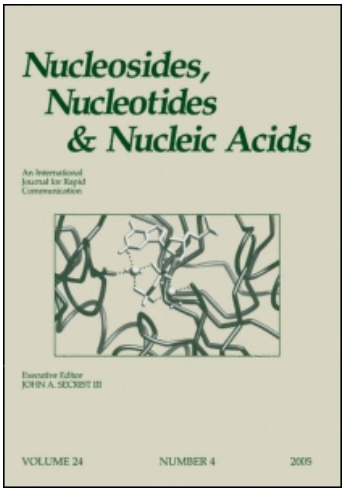

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Rapid Routes of Synthesis of Oligonucleotide Conjugates from Non-Protected Oligonucleotides and Ligands Possessing Different Nucleophilic or Electrophilic Functional Groups

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(10–12), 1943–1965 (2000)

RAPID ROUTES OF SYNTHESIS OF OLIGONUCLEOTIDE CONJUGATES FROM NON-PROTECTED OLIGONUCLEOTIDES AND LIGANDS POSSESSING DIFFERENT NUCLEOPHILIC OR ELECTROPHILIC FUNCTIONAL GROUPS

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ABSTRACT. Optimized methods are described for post-synthetic conjugation of non-protected oligodeoxyribonucleotides to different ligands. Methods for the terminal functionalization of oligonucleotides by amino, sulfhydryl, thiophosphate or carboxyl groups using different chemical reactions and linkers in both organic and aqueous media are described and compared. Experimental conditions for subsequent coupling of ligands containing aliphatic and aromatic amines, aromatic alcohols, carboxylic, sulfhydryl, alkylating, aldehydic and other reactive nucleophilic and electrophilic groups to oligonucleotides were established, including covalent linkage to other oligonucleotides.

INTRODUCTION

Oligonucleotides, which can form complexes with RNA by complementary base pairing (anti-sense strategy [1-4]) or with DNA by triple helix formation (anti-gene strategy [5-7]), represent a promising class of potential therapeutic agents acting directly on gene expression. However, several serious problems were identified from the very

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ABBREVIATIONS: EDAC, *N*-ethyl-*N*-(3-dimethylaminopropyl) carbodiimide; MGB, 1-methyl-4-[1-methyl-4[1-methyl-4[1-methyl-4[methylcarbonyl]aminopyrrol-2-carbonyl]aminopyrrol-2-carbonyl]aminopyrrol-2-carbonyl]-aminopyrrol -2-carboxylic acid; BIQ-1, 6-[[3-aminopropyl]amino]-10-amino-13H-benzo[6,7]indolo[3,2-c]quinoline; BIQ-2, 6-[[3-dimethylamino)-ethyl]amino]-11-amino-13H-benzo [6,7] indolo[3,2-c]quinoline; EDTA, ethylenediamine tetraacetate; DMF, dimethylformamide DMSO, dimethylsulfoxide; CTAB, hexadecyltrimethylammonium bromide (CTA salt, hexadecyltrimethylammonium salt); DTT, dithiothreitol; TLC, thin layer chromatography; MES, 2(N-morpholino)ethanesulfonic acid

beginning of the research in this field. Among them, the low stability of oligonucleotides in biological media, their poor penetration into cells as well as problems associated with their intracellular traffic and the reversibility of complex formation could be listed in the first place.

The conjugation of oligonucleotides to ligands of different chemical nature is one of the possible ways to solve some of these problems. Besides obvious terminal protection from cellular exonucleases and phosphatases by bulky moieties [8], an improvement of biological properties of oligonucleotides due to the attached ligands was observed. Numerous examples are known from the literature. Thus, attachment of hydrophobic residues [9,10] or specific peptides [11-15] enables better penetration of oligonucleotides into cells. Conjugated intercalators such as acridine [16], benzopyridoindole [17] and benzopyridoquinoxaline [18] derivatives, considerably stabilize duplexes and triplexes. To render the action of antisense or anti-gene oligonucleotides irreversible, conjugates with chemically active substituents [19-21], photosensitizers [22-26] or DNA-cleaving agents such as Fe^{2+}- or Cu^{+} -coordinating molecules (EDTA, phenantroline, porphyrins) [27-31] have been synthesized.

To obtain this large number of modified oligonucleotides, a variety of synthetic methods has been developed. In most of them the ligand is introduced during solid-phase synthesis of the oligonucleotide, either as a phosphoramidite derivative or as a modified nucleoside phosphoramidite containing a linker with a reactive group [32,33].

Custom synthesis of oligonucleotides was developed by several companies during the last 10-15 years. For numerous laboratories it is much easier to buy an oligonucleotide preparation ready-to-use than to develop a new synthetic group. Moreover, solid-phase synthesis methods cannot follow with a reasonable delay all the new ideas appearing in the field. This is why the "post-synthetic" introduction of ligands into deprotected oligonucleotides has been attracted much interest. Attachment of amines to a terminal phosphate upon activation of the latter by either dicyclohexyl carbodiimide [34] or the water-soluble *N*-ethyl-*N*-(3-dimethylaminopropyl) carbodiimide (EDAC) [35,36] has been described. In order to avoid harmful action of carbodiimides on the non-protected bases of nucleic acids, the oxidation-reduction condensation with triphenylphosphine and dipyridyl disulfide [37-39], which was originally developed for the

synthesis of peptides [40], was applied for the conjugation of nucleophilic amino groups. Amines have also been attached to aldehyde groups obtained upon hydrolytic cleavage of a glycosidic bond (e.g. upon depurination [41-43] or action of uracyl glycosylase [44]) or to sugars containing an α-diglycolyc group after periodate oxidation [45,46].

