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119. Synthesis of Oligonucleotides Carrying Anchoring Groups and Their Use in the Preparation of Oligonucleotide-Gold Conjugates.

by **Beatriz G. de la Torre[a]), Juan Carlos Morales[a]), Anna Aviñó[a]), Daniela Iacopino[b]), Andrea Ongaro[b]), Donald Fitzmaurice[b]), Deirdre Murphy[c]), Hugh Doyle[c]), Gareth Redmond[c])** and **Ramon Eritja[*a]).**

[a]) Department of Molecular Genetics. Institut de Biología Molecular de Barcelona. C.S.I.C., Jordi Girona 18-26, E-08034 Barcelona. Spain. (phone: +34(93)4006145; fax: +34(93)2045904; e-mail : recgma@cid.csic.es)

[b]) Department of Chemistry. University College of Dublin. Belfield, Dublin 4, Ireland.

[c]) National Microelectronics Research Center (NMRC), Lee Malting, Cork, Ireland

Dedicated to Prof. Dr. *Wolfgang Pfleiderer* on the occasion of his 75th birthday

Oligodeoxynucleotide conjugates **1-15** carrying anchoring groups such as amino, thiol, pyrrole, and carboxyl groups were prepared. A post-synthetic modification protocol was developed. In this method 2'-deoxy-O^4-(*p*-nitrophenyl)uridine-3-phosphoramidite was prepared and incorporated in oligonucleotides. Following assembly of the sequence, the modified nucleoside was made to react with different amines carrying the anchoring groups. At the same time protecting groups were removed to yield the desired oligonucleotide conjugates. In a second approach, amino, thiol and carboxylic groups were introduced into the 3'-end of the oligonucleotides by preparing solid supports loaded with the appropriate amino acids. Oligonucleotide-gold conjugates were prepared and their binding properties were examined.

Introduction.- Oligonucleotide carrying gold nanoparticles were first described in 1996 [1, 2]. These conjugates have been shown to form networked arrays [1, 3, 4] and to form predetermined dimeric and trimeric assemblies [2, 5]. The special properties of gold nanoparticles linked to oligonucleotides has attracted large interest due to their potential use in gene analysis [6-11]. In addition, oligonucleotides have been linked to monodispersed CdSe/ZnS quantum dots [12, 13] and core-shell Ag/Au nanoparticles [14].

The preparation of oligonucleotide-gold nanoparticle conjugates is usually performed by reaction of oligonucleotides carrying thiol or dithiane androsterone [8, 9] with either citrate- [1,3,7-11,14] or phosphine-[5,15] stabilized gold nanoparticles or nanogold carrying maleimido groups [2]. Dithiothreitol (DTT)-stabilized CdSe/ZnS quantum dots were also activated with carbonyl diimidazole to further react with

oligonucleotides carrying amino groups [13]. Oligonucleotides carrying amino and thiol groups are extensively used for the preparation of conjugates that carry a large variety of functionalities including fluorochromes, biotin, intercalators, enzymes, and reactive groups [16] as well as for producing oligonucleotide microarrays [17]. Electrochemically-addressed DNA matrixes can also be produced by using oligonucleotides carrying pyrrole groups. These oligonucleotides can be anchored onto a conducting polymer film by electrooxidization of a mixture of pyrrole and pyrrole-bearing oligonucleotides [18]. Finally, a description is given of the preparation of oligonucleotides carrying a carboxyl group. The specific reactivity of the carboxyl group was further used to prepare oligonucleotide-daunomycin conjugates [19].

Anchoring groups such as amino-, and thiol-groups can easily be introduced at the 5'end of oligonucleotides using phosphoramidites derived from aminoalcohols and mercaptoalcohols [16]. The introduction of anchoring groups at the 3'end is not so simple. Special linker molecules are needed to introduce the desired group between the succinyl linker and the oligonucleotide. Aminodiols and dithio compounds are the most commonly used linkers for the preparation of oligonucleotides carrying amino and thiol groups at the 3'end. Very little data is available on the preparation of oligonucleotides carrying pyrrole or carboxylic groups at the 3'end [18, 19].

This paper describes the use of post-synthetic modification protocols (or the convertible nucleoside approach [20]) to prepare oligonucleotide carrying amino, thiol, pyrrole and carboxylic groups at predetermined sites. This strategy involves a 2'-deoxyuridine derivative that carries a good leaving group (O^4-*p*-nitrophenyl, [21]) being incorporated at specific oligonucleotide sites and converted into a modified nucleobase, which carries the desired anchoring group by nucleophilic attack of the appropriate

amine (*Scheme 1*) while the other protecting groups are also removed at the same time. In a second strategy trifunctional amino acids were added at the 3'end of the oligonucleotide through the use of special solid-supports [22]. After the protecting groups are removed, the amino acid side chain generates the desired amino, thiol or carboxyl groups. Oligonucleotide-gold nanoparticle conjugates were also prepared and a report is given of their binding properties.

Results.- 1. *Synthesis of oligonucleotides carrying anchoring groups by the post-synthetic modification approach*. A derivative of 2'-deoxyuridine (dU) was selected as a convertible nucleoside [20]. For this purpose, the O^4-(*p*-nitrophenyl) derivative of dU was prepared, as is described elsewhere [21]. The O^4-(*p*-nitrophenyl) dU derivative was incorporated on controlled pore glass (CPG) in accordance with standard protocols [23, 24]. The support carrying the O^4-(*p*-nitrophenyl)dU derivative was used to prepare two oligonucleotide sequences: A) 6mer: 5' TAG CTU^{Np} 3' and B) 17mer: TAG CTT GAC GAT AGG TU^{Np} 3' being U^{Np}= O^4-(*p*-nitrophenyl) dU.

Phosphoramidite derivatives protected with the *tert*-butylphenoxyacetyl group were used to incorporate the natural bases. These groups are more labile than standard groups and are removed by amines and at the same time that the O^4-(*p*-nitrophenyl)–dU derivative is being modified. The use of these protective groups prevents cytosine modification, which has been described when amines other than ammonia are used to remove protective groups in DNA synthesis [20]. A similar strategy has been successfully used to prepare N^2-substituted guanine derivatives [25].

