the crude 60mers. The oligonucleotide made by the present method gives a chromatogram showing only feeble spots or peaks due to short-chain DNAs, which are not negligible with the reference sample. The bio-image analysis (ref. 14) estimated the 60mer content of the crude products to be 70% (allyl-AOC) and 20% (conventional). The former value is consistent with that expected from the coupling yield, indicating that the removal of 104 allylic protective groups and support-detachment have been achieved in near quantitative yield. The target DNA can be easily purified on the solid support by simple washing; tedious, time-consuming chromatography is no longer necessary. The capability to directly prepare solid-anchored DNA oligomers is particularly noteworthy (ref. 15). Its applicability in molecular biology and diagonostics is enormous. This method can be used, for instance, for affinity chromatography for analysis and isolation of specific complementary DNA (cDNA) and RNA sequence, enrichment of desired genes in a cDNA library, solid-phase amplification of DNA, purification of DNA binding proteins, diagonosis of infections and genetic diseases, etc.

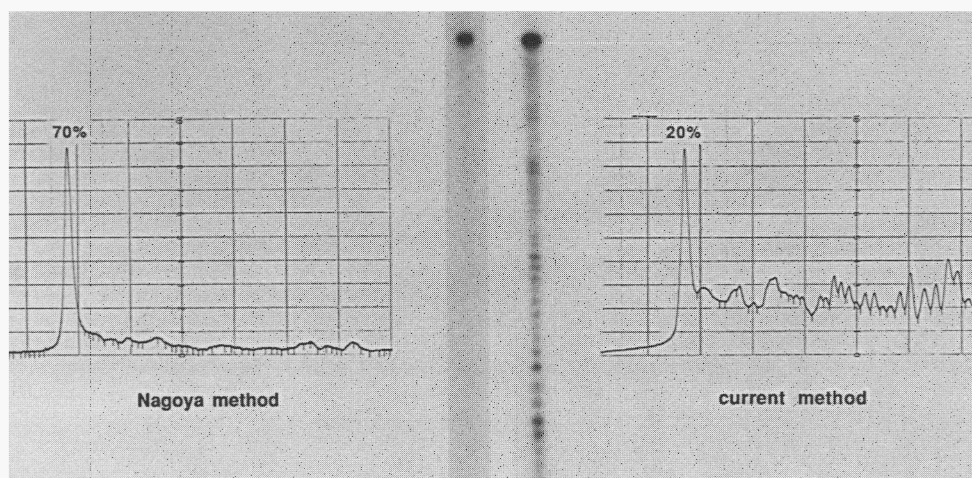


Fig.1. Autoradiograms of the crude ^{32}P 5'-phosphorylated 60mer **33**.

Acknowledgement

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