About ten years ago we published a paper where we reviewed some of the techniques that could be used in the synthesis of oligonucleotide conjugates [38]. At that time the applied methods were limited to the introduction of highly nucleophilic sulfhydryl or aliphatic amino groups followed by the reaction with electrophiles in organic media. The objective of the present work was to elaborate a versatile and simple set of conjugation methods available for groups with minimal chemical experience. The elaborated protocols allow the attachment of commercially available non-protected oligonucleotides (both phosphodiesters and phosphorothioates) to a great variety of ligand functional groups (carboxyl, hydroxyl, aldehyde, thiol and aliphatic or aromatic amino groups) in both organic and aqueous media. The coupling is possible directly to the oligonucleotide or *via* a specific linker. The length of the linker, the stability of the linkages in different conditions (pH, solvents, etc.), the position of the activated functional group could be varied. No damage to nucleotide heterocyclic bases or internucleotide linkages was observed under these procedures.

In addition, a catalytically active nucleic acid (aptamer) [47] can be considered as a catalytic ligand that might be attached to an oligonucleotide. Therefore we describe a simple method for the synthesis of oligonucleotide dimers, which could be applied to link together oligonucleotide "monomers" with parallel or antiparallel orientations.

EXPERIMENTAL PROCEDURES

Chemicals:

All the reagents and solvents were purchased from Sigma - Aldrich - Fluka and used without further purification, except dimethylsulfoxide and triethylamine. The latter were distilled under reduced pressure in the presence of dry NaOH, ninhydrin being added to triethylamine to remove traces of primary amines. The model phosphodiester oligonucleotides TTTTCTTTTCCCCCCT (1) and TTTTC^{me}TTTTC^{me}C ^{me}C ^{me}C ^{me}C ^{me}C ^{me}T (2), and phosphorothioate

CGTGATGTTCACCTC (3) were synthesized by Eurogentec (C^{me} is 5-methylcytosine). The first two oligonucleotides could form a triple helix with two genes of HIV proviral DNA [48], the last one is an antisense oligonucleotide targeted to the luciferase reporter gene [49]. Triplex-specific intercalators 6-[[3-aminopropyl]amino]-10-amino-13H-benzo[6,7]indolo[3,2-c]quinoline (BIQ-1) and 6-[[3-dimethylamino)-ethyl]amino]-11-amino-13H-benzo [6,7] indolo[3,2-c]quinoline (BIQ-2) were synthesized and kindly provided by Drs. C.H.Nguyen and P.Schmidt (Institute Curie, Orsay). Minor groove binder (MGB) 1-methyl-4-[1-methyl-4[1-methyl-4[1-methyl-4[methylcarbonyl]aminopyrrol-2-carbonyl]aminopyrrol-2-carbonyl]aminopyrrol-2-carbonyl]-aminopyrrol -2-carboxylic acid was a kind gift of Dr. A.Sinyakov (Novosibirsk). 5,10,15,20-tetra(p-hydroxyphenyl)-21H,23H-porphyne and 4-(N,N-dihexadecylamino)-4'(8-iodo-3,6-dioxaoctyloxy)chalcone were synthesized by C.Gosse (Ecole Normale Supérieure, Paris). The structures of the ligands are shown on Fig.1.

Analysis of reaction yields and purification of products.

Electrophoresis.

All the conjugates were analyzed by denaturing gel electrophoresis in 20% polyacrylamide gel / tris-borate - EDTA buffer in the presence of 7 M urea and visualized by UV-shadowing. In all described cases a single retarded band of the product was observed. The yield of the conjugate was considered as quantitative when no band of initial oligonucleotide was visible.

HPLC.

All the conjugated products were analyzed and purified, if necessary, by HPLC on a Lichrosorb C-18 column 250x4 mm (System CR-4A Chromatopack, Shimadzu). A 30 min gradient of acetonitrile 5-40% in 0.02 M ammonium acetate was applied for elution of the product. In all cases a retardation of the product peak compared to the starting oligonucleotide one was observed varying from 2 to 7 minutes (at a flow rate of 1 ml/min). For highly hydrophobic compounds (chalcones or fullerenes) HPLC purification was not possible due to irreversible adsorption of the product on the column. In this case electrophoresis on a 1% agarose gel in standard tris-acetate buffer was used.

Ion exchange chromatography was carried out on an Alltech SAX Spherisorb column (250x4,6 mm) in a linear gradient of $LiClO_4$ 0 - 1 M in 30% acetonitrile.

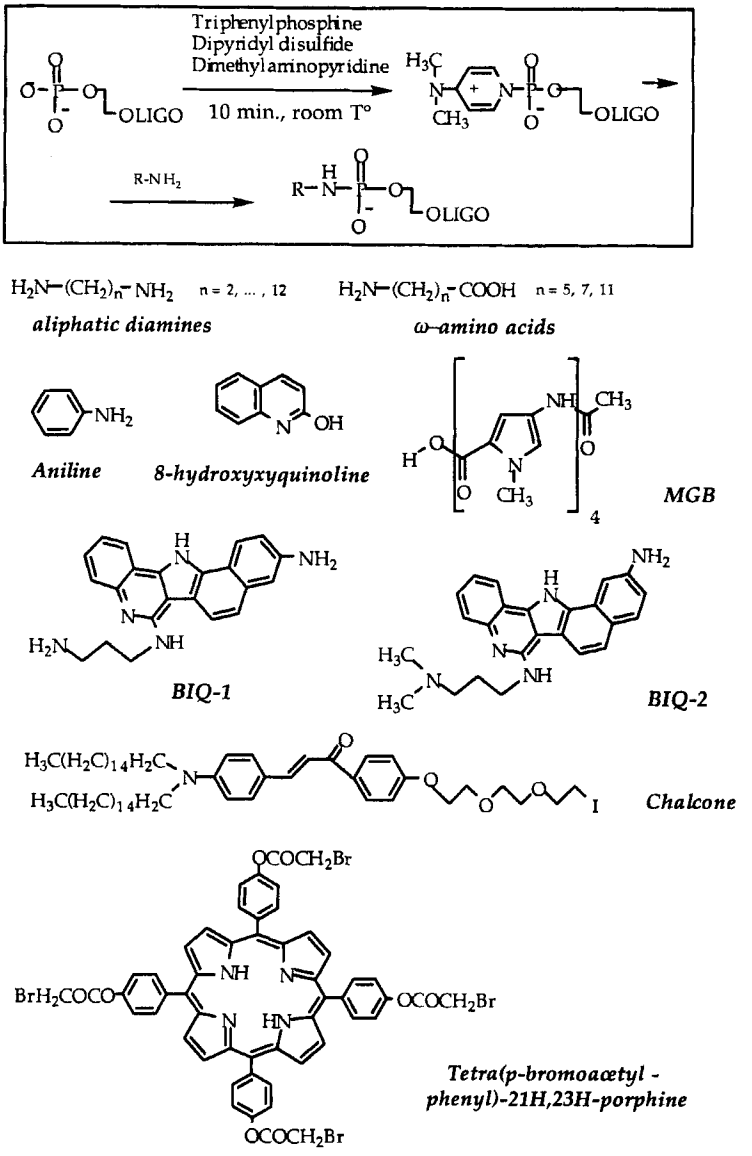


FIG. 1. Activation of the terminal phosphate of oligonucleotides and ligands used as model substances for the synthesis of conjugates.