Aliquots of the hexamer sequence A were treated with the following amines (1 M aqueous solution): I) cystamine (NH_2-CH_2-CH_2-S-S-$CH_2CH_2NH_2$), II) *N*-(2-

aminoethyl)pyrrole [26], III) 2,2'-[(ethane-1,2-diyl)bis(oxy)]bis(ethynamine) (NH_2-(CH_2-CH_2-O)$_2$-CH_2-CH_2-NH_2), IV) hexane-1,6-diamine, V) triglycine (NH_2-(CH_2CONH)$_2$-CH_2-COOH) and a thiol VI) 6-mercaptohexanol. The treatment with amine I yielded the hexamer **1** carrying a thiol group protected with as disulfide. Amine II generated the hexamer **2** carrying a pyrrole group. Amines III and IV generated hexamers **3** and **4** carrying an amino group linked through a polar (III) or apolar (IV) linker. Amine V produced the hexamer **5** carrying a carboxyl group. The thiol VI was selected to check whether a thiol group could also be used to incorporate molecules into the hexamer sequence. After the treatment, the resulting solutions were dry concentrated and desalted on a Sephadex G-25 column. The oligonucleotide-containing fractions were pooled and purified by reversed-phase HPLC. Mass spectrometry showed that the desired oligonucleotides (**1**-**5**) were obtained in all cases except one (*Table 1*). Treatment of the support with 6-mercaptohexanol did not yield any detectable oligonucleotide. Our belief is that the lack of product was due to 6-mercaptohexanol not being able to cleave the oligonucleotide-support bond. However, the strong yellow colour of the solution did indicate that *p*-nitrophenol was released. Following the thiol treatment, ammonia was then used to break the linkage between the oligonucleotide and the support. The two-step treatment was successful and the hexamer **6** was also obtained (*Table 1*). In addition to the desired oligonucleotide there were variable amounts (10-20%) of the hexamer resulting from hydrolysis of the *p*-nitrophenyl group. In order to prevent the hydrolysed product from forming, water was replaced with dry acetonitrile to dissolve the amine IV (1 M solution). In this case only the desired hexamer was obtained and no hydrolysed product was detected by HPLC, enzyme digestion or mass spectrometry.

Post-synthetic modification protocols were then applied to the 17mer sequence B to produce oligonucleotides carrying thiol, amino and pyrrole groups. In this case, sequence B was treated with three amines : I) cystamine (NH_2-CH_2-CH_2-S-S-$CH_2CH_2NH_2$), II) N-(2-aminoethyl)pyrrole and III) 2,2'-(ethylenedioxy)-diethylamine (NH_2-(CH_2-CH_2-O)$_2$-CH_2-CH_2-NH_2). The resulting oligonucleotides were purified by HPLC using standard trityl-on, trityl-off conditions. Treatment of sequence B with diamine I yielded oligonucleotide **7** in a 42% yield (*Table 2*). This yield is similar to that obtained by standard methods. In the case of the diamine III, the yield was lower (22%), and much lower with amine II. Oligonucleotides **7**-**9** were characterized by mass spectrometry and by analysis of the nucleoside composition after enzyme degradation.

Finally, the amount of excess amine necessary for the deprotection-modification reactions was determined. The support with hexamer A (200 nmol) was treated with decreasing amounts of hexane-1,6-diamine in acetonitrile: 1ml of 1 M solution and 0.1ml of 1 M, 0.1 M and 0.01 M solution (5000-5 molar amine/hexamer ratio). Good results were obtained with 5000-50 molar amine/hexamer ratio. At the 5 molar amine/ hexamer ratio the oligonucleotide was not released from the support. Subsequent treatment of the support with ammonia gave a mixture of three oligonucleotides in an equimolar ratio: the desired oligonucleotide (**4**), the hydrolysed product (hexamer with dU) and the product of the reaction of the convertible nucleoside with ammonia (hexamer with dC).

2. Synthesis of oligonucleotides carrying anchoring groups using trifunctional amino acids. The use of trifunctional amino acids to introduce anchoring groups at the 3'end of oligonucleotides was also studied. A recent protocol described for the stepwise

synthesis of oligonucleotide-peptide chimeras [22] was selected. First, a special support carrying the succinate derivative of 6-aminohexanol linked to amino-functionalized supports by a succinyl linkage was prepared (*Scheme 2*). Two amino-functionalized supports were used: controled-pore glass (CPG) and polystyrene-polyethyleneglycol (PEG-PS). The trifunctional amino acid was assembled on the 6-aminohexanol-succinyl-support using Boc-chemistry. To protect side chains, the ε-amino group of lysine was protected with the fluorenylmethoxycarbonyl (Fmoc) group, and the carboxyl group of glutamic acid and the thiol group were protected with the fluorenylmetyl (Fm) group. Coupling reactions were performed using triphenylphosphine and 2,2'-dithio-*bis*-(5-nitropyridine) [23] when CPG was used as solid support and Benzotriazol-1-yloxy)trispyrrolidino-phosphonium hexafluorophosphate (PyBOP) mediated coupling was applied with PEG-PS. After the incorporation of the appropriate amino acid, assembly of the oligonucleotide chain requires the presence of a hydroxyl group. The 4-hydroxypropionic linker carrying the dimethoxytrityl [$(MeO)_2Tr$] group for protection of the hydroxyl group [27] was used. The progress of amino acid and linker incorporation on PEG-PS supports was monitored by ninhydrin test [28]. This test was unreliable on CPG supports and for this reason a capping step was introduced after each coupling reaction. Standard phosphoramidites were used for oligonucleotide assembly. For synthesis of the oligonucleotide part, the standard synthesis cycle was used in the case of a CPG support and a special synthesis cycle with increased coupling time using PEG-PS. Although it has previously been reported that phosphoramidite coupling reactions on peptide-PEG-PS-supports are inefficient in acetonitrile due to poor swelling of the support, this

problem was overcome by using dichloromethane to dissolve the phosphoramidites [22]. However, attachment of just one single amino acid to the PEG-PS support produced no change in the solvents on phosphoramidite coupling.

Hexanucleotide sequences **10** and **11** were assembled according to scheme 2 on CPG- and PEG-PS- supports carrying Fm-protected Glu (*Table 3*). Following assembly of the sequences, standard ammonia deprotection was performed. In both cases, HPLC analysis of the resulting products gave one main product with the expected mass (*Table 3*). Supports carrying hexanucleotides **10** and **11** were alternatively treated first with a 0.5 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) solution (to remove the Fmoc group), followed by ammonia to prevent glutamine formation. HPLC profiles of the products obtained with or without DBU treatment were identical indicating that glutamine formation did not happen in at a detectable level (<10%) under standard ammonia deprotection. Likewise, oligonucleotides **12** and **13** were also assembled on CPG supports using glutamic acid as the amino acid component. These oligonucleotides were obtained in similar yields and purity (tested by gel electrophoresis, data not shown) as unmodified oligonucleotides. The carboxyl group functionalized oligonucleotides **12** and **13** are used for anchoring oligonucleotides to amino-modified gold electrodes (work in progress).