Spectrophotometry

All products were analyzed by UV-visible spectrophotometry on a Kontron Uvikon 923 instrument. The spectra of the products were compared with the sum of oligonucleotide and free ligand spectra. The conjugate was considered pure when a good coincidence and 1:1 ratio of the oligonucleotide and ligand absorption were observed.

Enzymatic 5'phosphorylation or thiophosphorylation.

Preparative enzymatic phosphorylation or thiophosphorylation of oligonucleotides on a milligram scale was carried out as described in previous papers [38,50], with small differences. Since preparative phosphorylation and thiophosphorylation of phosphorothioate oligonucleotides is quite slow, the time of incubation was increased. 2 mg (400 nmol) of the oligonucleotide were dissolved in 600 µl of kinase buffer (0.01 M $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, 0.05 M Tris HCl, pH 7.6). To this solution a 6-fold excess of ATP or ATP-γ-S was added. The reaction was started upon addition of T4-polynucleotide kinase (80 units). The mixture was incubated for 3 h at 37 °C for phosphodiesters, incubation was prolonged overnight at 4°C for phosphorothioates or 16 hours at room temperature for ATP-γ-S. Then the oligonucleotide was precipitated by addition of 0.2 M sodium acetate in ethanol, purified by gel filtration on Sephadex G-25, concentrated by butanol extraction and precipitated by ethanol/sodium acetate.

Attachment of amines or hydrazides to the 5′-phosphate group of oligonucleotides in organic and aqueous solutions.

A series of aliphatic diamines, beginning from diaminoethane up to 1,12-diaminododecane were conjugated to the model oligonucleotides with quantitative yields both in organic and water solutions. The protocol of Knorre et al. [39] was followed. 100-400 nmol of cetyltrimethyl ammonium (CTA) salt of the oligonucleotide and 5 mg (40 µmol) of 4-dimethylaminopyridine were dissolved in 50 µl of DMSO and mixed with 6.6 mg (30 µmol) of dipyridyl disulfide in 25 µl of DMSO and 7.9 mg (30 µmol) of triphenylphosphine in 25 µl of DMSO. After 10 min. of incubation at room temperature either 3-5 mg (25-60 µmols) of diamine were added directly into the reaction mixture or the activated oligonucleotide was precipitated with acetone/lithium perchlorate, washed with acetone and dissolved in an aqueous solution of diamine (3-5 mg in 100 µl). The mixture was incubated for 15 min. at room temperature and the conjugate was twice

precipitated with ethanol/sodium acetate. For higher amines (10-12 methylene groups) better results were obtained when the diamine was dissolved in 25-30% alcohol- water mixture. A majority of model reactions made use of hexamethylene diamine as a linker. The attachment of amino acids was always carried out in water or water-alcohol solution after removal of the activating agents by reprecipitation of the activated oligonucleotide. The same procedure was used to attach adipic acid dihydrazide.

The coupling of aromatic amines is possible under the same conditions in organic solutions, but with much longer incubation times. For example, conjugates of oligonucleotide 1 with aniline (100% yield after 1 day) and *para*-aminobenzoic acid (80% yield after 2 days) were obtained. However, the rate and yield of reaction depended on the nature of the amine and aromatic ring of the molecule.

Synthesis of the activated esters of carboxylic acids

In a typical procedure 9 μmol of the acid were dissolved in 80 μl of dry DMF. 1.5 mg (13 μmol) of *N*-hydroxysuccinimide were added to the solution. The reaction was started upon addition of 2.7 mg (13 μmol) of dicyclohexylcarbodiimide in 20 μl of dry DMF. The reaction mixture was incubated at room temperature. Progression of the reaction was monitored by thin layer chromatography. After 12 hours of incubation dicyclohexylurea was separated by centrifugation and washed with 20 μl of DMF. The combined DMF fractions were added in 20 μl portions to 1.5 ml of diethylether. The precipitated product was washed twice with 500 μl of ether, dried *in vacuo* and analyzed by thin layer chromatography on silica gel. The yield was quantitative.

Attachment of activated carboxyl groups to oligonucleotides carrying an amino group

The oligonucleotide with amino linker (50 nmol) was precipitated as cetyltrimethylammonium salt from water solution by 8% cetyltrimethylammonium bromide (CTAB). The dry compound was dissolved in 20 μl of dry DMSO. Then a 20-fold excess of the activated ester in 5 μl of DMSO or DMF solution was added. The reaction was stored at room temperature for 12 h. The conjugate was precipitated with acetone/$LiClO_4$, washed with acetone, dried, redissolved in 30 μl of water and the product isolated by HPLC.

Attachment of a carboxylic group to the oligonucleotide terminus

The activation of the 5′-terminal phosphate of the oligonucleotide (100-200 nmol) was carried out in 100 μl of DMSO as described above for the attachment of amines.

After 20 min the oligonucleotide was precipitated with acetone/LiClO4 and washed with acetone in order to remove any excess of activating agents. Then a solution of 6 mg (46 μmol) of 6-aminocaproic acid and 9 μl of triethylamine in 50 μl of water were added. The mixture was left for 2 h at room temperature. The oligonucleotide was then isolated by precipitation with acetone/LiClO4 and washed with acetone. The yield was quantitative.