Attempts to synthesize the hexanucleotide sequence C_6 on CPG support carrying Fmoc-protected Lys were not successful. This was possibly due, to the poor coupling efficiency of the linkers and amino acid. On the contrary, the tetranucleotide sequence **14** was assembled without any problems on PEG-PS carrying Lys(Fmoc) and, following ammonia deprotection, gave a main product with the correct mass (*Table 3*).

Finally, the dodecanucleotide sequence **15** containing Fm-protected Cys resulted from analogous synthetic steps. In this case, ammonia deprotection was performed with a mixture of concentrated ammonia and 50 mM DTT to prevent disulfide formation. A single peak in HPLC was obtained showing the expected mass (*Table 3*) and no dimer formation was observed even after several days with the HPLC-purified product kept in a solution without DTT.

3. *Synthesis and properties of oligonucleotide-gold nanoparticle conjugates*. First, phosphine-stabilized gold nanoparticles (1.4nm) carrying either a carboxyl group active ester (hydroxy-2,5-dioxopyrrolodine-3-sulfonic acid sodium salt, sulfo-NHS) or a maleimido group (NANOGOLD, *Nanoprobes Inc.*, USA) were selected to form defined conjugates carrying one oligonucleotide per particle (NANOGOLD particles statistically carry one reactive molecule incorporated into the phosphine shelf). Conjugation of maleimido-NANOGOLD to cysteine-oligonucleotide **15** was performed in 10% aqueous isopropanol. 0.1 M carbonate buffer (pH 9.0) was used for the conjugation of sulfo-NHS-NANOGOLD to amino-oligonucleotide **9**. Conjugates were analyzed and purified by 2% agarose gel electrophoresis (*Figure 1*) as described elsewhere [5, 15]. NANOGOLD is uncharged and for this reason remained near the wells whereas oligonucleotide-NANOGOLD conjugates are negatively-charged and move towards the anode. Conjugation efficiency was judged to be similar between cyteine-oligonucleotide **15** and the thiol-oligonucleotide **16** prepared with commercially available chemicals. These conjugates will be used for the self-assembly of well-defined gold nanoparticle architectures in solution [2, 5, 11] (work in progress).

In addition, oligonucleotides carrying thiol groups (**15-19**, *Tables 3, 4*) were reacted with citrate-stabilized gold nanoparticles (13nm) to obtain gold nanoparticles with several oligonucleotide molecules per nanoparticle [7]. Melting experiments with duplexes formed by complementary oligonucleotides **16** and **17** were studied by UV/Visible absorption spectroscopy. Marked changes in the optical absorption spectra observed as a function of increasing temperatures, included a substantial increase in the absorption at 260 nm and a shift (40nm) in the maximum position of the surface plasmon absorption mode of the Au nanoparticles (*Fig. 2a*). Both these features are in good agreement with previous studies, which assign these spectral changes to the binding of the derivatised Au nanoparticles with their complements [7]. *Fig. 2b* shows the temperature dependent melting of duplexes formed by sequences **16** and **17** at 260 nm with and without Au nanoparticles attached. When nanoparticles are present, melting transitions occur over a small temperature range and with a large absorbance change, producing sharp well defined melting transitions. In contrast, unmodified oligonucleotide duplexes melt over broad temperature ranges and produce comparatively small absorption changes.

Further studies were carried out to measure the effects of introducing a gap in the stability of a three component oligonucleotide system, (**20-23**). Two short oligonucleotides (**18** and **19**) attached to Au nanoparticles were hybridized to complementary oligonucleotides **20-23** with an increasing number of bases inserted into the target oligonucleotide sequence. As can be seen from *Figure 3* the introduction of unpaired bases into the target oligonucleotide results in a systematic lowering of the melting temperature. Again, the absorbance changes that occur during the melting of the three component oliognucleotide-nanoparticle conjugates are of greater magnitude

and occur over narrower temperature ranges than with unmodified oligonucleotides. Similar results were obtained when oligonucleotide **18** was replaced by cysteine-oligonucleotide **15** (*Figure 4*). Melting temperatures obtained with cysteine-oligonucleotide **15** were the same (within an error of 0.3 ºC) as melting temperatures obtained with oligonucleotide **18** prepared with commercially available compounds (*Table 5*).

Discussion. Oligonucleotides carrying several anchoring groups were prepared. The results obtained showed that O^4-(*p*-nitrophenyl)dU is a versatile compound for the preparation of modified oligonucleotides carrying a variety of additional functional groups including thiol, amino, pyrrole and carboxyl groups on the nucleobase. The O^4-(*p*-nitrophenyl)dU modified oligonucleotides allow the different linker molecules to be fixed at specific sites. This may be an advantage for finding the optimal characteristics of linker molecules without having to prepare a large number of derivatives. Moreover, side reactions may occur if aqueous conditions are used. Changes to anhydrous solvents may have a positive effect. Although the convertible nucleoside was located in this study at the 3'end, this methodology allows the convertible nucleoside to be assembled at any position on the oligonucleotide. O^4-(2,4,6-trimethylphenyl)-2'-deoxyuridine was previously used for similar purposes [20], and the use of a *p*-nitrophenyl as a more active leaving group in combination with the more labile *tert*-butylphenoxyacetyl (Tac) group for amino protection is preferred because the deprotection-modification reaction can be performed in one single step. Moreover, the intense yellow colour of the resulting *p*-nitrophenolate ion allows the development of nucleophilic substitution to be monitored.

The use of trifunctional amino acids to incorporate amino-, thiol- and carboxylic-groups at the 3'end of oligonucleotides was also studied. The use of base-labile groups protecting groups for the side chain functions of amino acids was compatible with oligonucleotide synthesis. The α-amino groups of the amino acids were protected with the Boc-group but these groups were removed prior to the oligonucleotide being assembled. Two linkers were needed to connect the amino acid to the desired oligonucleotide and 4-hydroxybutanoic acid and 6-aminohexanol, inexpensive compounds, were applied as starting materials. CPG and PEG-PS supports in general gave good results and were compatible with oligonucleotide synthesis without any change to the solvent used on the phosphoramidite coupling reactions.

Finally, the hybridisation properties of oligonucleotide gold nanoparticle conjugates were investigated and it was shown that the conjugates in general gave sharper and more defined melting transitions than unmodified oligonucleotides.

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Experimental Part

General. Phosphoramidites and ancillary reagents used during oligonucleotide synthesis were from *Applied Biosystems* (*PE Biosystems Hispania S.A.,* Spain), *Cruachem* (*Cruachem Ltd.,* Scotland) and *Glen Research* (*Glen Research Inc*., USA). Amino acid derivatives were from *Bachem* (*Bachem AG,* Switzerland) and *Novabiochem* (*Calbiochem-Novabiochem AG,* Switzerland). The rest of the chemicals were purchased from *Aldrich*, *Sigma* or *Fluka* (*Sigma-Aldrich Química S.A.,* Spain). Long chain amino controlled pore glass (LCA-CPG) was purchased from CPG (*CPG,* Inc., *New Jersey,* USA). Amino-polyethyleneglycol-polystyrene (PEG-PS) was purchased from *PerSeptive* (*PerSeptive Biosystems*, now *Applied Biosystems* USA). Solvents were from *S.D.S.* (*S.D.S.,* France). $(MeO)_2$Tr-protected-2'-deoxy-O^4-(*p*-nitrophenyl)-uridine was synthesized according to literature [21]. Gold nanoparticles (13 nm, citrate stabilized) were prepared as described elsewhere [7], and NANOGOLD (1.4 nm, phosphine stabilized) carrying maleimido and sulfo-NHS ester functionalities were purchased from *Nanoprobes* (*Nanoprobes, Inc*., USA).

Instrumentation. UV spectra were recorded on a UV-2301PC and a 2401 *Shimadzu* spectrophotometers. Mass spectra (matrix-assisted laser desorption ionization time-of-flight, MALDI-TOF) were provided by *Eurogentec* and Mass spectrometry service at the University of Barcelona.

Preparation of solid-supports. Solid-support carrying 2'-deoxy-O^4-(*p*-nitrophenyl)-uridine was prepared by reacting the 3'-hemisuccinate derivative of 5'-$(MeO)_2$Tr-2'-deoxy-O^4-(*p*-nitrophenyl)-uridine [24] with amino controlled pore glass (long chain amino-CPG) using triphenylphosphine and 2,2'-dithio-*bis*-(5-nitropyridine) [23] for condensation.

Solid supports (CPG and PEG-PS) carrying amino acids were prepared by sequential addition of the following compounds: 1) N^6-Boc-6-aminohexan-1-ol hemisuccinate prepared by reaction of commercially available N^6-Boc-6-aminohexan-1-ol with succinic anhydride and DMAP as reported elsewhere [24], 2) either Boc-Glu(Fm)-OH, or Boc-Lys(Fmoc)-OH, or Boc-Cys(Fm)-OH and 3) O^4-$(MeO)_2$Tr-4-hydroxybutyric acid *p*-nitrophenyl ester [27]. The addition of Boc-amino acids on PEG-PS was performed in dimethylformamide (DMF) using 5-fold excess of the appropriate amino acid, 5-fold excess of PyBOP and 10-fold excess of diisopropylethylamine (DIEA) for 1 h at room temperature. The removal of the Boc-protecting group was performed using 40% trifluoroacetic acid in dichloromethane. A neutralization step with 5% DIEA in dichloromethane and several washing steps were introduced between removal of the Boc-group and coupling. The coupling of the *p*-nitrophenyl ester of $(MeO)_2$Tr-protected 4-hydroxybutyric acid was performed in the presence of 1 equivalent of *N*-hydroxybenzotriazole (HOBt) using 5-fold excess. A negative ninhydrine test was obtained after several hours at room temperature. As a precaution acetylation of unreacted amino groups was performed by a 10-min treatment of the support with a mixture of acetic anhydride / DIEA/ DMF (1:1.7: 15.3 ml). The addition of Boc-amino acids to CPG supports was performed using triphenylphosphine and 2,2'-dithio-*bis*-(5-nitropyridine) as coupling activating agents [23]. The coupling of the *p*-nitrophenyl ester of $(MeO)_2$Tr-protected 4-hydroxybutyric acid was performed as described for PEG-PS supports.

Oligonucleotide Synthesis. Oligonucleotide sequences were synthesized on an *Applied Biosystems* DNA synthesizer model 392 (*Applied Biosystems*, USA). 5'-$(MeO)_2$-Tr-, (*tert*-butyl)phenoxyacetyl-protected-3'-(2-cyanoethyl-*N*,*N*-diisopropyl-

phosphoramidites) were used for the synthesis of oligonucleotides carrying the 2'-deoxy-O^4-(*p*-nitrophenyl)-uridine moiety. The rest of the sequences were prepared using standard (Bz- or ibu-protected) 3'-phosphoramidites. Standard 1-μmol-scale synthesis cycles were used. Supports were prepared as described below. Coupling efficiencies were higher than 98%.

In addition to compounds **1-15**, oligonucleotides carrying thiol groups at 5'end (**16, 17**, **19**) and the 3'end (**18**) were prepared using the phosphoramidite of $(MeO)_2$-Tr-protected 6-hydroxyhexyl disulfide and the CPG support functionalized with $(MeO)_2$-Tr-protected 3-hydroxypropyl disulfide (*Glen Research*, USA).

Deprotection of oligonucleotides. Oligonucleotide supports carrying 2'-deoxy-O^4-(*p*-nitrophenyl)-uridine were treated with 1ml (or less) of 1 M aqueous solutions of the appropriate amine (*Tables 1 and 2)* at 55 ºC overnight and the solution was filtered and concentrated to dryness. The solution of *N*-(2-aminoethyl)pyrrole contained 10% of dioxane. Cystamine was obtained from commercial sources in a hydrochloride form. One equivalent of triethylamine was added to the cystamine solution to neutralize the hydrochloride. Two different deprotection protocols were used for oligonucleotide supports carrying lysine and glutamic acid at the 3'end. In the first protocol supports were treated directly with 1ml of ammonia (overnight, 55ºC). In the second protocol supports were treated with 1ml of 0.5 M DBU solution in acetonitrile for 30 min at room temperature. The support was washed in acetonitrile, 1% triethylamine in acetonitrile and acetonitrile and dried. The resulting support was then treated with 1ml of concentrated ammonia (overnight, 55ºC). Oligonucleotide supports carrying either cysteine at the 3'end or oligonucleotides carrying the 6-mercaptohexyl group at the

5’end were treated overnight with 1ml of 50 mM dithio-DL-threitol (DTT) in concentrated ammonia at 55ºC.