Attachment of amines to the terminal carboxylic group of modified oligonucleotides

1. Activation with *N*-(3-dimethylaminopropyl)-N′-ethylcarbodiimide

Aliphatic amines

In a typical procedure 2.5 μmol of ligand carrying an aliphatic amino group (e.g. BIQ-1) were dissolved in 20 μl of DMSO. 5 μl of 1.2 mM water solution of aminocaproic acid-linked oligonucleotide (6 nmoles, 1/400 equiv.) were added and well mixed. The solution was cooled to 0°C and 1,3 mg (6,8 μmol) of *N*-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC) in 1 μl of water were added. The mixture was kept at room temperature overnight. The oligonucleotide was isolated by precipitation with ethanol/sodium acetate. The yield was quantitative.

Aromatic amines and alcohols:

a) in acid aqueous solution

In a typical procedure the aromatic amine (aniline or BIQ-2, 10 μmol) was suspended in 50 μl of water and dissolved as hydrochloride upon addition of hydrochloric acid. The solution of the carboxylic acid-modified oligonucleotide (50 nmol) and 10 mg (52 μmol) EDAC were added. The reaction mixture was stored at 4°C overnight. Then the oligonucleotide was precipitated by addition of acetone/$LiClO_4$, redissolved in 50 μl of water and for further purification re-precipitated with ethanol/sodium acetate. The yield was quantitative.

The same method was used to attach aromatic alcohols. 5 mg (34 μmol) of 8-hydroxyquinoline were suspended in 50 μl of water. HCl was added until the compound was dissolved as a salt. The reaction was carried out as described above. The yield was quantitative

b) in buffered aqueous/organic solution

The aromatic amine (BIQ-2, 5.4 μmol) was dissolved in 10 μl of DMSO. 3 μl of carboxylic acid-modified oligonucleotide (3 nmol) were added to this solution. The

reaction was started upon addition of 0.5 mg (2.6 μmol) of EDAC in 5 μl of 0.75 M MES buffer (pH 5.0) and left for 3 h at room temperature. The product was isolated upon addition of ethanol/sodium acetate, washed with ethanol, dried, redissolved in water and purified by HPLC. The yield was about 80%.

For attachment of aromatic alcohols in buffer conditions 5 mg (34 μmol) of 8-hydroxyquinoline were suspended in 100 μl of 0.75 M MES buffer (pH 5.0) and 70 μl DMF. 5 μl of aminocaproic acid-linked oligonucleotide (8-12 nmol) were added to this mixture. The reaction was started upon addition of 5 mg (26 μmol) of EDAC and then left at 4°C. After 3 h the oligonucleotide was precipitated with ethanol/sodium acetate, washed with ethanol, dried, redissolved in water and purified by HPLC. The yield was quantitative.

2. Activation of the carboxylic group of aminocaproic acid-linked oligonucleotides with dipyridyldisulfide and triphenylphosphine

The aminocaproic acid-linked oligonucleotide (10 nmol) was precipitated as cetyltrimethylammonium salt. The dry salt was dissolved in 10 μl of DMSO and 3.3 mg (15 μmol) of dipyridyldisulfide in 20 μl DMSO and 3.9 mg (15 μmol) of triphenylphosphine in 20 μl of DMSO were added. After 20 min at room temperature a solution of 2.7 μmol of aromatic amine (BIQ-2) in 10 μl of DMSO was added. After 2 h at room temperature the product was isolated by precipitation with ethanol/sodium acetate, washed with ethanol, dried, redissolved in water and purified by HPLC with double detection at 260 nm (oligonucleotide) and 320 nm (BIQ). The yield was about 70%.

Bromoacetylation of aromatic amines and alcohols

Reaction of acetylation with acetylhalides is widely used in organic chemistry. Here we just describe two examples that we used in our experiments.

Aromatic amines:

In a model reaction, a solution of 500 mg (489 μl, 5.37 mmol) of aniline in 5 ml of toluene was added under stirring to 541 mg (233 μl, 2.68 mmol, 0.5 equiv.) of bromoacetylbromide in 10 ml of toluene. A white precipitate (aniline hydrobromide) formed. After 1 h of stirring at room temperature the toluene was removed *in vacuo*, the remaining solid was dissolved in ethanol/water (9:1). In order to avoid hydrolysis of the bromoacetyl group two drops of 5 N H_2SO_4 were added. Upon addition of water a white

solid precipitated which was isolated and thoroughly washed with water. For further purification the product was again dissolved in ethanol and precipitated with water. ^{1}H-NMR (300 MHz, $CDCl_3$): δ= 4.00 (s, 2 H, CH_2), 7.15 (dd, J = 7.4 Hz, J' = 1.1 Hz, 1 H, 4-H), 7.34 (dd, J = 8.5 Hz, J' = 7.4 Hz, 2 H, 3-H, 5-H), 7.51 (dd, J = 8.5 Hz, J' = 1.1 Hz, 2 H, 2-H, 6-H), 8.13 (s, 1 H, NH).

Aromatic alcohols:

4 mg (5,9 μmol) 5,10,15,20-tetra(p-hydroxyphenyl)-21H,23H-porphine were dissolved in 150 μl of anhydrous dimethylformamide and 20 μl (37,6 mg, 239 μmol) of bromoacetyl chloride were added together with 5 μl of anhydrous triethylamine. The mixture was incubated 15 min at room temperature and the excess of acylating agent was removed by chloroform/water extraction. The product was purified on silica gel (Kieselgel 60, Merck) in 98% chloroform - 2% methanol, the first band was collected. Thin layer chromatography in chloroform revealed only one band of product with R_f close to 0,3.

Attachment of a thiol group to the oligonucleotide terminus

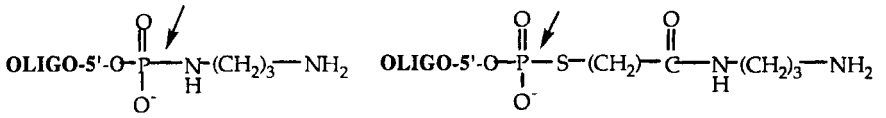
Coupling of cystamine was carried out using cystamine hydrochloride according to the method of Knorre et al. [39]. The attachment step was carried out in water solution, 5 μl of triethylamine were added to transform a cystamine salt into free base form. Reduction of the disulfide bond was done with tricarboxyethylphosphine or dithiothreitol. 3 μl of a 0.1 mM solution of tricarboxyethylphosphine (final concentration 3 mM, ca. 6 equiv.) or 10 μl of a 3 M aqueous solution of DTT (final concentration 0.3 M) were added to the solution of the cystamine-linked oligonucleotide (ca. 0.5 mM, 100 μl). After 1 h at room temperature the thiol-substituted oligonucleotide was precipitated with degased ethanol/sodium acetate. The thiol derivative was stored under nitrogen.

Attachment of the bromoacetyl-containing substances to thiol-substituted oligonucleotides or to a terminal thiophosphate group.

Reaction of compounds containing a bromoacetyl group with oligonucleotides containing a sulfhydryl or a thiophosphate group was described earlier [51,52].

Stability of conjugates in acid solutions.

The following conjugates of oligonucleotide 3 were used: 5'- [N-(3-aminopropyl)]-phosphoramidate of the oligonucleotide and 5'-[S-[N-(3-aminopropyl)]acetamide]-phosphorothioate of the oligonucleotide (Fig.2).



5'- [N-(3-aminopropyl)]-phosphoramidate *5'-[S-[N-(3-aminopropyl)]acetamide]phosphorothioate*

FIG.2. A. Oligonucleotide conjugates used for studies of comparative stability of P-N and P-S bonds in acid conditions. Acid-labile linkages are indicated by arrows.

50 μl of trifluoroacetic acid were added to 2 μl of a 1 mM solution of the oligonucleotides. After 1, 5, 10, 15 and 25 min of incubation at room temperature 1-5 μl aliquots were taken, re-precipitated by acetone/$LiClO_4$, dissolved in 20 μl of water and analyzed by ion exchange chromatography.

Covalent conjugation of two oligonucleotides

Method 1. 20-50 nmol of the first oligonucleotide (designated A) bearing a terminal phosphate were activated by triphenylphosphine - dipyridyl disulfide as described above. In one ("organic") version, a 2 to 5 time excess of CTA salt of amino-linked oligonucleotide (designated B) in 50-100 μl of DMSO was added and incubated for 2 hours. In another ("aqueous") version the activated oligonucleotide was precipitated with acetone/$LiClO_4$ and washed with acetone. A 2-5 times excess of a second oligonucleotide (designated B) bearing a terminal amino group was dissolved in 2 M NaCl and 3 μl of triethylamine added. The pellet of activated oligonucleotides was redissolved in this solution and incubated for 1 h at room temperature. The conjugate was analyzed by electrophoresis and purified by the ion exchange chromatography. The yield was between 60 and 90%.

Method 2. The synthesis of oligonucleotides containing terminal sulfhydryl or thiophosphate group was described above. For the synthesis of symmetric dimers (3'-3' or 5'-5') of the same oligonucleotide 2 equivalents of iodine solution in water were added. A dimer was formed with quantitative yield. For coupling of two different oligonucleotides a sulfhydryl-containing oligonucleotide A (2 mM in 25 μl of water solution) was mixed with 50 μl of DMSO solution containing 1 mg of dipirydyl disulfide. After 1 h of incubation at room temperature the resulting product was re-precipitated twice with ethanol/sodium acetate. Degased water solution of oligonucleotide B containing a

sulfhydryl group (1 mM in 25 µl of 0.01 M tris-HCl, pH 7.6) was added to the pellet and the pellet dissolved. The mixture was incubated for 1 hour at room temperature and analyzed by electrophoresis and ion exchange chromatography. The yield of conjugate was quantitative.

RESULTS AND DISCUSSION

The aim of this work was to develop or adapt a series of simple and reproducible methods for the conjugation of non-protected oligonucleotides to a large number of different ligands. Thus, we used not only substances that could be useful for further biological applications (MGB, intercalators, porphyrins), but also model molecules without any obvious biological utility (aniline as aromatic amine, 8-hydroxyquinoline as aromatic alcohol, etc.).

The formulas of several ligands used in these studies are presented in Figure 1. A general list of functional groups that could be easily introduced into oligonucleotides and ligands are shown in Figures 3 and 4.

Attachment *via* terminal phosphate

The first strategy that we chose made use of the properties of terminal phosphomonoester groups (Fig. 4, f) of oligonucleotides because they have different chemical activity as compared with internucleotide phosphodiesters. Attachment of a 3'- or 5'-phosphate or thiophosphate group could be done during solid state synthesis. Alternatively, an enzymatic reaction catalyzed by polynucleotide kinase could be used to attach phosphate or thiophosphate group at the 5'-end of the oligonucleotide using ATP or ATP-γ-S as substrates, respectively.

The use of organic media for manipulation with oligonucleotides is not a problem since the cetyltrimethylammonium salts of oligonucleotides are soluble in many organic solvents, especially polar ones (it works well with methanol, DMSO, pyridine, DMF, much less with tetrahydrofuran, toluene, badly with chloroform). Alternatively, a direct mixing of the oligonucleotide in aqueous solution with the ligand in DMSO could be used in order to avoid preliminary transformation of the oligonucleotide into CTA salt. After addition up to 9 volumes of DMSO to an oligonucleotide aqueous solution the latter remains soluble.