Purification of oligonucleotides. The resulting products were dissolved in water and the solution was desalted on a Sephadex G-25 column (NAP-10, *Pharmacia*, Sweden). The oligonucleotide-containing fractions were analyzed and purified by HPLC, as follows: Column : PRP-1, 10 μm (*Hamilton*, USA) (305 x 7mm), flow rate 3ml / min. A 20min linear gradient from 15 to 55% acetonitrile over 100 mM aqueous triethylammonium acetate was used for the oligonucleotide carrying the $(MeO)_2$-Tr group. After removal of the $(MeO)_2$-Tr group with 80% acetic acid (30min) the resulting oligonucleotides were purified on the same column using a 20min linear gradient from 5 to 25% acetonitrile over 100 mM aqueous triethylammonium acetate. Purified oligonucleotides were analyzed by mass spectrometry (MALDI-TOF). The results are shown in Tables 1-3.

Enzymatic analysis of oligonucleotides. Oligonucleotides prepared by postsynthetic modification (0.5-1 OD units) were incubated in 50 mM tris HCl pH 8.0 and 10 mM $MgCl_2$ with snake venom phosphodiesterase (*Pharmacia Biotech*, Sweden) and bacterial alkaline phosphatase (*Pharmacia Biotech*, Sweden) in a total volume of 0.03ml at 37ºC overnight. The resulting mixture was diluted and analyzed by HPLC. Column : C-18 Nucleosil, 5 μm (250 x 4 mm), flow rate 1ml/min. A 20 min linear gradient from 5 to 22% acetonitrile over 20 mM aqueous triethylammonium acetate was used, followed by 5 min of isocratic conditions at 22% acetonitrile . Retention times observed under these conditions were dC 3.6 min, dU (hydrolysis product) 4.3 min, dG

6.1 min, T 7.0 min, dA 9.7 min. Modified dC derivatives: N^4-(2-pyrrolethyl)-dC 15.8 min, N^4-{2-[2-(2-aminoethyl)oxyethyl]oxyethyl}-dC 11.4 min, N^4-(2-(2'-aminoethyl)dithioethyl)-dC 8.1 min, N^4-(6-aminohexyl)-dC 12.6 min, S^4-(6-hydroxyhexyl)-4-thio-dU 24.0 min (UV max at 330 nm).

Preparation of Au nanoparticles and Oligonucleotides prior to conjugation. Au nanoparticles were prepared by the citrate reduction of $HAuCl_4$ as described elsewhere [7]. Nanoparticles were analysed by UV-Vis absorption spectroscopy (*Shimadzu* 2401 spectrophotometer) and TEM (*JEOL* JEM 1200-EX). Oligonucleotides were desalted prior use on a NAP-10 (*Pharmacia*) column using a 10 mM sodium phosphate buffer (pH 7) as solvent. The resulting fractions were analysed by UV-Vis.

Preparation of oligonucleotide-gold conjugates. Conjugation of maleimido-NANOGOLD to thiol-oligonucleotides was performed by following the recommendations of the suppliers'. Aliquotes of liophylized maleimido-NANOGOLD (6 nmols) were mixed with thiol-oligonucleotides (6 nmols) dissolved in 10% aqueous isopropanol. The resulting mixtures were kept overnight at room temperature and the resulting solutions were stored in the refrigerator until further use. A similar protocol was used for the conjugation of amino-oligonucleotides to sulfo-NHS-NANOGOLD. In this case 0.1 M carbonate buffer (pH 9.0) was used as a reaction buffer. Conjugates were purified by 2% agarose gel electrophoresis (0.5xTBE) as described elsewhere [5, 15].

Furthermore, oligonucleotide carrying thiol groups were also conjugated to citrate-stabilized gold nanoparticles. The desalted thiolated oligonucleotide was removed from

the freezer and allowed to thaw. The optical absorbance of both the oligonucleotide and nanoparticle samples was measured. The nanoparticles and oligonucleotide were mixed in appropriate amounts and the entire solution was brought to 10 mM sodium phosphate buffer (pH 7). After one day, the solution was brought to 0.1 M NaCl concentration and allowed to stand at room temperature for another 40 hours. After this time, the solution was centrifuged at 13,200 rpm for 30 min. Supernatant was removed and the reddish solid at the bottom was dispersed in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 7) solution (the volume added was similar to that removed). This procedure was repeated by applying 0.3 M NaCl and 10 mM phosphate buffer (pH 7). The solution was analysed by UV-Vis spectroscopy and the absorbance at 260nm was recorded for future use. The solution was stored at room temperature.

Melting experiments. Melting experiments were carried out using equimolar amounts of each oligonucleotide-gold nanoparticle conjugate in 0.3 M NaCl, 10 mM sodium phosphate buffer, pH 7. The solution was heated to 90º for 5 min and then allowed to cool to room temperature for 48 hours. The solution was transferred to a stoppered 1-cm path-length cuvette and UV-Vis spectra were recorded at 5º intervals while the sample was heated from 20-90º at 5ºC/5 min. Heating of the UV cuvette was performed with a Grant W6-KD precision thermostatic circulator attached to a Shimadzu constant temperature cell holder. Calibration spectra were recorded to determine the heat transfer efficiency to the cuvette. The data were collected as wavelength versus absorbance spectra and a plot of temperature versus absorbance at 260nm was drawn. The melting temperature of the systems was determined from these graphs.

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Table 1: Mass spectrometry analysis of the oligonucleotide resulting from the treatment of hexanucleotide-support 5'TAGCTU^{Np}-CPG 3' with amines or thiols.

Compound	Amine/thiol used	Yield	M (found)	Calc.
1	I) $(NH_2\text{-}CH_2\text{-}CH_2\text{-}S)_2$	35%	1903	1903
2	II) $NH_2\text{-}CH_2\text{-}CH_2$-pyrrole	10%	1861	1862
3	III) $NH_2\text{-}(CH_2\text{-}CH_2\text{-}O)_2\text{-}CH_2\text{-}CH_2\text{-}NH_2$	30%	1900	1899
4	IV) $NH_2\text{-}(CH_2)_6\text{-}NH_2$	25%	1868	1867
5	V) $NH_2\text{-}(CH_2CONH)_2\text{-}CH_2\text{-}COOH$	25%	1941	1940
6	VI) $HS\text{-}(CH_2)_6\text{-}OH$[a]	15%	1885	1885

[a]Ammonia was needed to release the hexamer from the support after it was treated with the thiol.

Table 2: Yield and mass spectra of oligonucleotides resulting from the treatment of 17mer-support TAGCTTGACGATAGGTU^{Np}-CPG 3' with different amines.