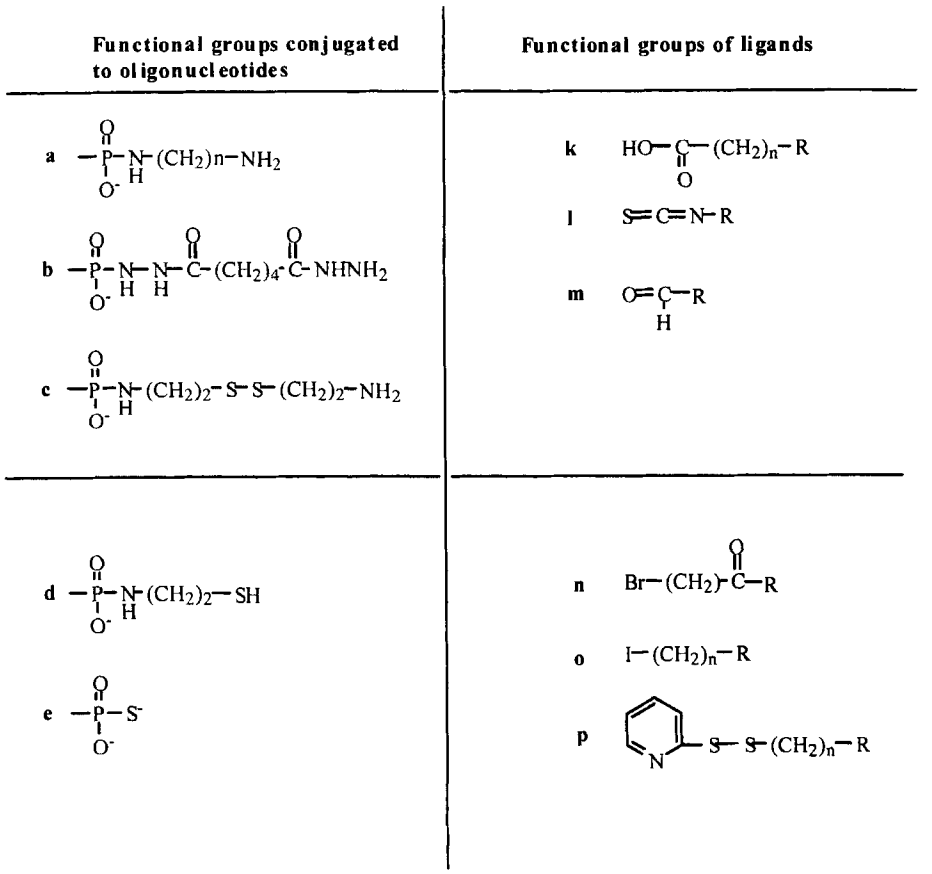


FIG. 3. Nucleophilic functional groups introduced at the 3'- or 5'-terminus of oligonucleotides (a-e) and corresponding electrophilic functional groups of ligands that could be used for conjugation (k-p)

Two different methods for terminal phosphate activation could be used: different carbodiimides (dicyclohexyl carbodiimide in organic solution or *N*-(3-dimethylaminopropyl)-N′-ethyl carbodiimide in water solution) and Mukaiyama reagents (triphenylphosphine / dipyridyl disulfide). However, carbodiimide activation needs a fairly long time and can affect nucleic acid bases after incubation times of more than 12 hours. Thus, it should be used with caution [36]. In contrast, the Mukaiyama reaction (perfectly described by Knorre et al. [39]) is rapid and does not affect nucleic acid bases and internucleotide phosphates or phosphorothioates (we tried also to apply this reaction

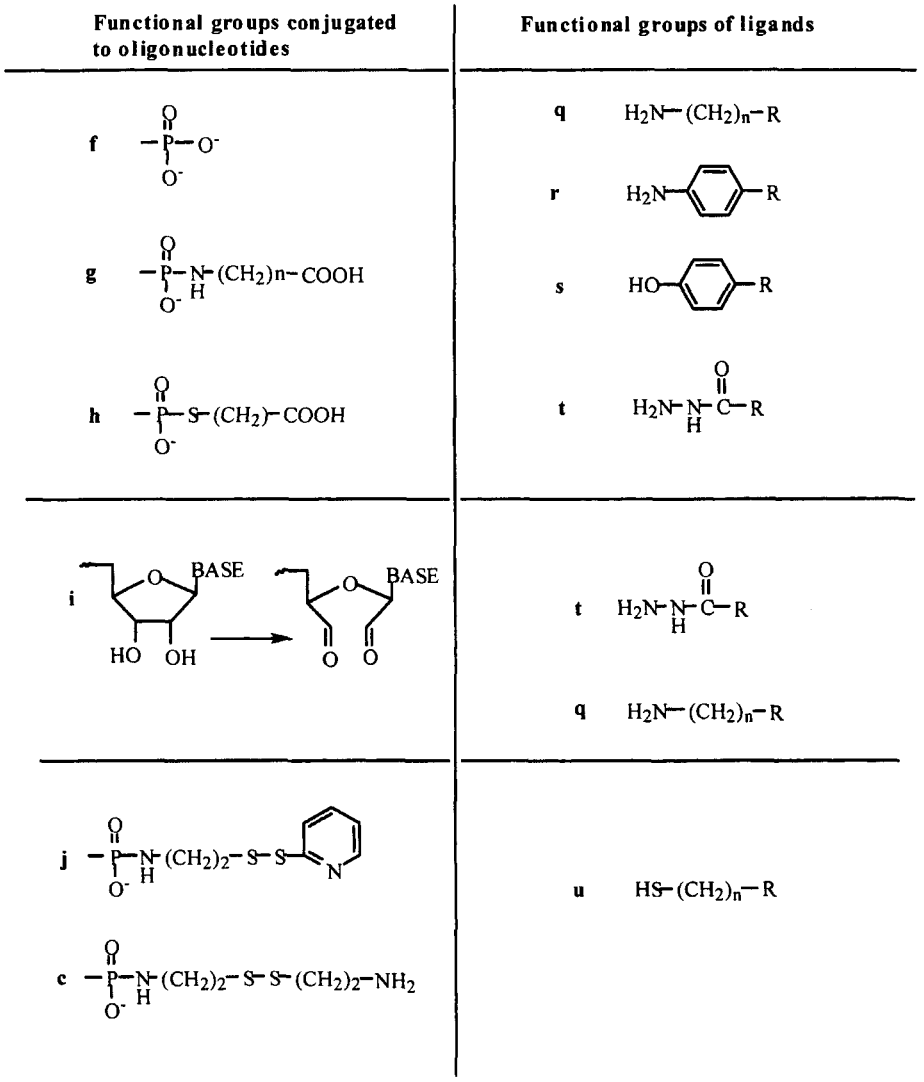


FIG. 4. Electrophilic functional groups introduced at the 3'- or 5'-terminus of oligonucleotides (c, f-j) and corresponding nucleophilic functional groups of ligands that could be used for conjugation (q-u)

to N3'=>P5' phosphoramidates, but in this case the internucleotidic phosphoroamidates seem to be affected). The yield of activation is quantitative, the use of dimethylaminopyridine or N-methylimidazole as catalyzer stabilizes the intermediate product, but this is not necessary. The reaction could be applied both to coupling of water-soluble and lipophilic nucleophiles. Because the activated intermediate is quite

stable, it could be isolated by precipitation and redissolved in water. As nucleophilic groups reacting with an activated phosphate, aliphatic and aromatic amines (primary and secondary) could be used (Fig. 4, q-t). The highest yield and rate of reaction could be achieved for aliphatic amines (Fig.3, a). If adipic acid dihydrazide was used instead of a diamine, a reactive hydrazide group was introduced (Fig. 3, b).

A special note must be added concerning aromatic amines (Fig.4, r). Firstly, the reaction requires much longer times (several hours) to proceed. Secondly, the reactivity of different amines is very different. For example, we succeeded in attaching aniline to the 5'-terminal phosphate of an oligonucleotide with a quantitative yield. But the aromatic 10-amino group of BIQ-1 (with protected aliphatic amino group) was much more inert, and we had to change the chemistry of conjugation and proceed via bromoacetylation (see below).

Attachment *via* a terminal amino or hydrazido group.

The attachment of aliphatic ω-diamines to the terminal phosphate group according to the method described above led to a nucleophilic amino group on the oligonucleotide terminus ready to react with electrophiles (e.g., activated carboxylic groups, isothiocyanates, alkylating agents or aldehydes with further reduction by sodium borohydrate [25,53]) (Fig. 3, k,l,m). If a hydrazide group was introduced instead of an amino group (Fig. 3, b), this provided us with an additional advantage since the latter is reactive under relatively acidic conditions (pH = 5), when the amino group was protonated and could not react with electrophiles. We have used such a hydrazide derivative to react with aldehydes, such as HEVP chlorin [25,53] (chlorin originated from **h**epta**e**thyl**v**inyl**p**orphyrin). In addition, the -C=N- linkage in hydrazones is much more stable than a similar linkage in Schiff bases and does not need borohydrate reduction.

In our experiments we used mainly N-hydroxysuccinimide esters of carboxyl-containing ligands; in several cases carbonyldiimidazole activation was used. Different ligands such as deutheroporphyrin, camptothecin [54], modified fullerenes [55], or even peptides under the form of N-hydroxysuccinimide activated esters could be attached to a terminal amino group by this method. Starting from N-hydroxysuccinimide ester of MGB, we synthesized a conjugate with oligonucleotide 2 with a yield of 46%. This conjugate was able to form a triplex with a target HIV proviral sequence [48] that possessed stability similar to that of the non-conjugated oligonucleotide (data to be published elsewhere).

Attachment *via* a terminal carboxyl group

In several cases when steric hindrance prevented the nucleophilic molecule from reacting with an activated terminal phosphate, a linker such as aminocaproic acid (or longer) could be attached (Fig. 4, g). A free carboxylic group becomes thus accessible at the oligonucleotide terminus separated from the terminal phosphate group by several carbon atoms. However, triphenylphosphine and dipyridyl disulfide must be removed from the mixture before the addition of non-protected amino acids to the activated oligonucleotide phosphate in order to avoid undesirable activation of the amino acid carboxyl group. Therefore the activated oligonucleotide was first precipitated and then dissolved in water or water/alcohol solution of the amino acid under basic conditions. The activation of the carboxylic group by Mukaiyama reagents could be carried out in the same conditions as that of the phosphate group. However, in several cases (amino-modified fullerenes and porphyrins) use of aminocaproic acid linker led to much higher yields of coupling compared to attachment to the terminal phosphate without a linker (data not shown). The rate and yield of the reaction between an activated carboxylic group and an amine is concentration-dependent, so the optimal concentrations of reagents described in Materials and Methods section must be respected.

Activation of a carboxylic group by carbodiimides was also more problematic than by Mukaiyama reagents. Water-soluble amines, or amines which were solubilized as hydrochlorides, could be attached to aminocaproic acid-linked oligonucleotides using the water-soluble EDAC. However, reactions with EDAC in the presence of nucleic acids were successful only in a narrow pH range (around 5) since we observed hydrolysis of the phosphoramide bond between the oligonucleotide and the amino acid under acidic conditions, and alteration of DNA bases by EDAC under basic conditions. Thus, a method was developed, using concentrated (0.5-0.75 M) MES buffer (pH 5.0) as a solvent, which appeared to be inert against EDAC and made the reaction possible at a controlled pH. In addition, carbodiimide activation permitted us to attach certain aromatic alcohols to an oligonucleotide in aqueous buffered solution (see Materials and Methods).

Activation of a terminal carboxylic group is also suitable for attachment of ligands *via* aromatic amino group. For example, the 11-amino group of BIQ-2 was quite reactive, and we succeeded in attaching this molecule to an aminocaproic-linked oligonucleotide 2

in a quantitative yield when carbodiimide activation was used and with 80% yield using the Mukaiyama reaction. This conjugate formed a very stable triple helix with a 29-mer fragment of HIV proviral DNA with a melting temperature of 55°C as compared with 32° for the non-conjugated oligonucleotide (data to be published elsewhere).