Compound	Amine/thiol used	Yield	M (found)	Calc.
7	I) $(NH_2\text{-}CH_2\text{-}CH_2\text{-}S)_2$	42%	5361	5360
8	II) $NH_2\text{-}CH_2\text{-}CH_2$-pyrrole	7%	5318	5318
9	III) $NH_2\text{-}(CH_2\text{-}CH_2\text{-}O)_2\text{-}CH_2\text{-}CH_2\text{-}NH_2$	22%	5375	5376

Table 3. Oligonucleotide sequences carrying trifunctional amino acids at the 3'end prepared in the present study.

Compound	Sequence 5'->3'	Solid support[a]	Yield	M (found)	Calc.
10	CCCCCC	Glu(Fm)CPG	22%	2066	2067
11	TTTTTT	Glu(Fm)PEG-PS	20%	2154	2157
12	GAGCTACTGAGTTACTGAGC	Glu(Fm)CPG	43%	n.d.	
13	GCTCAGTAACTCAGTAGCTC	Glu(Fm)CPG	40%	n.d.	
14	CCCC	Lys(Fmoc)PEG-PS	35%	1486	1488
15	TCTCAACTCGTA	Cys(Fm)CPG	40%	3947	3948

[a]Abbreviations: CPG: controlled pore glass, PEG-PS: polyethyleneglycol-polystyrene, Fm: fluorenylmethyl, Fmoc: fluorenylmethoxycarbonyl. n.d. not determined.

Table 4: Oligonucleotide sequences prepared using standard methods.

Compound	Sequence 5'->3'
16	Thiol-hexyl-CGAGTCATTGAGTCATCGAG
17	Thiol-hexyl-CTCGATGACTCAATGACTCG
18	TCTCAACTCGTA-propyl-thiol
19	Thiol-hexyl-CGCATTCAGGAT
20	TACGAGTTGAGAATCCTGAATGCG
21	TACGAGTTGAGACATCCTGAATGCG
22	TACGAGTTGAGACCATCCTGAATGCG
23	TACGAGTTGAGACCCATCCTGAATGCG

Table 5: Comparison of melting temperatures obtained with a DNA assembly made of three components, a target oligonucleotide sequence (**20-23**) with an increasing number of inserted bases and two short oligonucleotide-Au nanoparticle conjugates. In the first column, thiolated oligonucleotides **18** and **19** were used while cysteine-oligonucleotide **15** and thiolated-oligonucleotide **19** were used in the next column. Conditions 0.3 M NaCl and 10 mM sodium phosphate pH7.

Template	Number of insertions	Oligonucleotide **18** Tm(ºC)	Oligonucleotide **15** Tm(ºC)
20	0	51.8	51.2
21	1	47.6	47.5
22	2	47.4	47.0
23	3	46.4	46.7

LEGENDS

Scheme 1: Synthesis of oligonucleotides carrying anchoring groups by postsynthetic modification protocol.

Scheme 2: Synthesis of oligonucleotides carrying trifunctional amino acids at the 3'end. Lys (Fmoc), R= $(CH_2)_4$-NH-Fmoc. Cys (Fm), R= CH_2-S-Fm. Glu(Fm), R= $(CH_2)_2$-COO-Fm

Figure 1: 2% Agarose gel (0.5x Tris-Borate-EDTA (TBE)) of oligonucleotide-gold nanoparticle conjugates. The gel was run at 80 volts for 20 min. Lane 1: Maleimido-NANOGOLD. Lane 2: Reaction of cysteine-oligonucleotide **15** with maleimido-NANOGOLD. Lane 3: Reaction of thiol-oligonucleotide **16** with maleimido-NANOGOLD. Lane 4: Reaction of amino-oligonucleotide **9** with sulfo-NHS-NANOGOLD. Lane 5: Bromophenolblue and xylenecyanol dyes.

Figure 2: Hybridization and melting of oligonucleotide-gold nanoparticle conjugates. A) UV-spectra of duplex formed with oligonucleotide-gold nanoparticle conjugates **16** and **17**. B) Melting profiles at 260nm of duplex formed by sequences **16** and **17** with and without gold nanoparticles.

Figure 3: Melting profiles at 260nm of thiol-modified oligonucleotide- Au nanoparticle conjugates. A) Target oligonucleotide sequences (**20-23**) with an increasing number of inserted bases were mixed with unmodified oligonucleotide sequences **18** and **19**. B) Target

oligonucleotide sequences (**20-23**) were mixed with thiolated oligonucleotide sequences **18** and **19** attached to Au nanoparticles (0.3 M NaCl and 10 mM sodium phosphate pH 7).

Figure 4: Melting profiles at 260nm of cysteine-modified oligonucleotide- Au nanoparticle conjugate. Target oligonucleotide sequences (**20-23**) with an increasing number of inserted bases were mixed with oligonucleotides **15** and **19** attached to Au nanoparticles (0.3 M NaCl and 10 mM sodium phosphate pH 7).

Figure 1.

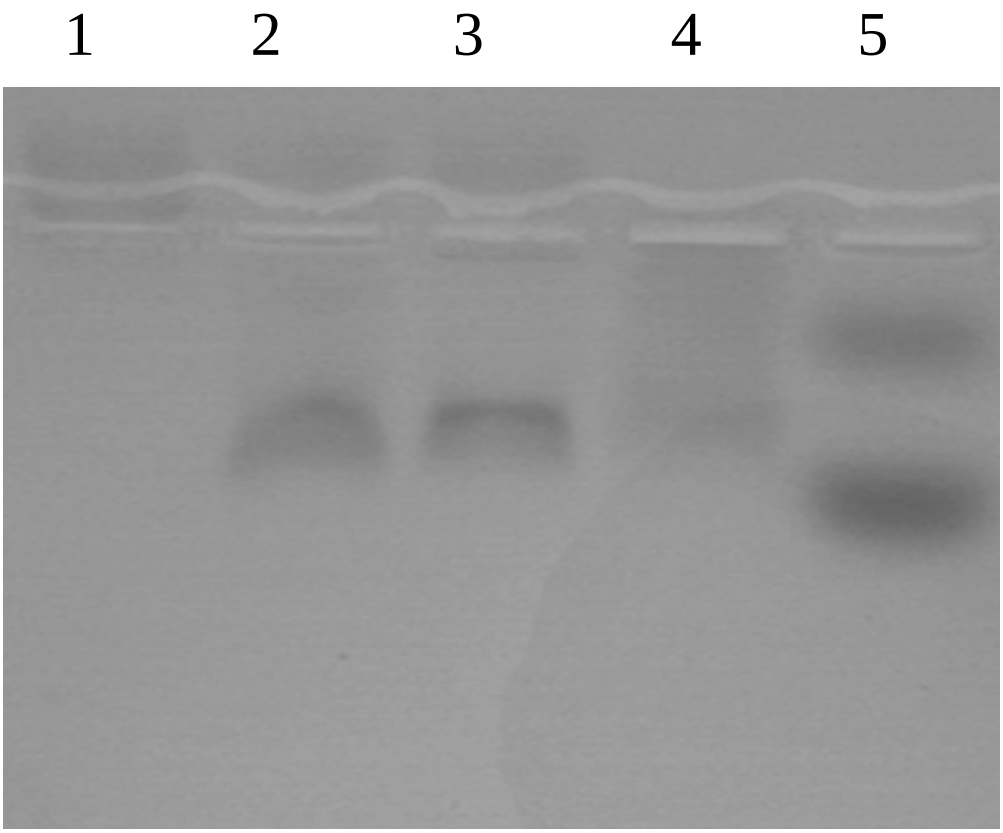


Figure 2.

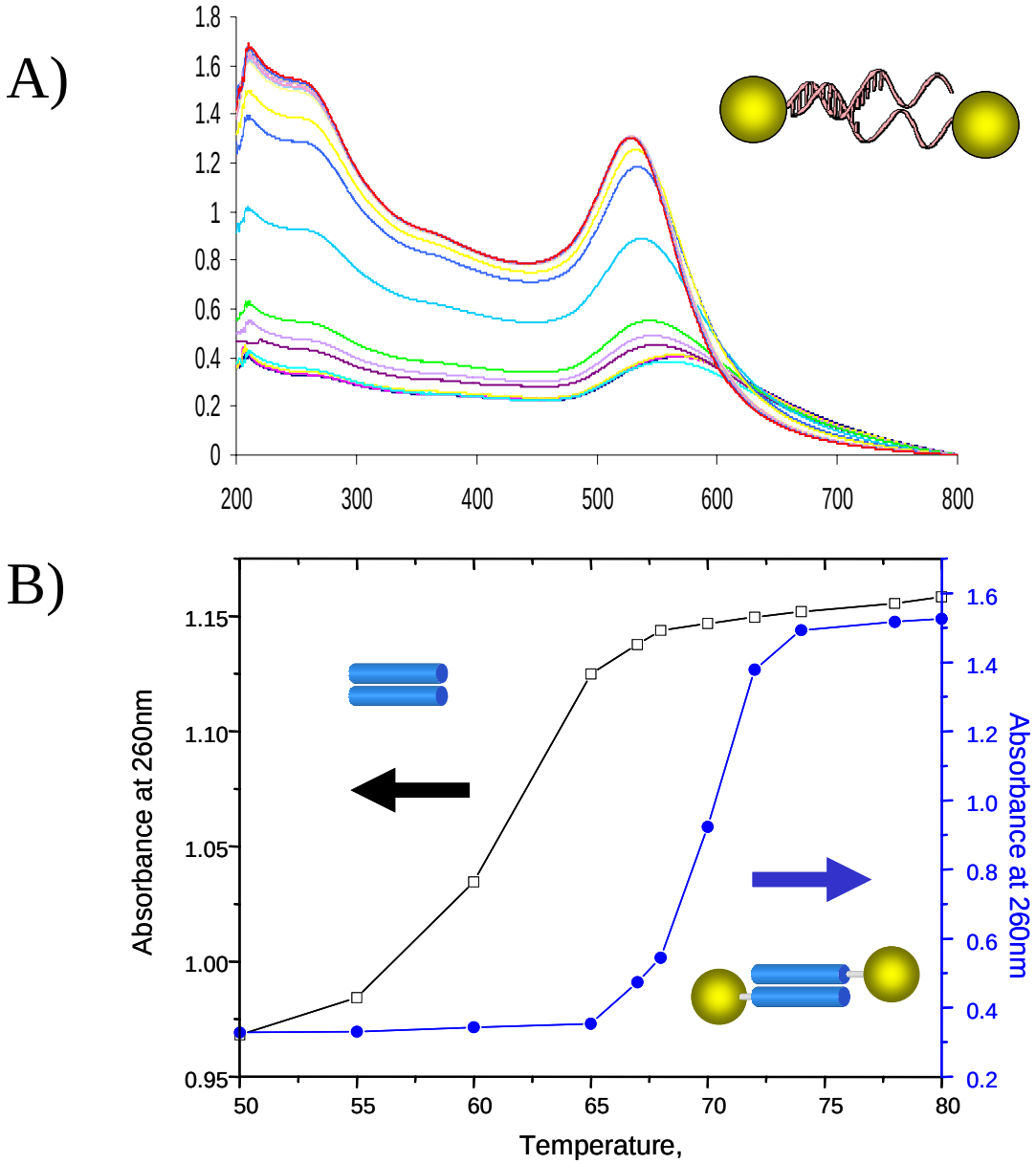
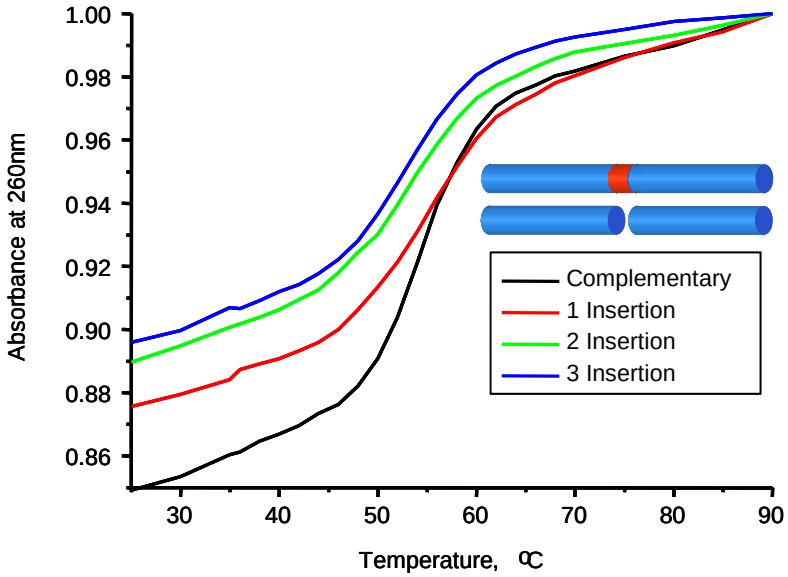

A)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
200
300
400
500
600
700
800
B)
Absorbance at 260nm
1.15
1.10
1.05
1.00
0.95
50
55
60
65
70
75
80
Temperature,
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance at 260nm

Figure 3.