Attachment *via* a terminal sulfhydryl group

Use of a terminal sulfhydryl group (Fig. 3, d) seems to be very attractive not only because this group is highly reactive and the chemistry of the reactions is very simple, but also because a reversible covalent fixation of the ligand to the oligonucleotide *via* a disulfide bond can be achieved. Covalent attachment of cystamine to the terminal phosphate (Fig. 3 and 4, c) of an oligonucleotide followed by reduction of the disulfide bond with dithiothreitol or tricarboxyethylphosphine led to a free SH group (Fig.3, d). When the sulfhydryl group is present, working under nitrogen or argon atmosphere is highly recommended in order to avoid dimer formation. This group could also be protected by formation of a mixed cysteamine-pyridyl disulfide (Fig. 4, j) after treatment of SH-containing derivative by excess of dipyridyl disulfide and isolation of the oligonucleotide conjugate by reprecipitation. In the last case the oligonucleotide SH-group is at the same time protected and activated for reaction with sulfhydryl-containing reagents, thereby forming a reducible disulfide bond (Fig.3, p). As examples, thiocholesterol [56] or cystein-containing peptides and proteins could be conjugated in this way.

A terminal sulfhydryl group can irreversibly react with many electrophilic moieties such as alkylhalides and other alkylating agents (Fig. 3, n,o) that are, under certain conditions, inert to the nucleic bases. This reaction is useful to attach many available reagents, including fluorescent or radioactive probes containing bromo- or iodoalkyl substituents, maleimides, p-chloromercurobenzoates and other SH-specific reagents (see, for example [15]). Synthesis of oligonucleotide conjugates with a fluorescent chalcone using a cysteamine-modified oligonucleotide and an iodoalkyl-modified chalcone has been already described and allowed us to synthesize an environment-sensitive fluorescent probe [57].

However, the alkylation of a terminal SH group is not compatible with phosphorothioate oligonucleotide chemistry. As expected, we observed considerable modification of internucleotidic phosphorothioates after treatment with bromoacetyl derivatives.

Attachment *via* a terminal thiophosphate. Stabilization against acid hydrolysis.

Use of a terminal thiophosphate group (Fig. 3, e) introduced chemically [51,58] or enzymatically [50] has already been described. This group is almost as reactive as a free sulfhydryl group. The additional advantage is an increased stability of conjugates under acidic conditions. It is well known that a phosphoramidate linkage is acid-labile and quickly hydrolyses at pH lower than 4 [38]. This can be an important drawback when acid deprotection of the synthesized conjugate or activation by EDAC under acidic conditions are used.

A thiophosphate linker possesses most of the properties of a sulfhydryl group, but does not contain a labile P-N linkage as cysteamine conjugates (Fig.2). Treatment of a thiophosphate by bromoacetic acid leads to a thioglycolic acid group joined to the terminal phosphate via a thioether bond. When the aminocaproic acid conjugate exhibited a half-life in trifluoroacetic acid of approximately 6 minutes, the half-life of phosphorothioether -linked thioglycolic acid was more than 1 hour. Thus, the reaction of a bromoacetic acid with a terminal phosphorothioate could be regarded as an alternative simple method to introduce a carboxylic group at the oligonucleotide terminus (Fig. 4, h).

Use of bromoacetylhalides for attachment of ligands possessing relatively inert amino groups or aromatic alcohols

Alcohols do not react with a terminal phosphate activated by the Mukaiyama reagents. In solid-phase synthesis, the activation of aliphatic alcohols was achieved by carbonyl diimidazole [59]. In certain cases the aromatic amino groups have a very low reactivity. For example, BIQ-1 could be conjugated to an oligonucleotide only *via* its aliphatic amino group, but we did not succeed in attaching a BOC-protected molecule *via* the aromatic amino group. In this case there is a simple solution when all the other reactive groups of the ligand are protected. The amino- or hydroxyl group can be acylated with bromoacetylbromide or bromoacetylchloride in an inert solvent. The reaction proceeds usually with equimolar quantities of bromoacetylhalide and gives a quantitative yield of the desired product (Fig. 3, n). Then the derivative can be easily conjugated to sulfhydryl- or terminal thiophosphate-containing oligonucleotide as described earlier.

For example, a conjugate between 5,10,15,20-tetra(p-bromoacetylphenyl)-21H,23H-porphine and a cysteamine-linked oligonucleotide was achieved in quantitative yield in 10-fold excess of tetra-bromoacetylated porphyrin. The three remaining bromoacetyl groups on the porphyrin moiety could be used for coupling of other nucleophilic reagents.

Synthesis of conjugates between two different oligonucleotides

In some cases it could be interesting to attach to each other two different oligonucleotides with different polarity (5'-5'; 3'-3', 5'-3') and different length of linker between the two oligonucleotide molecules. For example, one oligonucleotide could be a specific address recognizing a nucleic acid sequence or structure, the second could be a primer of transcription or could participate in recombination. The second oligonucleotide could also possess a catalytic activity (after selection from a pool of oligonucleotides by the SELEX method). Assuming that R on Figure 3 and 4, k-u, could be the second oligonucleotide, we tried to link two different oligonucleotides together. We elaborated two simple procedures of conjugation, polarity being dependent on the position of the terminal phosphate (5' or 3'). In one method (*via* a diamine linker), the linkage between oligonucleotides is irreversible and the length of linker could be varied. The other method (*via* S-S bond) gives better yields of conjugate and retains the possibility to cleave the linkage between the two oligonucleotides under reductive conditions. The general problem of this type of synthesis is steric hindrance and electrostatic repulsion of oligonucleotides. Therefore a high ionic strength is necessary in an aqueous solution.

Conclusion

The methods presented in this survey enabled us to conjugate ligands with different reactive groups to oligonucleotides, either with or without linkers. The length of the introduced linker and its reactivity could be varied in a wide range providing the possibility to choose the appropriate linker to be reacted with a specific ligand. Electrophilic ligands could be conjugated to amino or sulfhydryl modified oligonucleotides, nucleophilic ones could be attached directly to a terminal phosphate or to a carboxyl group connected to the oligonucleotide. Highly reactive nucleophilic or electrophilic groups could be attached selectively to the oligonucleotide termini without alteration of the non-protected nucleic acid bases.

For natural phosphodiesters all the described methods are suitable, and a majority of them are also suitable for PNA and RNA (except synthetic methods requiring basic conditions in aqueous solutions for the latter). Certain limitations are imposed by some synthetic backbones such as phosphorothioates and phosphoroamidates.

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