A)


1.00
0.98
0.96
0.94
0.92
0.90
0.88
0.86
Absorbance at 260nm
30
40
50
60
70
80
90
Temperature, ℃
Complementary
1 Insertion
2 Insertion
3 Insertion

B)

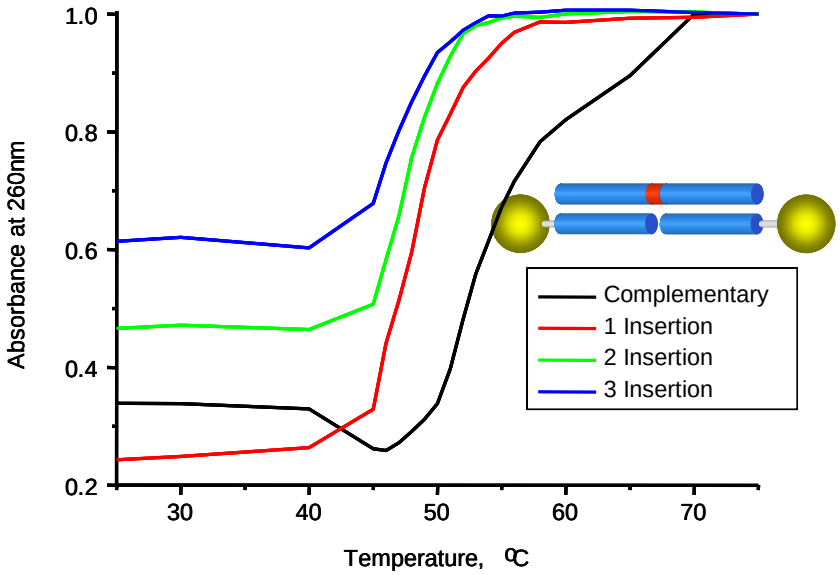
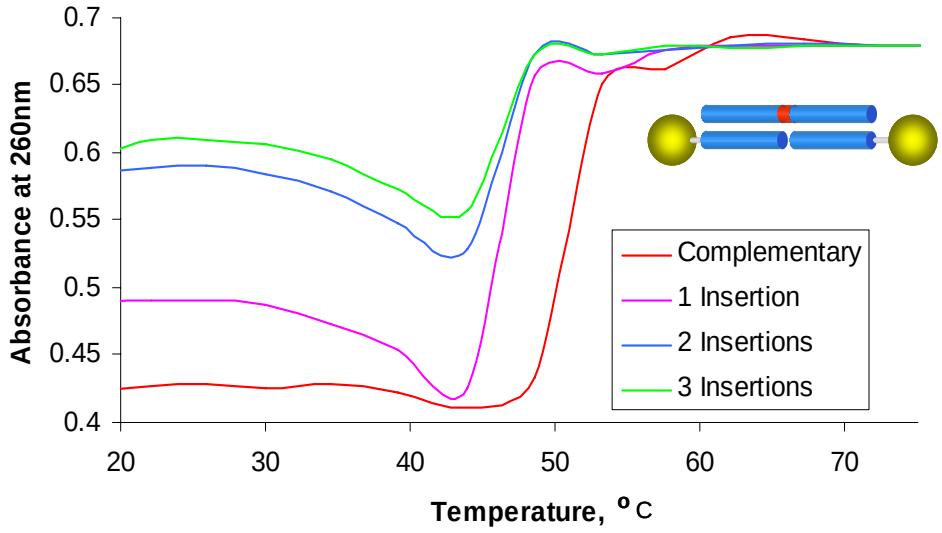
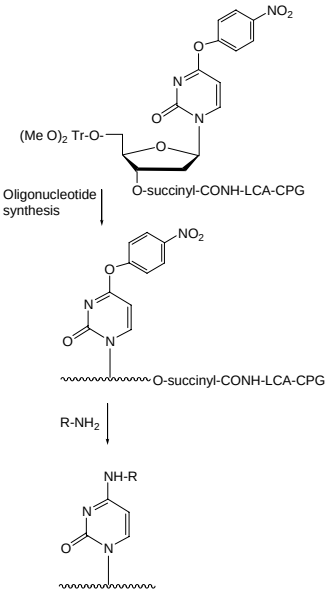

1.0
0.8
0.6
0.4
0.2
Absorbance at 260nm
30
40
50
60
70
Temperature, ℃
Complementary
1 Insertion
2 Insertion
3 Insertion

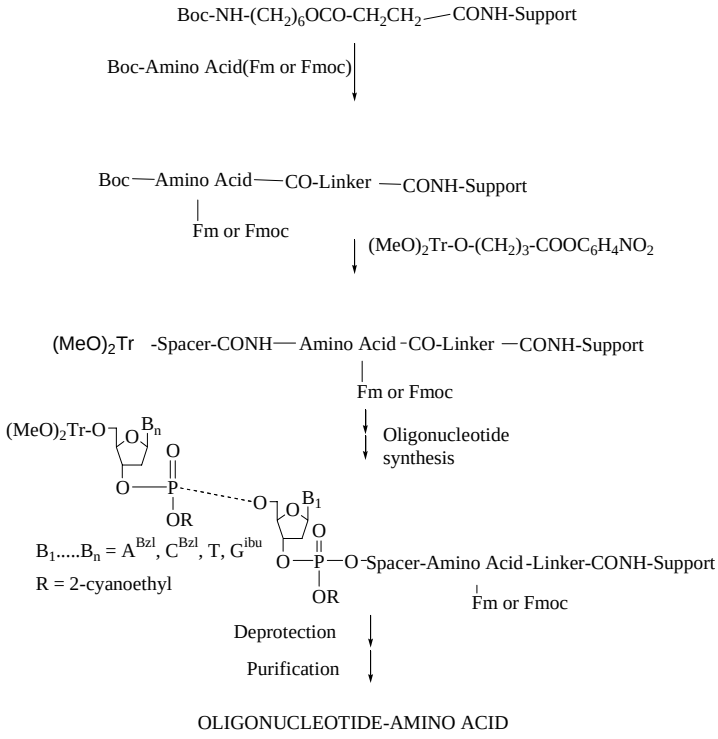
Figure 4.



Scheme 1.



Scheme 1



Scheme 2